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Insights into the olfactory system of the ectoparasite *Caligus rogercresseyi*: Molecular characterization and gene transcription analysis of novel ionotropic receptors

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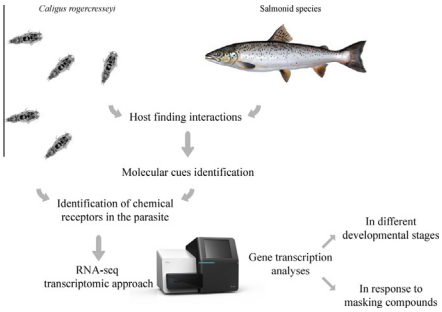
HIGHLIGHTS

- Novel ionotropic receptors were found at transcriptomic level in *Caligus rogercresseyi*.
- Expression analyses of these transcripts showed a relation with olfactory reception.
- Potential implication of these receptors in host-finding process is discussed.

GRAPHICAL ABSTRACT



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ABSTRACT

Although various elements of the olfactory system have been elucidated in insects, it remains practically unstudied in crustaceans at a molecular level. Among crustaceans, some species are classified as ectoparasites that impact the finfish aquaculture industry. Thus, there is an urgent need to identify and comprehend the signaling pathways used by these in host recognition. The present study, through RNA-seq and qPCR analyses, found novel transcripts involved in the olfactory system of *Caligus rogercresseyi*, in addition to the transcriptomic patterns expressed during different stages of salmon lice development. From a transcriptomic library generated by Illumina sequencing, contigs that annotated for ionotropic receptors and other genes implicated in the olfactory system were identified and extracted. Full length mRNA was obtained for the ionotropic glutamate receptor 25, which had 3923 bp, and for the glutamate receptor ionotropic kainate 2, which had 2737 bp. Furthermore, two other transcripts identified as glutamate receptor, ionotropic kainate 2-like were found. *In silico* analysis was performed for the transcription expression from different stages of development in *C. rogercresseyi*, and clusters according to RPKM values were constructed. Gene transcription data were validated through qPCR assays in ionotropic receptors, and showed an expression of glutamate receptor 25 associated with the copepodid stage whereas adults, especially male adults, were associated with the kainate 2 and kainate 2-like transcripts. Additionally, gene transcription analysis of the ionotropic receptors showed an overexpression in response to the presence of masking compounds and immunostimulant in salmon diets. This response correlated to a reduction in sea lice infection following in vivo challenge. Diets with masking compounds showed a decrease of lice infestation of up to 25%. This work contributes to the available knowledge on

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chemosensory systems in this ectoparasite, providing novel elements towards understanding the host-finding process of the salmon louse *C. rogercresseyi*.

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1. Introduction

In recent years, the salmon louse *Caligus rogercresseyi* has become a major cause for economic losses in the Chilean salmonid aquaculture industry (Costello, 2009). There is also continued concern over the impact of sea lice on wild fish populations and the environment (Heuschele and Selander, 2014). Control measures have been reliant upon the use of a number of chemotherapeutants since the 1970s. Reduced efficacy has now been reported for many of these compounds (Bravo, 2010). Further methods are required to effectively control sea lice, in conjunction with medicines. Methods to reduce initial attachment by disrupting host location been identified as additional control mechanisms (Mordue Luntz and Birkett, 2009).

Previous studies have demonstrated that the sea lice species such as *Lepeophtherius salmonis* have developed physical mechanisms to locate the host, such as water temperature (Heuschele and Selander, 2014), changes in the salinity (Genna et al., 2005), and the water currents produced by swimming fish (Heuch et al., 2007). In addition this ectoparasite also recognizes chemical cues, such as the semiochemicals produced by the target species (Bailey et al., 2006; Hull et al., 1998). In order to recognize these cues, sea lice have developed recognition mechanisms composed by various receptors that interact with semiochemicals, as derived from different sources such as fish odor, suggesting the role of these receptors in the host-finding process (Heuschele and Selander, 2014). Sea lice have advanced olfactory and contact chemoreceptors that are capable of accurate identification of specific host molecules (Hull et al., 1998; Genna et al., 2005; Fields et al., 2007; Mordue Luntz and Birkett, 2009). The main olfactory receptors of *L salmonis* are found on the first antennae (antennules). Electrophysiological recordings from the antennules have shown that receptors respond to seawater conditioned with host and non-host odours (Ingvarsdóttir et al., 2002; Fields et al., 2007). Ablation of the distal tip of the chemosensory antennules significantly reduced host-finding ability in *L. salmonis* adult males (Hull et al., 1998).

The molecular basis for the recognition of chemical cues in ectoparasites remains practically unknown. Despite the relatively high amount of knowledge available on the process of sensing odorants in vertebrates, in invertebrates studies on the molecular pathways associated with this process have only been characterized in a few model species such as *Drosophila*, but very little is known for other species not belonging to the insect taxa (Touhara and Vosshall, 2009). A wide array of receptors involved in the recognition of chemical cues has been characterized in vertebrates. For example, up to 1500 members of odorant receptors (OR) have been identified in mammals, but in fish only 100 OR genes have been discovered (Niimura and Nei, 2005). Meanwhile, efforts to identify homologous vertebrate OR sequences in arthropods have failed.

Nonetheless, novel OR have been elucidated in insect species, such as in the fruit fly *Drosophila melanogaster*, where 60 OR genes have been found on a genomic level (Robertson et al., 2003). These genes encode for a transmembrane-domain receptor family, which is mainly expressed in the neurons of insect olfactory organs, such as the antennae and maxillary palps (Vosshall et al., 1999). Regarding other invertebrates, such as crustaceans, no evidence has been found for OR genes orthologous to those receptors described in insects (Corey et al., 2013). However, diverse receptors and other genes related to the recognition of semiochemical cues have been identified in the lobster, which are similar to the family of ionotropic receptors (IRs) in other taxa (Hollins et al., 2003). The structural similarity between insect IRs and some described for crustacean ionotropic glutamate receptors (IGluRs) suggests the potential olfactory function of these IGluRs (Benton et al., 2009). A recent study discovered novel IRs in lobster that showed expression patterns related to the olfactory tissues and which were highly similar to the IR25a subunit of *Drosophila* (Corey et al., 2013). This evidence suggests the existence of these novel receptors in a wider range of species among arthropods. Regarding gene expression analyses of IR expression, knowledge how these receptors are modulated in invertebrates is still incomplete. In insects, it is known that IRs have an expression mainly related to antennae and then to olfactory sensory neurons (OSNs), but there are few reports on how its expression is modulated, which is due to the scarcity of loss-of-function or miss expression studies (Rytz et al., 2013). Meanwhile, various IRs have been identified on a transcriptional level in the olfactory organs of crustaceans, such as lobster (Stepanyan et al., 2006).

The main goal of the current study was to characterize novel transcripts involved in odor reception in the ectoparasite *C. rogercresseyi*. Four novel transcript related to IRs were characterized in this species. Even though the similarities between these transcripts and orthologous sequences from the lobster and insects were not great, conserved domains related to the olfactory reception process were found, and these structures were modeled in three dimensions. The transcripts identified in this study had similarity with the 25a subunit of *Drosophila* IR (IR25a) and with other kainate receptors. Moreover, the identified sequences were associated with conventionally established Gene Ontology (GO) annotation terms such as "signal transducer activity" and "receptor activity". Furthermore, *in silico* and qPCR gene transcription analyses evidenced differential expression patterns during the varied larval and adult stages of development in the salmon louse. The results discussed in this study provide the first insights towards understanding the molecular aspects, on a transcriptomic level, for the host finding process of *C. rogercresseyi* from the olfactory recognition point of view. Given the economic and environmental implications that the existence of this ectoparasite has for Chile, efforts made towards understanding these processes on a molecular level are fundamental towards the improved control of sea lice infections.

2. Materials and methods

2.1. Salmon lice culturing in controlled laboratory conditions

Ovigerous specimens of *C. rogercresseyi* were collected from recently harvested fish in Puerto Montt, Chile. Individuals were transported to the laboratory on ice, their egg strings were then removed and placed in culture buckets supplied with seawater flow at 12 °C with a gentle aeration. Eggs were allowed to hatch and develop until the infectious copepodid stage, at which point they were harvested for RNA extraction and cDNA library construction. The culture procedure was carried out according to Bravo (2010).

2.2. Transcriptome sequencing of C. rogercresseyi

Samples were collected according to the different developmental stages of the salmon louse. The selected stages corresponded to

Table 1
Primers used in this study for qPCR gene transcription analysis.

Primer name	Transcript	Primer sequence	*T* (annealing)	PCR product
Cr_IR-2473_1F	*IR25a*	TTGGGTAGTCTAGAGTCGGC	60 °C	98 bp
Cr_IR-2473_1R		ACAAACTCGATGGCAGTGTT		
Cr_IR-2843_1F	*IR_K2*	TTTCCAAAGGGAGGGCTTTC	60 °C	130 bp
Cr_IR-2843_1R		TTCACTGATAAGTCCTGCGC		
Cr_IR-5740_1F	*IR_K2-like1*	CCATCGAGTACAACGTGGAG	60 °C	110 bp
Cr_IR-5740_1R		GGCCCGAGTATAAGGAGAGT		
Cr_IR-13520_1F	*IR_K2-like2*	TTGTCAAGTTCACAGGAGCC	60 °C	92 bp
Cr_IR-13520_1R		CTCAATGTCCAACGTCGGAT		

four larval stages, which were nauplius I, nauplius II, copepodid (the infective stage), and chalimus (pool of III and IV stages), and to male and female adults. Ten individuals from each stage were collected and pooled separately, with two pools for each stage. Then, total RNA was extracted from pools using the RiboPure™ kit (Ambion, Life Technologies™, USA) according to the manufacturer's instructions. Quantity, purity, and quality of isolated RNA were measured in TapeStation 2200 (Agilent Technologies Inc., Santa Clara, CA, USA) using the R6K Reagent Kit according to the manufacturer's instructions. Subsequently, double-stranded cDNA libraries were constructed using the TruSeq RNA Sample Preparation Kit v2 (Illumina, San Diego, CA, USA). Two biological replicates for each sample pool ($n = 10$) were sequenced by the MiSeq (Illumina) platform using sequenced runs of 2×250 paired-end reads at the Laboratory of Biotechnology and Aquatic Genomics, Interdisciplinary Center for Aquaculture Research (INCAR), University of Concepción, Chile.

2.3. Identification of IRs from RNA-seq data

The data generated through RNA sequencing were processed using the CLC Genomic Workbench software (Version 6.1, CLC Bio, Aarhus, Denmark). Briefly, raw data from each pool were trimmed separately and read sequences were then *de novo* assembled into a single file comprised by the six stages studied using the following settings: mismatch cost = 2, insert cost = 3, minimum contig length = 400, similarity = 0.8, and trimming quality score = 0.05. Subsequently, sequences assembled into contigs were annotated using the tBLASTn algorithm against the non-redundant GenBank database. Additionally, contigs were annotated according to Gene Ontology (GO) categories using the open-source software Blast2GO (Conesa et al., 2005).

From the annotated data, contigs that showed positive BLAST hits with ionotropic receptors were extracted for further analyses. New BLASTn and BLASTx analyses were performed with these contigs against non-redundant GenBank, EMBL, RefSeq, and DBJJ databases to confirm annotation. The similarity threshold for selecting contigs was established with a cutoff *e*-value of 1E-10. From the correctly annotated contigs, the untranslated regions (UTR) and open reading frames (ORF) were identified using the Geneious software (Version 6.5, Biomatters Ltda., Auckland, New Zealand). Nucleotide sequences were translated into protein sequences using the same software. Then, multiple alignments were performed with the *Ionotropic receptor subunit 25a* and *Ionotropic kainate receptors* so as to compare sea louse sequences obtained in this study with other sequences reported in various species.

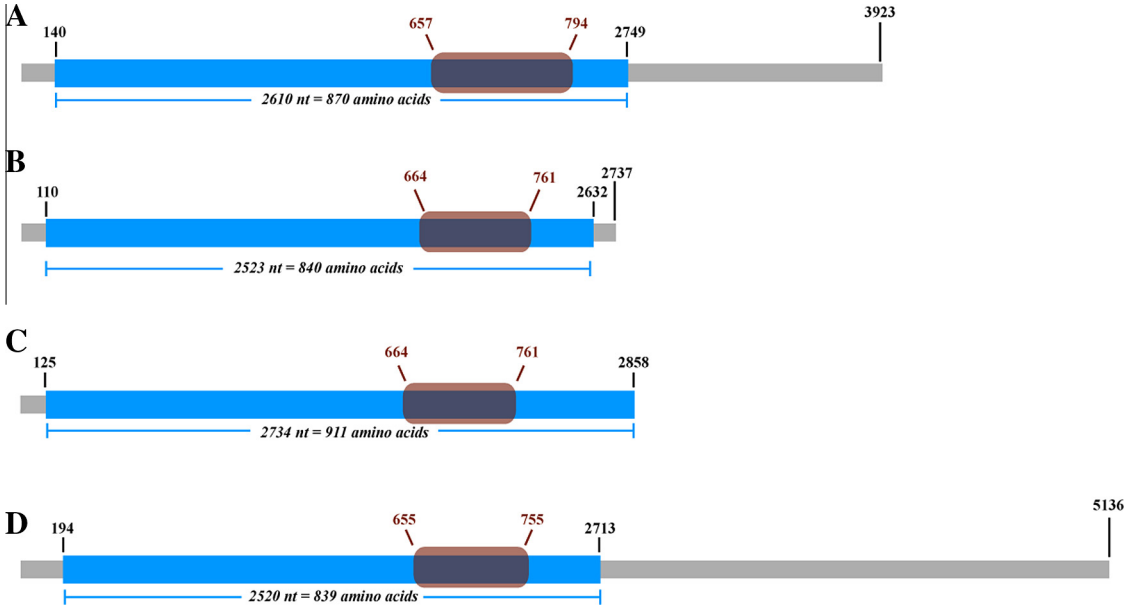

Fig. 1. Schematic structure of ionotropic receptors transcripts identified in *Caligus rogercresseyi*. Gray rectangles correspond to untranslated regions, blue rectangles to coding regions, and brown rounded-borders rectangles to the position of the most representative domain found along the transcript. Black numbers correspond to the start and end position of the coding region, and to the final position of the complete mRNA identified (number of nucleotides). Brown numbers correspond to the start and final position (number of amino acids) of the domains in the coding region. (A) *Ionotropic receptor 25 subunit α*, (B) *ionotropic receptor kainate 2*, (C) *ionotropic receptor kainate 2-like* (1), (D) *ionotropic receptor kainate 2-like* (2). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

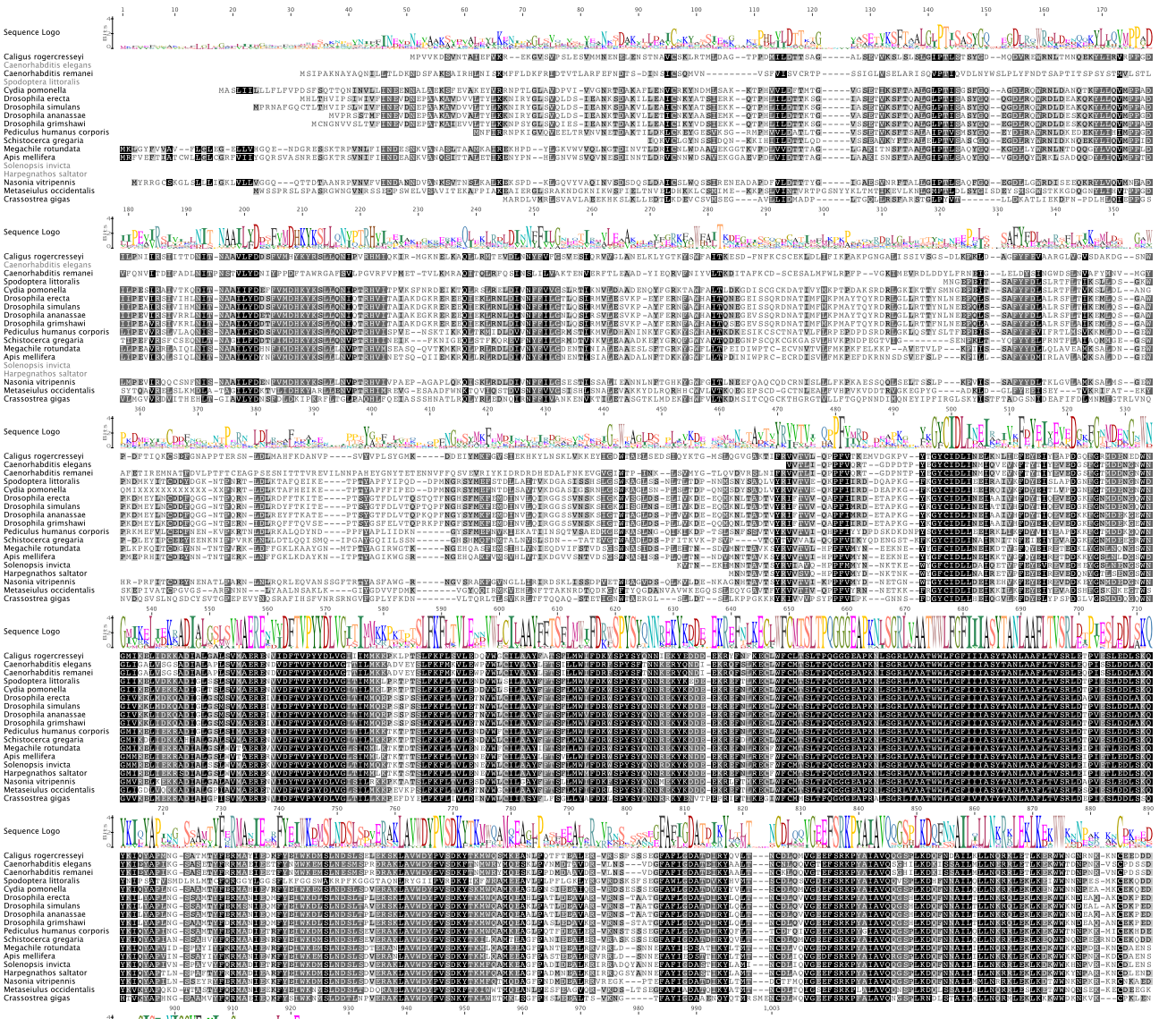

Fig. 2. Multiple alignments of protein sequences of *Ionotropic receptors subunit 25a*. The algorithm used was ClustalW, with a gap on cost equal to 10 and a gap extend cost of 0.1. Genbank accession number of sequences used are AF318611 (*Caenorhabditis elegans*), XP_003109814 (*Caenorhabditis remanei*), ADR46479 (*Spodoptera littoralis*), AFC91757 (*Cydia pomonella*), XP_001968713 (*Drosophila erecta*), XP_002078163 (*Drosophila simulans*), XP_001962492 (*Drosophila ananassae*), XP_001993170 (*Drosophila grimshawi*), XP_002423441 (*Pediculus humanos corporis*), AHA80143 (*Schistocerca gregaria*), XP_003703813 (*Megachile rotundata*), XP_006570896 (*Apis mellifera*), EFZ20266 (*Solenopsis invicta*), EFN76792 (*Harpegnathos saltator*), XP_001603703 (*Nasonia vitripennis*), XP_003743738 (*Metaseiulus occidentalis*), EKC42245 (*Crassostrea gigas*).

2.4. Analyses of IR sequences

Translated sequences were analyzed using different tools from the PSIPRED Server (Buchan et al., 2013). From this server, GO terms related to the molecular function category were found in the four transcripts selected as IRs. Meanwhile, DomSerf analyses were performed to identify the conserved domains of the protein sequences inputted into the server. Domains modeling was carried out based on previously reported CATH domains and by using PSI-BLAST and pDomTHREADER in the PSIPRED server. Additionally, the MEMSAT tool was used to determine the location of peptides in the extracellular, transmembrane, and cytoplasmic regions (Nugent and Jones, 2009).

2.5. In silico gene transcription analysis of IRs

Unassembled sequences from each of the six sequenced stages were used to calculate the relative gene transcription levels of the IRs identified in this transcriptomic analysis. For this, sequences obtained for IRs in previous steps were used as references to map the raw data of each dataset. Each contig annotated as an IR was used in this analysis. Using the CLC Genomic

Table 2
GO terms belonging to molecular function category related to the ionotropic receptors sequences identified in salmon louse.

GO term	Name	IR25a	IR_K2	IR_K2-like1	IR_K2-like2
GO:0004672	Protein kinase activity				X
GO:0004871	Signal transducer activity	X	X	X	X
GO:0004872	Receptor activity	X	X	X	X
GO:0004888	Transmembrane signaling receptor activity		X		X
GO:0005215	Transporter activity	X	X		
GO:0005261	Cation channel activity	X	X		
GO:0005509	Calcium ion binding				X
GO:0005524	ATP binding	X		X	
GO:0015075	Ion transmembrane transporter activity	X	X		X
GO:0015267	Channel activity	X	X		

Workbench software, values for the reads per kilobase per million mapped reads (RPKM) were separately calculated from the mapping sequences obtained for each stage. Calculated data were normalized by quantiles to avoid bias from the sequencing process. To visualize the results, a hierarchical clustering of features was performed for the dataset, and a heatmap was then constructed in order to plot significant differences of gene transcription between the developmental stages of sea lice.

2.6. Validation of gene transcription through quantitative PCR

The four transcripts used for molecular characterization were also used to validate gene transcription data through quantitative PCR (qPCR) analysis. For this, suitable primers for qPCR analysis were designed using the Geneious software (see Table 1 for primer sequences). Then, single stranded cDNA were synthesized from each of the RNA pools used in transcriptomic sequencing with the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Glen Burnie, Maryland, USA). Since qPCR runs require optimal reaction efficiencies to generate reliable values, dynamic ranges were calculated, which consisted in serial dilutions of each cDNA that were then amplified by using primers designed for each transcript. Dynamic ranges were calculated from 80 ng of cDNA and five serial dilutions with a serial factor of 1:5. Each qPCR was carried out in a total volume of 10 μL using the Maxima SYBR Green/ROX qPCR Master mix (Thermo Scientific, USA) following the manufacturer's instructions. Once an efficiency value ranging between 90% and 110% was obtained, the relative abundance of transcripts was calculated using the ΔCt method, with the expression of the β-tubulin gene used as an endogenous control. This housekeeping gene was used since it was characterized as an stable gene in different developmental stages of *C. rogercresseyi* in a previous work (Gallardo-Escárate et al., 2014).

Finally, relative quantification runs were performed using all samples and the same kit mentioned above. Each reaction was performed in a StepOne™ Plus Thermocycler (Applied Biosystems, Life Technologies, San Diego, USA). The conditions used for qPCR runs were as follows: a holding stage of 10 min at 95 °C, followed by 40 cycles of denaturation for 30 s at 95 °C, and annealing/extension for 60 s at 60 °C. After the qPCR cycling stage, a melting curve analysis between 60 and 95 °C was used to visualize the inexistence of unspecific amplifications or primer dimers.

2.7. Gene transcription analysis of IRs in response to the presence of other components in salmon diet

mRNA relative levels of the four transcripts characterized in this study were evaluated in assays where masking compounds and immunostimulants were incorporated in salmon diets. Five experimental groups of *Salmo salar* were acclimated in separate tanks (N = 10 fish per tank) with identical salinity, oxygen concentration, and temperature during 3 weeks. During this period, each group were fed with differential diets: 0% of additive, 1%, 2% or 3% of masking compounds, and 3% of masking compound in combination with an immunostimulant. After the 3 weeks each group were infested with salmon louse in the copepodid stage. 3 days post infestation, copepodid samples were taken to extract RNA and measure the mRNA levels of IRs through qPCR using the same conditions mentioned in point 2.6.

3. Results

3.1. Molecular characterization of IRs in C. rogercresseyi

Nine contigs annotated as ionotropic receptors were found in the transcriptome of *C. rogercresseyi*. However, some contigs had the same annotation and/or redundant residues. Given this, four contigs were finally selected on the basis of length (mRNA completeness) and *e*-value (BLAST hits). One of these transcripts corresponded to the *IR25a*, while the remaining three corresponded to *IRs* (*IR_K2*, *IR_K2-like1*, and *IR_K2-like2*).

Regarding *IR25a*, *C. rogercresseyi* had a transcript 3923 bp in length, where 140 bp corresponded to the 5′UTR region, 2610 bp to the ORF, and 1174 bp to the 3′UTR region. This complete mRNA sequence for *IR25a* was deposited into the GenBank database under the accession number KJ002537. The coding region encoded for a deduced protein sequence of 869 amino acids. Between amino acids 657 and 794, a conserved domain corresponding to the periplasmic binding protein-like II superfamily was found. The obtained *e*-value for this model was 3E-18, and three transmembrane-interacting regions were found in this transcript (Fig. 1 and Fig. S1). Furthermore, multiple alignments of protein sequences showed high levels of conservation tending towards the C-terminal region of proteins, especially in the region composed by the previously mentioned CATH domain (Fig. 2). The molecular features of deduced protein are shown in Supplementary Table 1. The GO terms found for the *IR25a* sequence were associated with channel, transporter, and transmembrane signaling receptor activities (Table 2).

The remaining three genes had BLAST hits with kainate IRs, specifically with the previously described *Ionotropic kainate receptor 2* and *Ionotropic kainate receptor 2-like* sequences. The first transcript, designated *IR_K2*, was composed of 110 bp in the 3′UTR, 2523 bp in the ORF, and 105 bp in the 5′UTR. The complete mRNA length was 2737 nt. The ORF encoded for 840 amino acids, and a domain related to the same protein as that found for *IR25a* was present between amino acids 664 and 761. Besides this, four transmembrane regions were found along the IR_K2 protein sequence (Fig. 1 and Fig. S2). GO terms associated with *IR_K2* were related to the same molecular functions as *IR25a* (Table 2).

The second transcript, *IR_K2-like1*, was partially characterized and showed an mRNA length of 2858 nt. The third transcript was

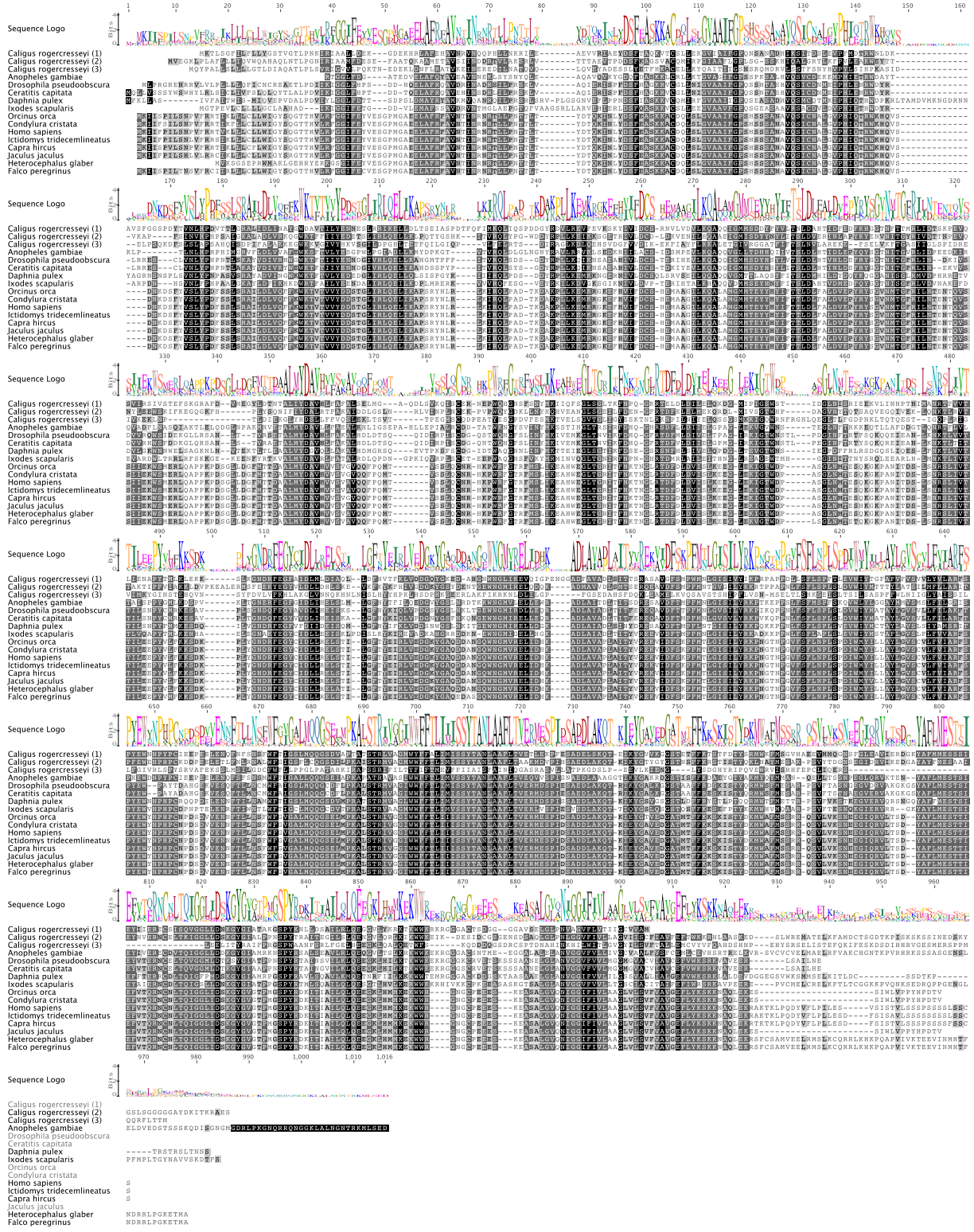

Fig. 3. Multiple alignment of protein sequences of *Ionotropic receptors subunit 25a*. The algorithm used was ClustalW, with a gap on cost equal to 10 and a gap extend cost of 0.1. Genbank accession number of sequences used are XP_559460 (*Anopheles gambiae*), XP_001358619 (*Drosophila pseudoobscura*), XP_004531079 (*Ceratitis capitata*), EFX90374 (*Daphnia pulex*), XP_002410444 (*Ixodes scapularis*), XP_004264828 (*Orcinus orca*), XP_004673787 (*Condylura cristata*), NP_001159719 (*Homo sapiens*), XP_005335944 (*Ictidomys tridecemlineatus*), XP_005684702 (*Capra hircus*), XP_004660659 (*Jaculus jaculus*), XP_004894420 (*Heterocephalus glaber*), XP_005244103 (*Falco peregrinus*).

IR_K2-like2, which had a complete mRNA sequence of 5136 nt. This transcript was composed by 194 bp in the 3′UTR, 2520 bp in the ORF, and a long 5′UTR region with 2423 bp. The ORF encoded for 839 amino acids. Both *IR_K2-like* transcripts presented the same domain as that found in *IR25a*, in addition to three transmembrane regions along the amino acid sequence (Fig. 1, Figs. S3 and S4). Regarding GO terms, *IR_K2-like1* presented only three that were related to the previously described molecular functions, while

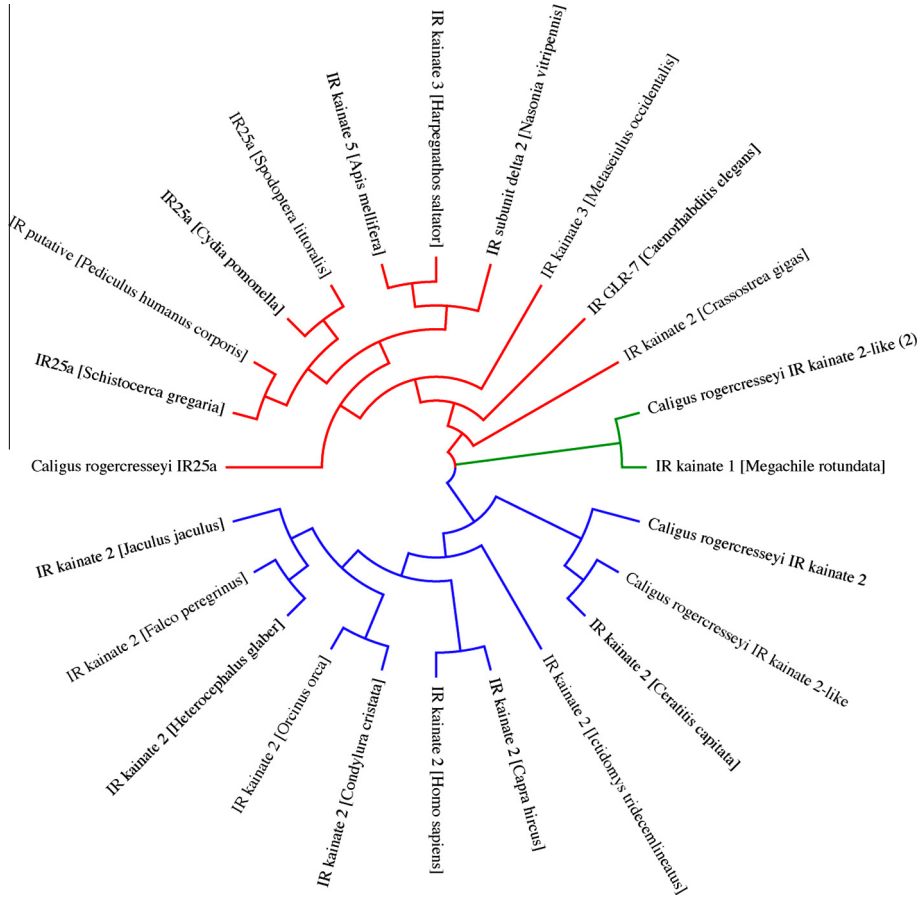

Fig. 4. Genetic distances tree of ionotropic receptors translated sequences of different species based on Jukes–Cantor model and Neighbor-Joining method. No outgroup was used in this analysis.

IR_K2-like2 presented six, including the protein kinase activity category (Table 2).

When comparing these three transcripts with those of other species, the same pattern was observed, where the regions closer to the C-terminal end were the most similar between the different transcripts previously described (Fig. 3). In this case, *IR_K2* was more similar to other *ionotropic kainate receptors* than the *IR_K2-like* transcripts. These three sequences were submitted to the GenBank database (Accession numbers KJ002538 for *IR_K2*, KJ002539 for *IR_K2-like1*, and KJ002540 for *IR_K2-like2*). The relation of *C. rogercresseyi IRs* with this kind of receptors in other species separated the genes in clear clades according to its genetic distances (Fig. 4).

3.2. IR gene transcription analyses in C. rogercresseyi

Gene expression patterns of *IRs* were measured through *in silico* and qPCR analyses among six different stages of the *C. rogercresseyi* life cycle. These stages corresponded to two free-swimming larvae stages (nauplius I and nauplius II), one infective stage (copepodid), one young stage (chalimus), and two adult stages (males and females). For the *in silico* RNA-seq analysis, nine contigs were used. Five of these showed differential expression levels among the sequenced stages (Fig. 5). Four of these contigs had higher expression levels in the earlier stages before showing decreased mRNA levels in adults. Meanwhile, *IR_K2-like2* had the opposite pattern and was more expressed in adults.

For qPCR analysis in different stages of sea lice development, the four characterized transcripts were assayed using specific primers (Fig. 6). In this case, the gene transcription of *IR25a* showed a strong relation to the infective stage (copepodid). However, the *ionotropic kainate receptors* showed a pattern similar to that obtained with RNA-seq analysis, and higher expression values were obtained in male adults. Significant differences were found for the four transcripts ($p < 0.05$).

In relation with the qPCR results in the experiment with differential salmon diets, the combination of 3% masking compound and immunostimulant promoted an overexpression in the four transcripts. Furthermore, the diet with 3% of masking compound stimulate only the expression of *ionotropic kainate 2-like* transcripts, but no significantly differences were found in the other two transcripts. In contrast, 1% of masking compound diet caused a decrease in IRs mRNA relative levels (Fig. 7). Following in vivo challenge experiments all the tested diets with masking compounds showed a decrease of lice infestation up to 25% (Fig. S5).

4. Discussion

4.1. IRs in the salmon louse C. rogercresseyi

The identification of olfactory-related genes in *C. rogercresseyi* was possible through the application of transcriptomic sequencing to the different developmental stages of this species (unpublished data). From this transcriptomic data, various contigs similar to previously reported IRs were found. The present study did search for other transcripts related to the olfactory transduction pathway, such as the odorant receptor and odorant binding proteins reported in insects (Leal, 2013; Zhou et al., 2014), but these type

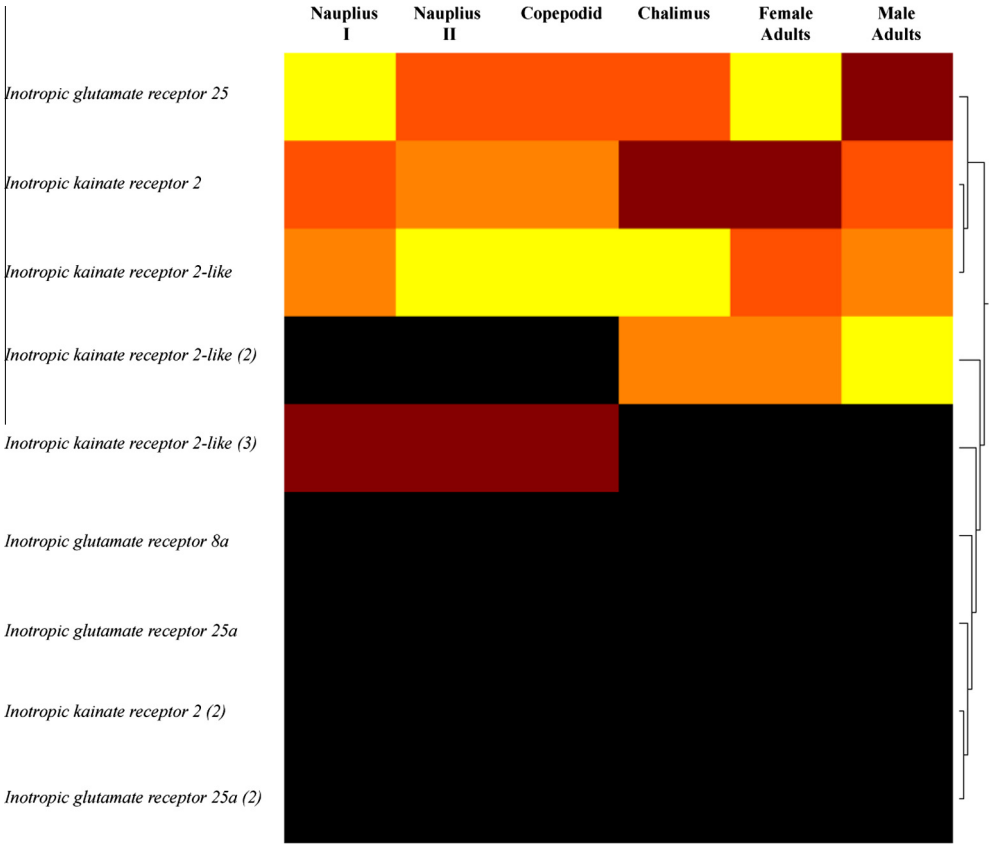

Fig. 5. *In silico* gene transcription analysis of contigs annotated as ionotropic receptors in *C. rogercresseyi*. Hierarchical clustering of RPKM values is shown in a heat-map constructed with normalized values by quantiles. Black: low levels of gene expression, Red: medium levels of gene expression, yellow: high levels of gene expression. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of genes were not found in the salmon louse transcriptome. Given this result, efforts were redirected towards finding and characterizing IRs in this species, especially as previous works have described the implications of these genes in the olfactory transduction of crustaceans (Corey et al., 2013). However, not all of the contigs found with positive BLAST hits for IRs exhibited the high quality read levels needed in the respective assembly, and/or these did not show differential expression levels in the different lifecycle stages of salmon lice (Fig. 5). Following these observations, only four transcripts were characterized on the basis of better coverage, *e*-values, and differential expression levels. We understand that the low expression levels of other transcripts do not indicate absence of those receptors, but we think other approaches have to be applied to evidence their relation with the host-finding process at a biological level. Therefore, we suggest that other approaches, for instance some gene-silencing techniques could be applied to establish some relation between molecular and functional processes.

Before the publication of the present study, no complete mRNA sequences for IRs or other kind of olfactory receptors had been characterized in sea lice. Public databases only offered a set of partial EST sequences for *Lepeophtheirus salmonis*, but these sequences were not directly labeled as IRs. Instead, the authors of this previous study named these sequences "Isal-pac," and the features claimed that Isal-pac was similar to glutamate receptor, kainates. Due to the scarcity of IRs sequences in species genetically close to the salmon louse, the IR sequences found in the present study had to be confirmed through molecular alignments with a wide array of species, through the identification of GO terms, and, mainly, through the identification of domains in the deduced protein. From this, domains related to the periplasmic binding protein-like II superfamily were found in the binding cores of two IGluRs, which provides the first evidence for the selective mechanisms of ionotropic kainate receptors (Mayer, 2005). In this context, the GO terms identified in Table 2, suggest functions related to ionotropic receptor, or even other kind of receptors that could be implicated in the olfactory signaling pathway. In specific we found GO terms associated with ionotropic receptors such as "receptor activity" and "signal transducer activity".

As was previously mentioned, there are only few efforts to evaluate gene transcription of IRs. In fact, the present work represents the first effort to evaluate on a transcriptional level the expression of IRs throughout the developmental stages of any crustacean. An interesting expression pattern was shown for the *IR25a* transcript of *C. rogercresseyi*, which was strongly associated with the infectious stage of development (Fig. 6). Since the parasitic stage of the sea louse begins at and the host is recognized during this stage, future studies should recognize the importance of understanding, on a molecular level, the transcripts which are upregulated during this process. Nevertheless, new techniques have to be applied to the understanding of the expression patterns of IRs in *C. rogercresseyi*. Firstly, because (discrepancies were obtained) in the expression patterns of transcripts measured by RNA-seq and qPCR runs, and also because transcriptional patterns do not show what is happening at translational level. Regarding the first issue, the technical discrepancies in the patterns of some transcripts could be based on the quantification process of different approaches. RNA-seq analysis is a direct quantification of mRNA abundance, while the quantification method used in this work by qPCR implied a relative quantification of transcripts abundance. In relation with the

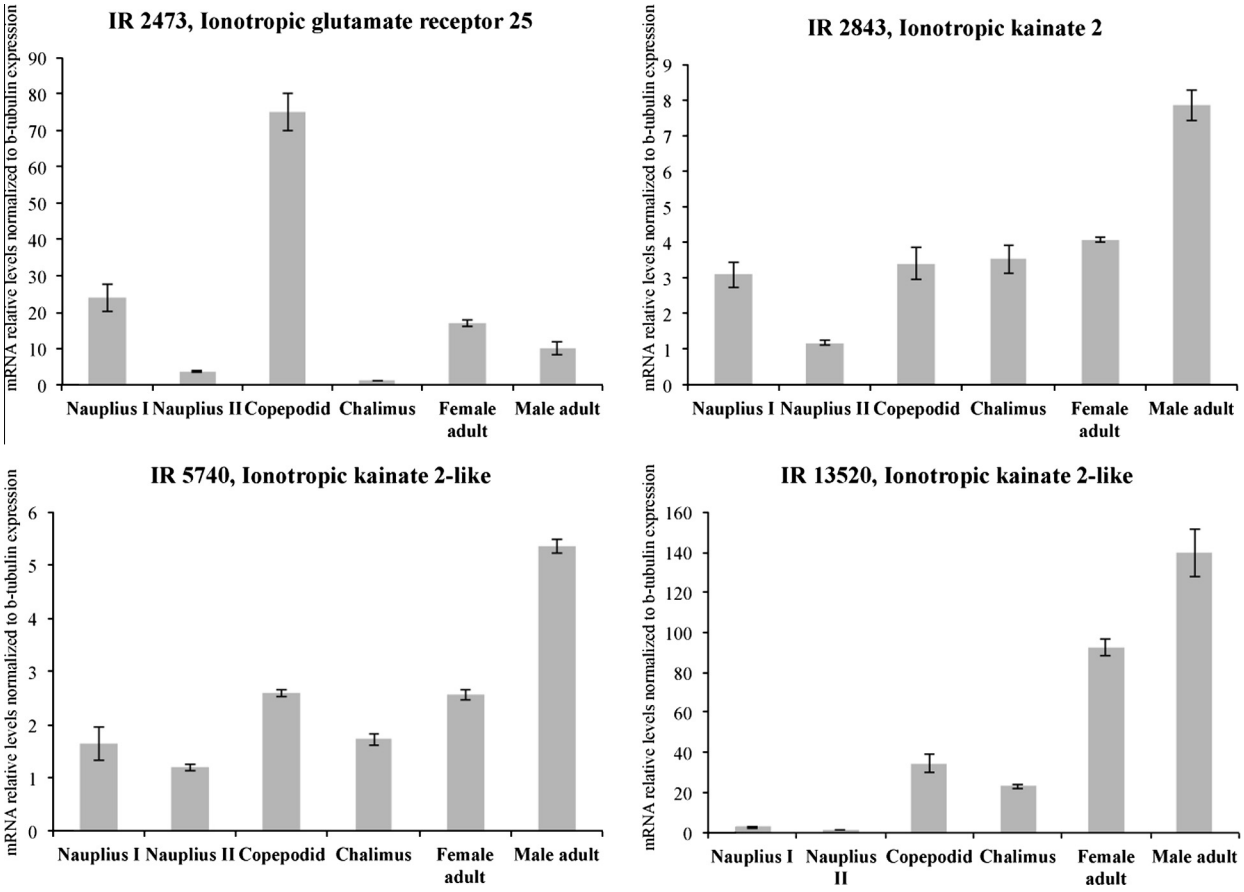

Fig. 6. Gene transcription analysis evaluated by qPCR from ionotropic receptors in different developmental stages of sea lice, *C. rogercresseyi*. Data were normalized by the expression of the endogenous control *b-tubulin*.

second issue, only the regulation of these genes at transcriptional level was measured, and then further evidences to confirm that these patterns are really happening at translational level are needed. Although all of this, we suggest that presenting differentially expressed ionotropic receptors transcripts in different stages of sea lice, due to its functional implications that could have, is a valuable contribution to the current knowledge of molecular mechanisms that salmon louse applied to find the hosts. But additionally to this we propose, in a second stage of this work, to evaluate the translational regulation of these receptors to confirm their expression level and to have all the evidences necessary to establish direct relations between molecular functions and host-finding mechanisms of this species. Currently we are developing gene-silencing methods to infer these relations, and we hope to publish these evidences soon.

Regarding the expression against the presence of diet additives, this is the first time that *ionotropic receptors* are evaluated in the presence of masking compounds and/or immunostimulant, therefore our results are considered as valuate data in order to contribute to the understanding of the biological function of these kind of receptors. Nonetheless, as is the first time that this evaluation is performed, other experiences to share or compare the results obtained in this work remain unknown. Although this fact, we propose that the overexpression of transcripts in presence of 3% masking compounds (in the case of *Ionotropic kainate 2-like receptor*) and the combination of 3% masking compound and immunostimulant (in all the transcript assayed), suggest a relation between these novel receptors in sea lice and olfactory mechanisms. Novel evaluations are needed to correlate this mechanism, but these results give the first hints to select these transcripts as potential candidates for further researches in this context.

4.2. Implications on the molecular study of the olfactory system in sea lice

Not only are economic and environmental problems implicated in salmon lice infection, but a resistance in this ectoparasite to chemotherapy drugs is becoming a concerning issue (Denholm et al., 2002). Some authors suggest "push–pull" trap strategies through the use of varied semiochemicals as a method for controlling sea lice infection. This management method is based in the use of semiochemical cues from kairomones, non-host kairomones, and/or pheromones as a way to attract the parasites into traps (Mordue Luntz and Birkett, 2009) or repel the lice from fish through the use of in-feed, host-masking compounds. When integrated within an integrated pest management programme diets containing host masking-compounds could make an important contribution to sea lice control.

While one of the main focuses of research for the authors of the present study is to understand the transcriptomic basis for host recognition in the ectoparasite *C. rogercresseyi*, it is understood that characterizing transcripts associated with the olfactory system and measuring expression patterns will not be enough to solve all of the still unanswered questions. Although this is the first descriptive insight for semiochemical reception in this species, other techniques and trials are currently being implemented with the aim of improving the understanding of this process. For example, molecular probes are being developed that will evaluate through *in situ* hybridization the expression pattern of transcripts involved in the detection of kairomones in different organs/tissues of the parasite. This is a difficult challenging given the small size of the species and the need for a high quantity of biological material from smaller samples. In parallel, new trials are being prepared to

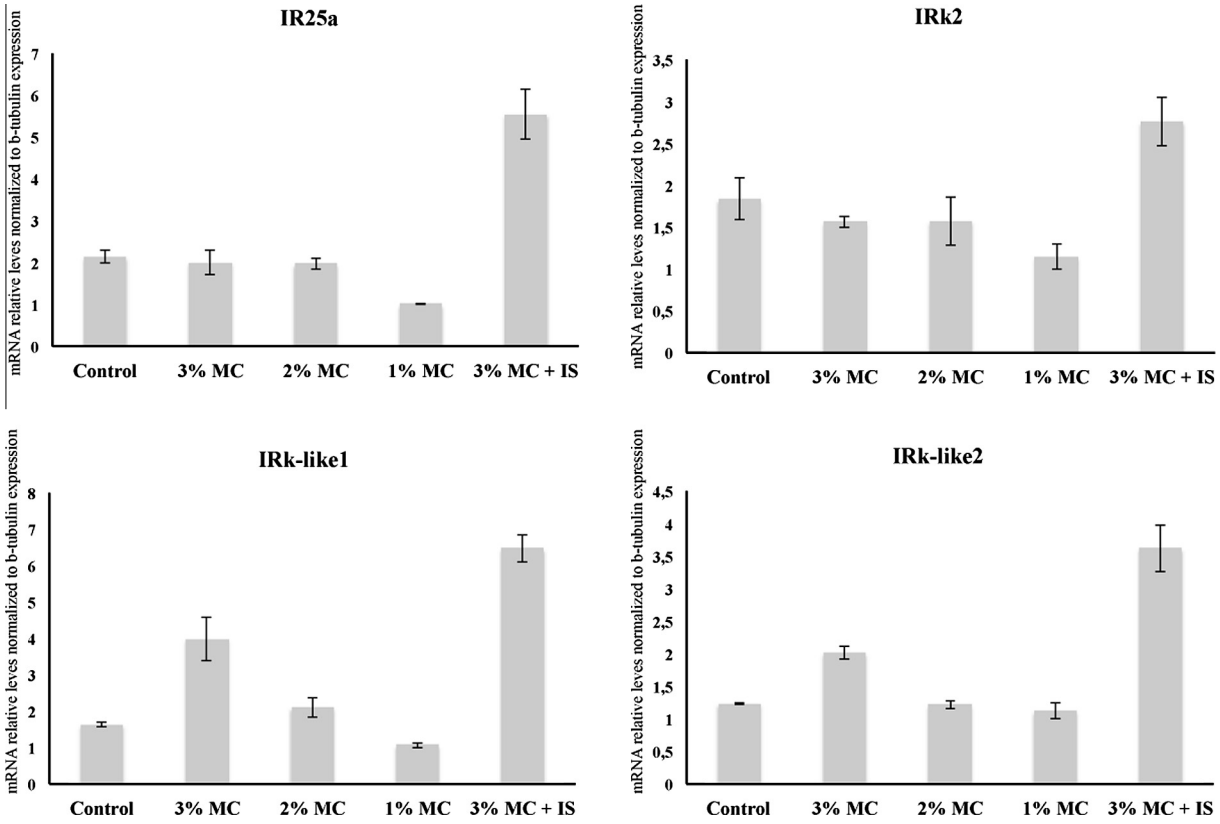

Fig. 7. Gene transcription analyses of ionotropic receptors in response to the presence of masking compounds in salmon diets. The mRNA relative levels (*y* axis) were measured in copepodids infesting salmons fed with diets composed by different concentration of masking compounds and immunostimulant. MC = masking compound, IS: immunostimulant. Data were normalized by the expression of the endogenous control *b-tubulin*.

determine the transcriptomic regions associated with the stimulation of semiochemicals in the larval stages of this species. It is believed that these efforts are necessary to understand the real basis of host recognition in this species. Given the urgent need to control ectoparasite infections, this and similar lines of study should be a priority for scientists working in this field.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.exppara.2014.08.003.

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