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# Sistema quimiosensorial del ectoparásito *Caligus rogercresseyi*: Una aproximación transcriptómica para comprender el proceso de identificación de peces salmónidos hospederos

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*A mi familia*

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Resumen

La comunicación química es un proceso fundamental para las interacciones hospedero-parásito en el medio marino. Especies parásitas del medio marino, como es el piojo de mar *Caligus rogercresseyi*, identifica señales químicas desde el ambiente (semioquímicos), para reconocer hospederos correctos para infectarlos y continuar su ciclo de vida. Sin embargo, los mecanismos moleculares asociados al sistema quimiosensorial del parásito, que favorecen la infección de peces hospederos, permanecen desconocidos. El objetivo de este estudio fue caracterizar las vías de señalización molecular de *C. rogercresseyi* involucradas en el reconocimiento de señales químicas proveniente de peces que puede actuar como atractantes (kairomonas). Para ello, se aplicó evaluaciones moleculares (secuenciación masiva), morfológicas, fisiológicas y conductuales sobre los parásitos, las cuales permitieron describir la relación entre su sistema quimiosensorial y kairomonas producidas por salmones hospedero. Se identificó un conjunto de genes relacionados con el sistema quimiosensorial de *C. rogercresseyi* correspondientes a receptores ionotrópicos y metabotrópicos, altamente relacionados con la etapa infectiva del parásito (copepoditos) mediante secuenciación masiva de todos los estados de desarrollo del parásito. Mediante NGS se determinó que estos receptores modifican sus patrones de expresión en Caligus cuando están en presencia de compuestos enmascarantes de señales químicas. Por otra parte, estos receptores aumentaron sus niveles transcripcionales en presencia de un péptido del pez, cathelicidina-2 (Cath-2), el cual se sugiere como kairomona para el piojo de mar al gatillar respuesta asociada a la atractancia en los parásitos. Finalmente, fue posible silenciar por RNA interferentes la expresión de uno de los receptores quimiosensoriales más importantes del parásito, el receptor ionotrópico 25a (IR25a), inhibiendo el comportamiento de búsqueda del hospedero por parte del piojo de mar. El principal aporte de esta tesis fue caracterizar estructural y funcionalmente el sistema quimiosensorial de *C. rogercresseyi*, e identificar un compuesto clave provenientes del pez que favorecen el reconocimiento de hospedero, como es Cath-2. La investigación aplicada a entender el ciclo de vida del parásito, y particularmente la identificación de peces hospedero en el medio marino a nivel molecular, puede propender al desarrollo de nuevos métodos de control de este parásito, los cuales son necesarios en la acuicultura nacional dada la alta resistencia farmacológica que presenta este patógeno.

Abstract

Chemical communication is an essential process for host-parasites interactions in the marine environment. Ectoparasite species of the marine ecosystem such as the sea lice *Caligus rogercresseyi*, identify chemical cues from the environment (semiochemicals), to recognize proper hosts to infect them and complete their lifecycles. However, the molecular mechanisms that are associated with the chemosensory system of the parasites, which promote host-recognition and infestation remains unknown. The goal of this study was to characterize the molecular pathways involved in chemosensory system in *C. rogercresseyi*, that develop to recognize chemical cues from host fishes (kairomones). Herein, molecular (next-generation sequencing), morphological, physiological and behavioral analyses were conducted on the parasites to establish and describe the relation between its chemosensory system and kairomones produced by salmon hosts. A group of genes related to the chemosensory system of *C. rogercresseyi* corresponding to ionotropic and metabotropic receptors were identified from RNA sequencing of the developmental stages of the parasites. RNA-seq analyses also revealed specific expression patters of chemosensory-related genes in presence of masking compounds (chemical cues blocking compounds), which were added to salmon fed as additives. On the other hand, these receptors were overexpressed after exposure to the salmons' peptide cathelicidin-2 (Cath-2), which is suggested as a kairomone for sea lice due to the attractant behavior of the parasites. It was also conducted gene silencing by RNA interference of one of the most representative chemosensory receptor of sea lice, the ionotropic receptor 25a (IR25a), inhibiting host-seeking behavior in the parasites. The main contribution of this study was the structural and functional characterization of the *C. rogercresseyi* chemosensory system, and also the identification of a novel compound as a key chemical cue with chemoattractant properties that favored host-recognition, which is Cath-2. Research aimed to uncover the mechanisms behind sea lice lifecycle, and particularly host-recognition mechanisms at molecular level, could give the basis for the development of novel control methods for this parasite, which are urgently needed by the national salmon farming industry due to the emerging pharmacological resistance of this pathogen.

# 1. INTRODUCCIÓN

## 1.1. Comunicación química y su rol en el ambiente marino

Las señales químicas o "semioquímicos" han sido cruciales para la evolución de diversas especies de animales en el ecosistema marino (Hay 2009; Lindsey and Lasker 1974; Steiger et al. 2011). Estas señales permiten la comunicación química entre organismos, permitiendo favorecer conductas que son cruciales para el desarrollo de las especies, como lo son la búsqueda de hábitat, reproducción, migración, interacciones predador-presa y parásito-hospedero (Brönmark and Hansson 2012; Paul and Ritson-Williams 2008). Dentro de las especies marinas más estudiadas al respecto se encuentran los copepodos, los cuales se comunican a través de diversas señales químicas intra o inter-específicas (Heuschele and Selander 2014; Preisser et al. 2005). Este grupo de crustáceos acuáticos ha desarrollado diversas estructuras receptoras que permiten identificar diversas señales químicas. Dentro de ellas, las más descritas son las *sensilla*, las cuales son estructuras simples donde las más comunes se conocen como sensilla unimodal (Barrientos 1980; Fields et al. 2007). También existen otras capaces de detectar señales químicas y físicas al mismo tiempo, y se conocen como Sensilla bimodales (Paffenhöfer and Loyd 1999). La evolución de estas estructuras le han permitido a los copepodos, comunicarse mediante el reconocimiento de semioquímicos que entregan distintos tipos de información. En el caso de señales químicas producidas por la misma especie que detecta la señal, o bien feromonas, existen más de 20 especies en las cuáles se han caracterizado distintos tipos de moléculas de esta naturaleza (Yen and Lasley 2010). Estas feromonas pueden permitirle a copepodos machos encontrar a hembras para el apareamiento mediante persecusión de estas señales (Seuront 2013). En otros casos les proporcionan información de características específicas de las hembras, como puede ser por ejemplo la preferencia por hembras vírgenes en algunas especies de copepodos como estrategia reproductiva (Heuschele and Kiørboe 2012). Pero en copepodos marinos, las feromonas no tienen información valiosa sólo para su reproducción, sino que también para otros propósitos como es la identificación de parentesco entre animales de la misma especie (Lazzaretto et al. 1990). En cuanto a la detección de semioquímicos provenientes de otras especies, o bien kairomonas, es mucho menor la cantidad de conocimiento al respecto para

este grupo de especies marinas. Una de las funciones de las kairomonas es prevenir de la presencia de especies predadoras, y aunque no existe conocimiento aun de que moléculas en específico podrían entregar este tipo de información, si se sugiere que kairomonas podrían indicar densidad de predadores en un área determinar a ciertas especies de copepodos (Buskey et al. 2012). Un tipo de conducta que sugiere la presencia de este tipo de kairomonas es el cambio de conducta de nado y pigmentación que realizan algunos copepodos para ocultarse de sus predadores (Hylander et al. 2012). Respecto de otras funciones, en especies parasíticas como el piojo de mar *Lepeophtheirus salmonis* se ha reportado que las kairomonas le permiten identificar hospederos correctos en el medio marino (Mordue Luntz and Birkett 2009).

1.2. Sistemas quimiosensoriales a nivel molecular: receptores olfativos

Las bases moleculares del reconocimiento de semioquímicos del medio ambiente permanece prácticamente desconocido en invertebrados marinos. A nivel general, existe mucha más información respecto a los procesos de reconocimiento de semioquímicos (gustativos y olfativos) en especies de vertebrados que invertebrados. De estos últimos la mayoría de los trabajos han estado enfocados en la especie modelo *Drosophila melanogaster*, u otras similares, pero existen muy pocos estudios enfocados en describir estos procesos en especies de invertebrados que no sean insectos (Touhara and Vosshall 2009). Por otra parte, en vertebrados mamíferos se ha descrito una amplia gama de receptores quimiosensoriales. Dentro de los más relacionados con la detección de semioquímicos existe la familia de los receptores olfativos (OR por su sigla en inglés), los cuáles en mamíferos tienen alrededor de 1500 miembros descritos, mientras que en peces, no alcanzan a ser más de 100 que se han descrito hasta la actualidad (Niimura and Nei 2005). Mientras tanto, los esfuerzos que se han dirigido para identificar secuencias homólogas a estos ORs en especies de artrópodos marinos han fracasado.

Adicionalmente, nuevos receptores olfativos han sido descritos en especies de insectos. En *D. melanogaster*, 60 genes similares a ORs han sido descritos a nivel genómico (Robertson et al. 2003). Estos genes codifican para proteínas que incluyen un dominio transmembrana característico de familias de receptores y que se expresan generalmente en las células neuronales de los órganos olfativos de insectos, tales como las antenas y los palpos maxilares

(Vosshall et al. 1999). En relación con invertebrados marinos, como por ejemplos los crustáceos, no existe evidencia de que se hayan reportado genes ortólogos a los ORs descritos en insectos, y los estudios que han intentado identificarlos no han tenido éxito (Corey et al. 2013). Sin embargo, receptores del tipo ionotrópico han sido identificados en la langosta americana, *Homarus americanus*, los cuales fueron similares a los receptores ionotrópicos de glutamato (IGluRs) en otras especies de invertebrados (Hollins et al. 2003). Estos receptores, llamados simplemente receptores ionotrópicos (IRs), resultaron ser similares estructural y funcionalmente a genes con actividad quimiosensorial en insectos. Como existen evidencias experimentales en las que responden ante la presencia de moléculas descritas como semioquímicos, sugieren un rol en la respuesta olfativa (Benton et al. 2009). Adicionalmente, un estudio reciente sugiere que nuevos IRs encontrados en langosta espinosa, *Panilurus argus*, presenta patrones de expresión relacionados con los tejidos y órganos olfatorio, y los cuales fueron significativamente similares a los correceptores IR25a e IR 8a de *Drosophila* (Corey et al. 2013). En relación con análisis referentes a la modulación de estos genes, aún se desconoce con certeza los mecanismos que regulan los niveles de expresión de IRs, sobretodo en especies que no sean insectos. Sin embargo, los estudios en *Drosophila* sugieren que estos genes están relacionados con el funcionamiento de las neuronas sensibles a olor (OSN), aunque aún no se ha confirmado definitivamente su función debido a la carencia de estudios que bloqueen el gen, o que lo utilicen en modelos *in vitro* e *in vivo* (Rytz et al. 2013). Mientras tanto, en crustáceos se ha detectado que IRs están relacionados, en términos de expresión génica, con los órganos olfativos, especialmente en langostas (Stepanyan et al. 2006).

### 1.3. El ciclo de vida de *Caligus rogercresseyi*: implicancia del reconocimiento de hospedero en el éxito de la infección

El ectoparásito marino conocido como piojo de mar, *Caligus rogercresseyi*, se ha convertido en una de las principales causas de pérdidas económicas en la industria productora de salmones en Chile, producto de la enfermedad conocida como caligidosis. Se reporta que al año 2009 a nivel mundial existieron pérdidas de alrededor de 180 millones de dólares, de los cuáles el 90% correspondía a los costos asociados a las estrategias de control de la enfermedad

(Costello 2009). Sin embargo, esta cifra ha sido corregida el año 2017 a 463 millones de dólares anuales (Abolofia et al. 2017).

El ciclo de vida de *C. rogercresseyi*, comprende 3 etapas larvales (naupilus I, naupilus II y copepodito), 4 etapas juveniles (chalimus I – IV) y una etapa adulta (macho o hembra). Dentro de las etapas de desarrollo larval ocurre el proceso de identificación del hospedero propicio para la infestación y el asentamiento en la etapa infectiva (copepodito); luego desde esta etapa hasta el desarrollo a juveniles existe la respuesta a los mecanismos de defensa de los hospederos, y la alimentación del mucus del pez; para luego en la etapa adulta reproducirse (González and Carvajal 2003). Estudios previos han mostrado que especies de piojo, como por ejemplo *Lepeophtheirus salmonis* que habita en peces del hemisferio norte, han desarrollado mecanismos físicos de reconocimiento del hospedero. Entre estos identificar la temperatura del agua a la que habitan las especies de peces blanco, identificar los cambios en la salinidad del medio marino y detectar las pequeñas variaciones en las corrientes generadas por el nado de los peces (Genna et al. 2005; Heuch et al. 2007; Heuschele and Selander 2014). Por otra parte, también se han descrito mecanismos de identificación de hospedero por detección de semioquímicos (Bailey et al. 2006; Hull et al. 1998). Se ha descrito la presencia de quimiorreceptores avanzados en el piojo de mar *L. salmonis*, que son capaces de identificar moléculas específicas de distintas especies de hospedero (Fields et al. 2007; Genna et al. 2005; Hull et al. 1998; Mordue Luntz and Birkett 2009). Los principales receptores quimiosensoriales descritos en la especie de piojo de mar del hemisferio norte han sido encontradas en las anténulas (ubicadas en la cabeza del parásito). La actividad neuronal de las anténulas aumenta, según lo descrito por ensayos electrofisiológicos, ante la presencia de agua acondicionada de salmones de distintos hospederos (Fields et al. 2007; Ingvarsdóttir et al. 2002a). Por otra parte, existe evidencias de que la remoción de las anténulas de esta misma especie por ablación genera una reducción en la capacidad de encontrar el hospedero por parte de los machos de piojo de mar, aunque luego el parásito es capaz de recuperarla y seguir identificando semioquímicos al estar en contacto con el hospedero (Hull et al. 1998). En cuanto a la especie que afecta la industria chilena, *C. rogercresseyi*, se ha descrito que existe un comportamiento de preferencia a aguas acondicionadas con peces salmónidos, lo cual sugiere que también existe un reconocimiento de naturaleza química para la infestación

exitosa por parte del parásito (Pino-Marambio et al. 2007). Sin embargo, las moléculas producidas por los salmones, que pueden ser detectados por *Caligus rogercresseyi* (actuando como kairomonas), permanecían desconocidas hasta la realización del presente estudio. Una aproximación son los compuestos apolares de bajo peso molecular isoforona y 6-metil-5-hepten-2-ona, producidos por salmón del atlántico que generan atractancia sobre *Lepeophtheirus salmonis* (Bailey et al. 2006). Sin embargo, dado el aumento de recursos genómicos para salmón del atlántico, se sospecha que en la actualidad se podría identificar mayor cantidad de moléculas con alta especificidad que podrían funcionar como kairomonas sobre *Caligus rogercresseyi*. En tal caso, se debería enfocar el estudio a compuestos de naturaleza peptídica que tengan las propiedades químicas necesarias para ser identificadas como kairomonas por el parásito.

### 1.4. Péptidos producidos por peces salmónidos: péptidos antimicrobianos

Los péptidos antimicrobianos (AMP) están dentro de los mecanismos más estudiados de defensa contra patógenos (Bulet et al. 2004). Los peces producen diversos tipos de AMP, incluyendo defensinas y catelicidinas, además de otros específicos para el grupo como son las piscidinas (Masso-Silva and Diamond 2014). En cuanto a peces salmónidos, éstos producen AMPs como los mencionados anteriormente, así como también hepcidinas y AMPs derivados de histonas. Todos estos forman en conjunto una de las bases principales de los mecanismos de defensa contra patógenos (Valero et al. 2013). De estos AMPs, el grupo de las catelicidinas podrían estar involucradas también en la defensa a largo plazo, como se ha descrito en truchas (Casadei et al. 2015). Respecto de este grupo, las catelicidinas han sido ampliamente estudiadas en diversas especies de vertebrados, pero los peces salmónidos tienen características propias a nivel estructural, lo que le pude conferir nuevas funciones (Zhang et al. 2015). Esta clase de AMP está altamente asociada a la respuesta de salmón del Atlántico a infecciones bacterianas como la yersiniosis (Bridle et al. 2011). Las catelicidinas también se encontraron entre los genes que se activaron en salmones infectados con el ectoparásito piojo de mar *Lepeophtheirus salmonis*, mientras que otros AMPs como defensinas decrecieron sus niveles de expresión (Krasnov et al. 2012). Sin embargo, aún se desconoce cuál es el efecto

que estos AMPs específicos de salmónidos pueden tener sobre patógenos como el piojo de mar.

### 1.5. Impacto del presente estudio en el desarrollo de la acuicultura nacional

A partir de la última década, Chile se ha convertido en uno de los líderes mundiales de la producción de salmones en cultivo, siendo el segundo mayor productor sólo detrás de Noruega. Sin embargo, nuestra industria salmonera depende casi exclusivamente de la producción de salmón del Atlántico *Salmo salar* (Niklitschek et al. 2013). La industria acuícola nacional ha sido fuertemente impactada por el parásito conocido como piojo de mar, el cual a nivel mundial impacta salmones en cultivo y poblaciones naturales de otros peces endémicos (Torrissen et al. 2013). En Chile, son grandes las pérdidas económicas, y el impacto ambiental asociado a la presencia del piojo de mar *C. rogercresseyi*, lo cual incluso afecta la opinión pública que se tiene respecto de la acuicultura (Costello 2009; Guo and Woo 2009). La mayoría de los costos asociados al control del piojo de mar, y una de las principales causas de su impacto global, es debido a que se utiliza principalmente drogas antiparasitarias para tratar las infestaciones ocasionadas por este parásito, los cuales atacan procesos clave del parásito como son la muda y su sistema nervioso central (Burridge et al. 2010). Estos tratamientos antiparasitarios han comenzado a perder efectividad en la industria nacional, emergiendo resistencia por parte del parásito (Bravo et al. 2010). Otro tratamiento que se ha probado consiste en la aplicación de prototipos de vacuna (Carpio et al. 2011; Ross et al. 2012), los cuales no han tenido suficiente reproducibilidad entre tratamientos, por lo que no se asegura su escalamiento a nivel productivo. Adicionalmente se han probado aditivos alimenticios en dietas de salmones en cultivo, los cuales tienen propiedades antiparasitarias mediante inmunoestimulación del hospedero (Núñez-Acuña et al. 2015; Poley et al. 2013). Sin embargo, este tipo de tratamiento aún está en desarrollo debido a la constante búsqueda de nuevos compuestos con las propiedades deseadas. Una nueva alternativa propuesta se basa en el desarrollo de trampas de parásitos utilizando compuestos atractantes de piojos de mar y así evitar o dificultar el proceso de identificación de hospedero, lo cual es viable dado que se conoce que este copépodo tiene comportamiento de atractancia a ciertas señales químicas provenientes de peces hospedero (Mordue Luntz and Birkett 2009). No obstante, el uso de

compuestos enmascarantes, semioquímicos que enmascaran las propiedades organolépticas de otros repeliendo patógenos y pestes, en diversas industrias alimenticias (incluida la piscícola), sugiere que podría utilizarse en la salmonicultura soluciones basadas en este tipo de compuestos (Cook et al. 2007; Horowitz and Ishaaya 2016). Sin embargo, se requiere de conocimiento profundo respecto de los mecanismos moleculares en los que se basa el reconocimiento de hospedero en *C. rogercresseyi*, previo a su aplicación en tratamientos preventivos.

Este estudio se basa en la identificación de aquellas moléculas clave en el proceso de identificación de hospedero por parte del piojo de mar *C. rogercresseyi*. Esto implica tanto los receptores que el parásito posee para identificar semioquímicos en el ambiente, como también que kairomonas específicas de salmones logra detectar para localizar al hospedero. Eso último no es sencillo, dada la escaza información que existe de potenciales moléculas que podrían estar actuando como kairomonas en el medio marino. Una posibilidad está en la aplicación de nuevas herramientas genómicas para identificar transcritos asociados a la infección en órganos target de estas infecciones parásitas. Existen estudios describiendo los péptidos más abundantes en las zonas de la piel de peces que están afectadas por infestaciones de *Caligus*, encontrando un amplio reportorio de péptidos relacionados con la zona de contacto entre el parásito y el hospedero (Tasumi et al. 2015). Dentro de este repertorio se ha identificado que la mayoría de los transcritos están asociados con la degradación de moléculas estructurales de las membranas celulares como por ejemplo colágeno, colagenasas, metaloproteínasas y fibronectina. No obstante, recientes estudios también han mostrado significativos incrementos en la producción de péptidos antimicrobianos (AMPs) en la piel de peces afectados por diversos patógenos, incluyendo el piojo de mar (Bridle et al. 2011; Gomez et al. 2013; Holm et al. 2015). Cabe señalar que múltiples evidencias sugieren que los AMPs tienen una amplia gama de funciones en la célula, que van desde el ataque directo a patógenos a la modulación de la respuesta inmune innata y de otras señales celulares (Rajanbabu and Chen 2011). La amplia gama de funciones que poseen los receptores quimiosensoriales y que los AMPs desempeñan en especies parásitas y hospederas, y la posibilidad de interacción inter-específica que puede existir entre estas moléculas, permiten hipotetizar que péptidos pequeños del tipo AMP provenientes del hospedero podrían actuar como kairomonas (Masso-Silva and Diamond

2014). Adicionalmente, se ha descrito que receptores de membrana acoplados a proteínas G (GPCR), como por ejemplo IGluRs y otros receptores de glutamato como los receptores metabotrópicos (mGluRs), son también capaces de reconocer señales de naturaleza peptídica como neuropéptidos y otros péptidos pequeños (Grunder and Assmann 2015; Pomierny-Chamioło et al. 2014). A su vez estos receptores son importantes elementos de vías de señalización sináptica de células con función nerviosa (Huang and Thathiah 2015), lo que sugiere un rol adicional a la identificación de semioquímicos, y que estaría asociado con la transmisión de señales celulares para gatillar diferentes repuestas a nivel celular frente a un determinado estímulo. La evaluación de la interacción entre AMPs y receptores quimiosensoriales en piojo de mar podría entonces explicar con bases moleculares el proceso de reconocimiento de hospedero por parte de este parásito.

## 2. HIPÓTESIS Y OBJETIVOS ESPECÍFICOS

### 2.1. Hipótesis

El reconocimiento de peces hospederos por parte de *Caligus rogercresseyi* es mediado a nivel molecular por receptores especializados, los cuales pueden ser modulados por moléculas específicas producidas por la especie hospedadora, así como por compuestos enmascarantes de señales semioquímicas.

### 2.2. Objetivos específicos

Objetivo 1. Identificar transcritos que codifiquen para receptores ionotrópicos, metabotrópicos y sus cascadas de señales en *Caligus rogercresseyi*.

Objetivo 2. Determinar el efecto que tienen moléculas que enmascaran semioquímicos sobre el funcionamiento del sistema quimiosensorial de *C. rogercresseyi*.

Objetivo 3. Evaluar si los péptidos antimicrobianos producidos por peces hospederos son capaces de activar el sistema quimiosensorial del piojo de mar.

Objetivo 4. Modular la interacción de reconocimiento del hospedero a través de interferencia a nivel transcripcional de genes claves del sistema quimiosensorial del piojo de mar.

## 3. MATERIAL Y MÉTODOS

Objetivo específico 1. Identificación de receptores quimiosensoriales en *Caligus rogercresseyi*

A partir de una librería transcriptómica realizada mediante secuenciación masiva (Illumina MiSeq) en el piojo de mar *Caligus rogercresseyi*, se identificaron transcritos relacionados con el sistema quimiosensorial de la especie. Esta librería consiste en secuenciación de RNA de todos los estados de desarrollo de la especie parásita: nauplius I y II, copepodito, chalimus I-IV y adultos de ambos sexos (Gallardo-Escárate et al. 2014).

A partir de secuencias disponibles en bases de datos públicas (NCBI-Genbank, EMBL, DDBJ), se construyó una base de datos con secuencias transcriptómicas relacionadas con el sistema quimiosensorial de artrópodos. Las secuencias de esta base de datos específica correspondieron a genes que, según literatura, están relacionados con el sistema quimiosensorial en artrópodos, tales como receptores olfativos y gustativos, receptores ionotrópicos, y otros receptores de membrana acoplados a proteínas G (GPCR). Mediante análisis BLASTs, se comparó las secuencias de esta base de datos con los transcriptomas de *C. rogercresseyi*. El criterio de selección para filtrar secuencias correctamente anotadas fue E-value < 1E-5.

Aquellas secuencias que fueron correctamente identificadas fueron utilizadas para análisis de expresión transcripcional mediante métodos *in silico* y por qPCR. Para los análisis *in silico*, se utilizó los datos transcriptómicos de los estados de desarrollo de *C. rogercresseyi* y se calculó los valores de RPKM con parámetros por defecto en el programa CLC Genomic Workbench (CLCBio v.9.0, Dinamarca). Mientras que para la validación por qPCR se utilizó muestras de ARN obtenidas a partir de los estados de desarrollo de la especie para realizar cDNA y amplificar utilizando primers específicos para cada gen. Las corridas de qPCR se realizaron en un equipo StepOnePlus (Applied Biosystems, Life Technologies, EEUU). Para ver detalle de estos procedimientos ver metodología en el Capítulo I.

Se construyó una base de datos específica para rutas genéticas del sistema quimiosensorial y neurotransmisión de artrópodos, a partir de rutas descritas en KEGG (Kyoto Encyclopedia of Genes and Genomes): transducción olfativa, sinapsis glutamatérgica, sinapsis gabaérgica, entre otras. Todos los genes involucrados en estas rutas fueron buscados en bases de datos públicas como Genbannk-NCBI, EMBL y DDBJ, específicamente para artrópodos. El conjunto de genes identificados fue comparado con dos bases de datos transcriptómicas para ser identificados en *C. rogercresseyi*: secuenciación masiva de RNA de ontogenia para la especie (Gallardo-Escárate et al. 2014) y de piojos expuestos a drogas antiparasitarias (Chávez-Mardones and Gallardo-Escárate 2015; Valenzuela-Muñoz et al. 2015). La identificación de estos genes en *Caligus rogercresseyi*, así como también los análisis de expresión génica fueron realizados de la misma manera señalada anteriormente. Para mayores detalles de la metodología de esta sección referirse al Capítulo II.

Objetivo específico 2. Evaluación del efecto de compuestos enmascarantes sobre el sistema quimiosensorial de *C. rogercresseyi*

Grupos de 60 peces de *Salmo salar* y *Oncorhynchus kisutch*, fueron divididos en tanques para evaluar su respuesta a dietas suplementas con compuestos enmascarantes (Fig. 1). Los grupos fueron separados según dieta suministrada: dieta comercial (control) o dieta suplementada con esmascarantes. A los 21 días se realizaron infecciones con 35 copepoditos por pez en cada grupo (por triplicado). Los parásitos fueron removidos de los peces, de ambos hospederos a los 1, 3, 7 y 14 días después de la infección. Las muestras obtenidas fueron fijadas en solución de fijación RNA Later (Ambion, USA), para posterior extracción de RNA y análisis de expresión génica de receptores quimiosensoriales utilizando la misma metodología expuesta en el punto anterior. Mayores detalles de esta sección en el Capítulo III.

Objetivo específico 3. Efecto de péptidos antimicrobianos sobre el desarrollo de filamento frontal del piojo de mar

El filamento frontal es una estructura que desarrollan las etapas infectivas del piojo de mar *C. rogercresseyi* para poder adherirse a la piel de peces hospedero (Pike and Wadsworth 1999). Se evaluó el desarrollo de este filamento en función de la presencia de compuestos sugeridos como atractantes para el parásito, que provengan del propio hospedero (kairomona). En este estudio, se evaluó si péptidos antimicrobianos (AMPs) expresados por salmones hospederos podrían actuar como kairomonas, bajo el supuesto que son péptidos pequeños que son capaces de expresarse en altas cantidades en la piel de salmónidos durante enfermedades infecciosas (Casadei et al. 2015; Valero et al. 2013). Los niveles de expresión *in silico* de AMPs se midieron en la piel de salmónidos, después de infección por *C. rogercresseyi*. Aquellos AMPs con mayor expresión de estos genes correspondió a dos péptidos de la familia cathelicidin: Cath-1 y Cath-2, los cuales fueron sintetizados químicamente y utilizados para las siguientes evaluaciones.

Grupos de 100 copepoditos fueron expuestos a 7 ppb de ambos péptidos en 1 L de agua de mar en un sistema de cultivo artificial que incluyó una matriz de agar + mucus de peces que sirvieron para que los parásitos se adhirieran a ella. Aquellos copepoditos que se adhirieron después de 48 h fueron extraídos y se observó el estado de desarrollo de su filamento frontal bajo microscopio, así como también se realizó extracciones de ARN a partir de ellos para medir niveles de expresión génica de receptores quimiosensoriales tal como la metodología del objetivo específico 1. Detalles de los métodos utilizados tanto para medir los niveles de expresión de AMPs en salmones, como para el ensayo de desarrollo del filamento frontal están disponibles en el Capítulo IV.

De los resultados a partir de la metodología descrita en el párrafo anterior, el péptido antimicrobiano que promovió el desarrollo del filamento frontal, y gatilló una sobre-expresión de genes quimiosensoriales, fue cathelicidina-2 (Cath-2). Por esta razón, este péptido se utilizó para evaluarlo como potencial kairomona para el piojo de mar. Mediciones neurofisiológicas fueron realizadas en anténulas de estos parásitos en presencia de Cath-2 disuelto en agua de mar en distintas concentraciones: 0,7; 7; 70 y 700 ppb, siguiendo una metodología descrita para el piojo de mar *Lepeophtheirus salmonis* (Fields et al. 2007). Actividad neuronal de los piojos expuestos al péptido fue medida directamente de quimio-receptores analizando la forma

de las longitudes de onda detectadas: picos de actividad, amplitud, frecuencia. Estos valores fueron contrastados con piojos expuestos sólo con agua de mar, utilizados como línea base de actividad neural.

Piojos de mar en estado copepoditos (N = 150) fueron expuestos Cath-2 disuelto en agua de mar en las mismas concentraciones señaladas en acuarios de 2 L de agua de mar. Se utilizaron análisis conductuales, específicamente *silhouette video photography* (SVP), para identificar respuestas asociadas al reconocimiento de hospedero en los parásitos (Fields et al. 2017). Los parásitos fueron estimulados con una combinación de estímulos químicos (Cath-2) y físicos (luz producida por lámpara de Xenon arc de 1000 W). Las respuestas medidas fueron: cantidad de copepoditos que nadaron hacia la señal, velocidad de nado después de la estimulación y tiempo que demoraron los copepoditos en responder. Adicionalmente, se evaluó la trayectoria de los copepoditos durante el nado en respuesta del estímulo, mediante el test variance ratio, con la finalidad de evaluar su direccionalidad (Charles and Darné 2009). Mayor detalle de los análisis de neurofisiología, como también de los conductuales de nado, se encuentra en el Capítulo V.

Objetivo específico 4. Modulación del sistema quimiosensorial del piojo de mar mediante silenciamiento de la expresión génica (RNAi)

Un oligonucleótido de 678 pb de tamaño, fue utilizado para silenciar la expresión del gen *IR25a* en piojo de mar, utilizando el kit MEGAscript™ T7 transcription (Ambion, USA). Los tratamientos de silenciamiento del gen por RNA interferente (RNAi) fueron realizados en estados larvales del parásito (nauplius I – II), siguiendo el protocolo descrito por Eichner et al. (2014), con modificaciones. Brevemente, se agregó un oligo RNAi del gen no relacionado *GFP* como control, y un último grupo control negativo (sin aplicación de ningún oligo) también fue agregado. El tiempo de exposición a los oligos fue de 16 h, en pequeñas cámaras con 25 animales en una concentración 20 ng/µL. Para confirmar la eficiencia de los tratamientos se midió los niveles de expresión génica de *IR25a* por RT-qPCR. Para mayores detalles del silenciamiento de este gen referirse a la metodología expuesta en el capítulo VI de la sección resultados.

Los animales tratados con RNAi fueron mantenidos en sistema de cultivo hasta que llegaran al estado copepodito. El efecto del silenciamiento de este gen fue evaluado mediante análisis de conducta de nado, con una combinación de estímulos físicos (luz) y químicos (Cath-2), utilizando la misma metodología aplicada en la sección anterior.

## 4. RESULTADOS

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

*Experimental Parasitology,* 145: 99-109, 2014.

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

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

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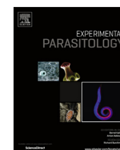

# Insights into the olfactory system of the ectoparasite *Caligus rogercresseyi*: Molecular characterization and gene transcription analysis of novel ionotropic receptors

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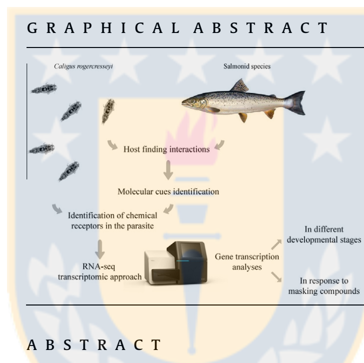
## HIGHLIGHTS

- 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 $2 \times 250$ 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.

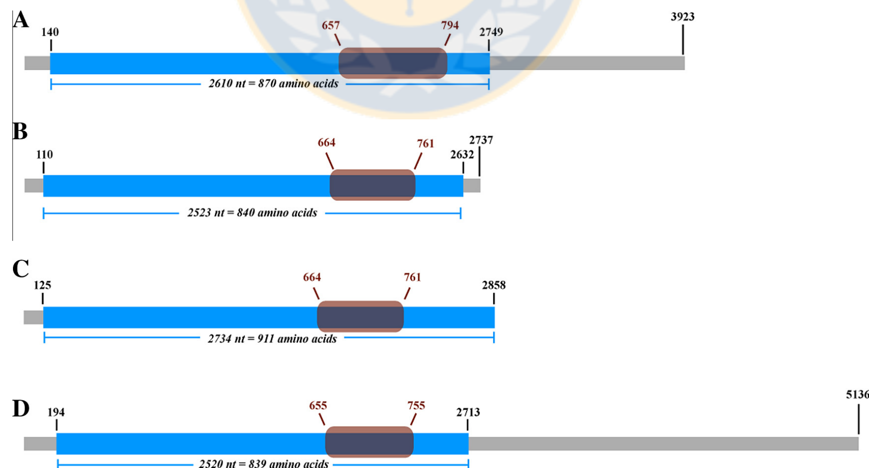


**Fig. 1.** Schematic structure of ionotropic receptors transcripts identified in *Caligus rogercresseyi*. Gray rectangles correspond to untranslated regions, blue rectangles to coding regions, and brown rounded-borders rectangles to the position of the most representative domain found along the transcript. 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.)

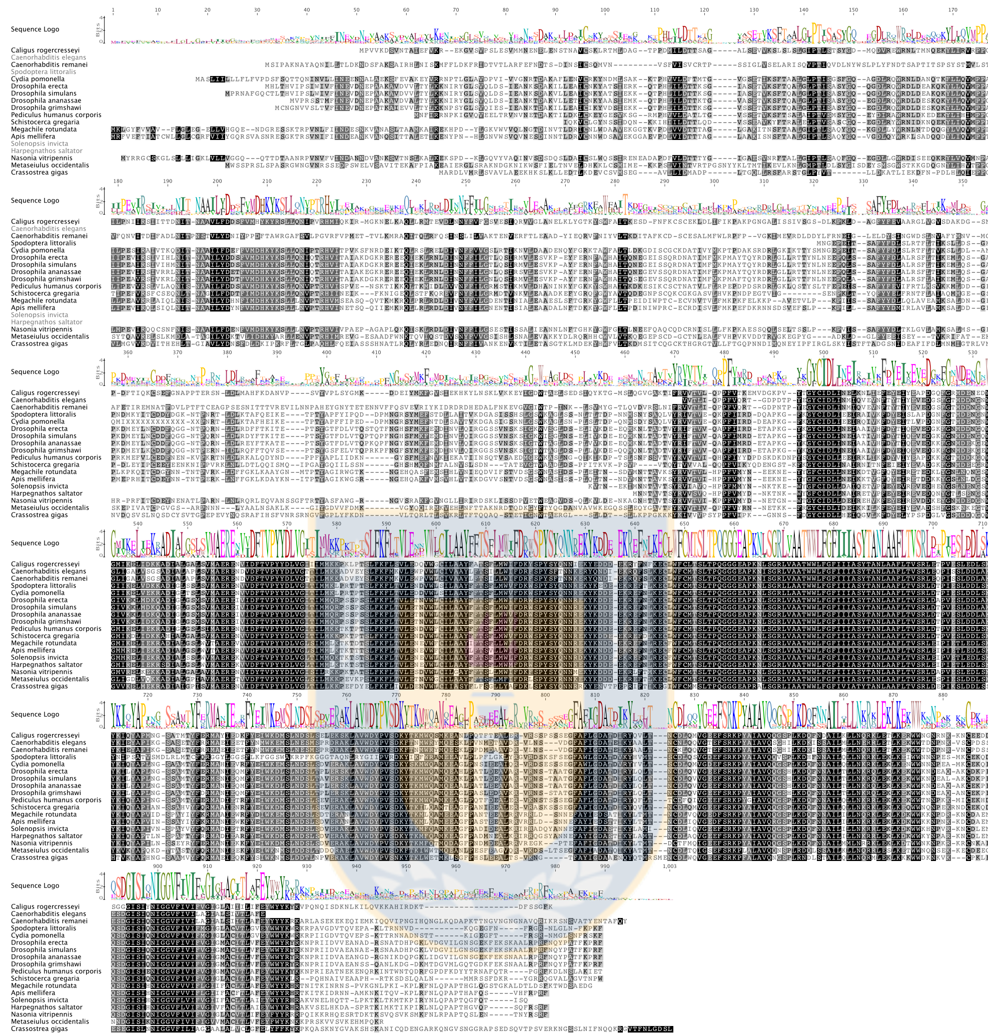


**Fig. 2.** Multiple alignments of protein sequences of *Ionotropic receptors subunit 25a*. The algorithm used was ClustalW, with a gap on cost equal to 10 and a gap extend cost of 0.1. Genbank accession number of sequences used are AF318611 (*Caenorhabditis elegans*), XP_003109814 (*Caenorhabditis remanei*), ADR64679 (*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

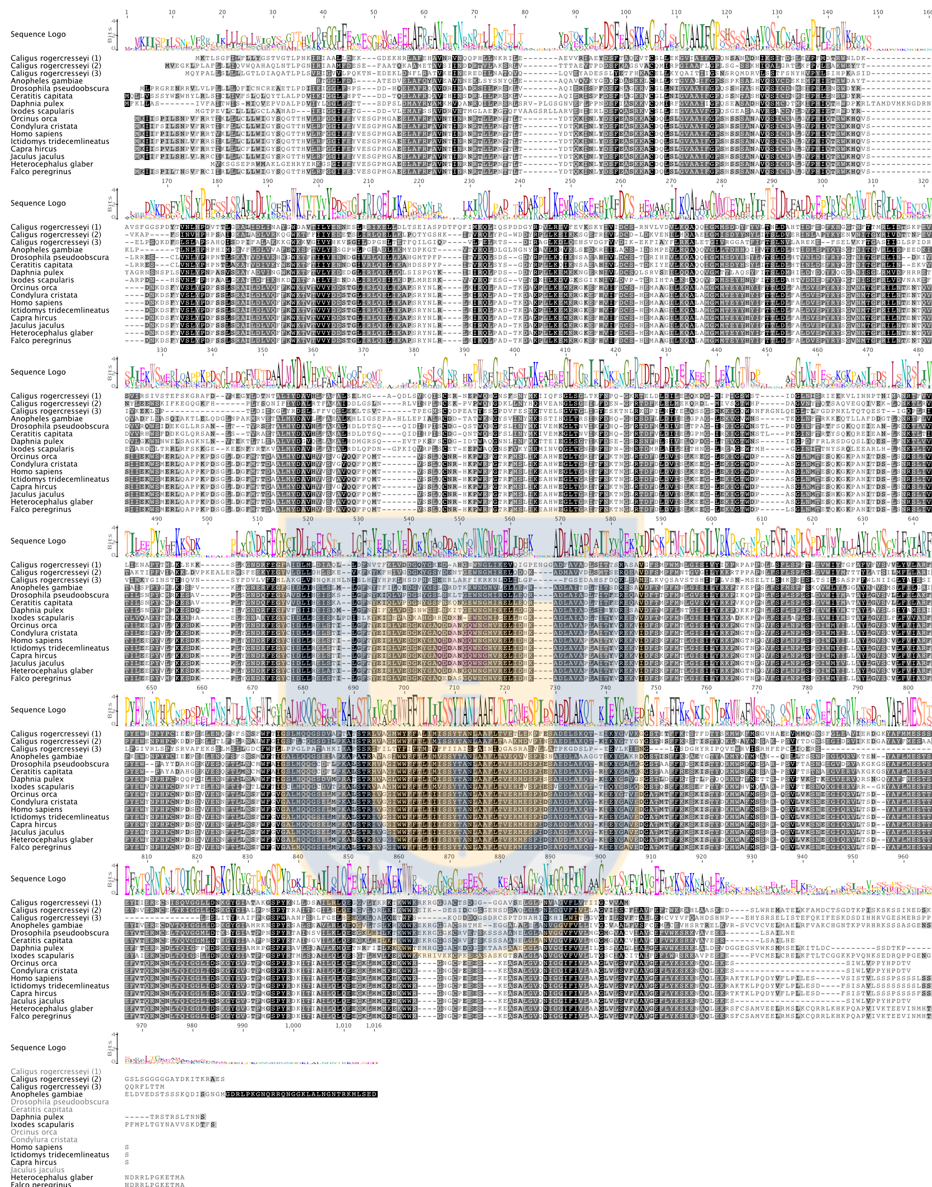

**Fig. 3.** Multiple alignment of protein sequences of *Ionotropic receptors subunit 25a*. The algorithm used was ClustalW, with a gap on cost equal to 10 and a gap extend cost of 0.1. Genbank accession number of sequences used are XP_559460 (*Anopheles gambiae*), XP_001358619 (*Drosophila pseudoobscura*), 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

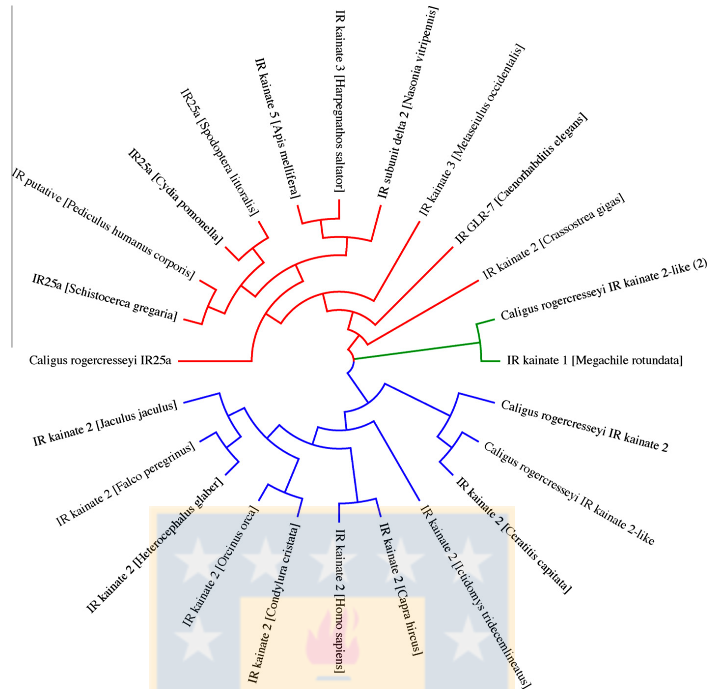


**Fig. 4.** Genetic distances tree of ionotropic receptors translated sequences of different species based on Jukes–Cantor model and Neighbor-Joining method. No outgroup was used in this analysis.

*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

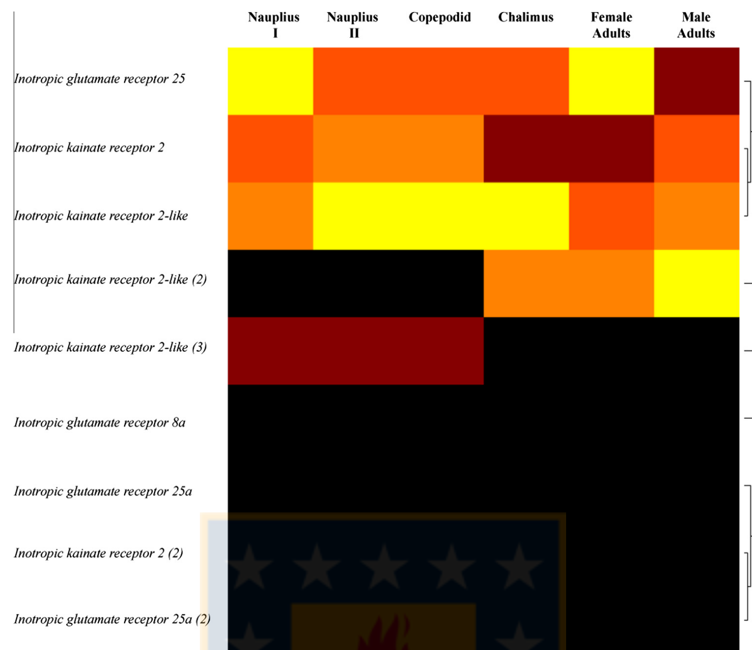


**Fig. 5.** *In silico* gene transcription analysis of contigs annotated as ionotropic receptors in *C. rogercresseyi*. Hierarchical clustering of RPKM values is shown in a heat-map constructed with normalized values by quantiles. Black: low levels of gene expression, Red: medium levels of gene expression, 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

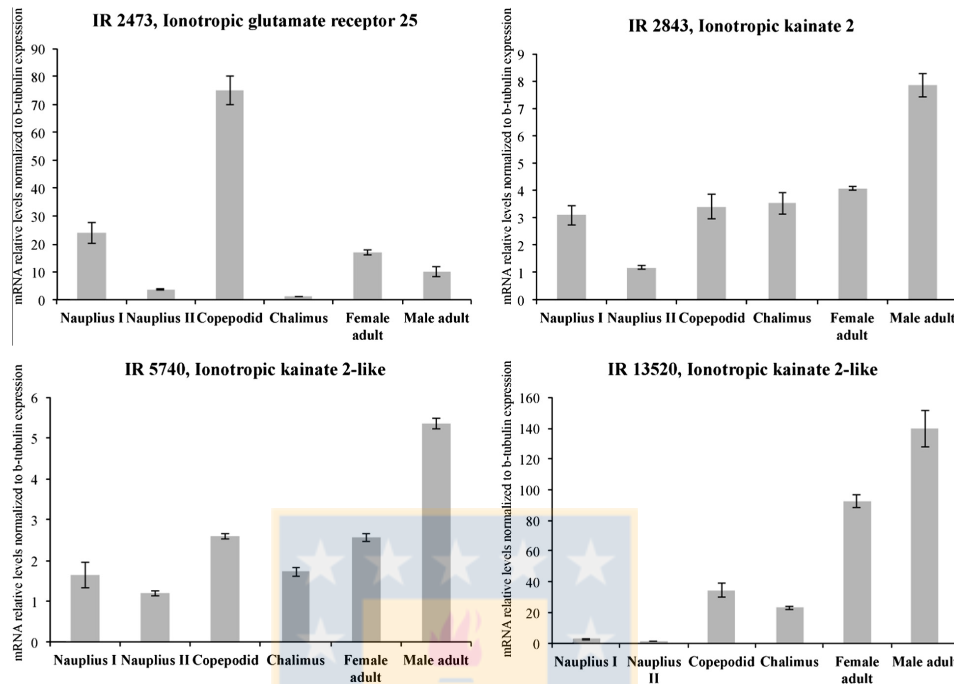


**Fig. 6.** Gene transcription analysis evaluated by qPCR from ionotropic receptors in different developmental stages of sea lice, *C. rogercresseyi*. Data were normalized by the expression of the endogenous control *b-tubulin*.

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

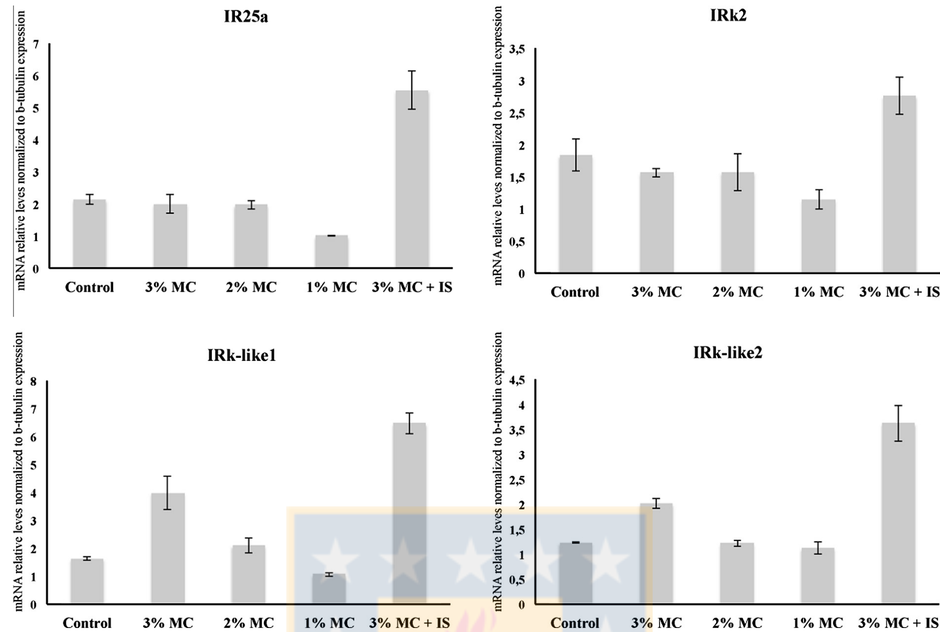


**Fig. 7.** Gene transcription analyses of ionotropic receptors in response to the presence of masking compounds in salmon diets. The mRNA relative levels (*y* axis) were measured in copepodids infesting salmons fed with diets composed by different concentration of masking compounds and immunostimulant. 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.

## Acknowledgments

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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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**CAPÍTULO II.** Pesticides Drive Stochastic Changes in the Chemoreception and Neurotransmission System of Marine Ectoparasites.

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Abstract

Scientific efforts to elucidate the mechanisms of chemical communication between organisms in marine environments are increasing. This study applied novel molecular technology to outline the effects of two xenobiotic drugs, deltamethrin (DM) and azamethiphos (AZA), on the neurotransmission system of the copepod ectoparasite *Caligus rogercresseyi*. Transcriptome sequencing and bioinformatics analyses were conducted to evaluate treatment effects on the glutamatergic synaptic pathway of the parasite, which is closely related to chemoreception and neurotransmission. After drug treatment with DM or AZA, stochastic mRNA expression patterns of glutamatergic synapse pathway components were observed. Both DM and AZA promoted a down-regulation of the *glutamate-ammonia ligase*, and DM activated a *metabotropic glutamate receptor* that is a suggested inhibitor of neurotransmission. Furthermore, the delousing drugs drove complex rearrangements in the distribution of mapped reads for specific metabotropic glutamate receptor domains. This study introduces a novel methodological approach that produces high-quality results from transcriptomic data. Using this approach, DM and AZA were found to alter the expression of numerous mRNAs tightly linked to the glutamatergic signaling pathway. These data suggest possible new targets for xenobiotic drugs that play key roles in the delousing effects of antiparasitics in sea lice.

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# Pesticides Drive Stochastic Changes in the Chemoreception and Neurotransmission System of Marine Ectoparasites

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**Abstract:** Scientific efforts to elucidate the mechanisms of chemical communication between organisms in marine environments are increasing. This study applied novel molecular technology to outline the effects of two xenobiotic drugs, deltamethrin (DM) and azamethiphos (AZA), on the neurotransmission system of the copepod ectoparasite *Caligus rogercresseyi*. Transcriptome sequencing and bioinformatics analyses were conducted to evaluate treatment effects on the glutamatergic synaptic pathway of the parasite, which is closely related to chemoreception and neurotransmission. After drug treatment with DM or AZA, stochastic mRNA expression patterns of glutamatergic synapse pathway components were observed. Both DM and AZA promoted a down-regulation of the *glutamate-ammonia ligase*, and DM activated a *metabotropic glutamate receptor* that is a suggested inhibitor of neurotransmission. Furthermore, the delousing drugs drove complex rearrangements in the distribution of mapped reads for specific metabotropic glutamate receptor domains. This study introduces a novel methodological approach that produces high-quality results from transcriptomic data. Using this approach, DM and AZA were found to alter the expression of numerous mRNAs tightly linked to the glutamatergic signaling pathway. These data suggest possible new targets for xenobiotic drugs that play key roles in the delousing effects of antiparasitics in sea lice.

**Keywords:** *Caligus rogercresseyi*; sea lice; deltamethrin; azamethiphos; glutamatergic synapse; metabotropic receptor; ionotropic receptor

## 1. Introduction

Chemical signals and cues constitute the main mechanisms of communication between animals in aquatic environments. Identifying these chemical communications and discriminating between evolved functions (signals) and unintentional releases (cues) are foremost challenges in aquatic biology. Importantly, by fully understanding the recognition systems of chemical communication in marine systems, knowledge regarding biotic interactions could advance more rapidly. Chemical cues play critical roles at every level in marine systems [1,2], but their presence in the environment and recognition by and impact on organisms are not completely understood. For most marine species, chemical cues determine whether they consume, fight with, run from, or mate with the cue-emitting organism, as well as whether they are eaten, infected, or overgrown by natural predators and/or parasites. The molecules involved in these processes are known as allelochemicals (interspecific communication) and pheromones (intraspecific communication) [3]. Besides chemical signals, which are intentionally released by the sender, receivers also react towards unintentionally released chemical cues [4]. While chemical signaling is usually advantageous to the signal-emitting organism, the release

of chemical cues usually results in either neutral or damaging effects. In contrast, allelochemicals, such as kairomones, are mostly beneficial to the sender and are often used in predacious or parasitic relationships [5].

In marine ectoparasites, allelochemicals facilitate significant biological processes such as kin recognition, foraging, host infection, and mate pairing [6,7]. Some kairomones that mediate host recognition in marine ectoparasites have been identified [8,9], but most evidence is limited to the Northern Hemisphere ectoparasite *Lepeophtheirus salmonis*. Regarding marine ectoparasites from the Southern Hemisphere, host recognition processes mediated by semiochemicals have been suggested for the sea louse *Caligus rogercresseyi* [10]. Notwithstanding these contributions in host recognition, the chemoreception process of marine parasitic species remains unclear, especially at a molecular level.

The chemosensory system in animals includes key downstream pathways mainly related to the recognition of chemical stimuli and transduction of neurotransmission signals, including of glutamate receptors, adrenergic receptors, and acetylcholine. Related to this, G protein-coupled receptors (GPCRs) represent the majority of membrane-bound proteins with known functions in signal neurotransmission and sensory perception [11,12]. Within the GPCRs, metabotropic glutamate receptors (mGluRs), play key roles in signal transduction, especially in fast synaptic transmission, such as in the glutamatergic synapse pathway [13]. In mammals, mGluRs include a family of proteins with seven transmembrane domains that are subdivided into three groups containing at least eight different proteins, all of which couple to G proteins [14]. In turn, ionotropic glutamate receptors (iGluRs) correspond to a group of ion channels with affinity for a diverse array of ligands [15]. Various members of the iGluR family have been described in insect species, and a subfamily of ionotropic receptors (named IRs) can bind different ligands, including some volatile chemicals [16]. Additionally, these IRs share structural amino acid components with iGluRs and have similar gene expression patterns as insect odorant receptors, suggesting a role in chemical communication [17,18]. Recently, a group of iGluRs was identified in *C. rogercresseyi*, thus indicating a potential role of this group in the chemosensory reception system [19]. However, the downstream signaling pathway of the chemosensory system has not been identified in sea lice. Furthermore, while some glutamate receptors are characterized as drugs targets [14,20], it is not clear whether delousing drugs are also able to trigger expressional changes in mGluRs and iGluRs. In this context, there is just a few studies evaluating the effect of these drugs to ectoparasite abilities to recognize hosts, and they exhibited contradictory results. In the bug *Trissolcus basalis*, an alteration of the host recognition behavior of the parasite after exposure to sublethal concentrations of deltamethrin has been reported [21]. In contrast, in the egg parasitoid *Anagrus nilaparvatae* there were no alterations in the host recognition mechanism after exposure to sublethal concentrations of the same drug [22]. Besides, there are no studies describing any behavior related to host identification in marine ectoparasites.

The marine ectoparasite *C. rogercresseyi* was used as a model in the current study. This copepod causes major economic losses to Chilean salmon farms [23] by inducing caligidosis, a disease mainly treated with delousing drugs such as pyrethroids and organophosphates [24]. The main focus of this study was to infer the effects of xenobiotic drugs on glutamatergic synapse-related genes in *C. rogercresseyi* through *in silico* analyses in transcriptomes exposed to deltamethrin (DM, a pyrethroid) or azamethiphos (AZA, an organophosphate). The methodological approach was based on a deep analysis of transcriptional performance (RNA-seq), which allowed the determination of the effects of the drugs at different transcriptomic levels by estimating total mRNA abundance, candidate gene transcription, transcriptional directional shifts, and mapping arrangement. The present study is innovative in that it evaluates a novel gene-signaling pathway in marine copepods while under antiparasitic drug treatment and incorporates new concepts derived from an *in silico* strategy.

## 2. Results

### *2.1. Transcriptomic Responses of Glutamatergic Pathway Components to Antiparasitic Treatment: A Global Comparison*

In the marine environment, information remains lacking to explain the underlying molecular basis and signal perception of the chemosensory system, as well as the effects that chemicals or drugs would have on it. Therefore, having established the existence of a chemosensory mechanism through the glutamatergic pathway, transcriptome analyses were conducted on adult sea lice exposed to either DM or AZA, both chemical antiparasitics used in the control of *C. rogercresseyi* in salmon farming. Through this, a core set of transcripts tightly linked to the glutamatergic synapse pathway was identified in response to DM and AZA.

The abundances of glutamatergic synapse pathway transcripts derived under DM and AZA treatment were significantly different in regards to the total number of differentially expressed transcripts and intensity (fold change > $|-2|$). In total, 176 transcripts were differentially expressed (Reads Per Kilobase of transcript per Million mapped reads, RPKM) between the treatment groups (88 DM and 88 AZA) and the control. Peak intensity was observed with AZA (Δ total RPKM of 243,241 in 88 transcripts as compared to the control, *versus* 230,106 for DM). The expression profiles obtained for glutamatergic synapse-related genes highlighted a marked contrast between sexes during the parasitic drug response. Specifically, a greater number and higher intensity (fold change) of induced transcripts were observed in female sea lice than in male specimens (Figure 1).

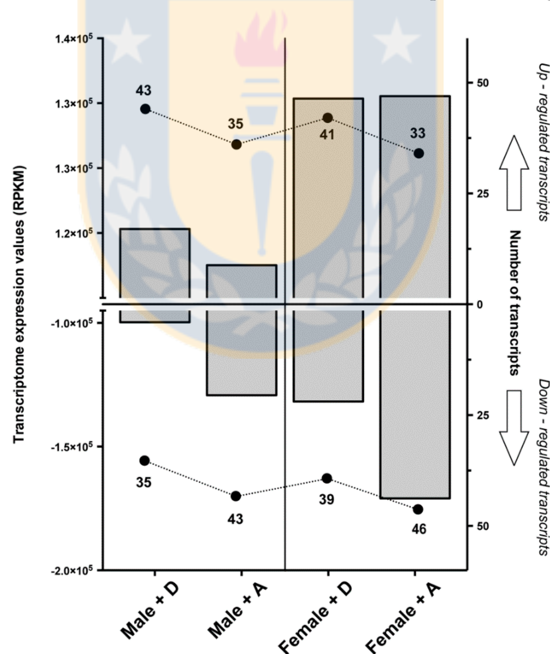


**Figure 1.** Total transcriptomic response of the glutamatergic synapse pathway in male and female *Caligus rogercresseyi* exposed to xenobiotic drugs. In the left *Y*-axis (gray bars), the sum of expression values for up- and down-regulated transcripts after drug exposure are shown. Values correspond to differences in Reads Per Kilobase of transcript per Million mapped reads (RPKM) values as compared to the same transcripts in the control condition. In the right *Y*-axis (plot points), the total number of up- and down-regulated transcripts after drug application are shown. +D: Deltamethrin, +A: Azamethiphos.

To determine whether delousing drugs simply shifted the global gene expression profile of the glutamatergic synapse-related transcripts, the directional shift of the regulated transcripts was measured within the pathway; in other words, up-regulation with one drug but down-regulation with the other drug was identified. Significant differences were recorded between AZA and DM in regards to gene shift, where eight shifts were found with DM, nine shifts with AZA, and eight genes with no shifts (Figure 2). Non-shifted transcripts corresponded to the down-regulated voltage-dependent calcium channel and to up-regulated genes by both drugs, including solute carrier family 8, members 3 (SN1) and 2 (GLS); protein phosphatase (PP2B); mitogen-activated protein kinase 1; phospholipase D; and the mGluR regulators ankyrin repeat domain (SHANK) and Homer. Regarding other receptor proteins, solute carrier family 1, member 2 (EEAT); ionotropic kainate receptors (KA); and mGluR 3–5 were shifted to DM, while the 7-transmembrane glutamate receptor domain containing protein (7mGluR) and ionotropic NMDAR receptors were shifted to AZA. Additionally, the G protein O and I subunits were shifted to DM, whereas the S unit was shifted to AZA (Figure 2). Although both drugs notably down-regulated glutamine synthetase, also known as glutamate-ammonia ligase (GLNS), some contigs related to this gene were up-regulated by DM. Furthermore, receptor activity was more closely associated with DM, especially for the mGluRs and solute carriers (Figure 3). Therefore, the different mRNA abundances were not a consequence of a random shift. Rather, these differences reflect drug-dependent changes in transcript abundance as part of the chemosensory response, suggesting an increased focus on transcription. This coordinated response indicates that delousing drugs can drive directionally juxtaposed variations in the mRNA abundances of the glutamatergic pathway.

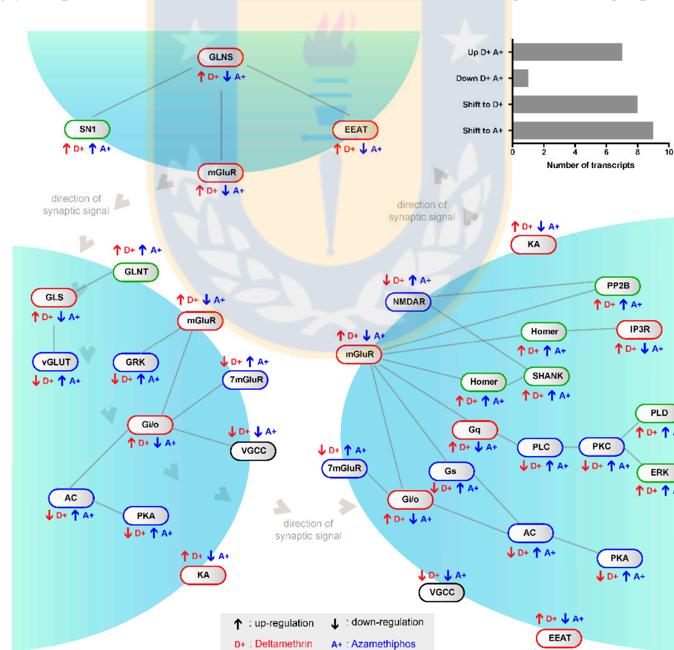


**Figure 2.** Modulation of glutamatergic synapse pathway transcription levels by xenobiotic drugs in *Caligus rogercresseyi*. Abbreviations are per established GeneCards nomenclature. Green border: genes up-regulated by both drugs; black border: genes down-regulated by both drugs; red border: genes up-regulated by deltamethrin but down-regulated by azamethiphos (*i.e.*, shift to deltamethrin); and blue border: genes down-regulated by deltamethrin but up-regulated by azamethiphos (*i.e.*, shift to azamethiphos).

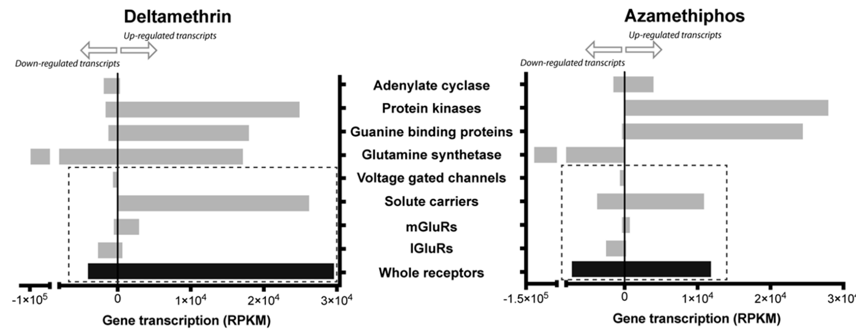


**Figure 3.** Expression levels of glutamatergic synapse-related genes grouped by molecular functions. Gene expression was measured as the sum of total RPKM after exposure to antiparasitic drugs as compared to control samples. Gray bars correspond to group of contigs clustered by gene family; black bar corresponds to the sum of all the groups that are related to receptors gene families. Dotted rectangle includes the gene families related to membrane receptors.

The global expression profiles of sea lice exposed to both DM and AZA were also assessed. A total of 119 transcripts scored positive for reliable annotation among all samples and were tightly linked to chemosensory components. These transcripts were used as statistical analyses that determined significantly different mRNA abundances between drug treatments. Principal components analysis (PCA) and hierarchical clustering were used to determine the contribution that each mRNA had in the response to DM and AZA treatments. Particularly, the DeDaL-PCA Cytoscape plugin combines classical and advanced data dimension reduction methods with the algorithms of network layout inside the Cytoscape environment. The genetic interactions between these genes and the epistatic profiles (computed only for this group of genes) were selected from de global expression profile (RNA-seq). Definitions for the chemosensory pathway were taken from the KEGG database.

Differences were obtained between the standard and DeDaL-PCA-based organic layouts for this network of genetic interactions (Figure 4). The DeDaL-PCA was computed without applying data matrix double-centering to take into account tendencies of genes to interact with a smaller or larger number of other genes, thereby estimating the effects of AZA or DM on the mRNA abundances of chemosensory components. Both PCAs clearly highlighted differences between the treatments and against the control group (Figure 4). In the standard PCA, the local glutamate receptors genes GLNS, EEAT, SN1, and GLS were distinctly positioned by DM and AZA treatments, a result similarly obtained by the DeDaL-PCA. Additionally, some genes, such as KA and mGluR, showed weak expression patterns in both treatments. For both treatments, the two principal factors together explained close to 99% of expressional variability. This suggests that both delousing treatments had a similar effect on the expression profile of chemosensory components.

### *2.2. mGluR Characterization in Caligus Rogercresseyi*

Two complete mRNA sequences were obtained for mGluRs in C. rogercresseyi. These sequences were termed Cr-mGluR-A and Cr-mGluR-B (GenBank Accession Numbers KT599917 and KT599918, respectively). BlastX analysis found Cr-mGluR-A similar to the mGluR3-like gene in diverse arthropod species (*E*-value = 0) such as Microplitis demolitor, Nasonia vitripennis, and Megachile rotundata. In turn, Cr-mGluR-B was similar to mGluR in Tribolium castaneum (*E*-value = $7.42 \times 10^{-68}$) and mGluR3 in Strongylocentrotus purpuratus (*E*-value = $1.96 \times 10^{-65}$).

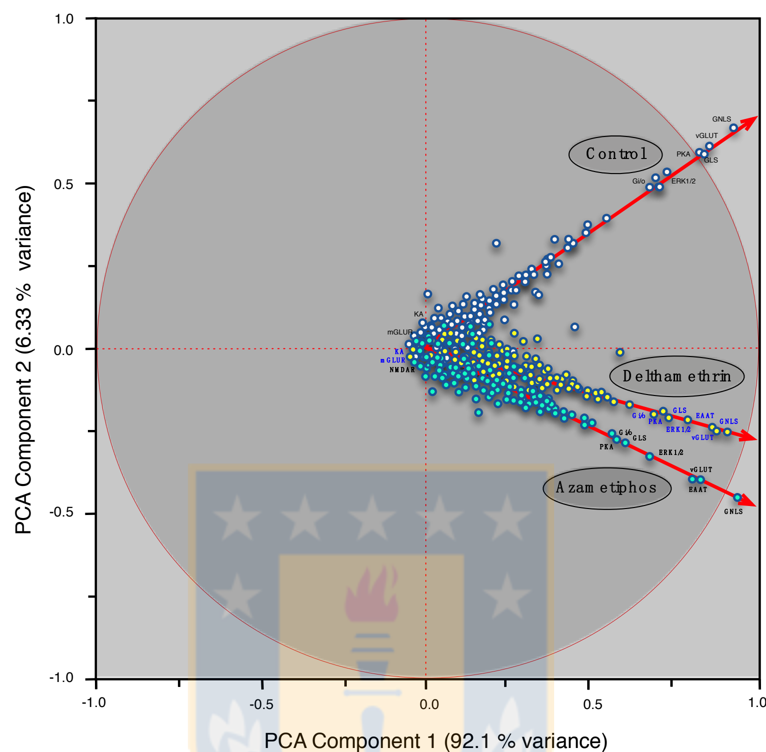


**Figure 4.** The DeDaL-PCA Cytoscape plugin was used to visualize the network of genetic interactions for downstream components of the glutamatergic pathway affected by delousing drugs. Different node colors indicate distinct mRNA regulation after drug exposures. A principal components analysis (PCA) was applied to the network-smoothed profile. DeDaL used the elastic map (elmap) algorithm for computing non-linear principal manifolds. A factorial map of the PCA was applied to data for components of the glutamatergic pathway, which are represented as colored circles for each treatment condition (white: control; yellow: deltamethrin; and blue: azamethiphos). The portion of the variance explained by the principal component is indicated in parentheses.

Both sequences exhibited coding sequences and the 3′ and 5′ untranslated regions. The complete sequence, coding sequences, and untranslated regions of Cr-mGluR-A were larger than Cr-mGluR-B (Figure 5A,B). In both Cr-mGluRs, nucleotide regions homologous to the mGluR domain 1 and to the 7-transmembrane sweet-taste receptor of 3 GPCR domain were found (Figure 5A). Structural differences were assessed with the PSIPRED v3.3 and DomSerf v2.0 tools, which showed the crystal forms of the Cr-mGluRs and provided information on the structural disposition of both proteins. The *in silico* crystallized structures of Cr-mGluR-A and Cr-mGluR-B assumed active conformations with well-defined canonical dispositions of $\alpha$-helixes and $\beta$-sheets in the first domain, but with dissimilar fragments in the second domain (Figure 5B). Finally, the conformations adopted by the $\alpha$-helixes and $\beta$-sheets differed from those seen in the mGluR structures of other organisms.

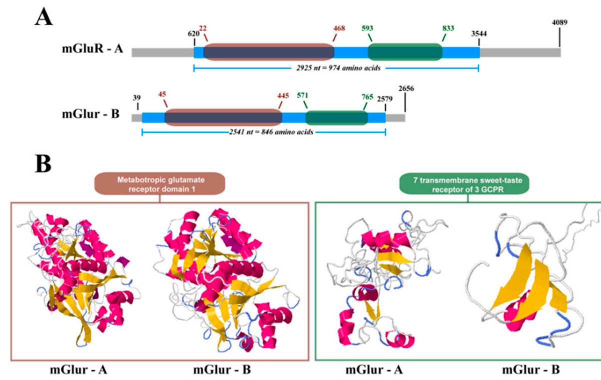


**Figure 5.** Structural characteristics of two metabotropic glutamate receptor (mGluR) sequences in *Caligus rogercresseyi*. (**A**) Sequence schemes for Cr-mGluR-A and Cr-mGluR-B, indicating the position of the open reading frame (blue rectangle), metabotropic glutamate receptor domain 1 (brown rounded-rectangle), and 7 transmembrane sweet-taste receptor of 3 GPCR (green rounded-rectangle); (**B**) Predictive model of both domains in each mGluR sequence. Brown and green lines correspond to the same domains described in **A**, respectively. In the models, yellow arrows correspond to beta sheets and pink regions to alpha helix.

### 2.3. Gene Transcription of Cr-mGluR after Drug Treatments and Mapping Arrangement Analysis

The gene transcriptions of Cr-mGluR-A and Cr-mGluR-B were evaluated through RT-qPCR and *in silico* (RPKM values) analyses (Figure 6A). Both transcripts were activated by DM exposure, showing expression levels 2-fold higher than control samples. In contrast, gene transcription levels did not vary following AZA treatment.

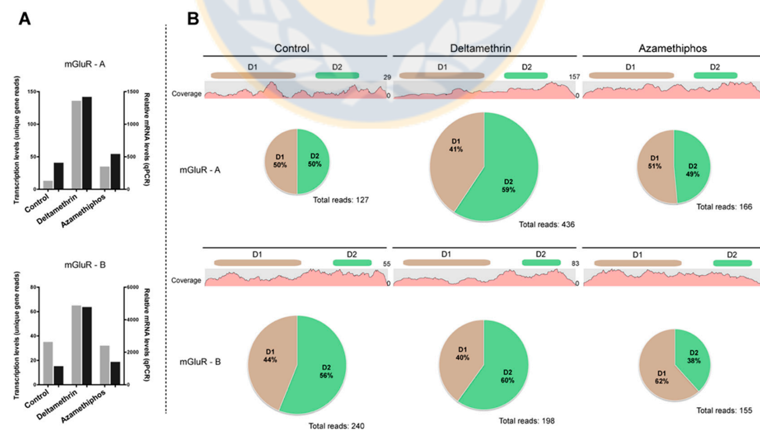


**Figure 6.** Expression levels of two mGluR genes in *Caligus rogercresseyi* treated with delousing drugs. (**A**) Validation of gene transcription levels by qPCR analyses. Gray bars correspond to expression changes obtained by *in silico* analysis, and black bars expression levels inferred by qPCR reactions; (**B**) Distribution of mapped reads in both mGluR transcripts after exposure to deltamethrin or azamethiphos. D1: metabotropic glutamate receptor domain 1; and D2: 7 transmembrane sweet-taste receptor of 3 GPCR.

To determine the impact of DM and AZA on the mapping arrangement of Cr-mGluR-A and Cr-mGluR-B, the distribution of reads in each domain was checked (Figure 6B). For Cr-mGluR-A, the control samples exhibited an equal distribution of mapped reads for both the mGluR 1 and the 7-transmembrane sweet-taste receptor of 3 GPCR domains. Exposure to DM resulted in a redistribution of mapped Cr-mGluR-A reads, with a 9% increase in the total number of reads mapped in the mGluR domain 1. However, AZA treatment did not result in variations to the mapping arrangement of Cr-mGluR-A as compared to the control. Regarding Cr-mGluR-B, mapping arrangement, as compared to the control, varied by domain, with 44% of mapped reads in the mGluR domain 1% and 56% in the 7-transmembrane sweet-taste receptor of 3 GPCR. Additionally, DM triggered a 4% increase in the total number of mapped reads for the 7-transmembrane sweet-taste receptor of 3 GPCR in Cr-mGluR-B, but AZA exhibited an opposite trend, increasing the mapped reads of the mGluR domain 1 by 18%.

## 3. Discussion

Chemical cues and signals are involved in every aspect of the copepod life-cycle, including in finding and assessing mates, determining and stabilizing dominance hierarchies, finding food, recognizing kin, foraging, and infecting a host [1]. However, characterizing chemical communication, recognition, and responses in the aquatic environment are challenging due to small chemical amounts and a complex background of other compounds. Cues and signals are transduced from the sense organs into sparse and stimulus-specific activity patterns across large populations of perception cells [25,26]. Nevertheless, the subsequent processing steps are poorly understood.

The complexity of the receptor recognition pattern could be driven by several factors (*i.e.*, size, landscape, and noise of chemical signal). Using the expressional pattern of the downstream glutamatergic pathway, a spatial *in silico* pattern of neuronal synaptic activation was constructed. Glutamate signaling includes mGluRs and iGluRs [13], both of which are implicated in the recognition of chemical communications. The impact that delousing drugs had on the *C. rogercresseyi* transcriptome was systematically assessed, revealing significant remodelling of the transcriptional profile, with particular divergence in the response between sexes. This sex-dependent finding is in agreement with previous results in *C. rogercresseyi* [27,28] and highlights the effects that DM and AZA can have on the magnitude of the transcriptomic response. First, in both cited studies most of the survivors were female individuals. Furthermore, both studies described a wide number of differentially expressed genes in both sexes, including more abundance of female-exclusive transcripts in the delthamethrin treatment. Besides, both drugs changed the expression pattern of different genes of the glutamatergic synapse pathway. As there were no other known variables, transcriptomic profile differences were assumed to be solely due to the chemical composition of the different drugs and, additionally, to male/female variations in drug recognition. This assumption was supported by variations in transcript numbers and intensity (Figure 1).

Comparisons including pathway-drug interactions indicated different treatment responses in regards to magnitude and intensity. These variations were identified at multiple levels, including for the expression of single mRNAs, the regulation of mRNA expression, and for the directional juxtaposition of shifts linked to increased variations in mRNA abundance, signifying a change in functional output (excited or inhibited, Figure 2) [11]. The obtained results suggest that DM promotes significant changes in specific Gi/Go subunits of the G protein that could drive the inhibition of glutamatergic synapse transmission [29–31]. This regulation might affect drug effectivity in relation to parasite survival. Specifically, DM rapidly killed both males (73%) and females (80%), while AZA killed only 60% of males and 46.7% of females. Supporting this observation on glutamatergic neurotransmission, both antiparasitics also promoted significant consequences in other nervous system elements, such as NOTCH and the ABC transporters [32]. There is increasing evidence that delousing drugs are able to control unknown regulatory elements of the nervous system, consequently indicating a close interaction between drugs and hypothetical drug resistance [33]. In this context, the present

study expands on the description of potential targets for DM an AZA, which was previously limited to specific genes, such as the acetylcholinesterase enzyme in the case of AZA [34].

In support of this, a significant effect of both drugs on GLNS mRNA transcript abundance was significantly affected by both DM and AZA (GLNS, Figures 3 and 4). As a multi-functional enzyme tightly related to drug-resistant phenotypes [35], GLNS catalyses the conversion of glutamate and ammonia into glutamine, which plays a key role in the activation of GPCRs, thus promoting glutamatergic synapse transmission [36]. In the present analysis, the importance of GLNS down-regulation could be in the lethality effect of xenobiotic drugs in this marine ectoparasite. Additionally, GLNS activity is pivotal to olfactory perception in other arthropods such as *Drosophila melanogaster* [37]. Therefore, the present results suggest that DM and AZA may also inhibit olfactory perception in *C. rogercresseyi*.

A notable effect on the neurotransmission and olfactory systems was triggered in *C. rogercresseyi* following xenobiotic drug exposure. Integrative computational and analytical assessments of transcriptome data [38] further support this observation. Other *in silico* approaches, such as those focused only on mRNA fragments, could be biased since gene expression measures are extrapolated from short sequences [39]. Full-transcript *in silico* analyses allow for inferring the presence of transcript variants and isoforms due to overlapping mRNA sequences [40]. The novel method applied in the present study was based not on developing an automatic tool to infer transcriptional changes at the deepest level, but rather on monitoring specific mRNA sequence mappings to detect differences in the read distribution arrangement of specific sequences as a result of drug exposure. For this, the mapping arrangement of Cr-mGluRs were assessed first due to their importance in signal transduction of the glutamatergic synapse [13]. To prevent incorrect interpretations of results, technical biases have to be discarded through strong bioinformatics approaches that incorporate the assembly of reads into contigs [41]. This computational and analytical method also discards other bias sources from the library construction procedure, which, in the case of Illumina sequencing, would include the use of random hexamers, selective adapter ligation, and a biased PCR system [42].

The observed mapping rearrangements might explain the biological effect of DM and AZA on the polymerase efficiency and transcriptional performance of the selected mRNAs. This was also observed in untreated individuals at different developmental stages (Figure S1). Detailed mapping analyses exhibited greater read depth at the 3′ untranslated region of transcripts, especially in samples exposed to DM (Supplementary Figures S2 and S3). The poly-A tails located at the end of 3′ untranslated regions allows subtractive hybridization to eliminate non-messenger RNA in the library construction process [42]. A technical limitation of RNA-seq analysis is that it can only display a time-dependent snapshot of the transcriptome. Therefore, short mRNA fragments present in the cytoplasm may also be retained as poly-A tails could attach to these sequences. This observation suggests that DM may generate primary and secondary effects on mRNAs transcription, and, in some cases, cell machinery appears to respond by activating post-transcriptional regulations. However, further analysis should be carried out to confirm this hypothesis. The present analyses highlight that the key functions of the sensorial and nervous systems are inhibited in distinct transcriptional phases that are strongly triggered by the delousing drugs DM and AZA. Although the deleterious effects of xenobiotics are somewhat defined in other marine parasites [43], the present results and methodology expand previous observations.

## 4. Materials and Methods

### *4.1. Bioassays and Transcriptome Sequencing*

Sea lice (*Caligus rogercresseyi*) were cultured under controlled laboratory conditions according to the protocol described by Bravo *et al.* (2010) [44]. After obtaining active male and female adults, bioassays were performed with the delousing drugs deltamethrin (AlphaMax™, PHARMAQ AS Chile Ltda., Puerto Montt, Chile) and azamethiphos (Byelice®, Bayer S.A. Chile, Santiago, Chile).

Bioassays and transcriptome sequencing were performed for DM according to Chavez-Mardones *et al.* (2014) [45] and for AZA according to Valenzuela-Muñoz *et al.* (2015) [28]. Briefly, adult male and female sea lice were incubated for 30 min in either DM (2 ppb) or AZA (3 ppb) and then transferred to a drug-free medium. Drug concentrations were selected according to previously obtained results [26,43]. Control sea lice were maintained under the same culture conditions as experimental sea lice but were not exposed to any drug. At 24 h post-treatment, survival rate and the number of immobilized lice were obtained for each assay. Surviving lice were transferred to cryogenic tubes containing RNAlater® (Ambion®, Thermo Fisher Scientific®, Waltham, MA, USA), fixed, and stored at −80 °C until molecular analyses. Sea lice RNA was extracted using the RiboPure RNA Isolation Kit™ (Ambion® Thermo Fisher Scientific®, Waltham, MA, USA), and cDNA libraries were constructed using the TruSeq RNA Sample Preparation v2 Kit (Illumina®, San Diego, CA, USA). High-throughput sequencing was conducted with an Illumina MiSeq™ System (Illumina®, San Diego, CA, USA) Male and female samples were separately sequenced for each group, and each group was sequenced twice (*i.e.*, two technical replicates).

### 4.2. In Silico Analyses of Glutamatergic Synapse Pathway in Sea Lice to Antiparasitic Drugs

Millions of short sequences obtained from Illumina sequencing runs for each assay were trimmed, filtered, and assembled into contigs following the methodology established in a transcriptomic characterization of *C. rogercresseyi* [46]. For MultiBlast analysis, sequences of glutamatergic synapse pathway genes from other arthropod species were used as references for identifying these genes in the *C. rogercresseyi* transcriptome. *In silico* analyses were performed using the CLC Genomic Workbench software (version 8.0, CLCBio®, Aarhus, Denmark). MultiBlast sequence results with *E*-values higher than $1 \times 10^{-20}$ were discarded. In cases where two or more sequences were related to the same gene, the sequences were aligned with the corresponding reference, and the sequence with the highest coverage and homology was selected. The selected contigs were used as new specific references for evaluating corresponding expression levels through the CLC Genomic Workbench RNA-seq module. The RPKM value was calculated for each contig in the different experimental groups. With this dataset, the total transcriptomic expression of the assessed genes was obtained by applying logic functions according to individual transcript expression patterns in a Microsoft Excel spreadsheet (Table S1). These analyses provided a count of the up- and down-regulated transcripts, as well as an account of respective total expressional changes.

Transcriptional directional shifts of genes from the glutamatergic synapse pathway were evaluated using the methodological approach described by Boltaña *et al.* (2013) [47]. A directional shift is defined as a variation in the transcriptional direction (up-regulation, down-regulation) of a single gene under different conditions. Directional shifts were classified within the obtained transcriptional data after exposure to DM or AZA, with variations inferred as compared to the basal expression levels of the control group.

### 4.3. Statistical Analyses

Hierarchical clustering analysis was performed using the Euclidean distance matrix and complete linkage method. Principal component analysis of gene ontology terms showed two-dimensional views, as retrieved from the DeDaL plugin of the Cytoscape environment [48]. This analysis was used to visualize the relatedness of all RNA-seq samples. All *p*-values were adjusted with a false discovery rate correction for multiple testing by the Benjamini-Hochberg method [49]. All genes with false discovery rate-corrected *p*-values $<0.05$ were considered significant. The expression of genes found to be significantly different between DM and AZA treatments were further characterized by hierarchical clustering analysis. Hierarchical clustering was based on the expression pattern across the sampled population, thereby identifying gene clusters with common expression profiles. Sample variances were homogeneous (normal distribution). Principal components analysis in DeDaL was computed using singular value decomposition, as described by Gorban and Zinovyev (2009) [50]. This allowed

using missing data values without pre-imputing the values, but data points containing more than 20% of missing values were filtered out from the analysis. DeDaL computes the first ten principal components if there are more than ten data points. DeDAL also computes the $k$ principal components if there is $k + 1$ data points, $k < 10$. After computing the principal components, DeDaL reports the amount of variance explained by each of the principal components. DeDaL is the first plugin able to construct biological network layouts from high-throughput data in the Cytoscape environment.

### *4.4. Structural Analysis of Cr-mGluRs*

From the filtered contigs, transcripts annotating for the metabotropic glutamate receptor were extracted. Six contigs were found and assessed through multiple alignments using the CLC Genomic Workbench software to infer if they could correspond to different transcripts. Two of these transcripts were selected as they encompassed the complete mRNA sequence, including coding sequences (open reading frame) and untranslated regions. These two sequences were termed Cr-mGluR-A and Cr-mGluR-B and were deposited in the NCBI GenBank Database (Accession numbers KT599917 and KT599918).

Protein domains and the 3D structural models of Cr-mGluR-A and Cr-mGluR-B were predicted with the online tool PSIPRED [51]. Specifically, the PSIPRED v3.3 and DomSerf v2.0 tools were used to predict the secondary structure of proteins and model the domains, respectively.

### *4.5. Gene Transcription and Imbalance Analysis of mGluR*

Gene transcription levels (*i.e.*, mRNA abundance) of Cr-mGluR-A and Cr-mGluR-B were evaluated by calculating RPKM values. These values were validated through quantitative PCR runs using specifically designed primers. The qPCR runs were performed in a StepOnePlus™ qPCR System (Applied Biosystems®, Foster City, CA, USA) using the $\Delta\Delta C_t$ quantification method. Reactions were conducted using the Maxima SYBR Green qPCR Master Mix™ (Thermo Scientific®, Thermo Fisher Scientific®, Waltham, MA, USA) following the manufacturer's protocol. Data were normalized against the expression levels of β-*tubulin*, which was previously validated as an endogenous control for this species [46].

Furthermore, a novel approach was used to infer the transcriptional changes of the Cr-mGluR-A and Cr-mGluR-B sequences resulting from exposure to DM or AZA. Specifically, the distributions of the mapped reads were evaluated in both Cr-mGluR transcripts. For each transcript, the nucleotide sequence homologous to each specific domain (abbreviated as D1 and D2) was extracted to separately map all of the reads obtained for each treatment (control, DM, and AZA). The mapped reads corresponding to each domain were counted for both Cr-mGluRs and for each treatment. Then, the percentage of reads mapped to domains was determined. To prevent bioinformatics bias, the extracted sequences were compared and then blasted against the complete database of *C. rogercresseyi* transcriptomes to determine fragment uniqueness.

## 5. Conclusions

Overall, both xenobiotic drugs caused notable changes in the expression patterns of glutamatergic synapse pathway components, leading to stochastic disorder in the mRNA abundances for most of the regulatory transcripts important to this pathway. Among these essential genes, metabotropic receptors and glutamate-ammonia ligase are considered key elements in understanding the effects of pesticides on marine ectoparasites. Forthcoming research should study transcriptome regulation of transcriptomes exposed to drugs, using a fine-tuning approach, such as the mapping arrangement.

**Supplementary Materials:** Supplementary materials can be found at http://www.mdpi.com/1422-0067/17/6/700/s1.

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**Author Contributions:** Gustavo Núñez-Acuña, Sebastián Boltaña and Cristian Gallardo-Escárate designed the research. Gustavo Núñez-Acuña and Cristian Gallardo-Escárate carried out the experiment. Gustavo Núñez-Acuña and Sebastián Boltaña analyzed data. Gustavo Núñez-Acuña, Sebastián Boltaña and Cristian Gallardo-Escárate wrote and revised the manuscript. All authors reviewed the manuscript.

**Conflicts of Interest:** The authors declare no conflict of interest.

## Abbreviations

| | |
|---|---|
| DM | Deltamethrin |
| AZA | Azamethiphos |
| mRNA | messenger RNA |
| GPCRs | G protein-coupled receptors |
| mGluR | metabotropic receptor |
| iGluR | ionotropic glutamate receptor |
| IR | ionotropic receptor |
| RPKM | reads per kilobase of transcript per million mapped reads |
| SN1 | solute carrier family 8, member 3 |
| GLS | glutaminase (solute carrier family 8, member 2) |
| PP2B | protein phosphatase 2B |
| EEAT | solute carrier family 1, member 2 |
| KA | ionotropic kainate receptor |
| 7mGLuR | 7-transmembranse glutamate receptor domain containing protein |
| GLNS | glutamine synthetase/glutamate-ammonia ligase |
| PCA | principal component analysis |
| KEGG | Kyoto Encyclopedia of Genomes and Genes |

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**CAPÍTULO III.** 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.

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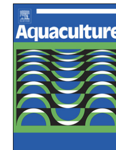

# In-feed additives modulate ionotropic receptor genes from the sea louse *Caligus rogercresseyi*: A comparative analysis in two host salmonid species

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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.

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## 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

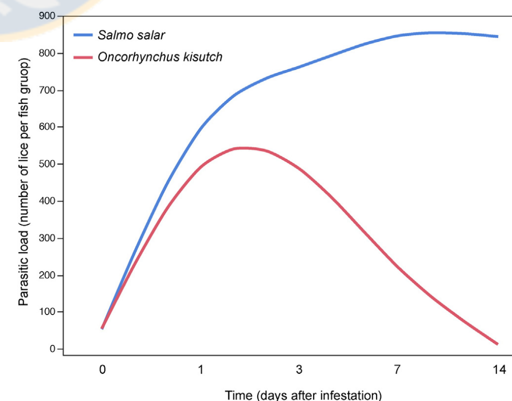



**Fig. 1.** Parasitic load data. Parasitic load measurements taken after sea lice infestation of Atlantic and Coho salmon. Y-axis corresponds to the total count of sea lice attached to fish for the entire group.

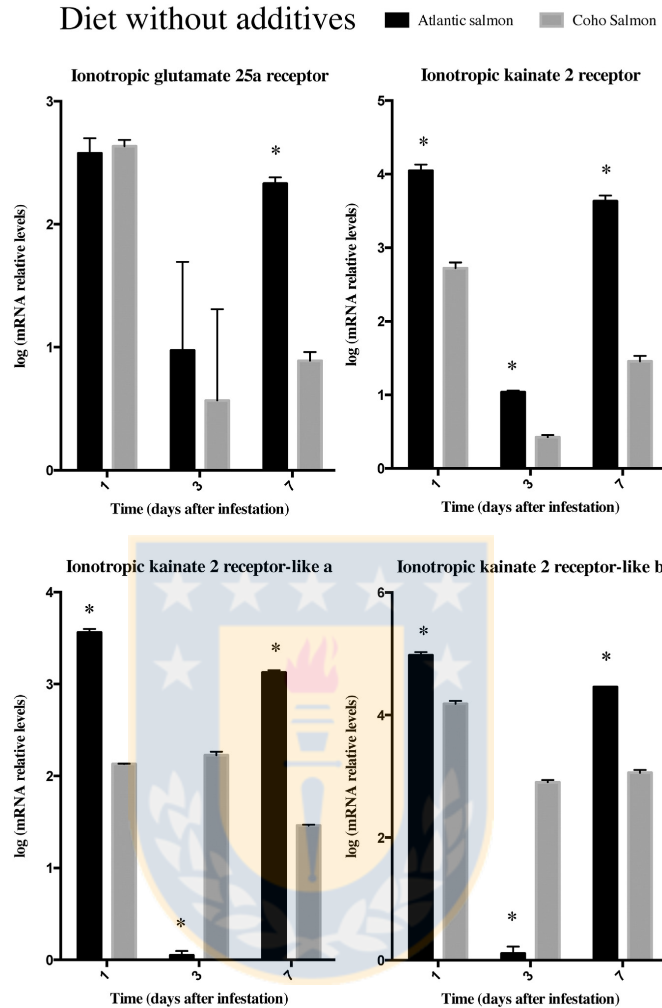



**Fig. 2.** *C. rogercresseyi* IGluR gene expression following the infestation of two fish species. Relative mRNA levels are presented in a logarithmical scale and correspond to the relative quantification of qPCR data using the *β-tubulin* gene as an endogenous control. Statistically significant differences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

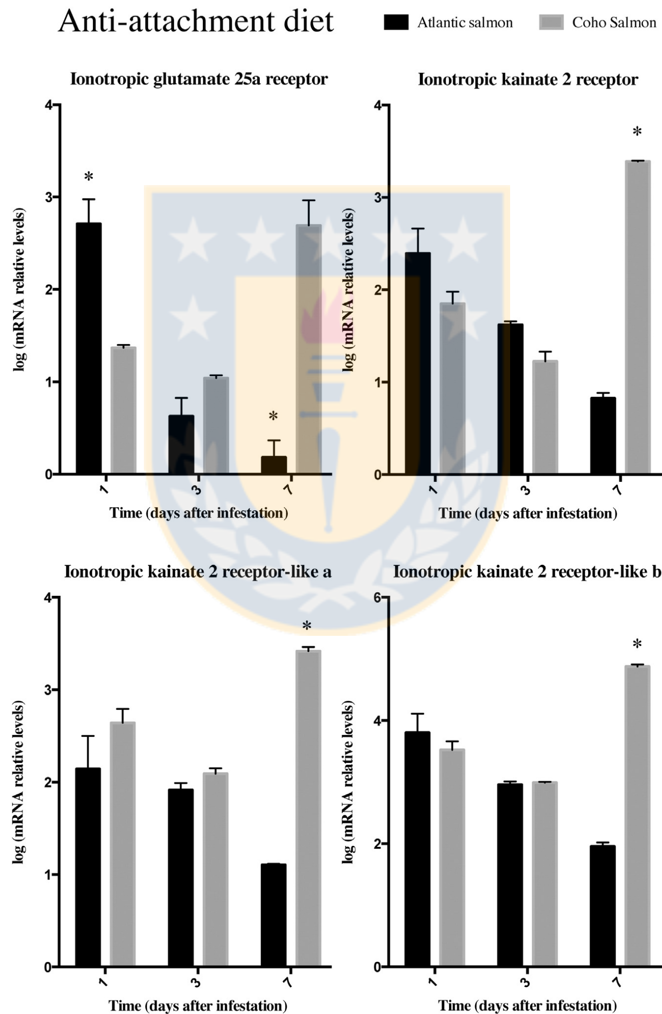



**Fig. 3.** Sea lice IGluR gene expression after exposure to anti-attachment diet salmon. Results are provided for two host species. The Y-axis corresponds to relative mRNA levels normalized to the *β-tubulin* gene. Statistically significant differences are shown with a * ($p < 0.05$).

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

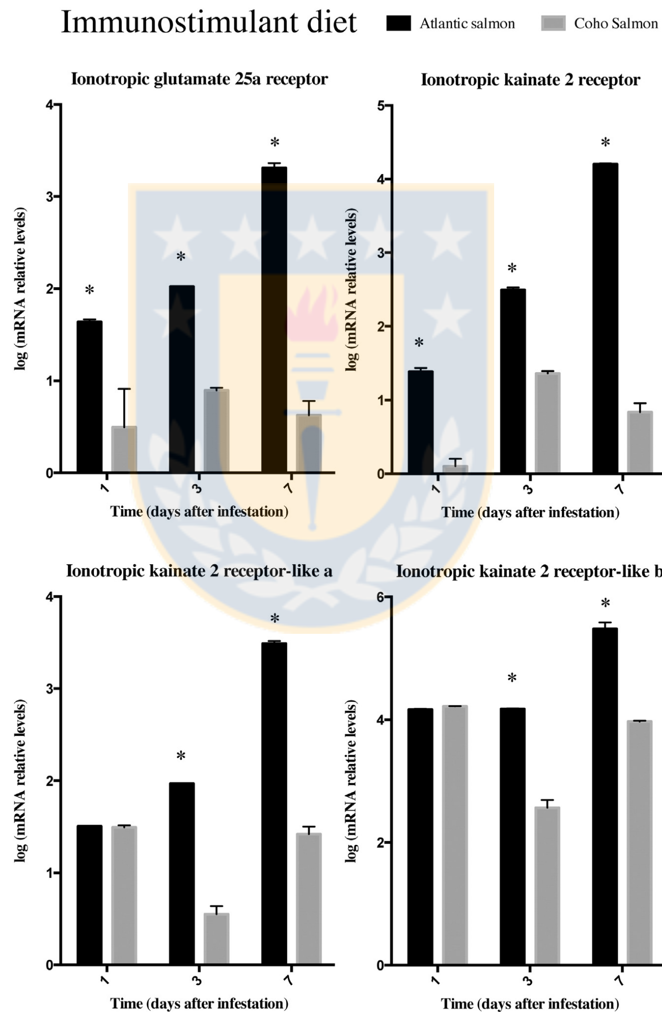
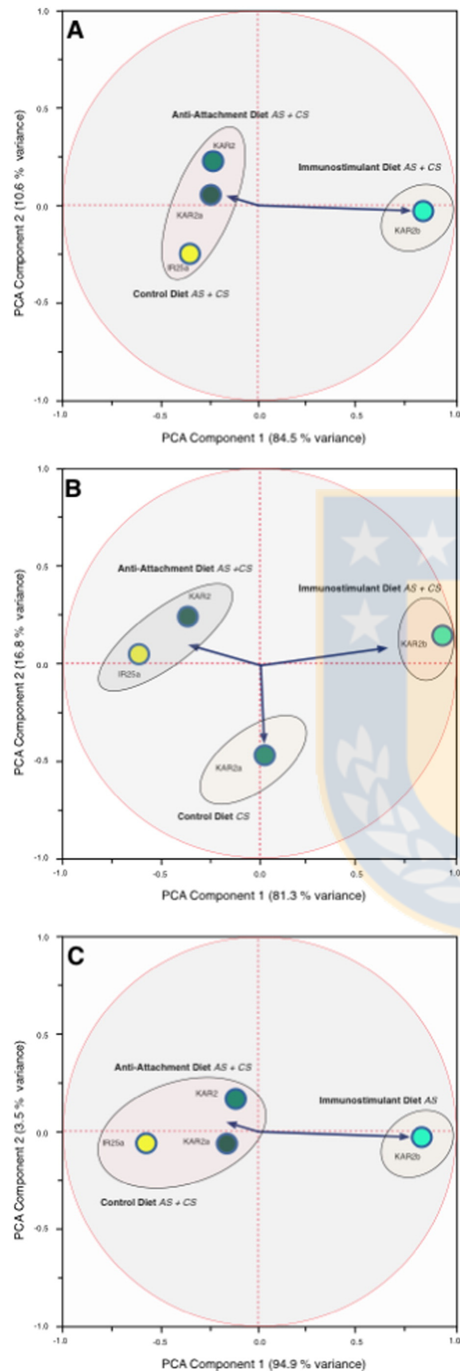



**Fig. 4.** Sea lice IGluR gene expression after exposure to immunostimulant diet salmon. Results are provided for two host species. Gene transcription levels corresponded to relative mRNA levels normalized to the *β-tubulin* gene in *C. rogercresseyi*. Statistically significant differences are shown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

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**CAPÍTULO IV.** Antimicrobial peptides from Salmon salar skin induce frontal filament development and olfactory/cuticle-related genes in the sea louse *Caligus rogercresseyi*.

*Aquaculture*, 464: 171-177, 2016.

Abstract

The discovery of key molecules involved in host-recognition has the potential to develop novel tools against the most prevalent pathogens in aquaculture. However, how mucosal surface compounds of teleost fish can modulate the attraction mechanisms of ectoparasites remains unknown. This study evaluated the effect of antimicrobial peptides (AMPs) highly expressed in *Salmo salar* and *Oncorhynchus kisutch* during the *Caligus rogercresseyi* infection. RNA-seq analysis from infested skin tissue revealed that Cathelicidin (CATHL) was the most AMPs expressed in comparison to other AMPs. To evidence morphological and transcriptional modulation of sea lice exposed to AMPs, copepodids were incubated with agar medium containing salmon mucus and CATHL1 or 2, or a combination of these. Interestingly, exposure to CATHL promoted the development of the frontal filament of sea lice, mainly CATHL2 peptide. Significant variations in transcript expressions were observed in chemosensory reception- and cuticle formation-related genes. Thus, copepodids exposed to CATHL2 showed significant increases in the mRNA abundance of cuticle formation genes and chemosensory receptors, mainly ionotropic kainate receptors. These results suggest that CATHL can trigger transcriptional responses in sea lice that are not directly linked with the effects of AMPs. The AMP-mediated activation of ionotropic kainate receptors in *C. rogercresseyi* raises novel questions regarding the molecular aspects of olfactory signal transduction in the host–parasite interactions.

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# Antimicrobial peptides from *Salmon salar* skin induce frontal filament development and olfactory/cuticle-related genes in the sea louse *Caligus rogercresseyi*

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ABSTRACT

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*Statement of relevance:* Understanding of molecules that promote the frontal filament during the sea lice infection can be used to develop novel control tools and to explore nutritional additives able to modulate the AMPs fish mucosal.

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## 1. Introduction

Sea lice infestations cause significant economic losses and social consequences for the salmonid aquaculture industry worldwide (Costello, 2009). In the Southern Hemisphere, the *Caligus rogercresseyi* sea lice species has the greatest impact. This species has three larval stages, four juvenile stages, and one adult instar stage marked by molting (Bravo, 2010). Understanding the copepodid larval stage is of particular importance to identify chemical molecules related to host recognition mechanisms (Núñez-Acuña et al., 2014; Pino-Marambio et al., 2007). Also in this stage, sea lice acquire a frontal filament that attaches to fish skin (Pike and Wadsworth, 1999). Cuticle formation genes were recently described in this species, and these may play a pivotal role in molting and cuticle formation between each developmental stage (Chávez-Mardones et al., 2016). While these processes are critical for successful sea lice development and infestation, it is unknown if host fish possess a defense response that could affect these processes.

The deployment of antimicrobial peptides (AMPs) is among the most studied defense mechanism against different pathogens (Bulet et al., 2004). Fish produce all of the major AMP classes, including defensins and cathelicidins, in addition to fish-specific peptides termed piscidins (Masso-Silva and Diamond, 2014). Regarding salmonids, AMP expressions, especially of cathelicidins, defensins, hepcidins, and histone-derived AMPs, form the basis for defense mechanisms against various pathogens (Valero et al., 2013). Of these AMPs, cathelicidins might be implicated in long-term antimicrobial response against pathogens in salmonid species, due to its response associated to continuous exposure to peptidoglycan in salmon trout (Casadei et al., 2015). On the other hand, some antimicrobial peptides, such as piscidins-3 have suggested a putative role against the ectoparasite *Ergasilus* infestations (Dezfuli et al., 2011). Regarding cathelicidins, these peptides correspond to a widely studied group of AMPs in vertebrate species, but with specific distinctive features in salmonid fishes, such as differences in their

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sequences and tridimensional structures, that could derive in novel functions in these species (Zhang et al., 2015). This class of AMPs was also highly associated to the response of Atlantic salmon to the bacterial infectious diseases known as Yersiniosis (Bridle et al., 2012). Furthermore, a previous study in Atlantic salmon showed that cathelicidin peptides are activated after sea lice infestation (*Lepeophtheirus salmonis*), while other AMPs such as defensin were suppressed (Krasnov et al., 2015). Nonetheless, there is no evidence regarding the effect that this class of AMPs could trigger in sea lice infesting salmon fishes.

The aim of this study was to assess if cathelicidins derived from salmonid species have the potential to interact with sea lice causing effects in its morphological development and attraction mechanisms. From *in silico* transcription analyses, highly expressed AMPs in infested salmons were identified. The amino acid sequences were used to synthesize AMPs, evaluating their effects on assays including sea lice at the copepodid stage. Evaluation on parasites was conducted through direct observation of frontal filament development by microscopy and expression analyses of selected genes in lice exposed to salmon AMPs. A group of genes related to cuticle formation was also used due to its relation to developmental process in this species (Chávez-Mardones et al., 2016). Furthermore, due to the involvement of the frontal filament structure in host recognition, particularly at the attachment event (between copepodid and chalimus stages), a group of genes related to the chemosensory system of sea lice, was also selected for this study (Núñez-Acuña et al., 2014). Interestingly, the cathelicidins exhibited a noticeable modulation on *C. rogercresseyi*, giving novel information of molecules present on fish mucosal with putative role in host-ectoparasite interaction.

## 2. Materials and methods

### 2.1. Gene transcription analyses of AMPs in fish skin

From NCBI Genbank database a group of salmon's AMPs sequences were obtained: cathelicidin (AY728057), defensin beta 4 (NM_001195169), defensin beta 3 (NM_001195183), hepcidin (XM_014170058) and NK-lysin (NM_001141110). The expression patterns of these AMPs were evaluated in transcriptome data obtained from salmonids species infested with sea lice *C. rogercresseyi* (unpublished data). Briefly, specimens of *Salmo salar* and *Oncorhynchus kisutch* species were used for a sea lice challenge, consisting in 35 copepodids/fish of *C. rogercresseyi*. Skin tissues samples were collected from both infested species at different sampling points: 0 (control), 7 and 14 days after infestation. Samples were fixed with RNA Later solution (Thermo Fisher Scientific, Waltham, MA, USA) and stored at −80 °C until RNA isolation, which was performed with the RNAeasy Mini Kit protocol (Qiagen, Hilden, Germany). From purified RNA, libraries for transcriptomic sequencing were made using the TruSeq RNA Library Preparation kit (Illumina, San Diego, CA, USA). Sequencing runs were conducted in a MISEQ platform (Illumina, San Diego, CA, USA).

To estimate AMP transcription levels, RNA-seq analyses were performed using default settings to calculate Transcripts per million reads (TPM) in the CLC Genomic Workbench software (version 9.0, CLCBio, Qiagen, Germany). Hierarchical clustering of TPM values was calculated in the same software and a heatmap was constructed based on Manhattan distances and a complete linkage to visualize expression changes.

### 2.2. AMP synthesis

From the expression analyses described in the previous point it was observed that cathelicidin peptide has the higher abundance and transcriptional differences among experimental groups. This peptide corresponded to cathelicidin previously reported in *S. salar*. For further analyses this gene was selected along with cathelicidin-2, which was also previously reported in the same species, for peptide synthesis (Chang et al., 2006). Both peptides were named CATHL1 and CATHL2 respectively and were chemically synthesized at GenicBio service (GenicBio Limited, http://www.genicbio.com). Sequences of peptides corresponded to RRSQARKCSRGNGGKIGSIRCRGGGTRL GGGSLIGRLRVALLLGVAPFLLDLSQINVMEIAFA for CATHL1 and RRGKPSGGSRGSKMGSKDSKGGWRGRPGSGSRPGFGSSIAGA-SGRDQGGTRNA for CATHL2 peptide. Purity and molecular weight of both peptides were determined by HPLC and mass spectrometry in the GenicBio facilities.

### 2.3. Copepodids and exposure assays

Ovigerous *C. rogercresseyi* females kept under laboratory conditions produced the 1400 copepodids used in the exposure assays. The copepodids were grouped ($n = 100$) and exposed to one of the following treatments: (1) CATHL1, (2) CATHL2, (3) both CATHL1 and 2, or (4) control group with no AMPs exposure. Three replicates of each group were used. Each group was incubated in a glass bottle with 400 mL of seawater and a Petri dish containing 30 g of agar with 1:9 of fish mucus as a source of stimulus for host recognition. To the CATHL1 and CATHL2 groups, 7 ppm of the respective AMPs was added. For the CATHL1&2 group, 3.5 ppm of each CATHL was added. All copepodids were incubated with constant aeration and 14 °C for 48 h. Then, the water was filtered; live copepodids were collected, and dead copepodids were counted and discarded.

### 2.4. Evaluation of frontal filament development

Frontal filament development in the collected copepodids was observed under a microscope. The following score of three categories were established: (A) lack of frontal filament, (B) internally formed frontal filament, and (C) fully developed and visible frontal filament. The percentage of sea lice in each category was calculated for each group. Two-way ANOVA analysis was conducted to evaluate statistical significant differences according to two variables: AMPs in each treatment and frontal filament stage. A multiple comparison of groups was performed against the control group (without AMPs) to identify where the differences occurred. *P*-values lower than 0.05 were established as cut-off to determine statistical significant differences.

### 2.5. RNA extraction and qPCR analyses

A group of cuticle formation-related genes and chemosensory transduction signal-related genes were obtained from previous publications in *C. rogercresseyi* (Chávez-Mardones et al., 2016; Núñez-Acuña et al., 2014). The selected cuticle formation genes were *Prolyl 4-hydroxlase* (P4H) and *Cuticle protein 1*, *2*, and *3* (Cut1, Cut2, Cut3). The selected chemosensory transduction genes were *Ionotropic kainate receptor 2* (KAR2), *Ionotropic kainate receptor 2-like b* (KAR2b), and *metabotropic glutamate receptor A* and *B* (mGluR-A, mGluR-B). Primer list can be found in Table 1.

To evaluate gene transcriptions, after 48 h exposure with the corresponding peptides total RNA were isolated from each group using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and manufacturer protocol. Sea lice within the same group were pooled to obtain high quality RNA. A total of 50 copepodids were pooled for each group to have enough RNA for further analyses. One pool was used for each experimental replicate (three replicates). Purity was measured using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and integrity was determined by agarose gel under denaturant conditions obtained by adding the MOPS running buffer. From 200 ng/μL of total RNA, cDNA was synthetized using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). For qPCR reactions, specific primer pairs were tested in 80 ng of cDNA using a 1:5 ratio to establish dynamic range and efficiency (efficiency in Table 1). The qPCR runs were performed with the StepOnePlus Real-Time PCR System (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) using the comparative ΔCt method. *β-tubulin*

**Table 1**
Primers list and characteristics for qPCR reactions.

| Gene | Primer sequence (5′ to 3′) | T° annealing (°C) | qPCR efficiency (%) |
|---|---|---|---|
| *KAR2* | TTTCCAAAGGGAGGGCTTTC<br>TTCACTGATAAGTCCTGCGC | 60 | 92.1 |
| *KAR2b* | TTGTCAAGTTCACAGGAGCC<br>CTCAATGTCCAACGTCGGAT | 60 | 92.27 |
| *mGluR-A* | CCCTCAAACATCCACCCAAA<br>GGAGTGGCTCATTTCCAGAG | 60 | 100 |
| *mGluR-B* | GTGCGATCTCTATGCTGGTC<br>GGCATTCCTCTTGTTGTCCA | 60 | 99.98 |
| *Cut1* | GCCTACAAGGAGGCACCCGCCGT<br>TTGAATGCGGCCGTCGGGGAGGG | 62 | 100 |
| *Cut2* | TGCCTACAAGGAGGCACCCGCCG<br>GAATGCGACGCTCGGGGAGGGCA | 66 | 100 |
| *Cut3* | AGGCTTGTATGGGGCAGGGGCGG<br>ACCGTTGCCCTTCCCGACGGACG | 66 | 108.67 |
| *P4H* | AGAATGCTCCACCACGACCTCAG<br>GGCGTTGAAGTCTGGACTCTTCCT | 63 | 100 |

was selected as the endogenous reference gene due to its stability, as determined by previous publications (Gallardo-Escárate et al., 2014; Núñez-Acuña et al., 2014). Each reaction was conducted in a final volume of 20 μL using the Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). The amplification cycle was as follows: 95 °C for 10 min, 40 cycles at 95 °C for 30 s, and 60 °C for 1 min, followed by a melting curve from 60 to 95 °C (annealing temperatures in Table 1). Statistical analysis was conducted through one-way ANOVA using the GraphPad Prism v6.0 software (GraphPad Software, Inc., La Jolla, CA, USA). Significant differences were established at $p < 0.05$.

### 2.6. Correlation and statistical analyses

A principal component analysis (PCA) of correlations was conducted using the relative mRNA abundance of each transcript (variables). Stages of frontal filament formation were used as supplementary variables. As these are categorical variables, the numbers of copepodids in each stage were used as numerical variables. The grouping variable was exposure to different CATHLs. Principal components were included with an eigenvalue >1 (Kaiser criterion). These analyses were performed in JMP v9.0 software (Statistical Discovery, SAS, Cary, NC, USA). Pearson's correlations of gene transcription levels and the number of copepodids in each frontal filament stage were estimated using JMP v9.0 software (Statistical Discovery, SAS, Cary, NC, USA). The correlation matrix was plotted using the Corrplot package (https://github.com/taiyun/corrplot) included in the R software (Tem, 2015).

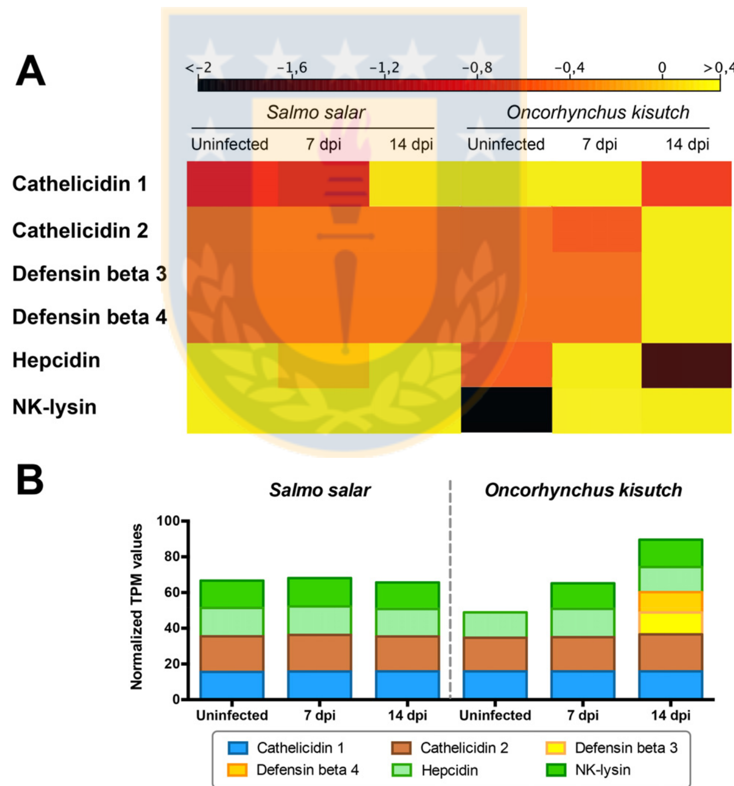


**Fig. 1.** Relative abundance of antimicrobial peptides messenger RNA in skin tissue of *Salmo salar* and *Oncorhynchus kisutch*. Gene transcription levels were calculated by measuring the TPM values through bioinformatic analyses on transcriptomic data. (A) Heatmap based on hierarchical clustering of TPM values throughout infestation times. Clustering was performed on Manhattan distances using a complete linkage. Color scaling ranges from lowest expression levels (black), to medium levels (red) and to highest expression levels (yellow). (B) Proportion of normalized TPM values of AMPs in each species at different sampling points. Normalization was done by scaling method. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

## 3. Results

### 3.1. AMP expression patterns in infested skin tissue of salmonid species

To verify if AMPs are being produced in salmon infested tissues, an evaluation of the expression patterns of six selected antimicrobial peptides was performed in transcriptome data obtained from Atlantic (*S. salar*) and Coho salmon (*O. kisutch*) infested with *C. rogercresseyi*. Differential expression results were obtained for each AMP during the 14 day-infestation trials. From 0 to 14 days, an increasing trend in the relative abundance of *Cathelicidin 1* was observed in Atlantic salmon, but a down-regulation in Coho salmon. In contrast, *cathelicidin 2* maintained its expression levels in Atlantic salmon, but is up-regulated at 14 days in Coho salmon. *Defensin beta 4* and *defensin beta* 3 exhibited a similar tendency than CATHL2, while other AMPs exhibit a different pattern including *hepcidin* and *NK-lysin*, which presented stable expression levels in Atlantic salmon, but an increasing trend in Coho salmon at 7 and 14 days after infestation (Fig. 1A). Overall, the relative abundance of all AMPs in Atlantic salmon maintained a similar expression pattern after infestation. Meanwhile, AMP expression pattern in Coho salmon while highly divergent, observing an increase in the expression levels after infestation (Fig. 1B).

### 3.2. Frontal filament development after AMP exposure

*Cathelicidin-1* (CATHL1) and *cathelicidin-2* (CATHL2) peptides were selected for chemical synthesis. Purity was 96.66% for CATHL1 and 95.46% for CATHL2. These peptides were used to evaluate their effect on sea lice frontal filament formation. Compared to the control group, the experimental copepodid groups exhibited differentiated frontal filament formation following exposure to CATHLs (Fig. 2). Exposure to these AMPs triggered frontal filament development in copepodids, increasing the number of maturing larvae in the slightly visible and fully developed stages. CATHL1 exposure increased the proportion of individual (copepodids) on score A from 10 to 38.9%, decreased the proportions of individuals on score B from 70 to 27.8% and increased the lice with score C from 20 to 33%. Meanwhile, CATHL2 exposure decreased lice with score A to 5%, with score B to 30%, and increased copepodids with score C to 65%. Overall, both peptides affected frontal filament formation in the copepodids, but this effect was the opposite between each other. Apparently, CATHL1 tends to inhibit or perhaps delay in frontal filament formation, but CATHL2 tends to promote its development. Combination of both peptides applied at the same time produced similar results than control group: 11.1% of copepodids with score A, 55.6% with score B and 33.3% with score C.

### 3.3. Gene expressions of olfactory and cuticle-related genes in *C. rogercresseyi*

Group differences for the assessed gene expressions were more notable than the differences recorded for the frontal filament stages (Fig. 3). Among the chemosensory signal transduction genes, ionotropic KAR2 and KAR2b showed strong up-regulations in larvae exposed to CATHL2. In turn, the chemosensory-related mGluR-A was less expressed in the CATHL2 group, whereas mGluR-B had a higher expression in the CATHL1&2 group, followed by CATHL2. Regarding the

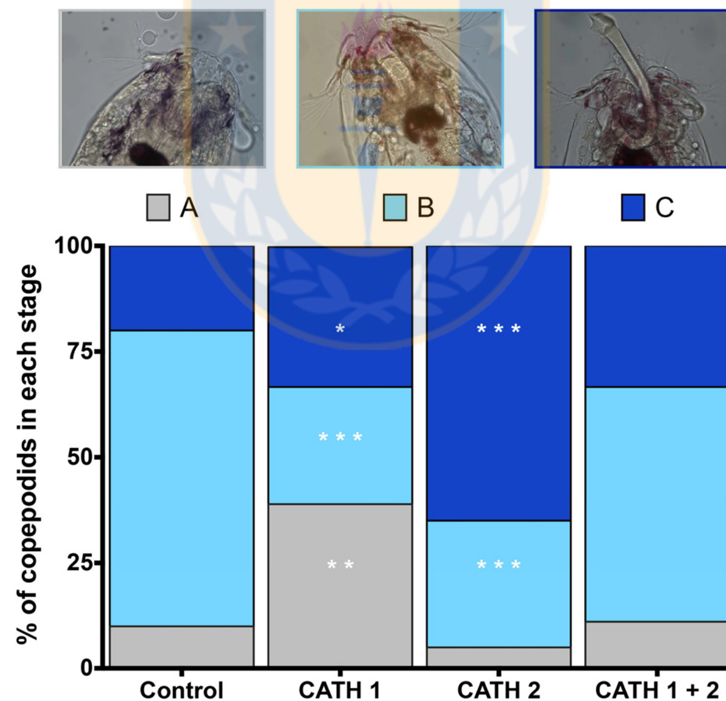


**Fig. 2.** Frontal filament formation in copepodids exposed to *Salmo salar* cathelicidins. Pictures show copepodid samples microscopically observed during four stages of frontal filament development. The graph corresponds to the number of copepodids at each stage of frontal filament development. (A) lack of frontal filament, (B) internally formed frontal filament, and (C) fully developed and exposed frontal filament. One-way ANOVA was applied to determine statistical significant differences, which are indicated by asterisks ($P$-value <0.05*, <0.01**, <0.001***).

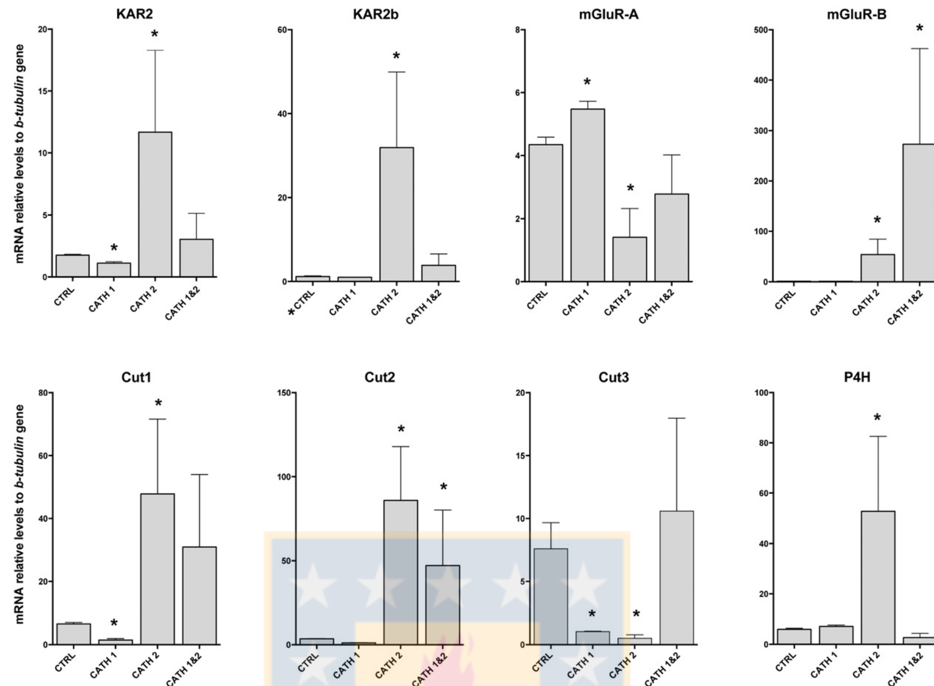


**Fig. 3.** Gene transcription levels for chemosensory receptors and cuticle formation proteins in copepodids exposed to *Salmo salar* cathelicidin. Expression levels were measured by qPCR reactions normalized by *β-tubulin* as an endogenous control. The Y-axis represents expression values quantified by the ΔΔCt method. Chemosensory-related receptors included ionotropic kainate receptor 2 (KAR2), ionotropic kainate receptor-like 2 (KAR2b), and metabotropic glutamate receptors A and B (mGluR-A, mGlur-B). Cuticle synthesis-related transcripts included cuticle proteins 1, 2, and 3 (Cut1, Cut2, Cut3) and prolyl 4-hydroxylase (P4H). Significant differences in respect to control group were set up at $p < 0.05$, and are marked with * symbols. Error bars represent standard deviation.

cuticle-related genes, most exhibited an expression pattern similar to the KAR receptors. The Cut1 and 2 genes exhibited higher expression in sea lice exposed to CATHL2 and CATHL1&2. The P4H gene presented a similar pattern, but expression was lower in the CATHL1&2 group. On the other hand, Cut3 was most expressed in CATHL1&2 and control group copepodids, while expression levels were significantly lower in sea lice exposed to CATHL2. In summary, most of the cuticle- and chemosensory transduction-related genes were associated with exposure to the CATHL2 peptide.

### 3.4. Correlations between variables

Principal component analysis explained 82.5% of correlation between gene expression and frontal filament stages (Fig. 4A). Exposure to CATHL2 was more related to greater frontal filament formation (slightly visible and fully developed) and to transcription for most of the assessed genes. Specifically, CATHL2 exposure was more related to KAR2, KAR2b, P4H, Cut1, and Cut2 transcripts. On the other hand, CATHL1 exposure was more related to a lack of frontal filament formation, and CATHL1&2 exposure was related to the expression of Cut3 and mGluR-B.

The constructed Pearson's correlation plot graphically depicted associations between each variable (Fig. 4B). The most significant positive correlations were found between fully developed frontal filaments and the transcriptional expressions of KAR2, KAR2b, and P4H. The most significant negative correlations were observed between fully developed frontal filaments and Cut3 expression, as well as between slightly visible frontal filaments and mGluR-A gene transcription. In summary, both the PCA and Pearson's correlation were consistent in associating the expression of most chemosensory reception- and cuticle-related genes with frontal filament formation and CATHL2.

## 4. Discussion

Over the last decade, the development of AMPs and polypeptides by fish species has been described as major defense mechanisms against pathogens. These molecules are expressed in secretions present in mucus produced in salmon skin (Noga et al., 2011; Rajanbabu and Chen, 2011). Relevant to this, *C. rogercresseyi* is a marine ectoparasite that feeds on fish skin and mucus (González and Carvajal, 2003), suggesting that this sea lice species is exposed to fish AMPs. However, little is known about the effects that AMPs might provoke in *C. rogercresseyi*.

The transcriptional expression of AMPs has exhibited differential responses in both host fish species (Fig. 1). Overall, temporal changes in gene expression were found with an increasing trend in Coho salmon. These findings are consistent with the hypothesis that explains resistance of Coho salmon to sea lice due to an early immune response (Johnson et al., 1992; Sutherland et al., 2014). Focusing in the parasite perspective, this is also related to an earlier defense response associated to sea lice that are infesting the susceptible species Atlantic salmon (Vera-Bizama et al., 2015).

This study was aimed on describing the effects of *S. salar* AMPs on chemosensory transduction and cuticle formation, two crucial processes in sea lice lifecycle development. These processes are also related to frontal filament formation, which allows salmon lice to attach to fish skin. Species from the *Caligus* genus have a consistent pattern of frontal

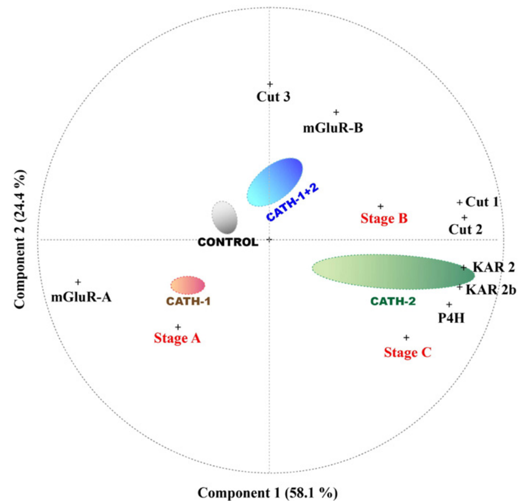


**Fig. 4.** Principal component analysis (PCA) of correlations between gene transcription levels, stage of frontal filament formation, and peptide exposure. Colored labels with a peptide name correspond to centroids of replicates in each treatment. The brown arrows correspond to gene expression levels, while green arrows indicate the frontal filament stages. Components 1 and 2 explained 82.5% of total variability. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

filament origin, formation, and function (Piasecki and MacKinnon, 1993; Pike and Wadsworth, 1999; Pike et al., 1993). Specifically, the frontal filament is preformed inside the copepodid body, posteriorly extruded, and finally attached to the skin of the fish host. Therefore, frontal filament formation in *C. rogercresseyi* is a good measurement of whether the sea louse is attempting host attachment. The present results suggest that exposure to fish AMPs, especially CATHL2, promotes frontal filament development. To further evaluate the effects of CATHLs in sea lice, gene transcription analyses were conducted.

Among the assessed genes, ionotropic kainate receptors were selected based on association with the chemosensory system (Núñez-Acuña et al., 2014). However, the relation of these receptors with chemosensory transduction in invertebrates remains under debate (Croset et al., 2010). The ionotropic kainate receptors belong to the G-protein-coupled receptor family and play a pivotal role in the neurotransmission of synaptic signals in various species (Krishnan and Schioth, 2015). Notably, a recent study described a novel class of ionotropic chemoreceptors able to bind semiochemicals (Benton et al., 2009). This class was first discovered in insects and corresponds to olfactory receptors that evolutionarily appeared before insect-exclusive olfactory and gustatory receptors (Benton, 2015).

A group of receptors from this novel class were found expressed in the olfactory organs of crustaceans (Corey et al., 2013). This finding opened the possibility of finding ionotropic receptors with olfactory functions in a number of marine species. Prior studies assessed a group of these genes and ionotropic kainate receptors in sea lice, finding expression in the presence of semiochemical-blocking compounds (Núñez-Acuña et al., 2014; Núñez-Acuña et al., 2016). Interestingly, the expression pattern of ionotropic receptors and kainate receptors in this current work was similar to the aforementioned studies. Overall, these evidences provide novel insights towards the understanding of the receptors implicated in the chemosensory systems of this marine invertebrate species.

In the present study, the kainate receptor expressions were highly associated with the presence of CATHL2, the same peptide that increased the levels of frontal filament formation (Fig. 4A–B). This suggests that a neurotransmission process might have been stimulated in response to the presence of specific molecules, such as salmon's AMPs. Unfortunately, the current study could not establish a direct link to the molecular mechanisms associated on this interaction. Future studies will be conducted to determine if binding proteins are involved in host–parasite chemosensory reception or, on the other hand, it is triggered as an immunological response during the infection process against AMP skin exposure.

Metabotropic receptors are also G-protein-coupled receptors and are some of the main neurotransmission mediators in the eukaryotic nervous system (Krishnan and Schioth, 2015). These receptors can mediate chemoreception in olfaction and gustation, but this is a slow response process that normally requires key enzymes to produce glutamate as a neurotransmitter, the presence of a second messenger, and the activation of G-proteins (Wicher, 2012). This might explain the different patterns of gene expression found between the assessed metabotropic and ionotropic receptors. Likewise, further studies are needed to determine the key enzymes and binding proteins involved in the molecular functions of G-protein-coupled receptors.

The evaluated cuticle formation genes had expression patterns similar to the ionotropic receptors (Fig. 3). This group of genes has been found in different arthropod species. In insects, there is great concern about cuticle formation-related proteins as these are associated with insecticide sensitivity or resistance (Vannini et al., 2014). Critically, cuticle thickness is related to pesticide resistance in insects (Lin et al., 2012). In relation to sea lice species, some cuticle formation-related genes were differentially expressed in resistant strains of *L. salmonis* when exposed to the insecticide emamectin benzoate (Carmichael et al., 2013). Interestingly, a wide set of cuticle-related genes have been found in *C. rogercresseyi*, and some are related to drug exposure (Chávez-Mardones et al., 2016). Nevertheless, the present study is the first to measure the abundance of cuticle-related transcripts in parasites exposed to host AMPs. Given the importance of cuticle formation in the subsequent molting stages of the sea lice lifecycle, expanding upon the present results and fully understanding the effects of the CATHLs produced by salmon hosts are important for the management of sea lice infestations.

Overall, both salmon's CATHL peptides have an opposite effect on infesting sea lice. While CATHL1 seems to inhibit the development of the frontal filament, CATHL2 might be promoting its formation. These trends are also supported by gene expression analyses. This is interesting considering that the general mechanism of AMPs molecular function consists in the defense against pathogens. The finding of an AMP with potential beneficial molecular interactions on an ectoparasite such as *C. rogercresseyi* opens the question about unraveled molecular functions of these compounds, or a molecular adaptation by the parasite. To answer this questions further studies relying on the molecular mechanisms of AMP in the host–parasite interactions context should be conducted. The cumulative knowledge in this field would be helpful to develop novel strategies for parasitic disease in salmon farming industry.

## 5. Conclusion

The obtained results suggest a strong relation between AMP exposure and the regulation of key transcripts involved in sea lice chemosensory and cuticle processes. Despite this advancement in knowledge, there is an urgent need to characterize binding proteins and downstream signaling pathways involved in these sea lice mechanisms. This information would aid in fully comprehending and possibly utilizing AMP production in fish as a strategy for defending against sea lice infestations.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.aquaculture.2016.06.023.

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The following are the supplementary data related to this article.

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**CAPÍTULO V.** The salmon antimicrobial peptide Cathelicidin-2 is a chemical fingerprint for sea lice host recognition system.

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Abstract

Chemical communication systems are essential for host-parasite interactions in all the environments. In marine environments, there are many species, such as parasitic copepods that uses chemicals cues to identify proper hosts. However, the molecular fingerprints (semiochemicals) that the parasites detect to identify hosts are not well described. Here we describe a novel kairomone for the marine copepod *Lepeophtheirus salmonis* (sea lice) host recognition, which is a salmon peptide (Cathelicidin-2), through a combination of electrophysiological, behavioral and transcriptomic analyses. Sea lice were exposed to different concentrations of Cathelicidin-2 (*Cath-2*) and chemoreceptors activity were measured using neurophysiological methods. Then, copepodids (infestive stage of sea lice) were exposed to the peptide and their swimming behavior to were analyzed through silhouette video processing (SVP) analyses. Expression patterns of genes related to the chemosensory perception of sea lice were also measured from RNA-seq analyses. Our resuts suggest that *L. salmonis* parasites can detect *Cath-2* diluted in seawater, as it is inferred by an increased frequency of chemoreceptors activity. Moreover, the peptide in the water elicited altered swimming behavior of copepodids, increasing the swim speed and changing the trajectories of the movement after they were stimulated. Chemosensory related genes were up-regulated in copepodids exposed to *Cath-2*. These combined evidences strongly suggest that *L. salmonis* parasites were more activated after being exposed to *Cath-2*, indicating a tightly link between this peptide and the chemosensory system of the parasite, which now can be described as a chemical fingerprint detectable by sea lice during host-recognition process.

1 **The Atlantic salmon (*Salmo salar*) antimicrobial peptide cathelicidin-2 is a**

2 **molecular host recognition cue for the salmon louse (*Lepeophtheirus salmonis*)**

3

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29 **Short title:** Cath-2 is a kairomone for sea lice host recognition

30 **ABSTRACT**

31 Chemical communication is essential for host-parasite interactions. In marine ecosystems,
32 obligate ectoparasites, such as sea lice, use chemical cues and other sensory signals to identify
33 appropriate hosts on which they depend to complete their life cycle. The chemical cues that
34 underlie host identification by the sea lice are not well described or characterized. Here, we report
35 a novel kairomone - the Atlantic salmon (*Salmo salar*) antimicrobial peptide cathelicidin-2 (Cath-
36 2) - for host recognition by the marine parasitic copepod *Lepeophtheirus salmonis*. *L. salmonis*
37 were exposed to 0, 7, 70 and 700 ppb of Cath-2 and neural activity, swimming behaviour and
38 gene expression profiles of animals in response to the peptide were evaluated. The
39 neurophysiological, behavioural and transcriptomic results were consistent: *L. salmonis* detects
40 Cath-2 as a water-soluble peptide released from the skin of salmon, triggering chemosensory
41 neural activity associated with altered swimming behaviour of copepodids exposed to the
42 peptide, and chemosensory-related genes were up-regulated in copepodids exposed to the
43 peptide. The host-recognition mechanism of *L. salmonis* appears to be activated by Cath-2,
44 indicating a tight link between this peptide and the salmon louse chemosensory system.

45

46 *Keywords:* Kairomone; chemical cue, Cath-2; sea lice; chemosensory system.

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54 **1. Introduction**

55 *Lepeophtheirus salmonis* (hereafter referred to as salmon lice) is an ectoparasitic copepod that
56 infests both wild and farmed salmonid fishes (mainly of the generi *Salmo*, *Salvelinus* and
57 *Oncorhynchus)*[1,2]. These parasites reside on the fish and feed on their mucus, tissue and blood,
58 reducing feed conversion efficiency and causing sores and immunosuppression[3]. Salmon lice are
59 a major disease problem in farming of Atlantic salmon (*Salmo salar*), costing the industry
60 millions of USD annually in direct losses to keep parasite loads below prescribed levels[3-5]. They
61 also play a role in the decline of some wild salmonid populations[3,6-11].

62 For ectoparasites with free-living life stages, such as *L. salmonis*, host-finding is a process
63 that is critical to complete the life cycle[12]. Thus, there has been strong adaptive-evolutionary
64 pressure for this species to develop reliable and sophisticated links to their hosts[13]. Salmon lice
65 use a combination of mechanical, visual and chemical sensory cues to identify and locate
66 potential hosts[14]. However, it has been suggested that chemical signals confer the highest
67 specificity for host recognition[15]. The detection of chemical cues released by the host organism
68 (kairomones) provides an adaptive advantage to the parasite. To detect kairomones, sea lice have
69 evolved chemical reception systems[15,16], including chemical receptors found on the antennules
70 located in the first antennae[17]. Antennules are responsive to chemical cues associated with
71 salmon hosts[18,19]. This suggests the presence of kairomone receptor proteins in these olfactory
72 structures. While general suites of chemical signals have been previously investigated[18], to our
73 knowledge, specific compounds that invoke physiological and behavioural changes in the salmon
74 lice are unknown.

75 The first description of kairomone receptor proteins in sea lice species was the finding of
76 *ionotropic receptor* (*IRs*) genes in *Caligus rogercresseyi*, which were responsive to the presence
77 of kairomone-blocking compounds[20,21]. These receptors were also linked to some

78 neurotransmission gene pathways, and had expression patterns related to the presence of
79 antiparasitic xenobiotic drugs[22]. A recent study described a group of new *IRs* genes in the salmon
80 louse, *Lepeophtheirus salmonis*, that are related to olfactory transduction, as demonstrated by *in*
81 *situ* hybridization and RNA-interference techniques[23]. These IRs are the only receptor proteins
82 with known function related to olfaction that have been found in any marine invertebrate
83 species[24,25]. The role of these receptors in olfaction was predicted from their structural similarity
84 to IRs found in insects which have known olfactory function[26]. Despite the diversity of genes
85 associated with the chemosensory system in the sea lice, the kairomones that they can identify
86 remain poorly known.

87 Salmon odour containing unidentified chemical cues triggers altered swimming behaviour
88 (related to host-seeking) in the sea lice *L. salmonis*[17,27], and in *C. rogercresseyi*, another species
89 of sea lice, which was attracted by the presence of salmon host odour[28]. *L. salmonis* also exhibit
90 chemoreceptive activity in the presence of salmon extracts from the mucous in the skin tissue,
91 which contains its odour[18]. However, the specific chemical cues that sea lice detect were not
92 identified in these studies. The first attempt to identify host-derived attractant molecules was the
93 annotation of cDNA libraries from the specific sites where the salmon is being infected by sea
94 lice, which showed up-regulation of various peptides and proteins[29]. Peptides related to the
95 antimicrobial peptide (AMP) class of cathelicidins appear to be involved in the sea lice host-
96 recognition system[22]. These AMPs have been tested by *in vitro* assays, producing significant
97 effects on frontal filament formation (which favour sea lice infection) and activation of
98 chemosensory-related genes. However, it has not yet been established that exposure to
99 cathelicidins dissolved in water modulates the neurophysiological responses or swimming
100 behaviour of sea lice.

101 The objective of this study was to determine whether AMPs related to the cathelicidin
102 family could act as kairomones that trigger host-recognition mechanisms in *L. salmonis*. To
103 achieve this, we (1) applied neurophysiological techniques to evaluate if the parasites can detect
104 AMPs as a kairomone in seawater, (2) observed swimming behaviour to determine if a host-
105 seeking response was triggered by exposure to salmon AMPs and (3) undertook transcriptomic
106 evaluation of *L. salmonis* genes to evaluate whether there was a chemosensory-related response
107 to the putative kairomone.
108

109 **2. Materials and methods**

110 *2.1. Sea lice culture*

111 Salmon lice, *Lepeophtheirus salmonis*, were obtained from Atlantic salmon reared at the Institute
112 of Marine Research's (IMR), Austevoll Research Station, Norway. Between 90 - 110 gravid
113 female lice were collected from infected salmon. Egg strings were separated from the female
114 using a scalpel and placed in a hatching container (100 cm in diameter fitted with a 100 um sieve
115 on the bottom). The egg chambers were suspended in a running seawater bath (20 L min-1) at
116 8°C under a 12 hr light: 12 hr dark photoperiod. Sieves were checked daily for the presence of
117 hatched nauplii. Unhatched egg strings were transferred to a new sieve, which was suspended in
118 the water bath. Sieves containing newly hatched nauplii were labelled with the date to generate
119 cohorts of lice of the same age. Larvae were observed under a microscope to evaluate
120 development until the copepodite stage was reached. Groups of copepodids of the same age were
121 kept separately for further analyses. Cultured lice were used for swimming behaviour,
122 neurophysiological and transcriptomics analyses (Fig. S1).
123

124 *2.2. Behavioural observations*

125 Swimming behavior was evaluated using a three-dimensional silhouette video photography

126 (SVP) system [14,30]. SVP image sequences were analysed by measuring three behavioural

127 variables: % activity (% of animals responding to the signal), swimming speed (velocity of

128 swimming hops in response to the light stimulus) and latency (the time between the start of the

129 light stimuli and when the animals initiated a hop). About 20 minutes of video (50 hz) was

130 recorded for each trial (StreamPix software v5.0, Norpix Inc., Canada). Frame-by frame analysis

131 (MANTRACK software (JASCO Scientific, Canada) was used to characterize the response of the

132 lice to the light signal. An intermittent light was used as a triggering stimulus in all the

133 behavioural experiments. The light was produced using a 1000 W Xenon arc lamp (Oriel

134 Instruments, USA) with an ON:OFF cycle of 13:47s. This ON:OFF frequency was used because

135 it produces a strong swimming response from copepodids[14]. Animals were exposed to 20

136 consecutive ON:OFF cycles. Light intensity (141 microwatts/cm) was recorded at the bottom of

137 the tank using an Ocean Optics Flame spectroradiometer. Data was extracted from the first 10 s

138 after the beginning of the light stimulus (ON) in each of the 20 cycles. The response of the

139 animals to the ON:OFF signal was evaluated for the three control group replicates to determine

140 when the swimming activity was most stable in the consecutive 20 cycles of stimulus. This

141 proved to be after 16 minutes. Therefore, the response to the ON:OFF signal at 16 minutes was

142 used to compare the responses among experimental groups. One-way ANOVA (P-value < 0.05)

143 was used to test for between group differences in the proportion of animals responding to light

144 and swimming velocity, while Tukey's post-hoc test was used to determine where the statistically

145 significant differences were.

146

147 *2.3. Selection of candidate antimicrobial peptides*

148 Peptides were obtained as described in a previous study for cathelicidin 1 and 2 (Cath-1 and
149 Cath-2) peptides[22]. In this study and additional peptide (hepcidin; Hep) was also included
150 because it has a similar function to Cath peptides (antimicrobial peptides). Synthesis, purification
151 and molecular weight measurement procedures were the same for these three peptides.

152 Selection of a compound with putative chemoattractant effect on lice was performed by
153 analysing the swimming behaviours of animals after exposure to the peptides. Groups of 150
154 healthy copepodids were used in each treatment and in the control. Each group was transferred to
155 a 20x20x20 cm glass aquarium containing 2 L of filtered seawater at 8 ºC. Animals were exposed
156 to one of five treatments before stimulation with light: (1) three classes of salmon antimicrobial
157 peptides (AMPs: Cath-1, Cath-2 and Hep), (2) whole-fish extract (WFX) and (3) 6-methyl-5-
158 hepten-2-one reagent (Sigma-Aldrich, USA). WFX preparation was as described in [18]. The 6-
159 methyl-5-hepten-2-one (6m-5h-2-one) compound is a ketone that was previously suggested as a
160 possible kairomone, which is putatively detected by *L. salmonis* during the host-recognition
161 process[31]. Each group of animals was exposed to the treatments for 5 minutes prior to stimulation
162 with the light. A sixth group of animals were placed into an aquarium without any compounds
163 and for 5 minutes before the first stimulus, which was considered as control group. Different
164 glass tanks were used for each experimental group to avoid cross-contamination of compounds,
165 and there were three replicates of every treatment group. Swimming behaviour observations were
166 conducted in order to determine the number of animals that responded to the light stimulus after
167 being incubated with the various test compounds. Selection of the candidate compound for
168 further evaluation was based on the peptide that triggered the strongest responses in the animals
169 (number of copepodids responding and swimming velocity). That compound was cathelicidin-2.
170

171 *2.4. Neurophysiological evaluation of sea lice exposed to cathelicidin-2*

172 Electrophysiological measurements and analyses were based on a technique modified from Fields
173 et al., 2007[18]. Adult female *L. salmonis* were removed from the skin of salmon cultured at the
174 Austevoll Research Station and maintained as described above. Animals were immobilized in a 4
175 cm diameter petri dish using insect pins (size-000) driven into Syl-Guard (a non-toxic silicon
176 elastomer: Sigma, USA). During the experiment, the antennules were exposed to a constant 1-2
177 ml/min flow rate of filtered seawater at 8º C in a movement-damped Faraday cage. Chemical
178 stimuli corresponded to three concentrations of Cath-2 AMP: 7, 70 and 700 ppb, while the
179 control group was filtered seawater. A 200 µm (outer diameter) capillary tube was positioned
180 next to the tip of the antennule to expose distal chemoreceptors to the different concentrations of
181 Cath-2. To record the neural activity of chemoreceptors, the antennule was perforated with 5 MΩ
182 tungsten electrode (FHC, Bowdionham ME, USA) with a 1 µm recording tip. The voltage signal
183 was normalized to a silver reference wire mounted in the water bath and amplified 10X using a
184 DC pre-amplifier and secondarily amplified up to an additional 10000X using an FHC Xcell -3
185 Microelectrode Amplifier (FHC- Bowdoinham ME, USA). Signals were pre-filtered for 50/60
186 cycle noise using a HumBug (Questscientific Instruments) and subsequently stored digitally at
187 96k Hz using commercially available software (Datawave, Fort Collins CO, USA) The data was
188 analyzed off-line using signal processing software (Datawave) and the neural responses sorted
189 based on their waveform characteristics (e.g. peak and valley amplitude, rise time, offset slope) in
190 order to identify individual neurons[32]. We used this analysis to determine instantaneous spike
191 frequency and the number of spikes occurring in response to each stimulus presentation. All
192 responses were corrected for background activity in response to filtered seawater and, where
193 appropriate, control solutions. A one-way ANOVA to test for differences between Cath-2
194 concentrations and control group.

195

196 *2.5. Silhouette video photography analyses of swimming behaviour after exposure to*

197 *different cathelicidin-2 concentrations*

198 Groups of 150 animals of the same age were obtained as described above. Four experimental

199 groups were used to determine the response of *L. salmonis* to different concentrations of Cath-2:

200 0 (control), 7, 70 and 700 ppb. Swimming behaviour of copepodids was assessed using the same

201 methods as described above, but this time reducing the intensity of the light stimulus in order to

202 obtain larger differences in responses that would be more related to the peptide that was added

203 than to the change in light intensity (assuming that less light would produce less activity in

204 copepodids, and the activity increase resulting from the chemical activator would thereby be

205 relatively stronger). The light stimulus was reduced by a factor of 100x using a neutral density

206 filter (ND2), resulting in an intensity of 1.4 microwatts/cm in the aquarium. Data recording,

207 extraction and analyses were performed as described above. All the trials were conducted in

208 triplicate.

209 After approximately 20 minutes of ON:OFF light stimulus, with the corresponding

210 incubation in Cath-2, all of the experimental groups were filtered using a vacuum suction and

211 filter paper in an Erlenmeyer flask to remove the seawater and all the animals were collected.

212 Copepodids were stored in different cryogenic tubes for each treatment group and fixed in RNA

213 Later solution (Ambion, USA) for transcriptomic sequencing analyses. This was conducted for

214 the three replicates of each group exposed to Cath-2 at different concentrations, including the

215 three control group replicates.

216

217 *2.6. Tracking the movement of sea lice after exposure to cathelicidin-2 using the*

218 *random walk test*

219 A variance ratio test (random walk test) was used to evaluate whether the parasite's movements
220 are responses to the stimulus or are just random[33]. This test is widely used to study random
221 variations and stochastic patterns[34].

222 The swimming paths of all of the individuals from the different experimental groups were
223 evaluated by calculating the displacement of every individual in x, y and z directions. These
224 calculations consisted of measuring the total distance per unit time in one direction: $q(i+1) - q(i)$,
225 where "*q*" corresponded to the direction x, y or z. The displacement of any individual was equal
226 to $\sqrt{(\text{distance-x})^2 + (\text{distance-y})^2 + (\text{distance-z})^2}$, while the total displacement of an individual is
227 the sum of the displacements during each time unit.

228 Displacement in each direction was tested using the variance ratio test as a time series,
229 comparing every time point with the subsequent point to evaluate if the path was following
230 stochastic behaviour (equations are provided in electronic supplementary material 1).
231 Comparisons of this test in the "z" direction indicated presence or absence of stochasticity in
232 vertical swimming (i.e. toward the ON:OFF light stimulus).
233

234 *2.7. Transcriptomic analyses of sea lice exposed to cathelicidin-2*

235 RNA extractions of the experimental groups were performed using the Trizol reagent (Invitrogen,
236 USA), following the manufacturer's protocol. In this case all the 150 copepodids were pooled
237 and RNA was obtained for the controls and for lice exposed to the different concentrations of
238 Cath-2. The isolated RNA was used for whole transcriptome sequencing previously described for
239 sea lice[35]. Briefly, quality of RNAs was measured in a TapeStation 2200 system (Agilent
240 Technologies, USA), concentrations in a QUBIT equip (ThermoScientific, USA). Double-strand
241 cDNA libraries were constructed using the CATS Total RNA-seq kit (Diagenode, Belgium), and

242 were quantified using the NEBNext Library Quant Illumina kit Master Mix for (NewEngland
243 BioLabs, USA). All of the libraries were diluted to 13.5 pM concentration and were sequenced in
244 a MiSeq platform (Illumina, USA), using a 250 x 250 paired-ends reads configuration and 500
245 sequencing cycles.

246 Reads obtained from high-throughput sequencing were trimmed by quality and adapters
247 were removed, using the Cutadapt phyton package [36], using a script detailed by the libraries
248 preparation kit manufacturers. Trimmed reads were used to perform a reference-based assembly
249 using the *L. salmonis* genome draft (https://licebase.org/) as a reference in the CLC Genomics
250 Workbench software (version 10.0, Qiagen Bioinformatics, USA) with the default parameters.
251 All the assembled mRNAs from the *L. salmonis* genome with coverage higher than 20 in their
252 assemblies were extracted and used as reference for further expression analyses. RNA-seq
253 analyses were performed to measure the expression levels of all of the contigs obtained by the
254 assembly in the four treatment groups using the same software. Expression levels were estimated
255 by calculating Counts Per Millions (CPM), and Kal's test was applied to calculate fold change
256 values for each Cath-2 concentration over the control group. Contigs having statistically
257 significant differences (fold change $> |4|$, p value $< 0.05$) against the control group, or among
258 different concentrations of the peptide, were extracted and annotated using the Gene Ontology
259 criteria (biological processes, molecular function and cellular components) using the Blast2GO
260 software (version 4.0.1, BioBam Bioinformatics S.L., Spain). Direct GO terms counting and GO
261 terms distributions evaluation were conducted using the same software. Three specific gene sets
262 were evaluated: chemosensory receptors, olfactory transduction and synapse. A group of genes of
263 these processes belonging to sea lice species (*L. salmonis* and *C. rogercresseyi*) were used as a
264 reference, which were obtained from previous studies [20,23]. The gene expression patterns of these

265 genes were measured in all of the experimental groups and statistical analyses were conducted

266 using the same tests and procedures as for the behavioural data.

267 Transcriptomic data is public in Sequence Read Archive (SRA) of NCBI Genbank

268 database. Accession number: SRP135658.

269

270

271 **3. Results**

272 *3.1. Identifying candidate peptide to test*

273 There was a significant effect of the addition of three peptides on the percent of the population

274 responding to the ON:OFF signal. Activity was reduced by 17.19% by Cath-1 ($p < 0.01$), 11.84%

275 by Hep ($p < 0.05$), and was increased by 7.83% by Cath-2 ($p < 0.05$) (Fig. 1A). There was a

276 significant increase in the swimming speed in the presence of Cath-2 (18.63 mm/s faster than

277 control animals, $p < 0.05$), and a significant speed reduction in the presence of Hep (9.51 mm/s

278 slower than control animals, $p < 0.05$) (Fig. 1B). There was a significant latency effect caused by

279 Hep (1.46 s more time to initiate the response than control animals, $p < 0.01$) (Fig. 1C). No

280 significant effect was observed in any of the three variables in the presence of WFX or 6m-5h-2-

281 one compounds. On this basis, Cath-2 was selected for further analysis.

282

283 *3.2. Neurophysiological response of sea lice to cathelicidin-2*

284 We recorded chemosensory responses from 7 chemosensory neurons in female *L. salmonis*. A

285 typical recording contained activity from 1 to 2 identifiable neurons. Not all neurons showed a

286 response to the chemical stimuli. In the 5 animals that responded to the chemical cues the

287 instantaneous spike frequencies to full strength Cath-2 ranged from approximately 10 to 38

288 spikes $s^{-1}$, with background activity ranging from 0 to 10 spikes $s^{-1}$. We found no neural activity

289 in response to the companion peptide (cath-1) suggesting that results are not a general response to
290 the peptide but rather specific to Cath-2.
291 There was a clear electrophysiological response of animals to Cath-2 with higher firing
292 frequency in the neural activity with increased concentration of the peptide (Fig. 2). Stimulation
293 by 700 ppb of the peptide caused the strongest bursts in neuron activity, eliciting a greater
294 response frequency in the chemosensory action potentials than other groups (Fig. 2A). In
295 addition, stimulation with 700 ppm Cath-2 was often accompanied by burst of strong motor
296 activity, in conjunction with visible movement of the antennule or the legs. The
297 neurophysiological response was repeatable within a single louse, showing little evidence of
298 adaptation carried over to subsequent trials. This observation suggested that the 60 s flushing of
299 the chamber was sufficient to remove stimuli from the previous trial. In general, the neural
300 activity for all the lice tested showed a rapid increase in firing frequency in response to an 'OFF–
301 ON' chemical signal and on average generated a 10 fold increase in firing frequencies to the
302 highest dose of the peptide. Individual neurons showed a pronounced dose response to
303 increasing concentrations of Cath-2 (Fig. 2B), and typically displayed a log-linear increase in
304 firing rate over the dose range. However, we found no indication that the neural activity saturates
305 at a concentration of Cath-2 above 700 ppb. The pooled data showed a robust log-linear dose-
306 response function over the 3 orders of magnitude increase in concentration of Cath-2. There is no
307 evidence that the animals responded to Cath-1 at concentrations up to 700ppb. A concentration of
308 0.7 ppb of Cath-2 showed a similar response as filtered seawater and Cath-1 suggesting it is at the
309 lower limit of detection for the animal.
310
311 *3.3. Swimming behavior of sea lice copepodids after exposure to cathelicidin-2*

The behaviour of the salmon lice was stimulated by the change in light from high intensity (1000W) to the OFF position after exposure to different concentrations of the Cath-2 peptide. Under this large difference in light intensity, there was a small and non-significant effect of Cath-2 concentration. These results suggest that the behavioural response of the animals may be saturated by the light stimulus, such that exposure to the peptide had little additional effect. However, after reducing the intensity of light signal by 2 orders of magnitude (ND2 filter), the swimming behavior of copepodids increased significantly with Cath-2 concentration (Fig. 3). At the lower light stimulus levels, the number of animals swimming towards the ON:OFF signal changed between control and the higher concentration of the peptide by 25-30%. Significant differences were found for both 70 ppb and 700 ppb of the compound (Fig. 3A). Swimming velocity increased from 32 mm/s (control group) to 60 mm/s (700 ppb Cath-2) (Fig. 3B). Significant changes in velocities were found for 70 ppb ($p$-value $< 0.05$) and 700 ppb ($p$-value $< 0.01$) compared to controls. In contrast, the latency time to respond to the ND2-light stimulus did not show significant changes with different Cath-2 concentrations, except for animals exposed at 700 ppb of the peptide (Fig. 3C).

### *3.4. Stochasticity of sea lice swimming behavior after exposure to cathelicidin-2*

There was a significant Cath-2 concentration-dependent reduction of animals exhibiting stochastic movement patterns: 71% of animals had stochastic movement in the control, 45% in those exposed to 7 ppb of Cath-2, 36% for 70 ppb and 50% for 700 ppb (Fig. 4A-B). Considering only vertical swimming, 50% of animals exhibited stochastic paths in the control, 36% in animals exposed to Cath-2 at 7 ppb, 9% at 70 ppb, and 8% at 700 ppb (Fig. 4C-D).

### *3.5. Transcriptome expression of sea lice in response to exposure to cathelicidin-2*

336 Hierarchical clustering of differentially expressed transcripts exhibited a unique cluster for the
337 control group and another grouping the expression of animals exposed to the peptide at the three
338 concentrations, indicating transcriptome expression patterns that depend on the presence of Cath-
339 2 (Fig. 5A). Statistical comparison revealed that more of these differentially expressed transcripts
340 were related to the exposure to Cath-2 at 700 ppb (409 transcripts with FC > |4|). Exposure to 70
341 and ppb 7 ppb of Cath-2 produced significant transcription expression in 252 transcripts and 289
342 transcripts, respectively. Common genes that were significantly expressed in the three conditions
343 corresponded to 34 transcripts, while most of the exclusively expressed transcripts were
344 associated with the 700 ppb exposure, corresponding to 302 transcripts (Fig. 5B). Furthermore,
345 there was a positive relation between the number of up-regulated transcripts and concentration of
346 the peptide. Down-regulated transcripts were also positively related to Cath-2 concentration (Fig.
347 5C). In total, 1684 transcripts were differentially expressed in the statistical comparisons of gene
348 expression among control and experimental groups.
349 Annotation (Gene Ontology criteria) of these transcripts revealed that the most important
350 biological processes varying across experimental groups were translation (24% of transcripts),
351 proteolysis (12.86%) and transmembrane transport (10%). A complete annotation list is presented
352 in supplementary materials file 2. Regarding molecular functions, the most enriched term was
353 structural constituents of cuticle (25.69%) and ribosome (14.68%), and ATP binding (14.68%).
354 In term of the cellular components, most of the differentially expressed contigs were had positive
355 hits with integral components of membrane (37.18%), the ribosomes (19.23%) and 10.26% in the
356 nucleus (Fig. 5D). Selected transcripts related to chemosensory receptors, olfactory transduction
357 and synapses, also were differentially expressed due to the presence of Cath-2 peptide (Fig. 5E).
358 The chemosensory receptor group included a subset of G-protein coupled receptors (GPCR) that
359 are related to the response to chemical stimuli: non-IGluR ionotropic receptors, variants of

360 metabotropic receptors, NMDA receptors, and chemosensory proteins. This class of membrane
361 receptors were highly up-regulated as Cath-2 peptide concentration increased in the treatments. In
362 contrast, other genes related to olfactory transduction had a different pattern. This subset included
363 all of the genes related to the olfactory transduction pathway but without including the membrane
364 receptors. In this case, the sum of the CPM values did not reveal clear differences. Other genes
365 whose expression was influenced by the Cath-2 treatment, related to the transduction of synaptic
366 signals, including the classic glutamatergic synapse and GABAergic synapse pathway; long-term
367 potentiation and depression; and other synapse transduction pathways, showed a similar trend to
368 the chemosensory receptors.

369

## 370 4. Discussion

371 *Lepeophtheirus salmonis* detect Cath-2 peptide, present at the Atlantic salmon skin and mucous,
372 when it is diluted in seawater. According to the results (Fig. 2), the threshold for the detection of
373 this peptide is likely between 7 and 70 ppb. This is a first step towards establishing an
374 ecologically meaningful response threshold for chemical detection of this peptide by free
375 swimming parasites from this species. Further characterization of the threshold sensitivity and
376 response range to Cath-2, and other putative kairomone, will clarify and quantify the role that
377 these cues play in *L. salmonis* host-seeking behaviour[37,38].

378 In an environment in which there are abundant and diverse chemicals that could act as
379 kairomone, signal filtering is critical for optimal detection and chemical communication[39]. The
380 molecular weights of the peptides used in this study were 6.11 kDa for hepcidin, 6.59 for
381 cathelicidin-1 and 5.22 for cathelicidin-2. These weights are in the range of water-soluble
382 compounds detected by *L. salmonis* (1 to 10 kDa)[18]. In addition, the approximate concentration
383 of amino acids, among other organic compounds, in the tissues of salmonids ranges from $10^{-5}$ to

384 $10^{-2}$ M [40]. These concentrations are consistent with the concentration detected by *L. salmonis*
385 based on previous physiological studies ($10^{-6}$ to $10^{-8}$ M)[18,31]. Combined, these results provide
386 support for the hypothesis that the peptide Cath-2 is a host-recognition signal.
387 Behavioural observation also suggest that Cath-2 is involved in host recognition by *L. salmonis*.
388 Swimming copepodids were stimulated when exposed to the Cath-2 peptide and showed a clear
389 concentration-dependent response (Fig. 3). This is consistent with the neurophysiological results
390 and with earlier studies that reported an increase in swimming speed after chemosensory
391 activation using salmon conditioned sea water[31]. Thus, these results support the role of the Cath-2
392 peptide as an activator of the chemosensory response in *L. salmonis*.
393 To further assess the effect of Cath-2 on the host-seeking behaviour of *L. salmonis*, we
394 evaluated the stochasticity level of their swim paths. Copepodids exposed to Cath-2 had lower
395 swim path stochasticity than control groups, suggesting that Cath-2 triggers a swimming response
396 directed towards the light stimuli. When the analysis was restricted to the vertical (z) axis of
397 movement (as has been done for *Daphnia pulex*)[41], this response was even clearer: most of the
398 stochasticity along the displacement on the z-axis was eliminated when the *L. salmonis*
399 copepodids were exposed to Cath-2, especially at higher concentrations. Thus, both
400 electrophysiological and behavioural observations strongly support the interpretation that Cath-2
401 is an important and potent host-related signal for *L. salmonis*. To evaluate whether Cath-2
402 activated the *L. salmonis* chemosensory system at molecular level, transcriptomic expression
403 analyses were conducted.
404 Exposing *L. salmonis* copepodids to Atlantic salmon Cath-2 triggered a broad differential
405 transcriptomic response that was concentration-dependent. This implies that more biological
406 processes were differentially expressed at the highest Cath-2 concentration. This is consistent
407 with the assumption that Cath-2 is a host signal for *L. salmonis* – a high concentration would

408 indicate host proximity and the parasite would respond by activating numerous gene pathways.
409 These results are analogous to those reported in other species, especially insects, that exhibit a
410 broad transcriptomic response when exposed to kairomone[42-44]. In aquatic environments, most of
411 the studies on gene expression in response to chemical cues have been conducted on *Daphnia*
412 *spp*., with results analogous to ours: an up-regulation of various genes[45,46]. Most of the
413 differentially expressed transcripts with GO annotation for molecular function in our study
414 belonged to genes related to the formation of the cuticle structure. This is consistent with a
415 previous study in which copepodids of the sea lice species *Caligus rogercresseyi* were exposed
416 to Cath-2, and cuticle-related genes were up-regulated[22]. These genes are related to the moulting
417 events[47], suggesting that the animals are activating these genes to accelerate their development in
418 response to the detection of a chemical cue indicating that a host is nearby.
419 According to Gene Ontology criteria of cellular components, most of the differentially
420 expressed genes code for proteins located in the integral component of cellular membrane. This is
421 consistent with gene expression results, because chemosensory receptors, such as ionotropic
422 receptors, are located in this membrane[26]. Congruently, a concentration-dependent gene
423 expression level was observed in these chemosensory-related genes, being consistent with
424 previous study in the sea lice species *C. rogercresseyi* exposed to the same peptide [22]. We also
425 found an up-regulation of *Ionotropic receptor 25a* gene in a Cath2 concentration-dependent
426 trend. This gene is one of the most studied olfactory receptor in sea lice species and other
427 invertebrates[48]. On the other hand, synapse-related genes, up-regulated in our experiment also in
428 a Cath2 concentration-dependent trend, are also relevant as indicators of neurotransmission
429 system in sea lice species[49]. These molecular findings are consistent with the electrophysiological
430 and swimming behavior analyses described above, strengthening the hypothesis that Cath2
431 peptide is acting as a kairomone during the host-recognition process of sea lice species.

432 The strength of the conclusions drawn in this study is based upon the inter-consistency of
433 the observations made at multiple biological levels: physiological, behavioural and molecular. *L.*
434 *salmonis* can detect Cath-2 peptide in sea water through activation of its chemoreceptors, and
435 also respond faster to a stimulus that promotes infestation (faster and more directed swimming),
436 and in parallel, a transcriptomic response related to chemosensory activation and transduction is
437 triggered. These three lines of evidence strongly support the conclusion that the antimicrobial
438 peptide Cathelicidin-2, which is produced by salmon, is a molecular fingerprint that sea lice use
439 as a kairomone during the host-recognition process.

440

441

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575

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584

585 **Authors contributions**

586 G.N.A. and C.G.E. conceived the idea. G.N.A., H.B., D.F., A.B.S. and S.S. designed the
587 experiments. D.F. performed the neurophysiological experiments and data analyses. G.N.A. and
588 S.S. performed the swimming behavior analyses and data analyses. G.N.A. performed the high-
589 throughput sequencing and bioinformatics analyses. I.O. conducted the variance ratio test to

590 evaluate stochasticity level of the movement and statistical analyses. G.N.A., C.G.E. and H.B.

591 wrote the article. All authors have commented and contributed to the manuscript. The authors

592 declare no conflicts of interest.

593

594 **Competing interests**

595 The authors declare no conflicts of interest.

596

597 **Figure Legends**

598 **Figure 1.** Swimming behavior analyses of *Lepeophtheirus salmonis* copepodids to full light

599 stimulus after incubation with five different compounds. A: percentage of copepodids

600 that respond to the stimuli; B: mean velocity of copepodids that responded to the light

601 stimulus in each group; C: median of the time that the copepodids spent before

602 responded to the light stimulus. Statistical differences relative to the control group are

603 identified as *, p-value < 0.05; **, p-value < 0.01. Control: group with no chemical in

604 the seawater, Cath-1: cathelicidin-1 peptide, Cath-2: cathelicidin-2, Hep: hepcidin,

605 WFX: whole-fish extract, 6m-5h-2one: 6-methyl-5-hepten-2-one.

606 **Figure 2.** Neurophysiological test of *Lepeophtheirus salmonis* chemoreceptor activity after

607 exposure to different concentrations of cathelicidin-2 peptide. A: normalized response of

608 neuronal activity (frequency of membrane potential recording) after exposure to the

609 peptide; B: recording of chemoreceptor activity after exposure to cathelicidin-2 at

610 different concentrations. Statistically significant differences relative to the control group

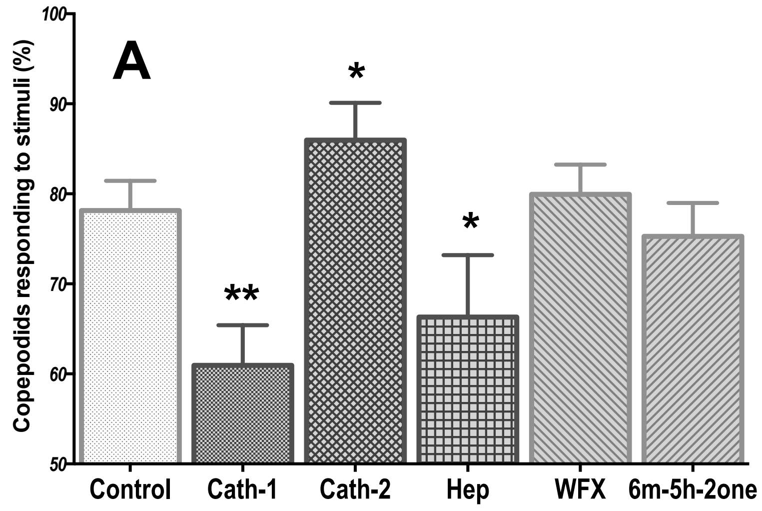
611 were identified at 70 and 700 ppb of cathelicidin-2.

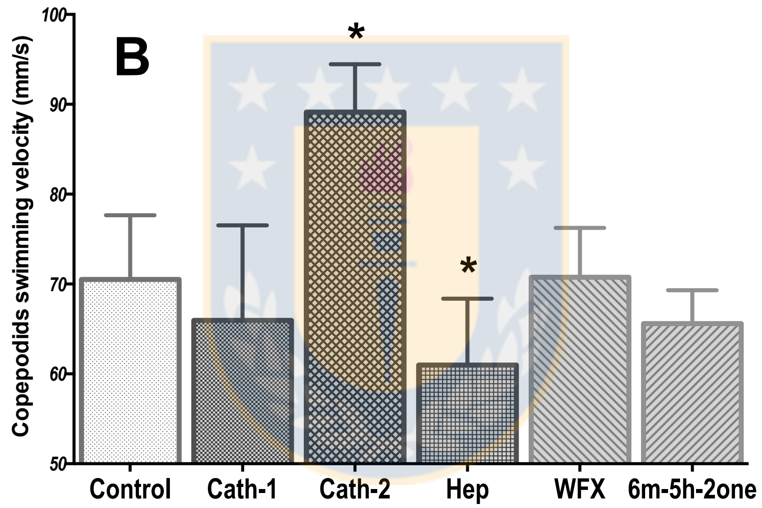
612 **Figure 3.** Swimming behavior analysis of *Lepeophtheirus salmonis* copepodids to reduced light

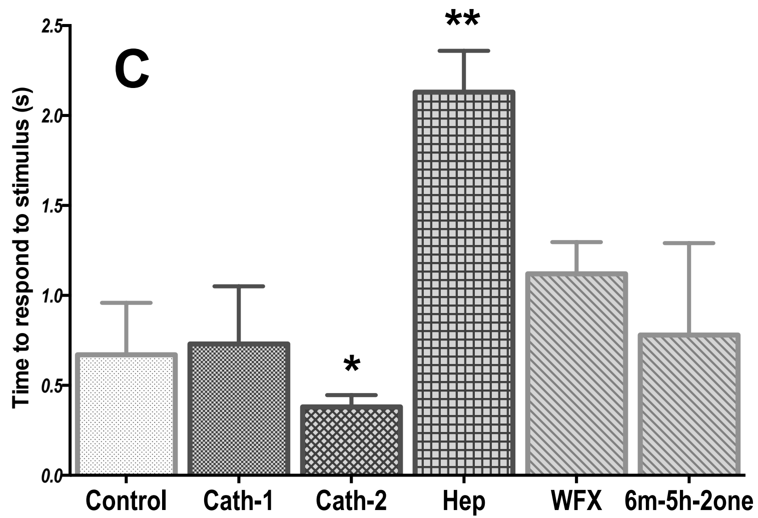
613 stimulus (ND2 filter) after incubation with cathelicidin-2 at three concentrations. A:

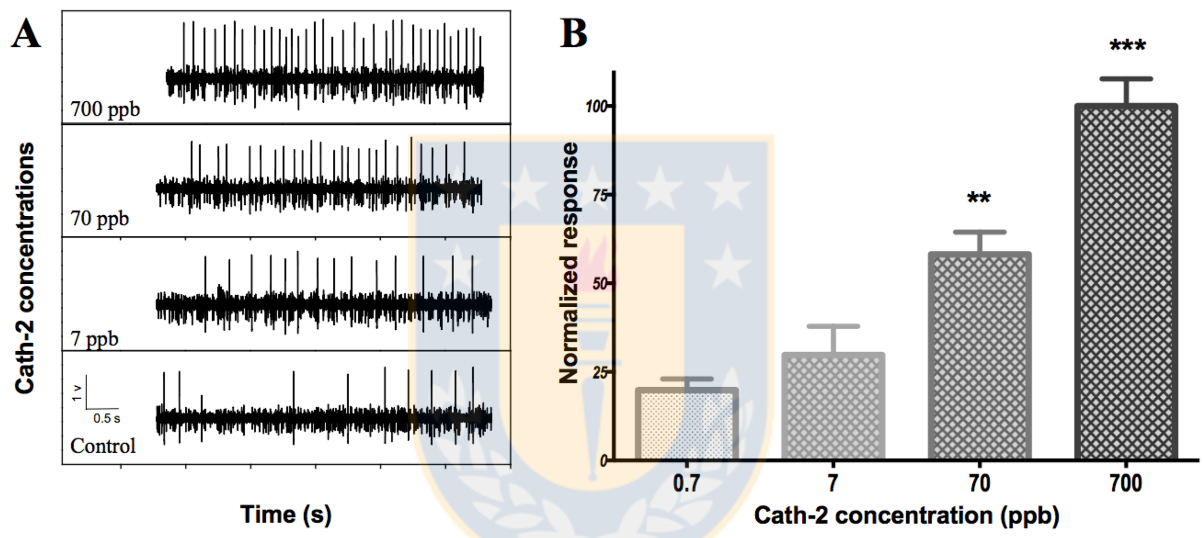
614 percentage of copepodids that respond to the stimulus; B: mean velocity of copepodids
615 that responded to light in each treatment group; C: median of the time that the
616 copepodids spent before responding to the light stimulus. Statistical differences relative
617 to the control group are identified as *, p-value < 0.05; **, p-value < 0.01. Control: no
618 chemical in the seawater.
619 **Figure 4.** Analyses of trajectories of *Lepeophtheirus salmonis* copepodids during the response to
620 the light stimulus after incubation with three concentrations of cathelicidin-2. A: 3D-
621 paths of animals responding to the light stimulus and with random walk (stochasticity) in
622 any of the component of the movement trajectory. Grey lines corresponded to paths with
623 no random walk in any component, red lines paths of animals with random walk in one
624 of the three components (x,y or z-axis), green lines animals with random walk in two
625 axes and yellow lines in the three components of the movement simultaneously. B: the
626 distribution of copepodids moving with random walk in 0 (grey), 1 (red), 2 (gray) or 3
627 (yellow) components. C: 3D-paths of copepodids to the light stimulus and with random
628 walk in the z-axis (vertical movement): grey lines indicate no random walk in the z-axis,
629 and blue indicates that the z-axis component has random walk during the light stimulus.
630 D: proportion of animals with presence (blue) or absence (grey) of random walk in the z-
631 axis.
632 **Figure 5.** Transcriptomic analyses of *Lepeophtheirus salmonis* copepodids exposed to different
633 concentrations of cathelicidin-2. A: heatmap showing hierarchical clustering of gene
634 expression (count per million, CPM values) linked by averages of manhattan distances
635 according to concentration of the peptide. B,C: number of significantly expressed
636 transcripts (fold change > |4| ; p-value < 0.05) in copepodids at each of the
637 concentrations of the peptide against the control group. D: top-ten most enriched gene

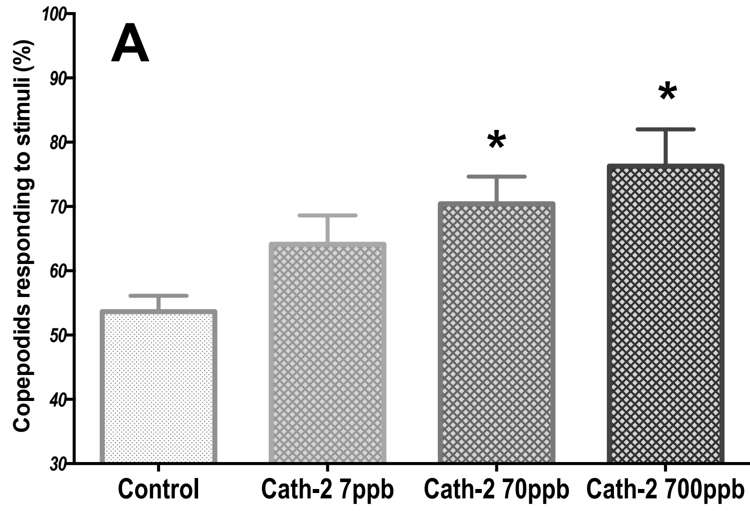
638 ontology (GO) terms according to biological processes, molecular functions and cellular
639 components of significantly expressed transcripts of any concentration of cathelicidin-2
640 relative to the control group. E: normalized expression values (CPM) of selected
641 transcripts.
642
643
644
645

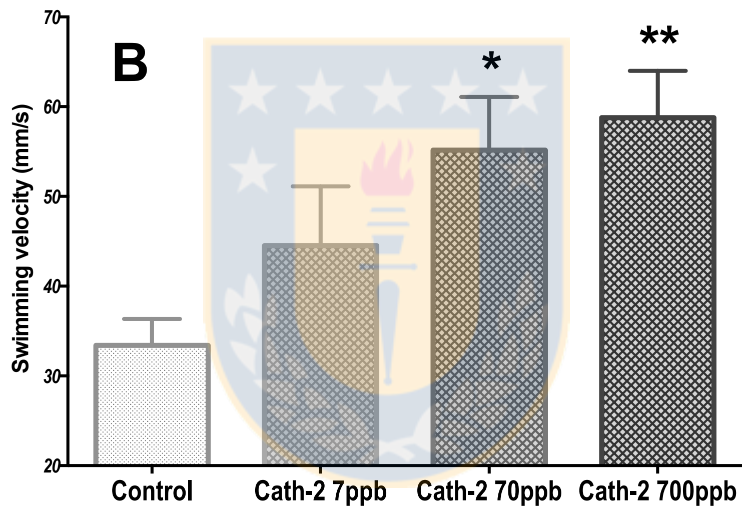

A
Copepodids responding to stimuli (%)
100
90
80
70
60
50
**
*
*
Control
Cath-1
Cath-2
Hep
WFX
6m-5h-2one

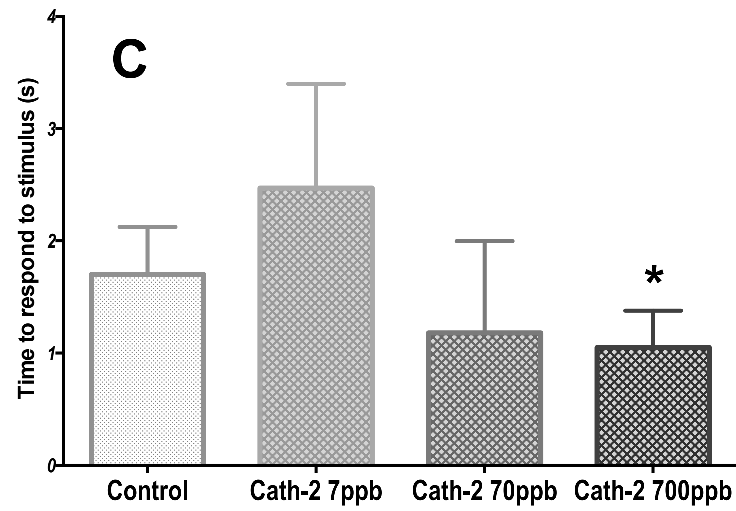

B
Copepodids swimming velocity (mm/s)
100
90
80
70
60
50
*
*
Control
Cath-1
Cath-2
Hep
WFX
6m-5h-2one

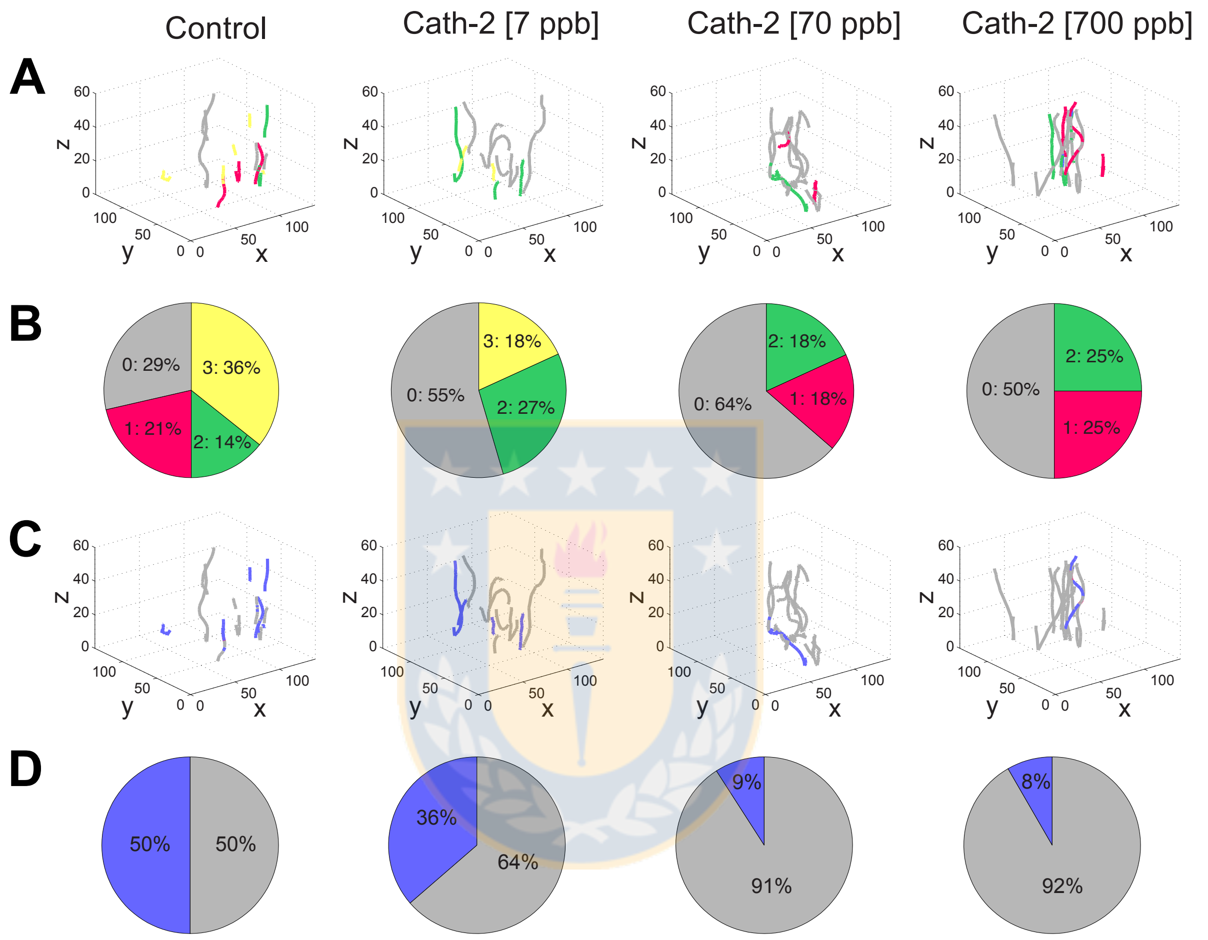

C
Time to respond to stimulus (s)
2.5
2.0
1.5
1.0
0.5
0.0
**
*
Control
Cath-1
Cath-2
Hep
WFX
6m-5h-2one

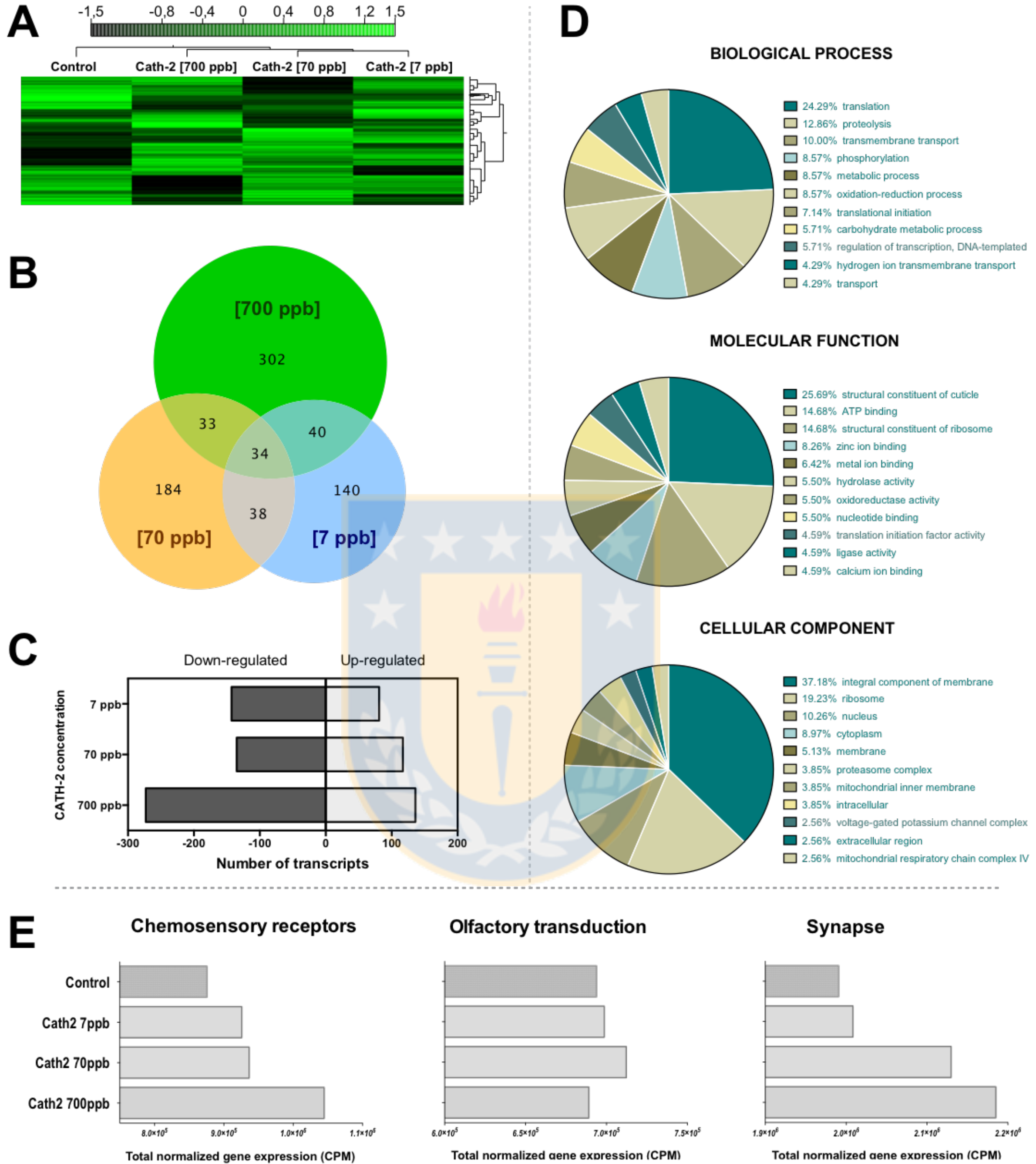

A
Cath-2 concentrations
700 ppb
70 ppb
7 ppb
Control
1 v
0.5 s
Time (s)
B
Normalized response
100
75
50
25
0
***
**
0.7
7
70
700
Cath-2 concentration (ppb)


A
Copepodids responding to stimuli (%)
100
90
80
70
60
50
40
30
*
*
Control
Cath-2 7ppb
Cath-2 70ppb
Cath-2 700ppb


B
Swimming velocity (mm/s)
70
60
50
40
30
20
*
**
Control
Cath-2 7ppb
Cath-2 70ppb
Cath-2 700ppb


C
Time to respond to stimulus (s)
4
3
2
1
0
*
Control
Cath-2 7ppb
Cath-2 70ppb
Cath-2 700ppb


Control
Cath-2 [7 ppb]
Cath-2 [70 ppb]
Cath-2 [700 ppb]
A
B
C
D
0: 29%
3: 36%
1: 21%
2: 14%
3: 18%
0: 55%
2: 27%
2: 18%
0: 64%
1: 18%
0: 50%
2: 25%
1: 25%
50%
50%
36%
64%
9%
91%
8%
92%



A
-1,5
-0,8
-0,4
0
0,4
0,8
1,2
1,5
Control
Cath-2 [700 ppb]
Cath-2 [70 ppb]
Cath-2 [7 ppb]
B
[700 ppb]
302
33
40
34
184
140
38
[70 ppb]
[7 ppb]
C
Down-regulated
Up-regulated
CATH-2 concentration
7 ppb
70 ppb
700 ppb
-300
-200
-100
0
100
200
Number of transcripts
D
BIOLOGICAL PROCESS
24.29% translation
12.86% proteolysis
10.00% transmembrane transport
8.57% phosphorylation
8.57% metabolic process
8.57% oxidation-reduction process
7.14% translational initiation
5.71% carbohydrate metabolic process
5.71% regulation of transcription, DNA-templated
4.29% hydrogen ion transmembrane transport
4.29% transport
MOLECULAR FUNCTION
25.69% structural constituent of cuticle
14.68% ATP binding
14.68% structural constituent of ribosome
8.26% zinc ion binding
6.42% metal ion binding
5.50% hydrolase activity
5.50% oxidoreductase activity
5.50% nucleotide binding
4.59% translation initiation factor activity
4.59% ligase activity
4.59% calcium ion binding
CELLULAR COMPONENT
37.18% integral component of membrane
19.23% ribosome
10.26% nucleus
8.97% cytoplasm
5.13% membrane
3.85% proteasome complex
3.85% mitochondrial inner membrane
3.85% intracellular
2.56% voltage-gated potassium channel complex
2.56% extracellular region
2.56% mitochondrial respiratory chain complex IV
E
Chemosensory receptors
Olfactory transduction
Synapse
Control
Cath2 7ppb
Cath2 70ppb
Cath2 700ppb
Total normalized gene expression (CPM)
Total normalized gene expression (CPM)
Total normalized gene expression (CPM)

**CAPÍTULO VI.** Silencing of ionotropic receptor 25a decreases chemosensory activity in the salmon louse *Lepeophtheirus salmonis* during the infective stage.

Submitted to *Aquaculture*.

Abstract

Chemoreception is critical for marine ectoparasites such as sea lice to identify and locate salmonid hosts. The molecular receptors that the parasite employs to detect host-specific chemical stimuli from hosts (kairomones) have not been well characterized. In the present study, the sea louse Ionotropic receptor 25a (IR25a) was evaluated as a chemical receptor for a specific kairomone isolated from the Atlantic salmon host. This was achieved by synthetizing RNA interference (RNAi) oligonucleotides by in vitro transcription and then exposing sea lice larvae (nauplii) to RNAi by soaking overnight. Silencing of the IR25a gene was confirmed by qPCR in experimental groups of knock-down copepodids (dsIR25a). Swimming behavior in the silenced group was observed to evaluate the activity level of lice after being incubated with the salmon cathelicidin-2 peptide, which has been identified as a host-specific kairomone. The dsIR25a group decreased expression levels of the corresponding silenced receptors and also decreased their activity level (swimming speed) by about 25% after activation by the kairomone. Since the swimming activity of sea lice copepodids is associated with the activation of the chemosensory system, these results indicate that the L. salmonis chemosensory perception system was not fully activated due to gene silencing. This demonstrates the role of ionotropic receptor 25a during host recognition by sea lice, information that could provide a basis for developing a method for sea lice control by disrupting host recognition.

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**Abstract**

Chemoreception is critical for marine ectoparasites such as sea lice to identify and locate salmonid hosts. The molecular receptors that the parasite employs to detect host-specific chemical stimuli from hosts (kairomones) have not been well characterized. In the present study, the sea louse Ionotropic receptor 25a (IR25a) was evaluated as a chemical receptor for a specific kairomone isolated from the Atlantic salmon host. This was achieved by synthetizing RNA interference (RNAi) oligonucleotides by in vitro transcription and then exposing sea lice larvae (nauplii) to RNAi by soaking overnight. Silencing of the IR25a gene was confirmed by qPCR in experimental groups of knock-down copepodids (dsIR25a). Swimming behavior in the silenced group was observed to evaluate the activity level of lice after being incubated with the salmon cathelicidin-2 peptide, which has been identified as a host-specific kairomone. The dsIR25a group decreased expression levels of the corresponding silenced receptors and also decreased their activity level (swimming speed) by about 25% after activation by the kairomone. Since the swimming activity of sea lice copepodids is associated with the activation of the chemosensory system, these results indicate that the L. salmonis chemosensory perception system was not fully activated due to gene silencing. This demonstrates the role of ionotropic receptor 25a during host recognition by sea lice, information that could provide a basis for developing a method for sea lice control by disrupting host recognition.



| | |
|---|---|
| **Keywords** | Salmon louse; IR25a; olfactory receptor; host recognition; RNA interference. |
| **Taxonomy** | Behavior Genetics, Gene Expression, Behavior |
| **Manuscript category** | Genetics |



| | |
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| **Corresponding Author** | Howard Browman |
| **Corresponding Author's Institution** | Institute of Marine Research |
| **Order of Authors** | Gustavo Nunez-Acuna, Cristian Gallardo-Escárate, Anne Berit Skiftesvik, David Fields, Howard Browman |


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Cover letter_dsIR25a_09042018_Aqua.docx [Cover Letter]

Highlights.doc [Highlights]

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Figs 1-2.docx [Figure]

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There are no linked research data sets for this submission. The following reason is given:
No data was used for the research described in the article

**INSTITUTE OF MARINE RESEARCH**
**Austevoll Research Station**
***Marine Ecosystem Acoustics Research Group***

09 April 2018

Dear Editor, Aquaculture,

Please find enclosed a manuscript entitled: “Silencing of ionotropic receptor 25a decreases chemosensory activity in the salmon louse *Lepeophtheirus salmonis* during the infective stage” for publication in Aquaculture. Please consider this manuscript as a Short communication.

The salmon louse *Lepeophtheirus salmonis* is a parasite that is one of the main threats to salmon production worldwide. This study describes the role of the Ionotropic receptor 25a (IR25a) in the chemosensory activity of *L. salmonis*. The study applied RNA interference in the free-living larval stages accompanied by observations of chemically mediated host finding behavior. The work demonstrates a tight link between the IR25a gene and the host-seeking behavior of the parasite. Hence, this paper provides novel knowledge that could be used to develop new strategies to control this parasitic species.

As Aquaculture is a widely read and highly valued journal in marine science, and considering that you have published works related to this before, we feel strongly that our study is appropriate.

Thank you for your consideration of our work. Please address all correspondence concerning this manuscript to us at our Institute and feel free to correspond with us by email.

Sincerely,

Howard I. Browman
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Austevoll Research Station
Saugeneset 16
5392 Storebø
Norway
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**Highlights**

- Sea lice ionotropic receptor function was determined by silencing assays.
- Silencing of IR25a gene suppresses host-seeking behabior in the parasite.
- Sea lice lost the capability of detecting kairomones in the water after RNAi treatment.
- Sea lice is a ectoparasite causing major impact in salmon culture worldwide.

# Silencing of ionotropic receptor 25a decreases chemosensory activity in the salmon louse *Lepeophtheirus salmonis* during the infective stage

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*These authors contributed equally to this work.

29 **ABSTRACT**

30 Chemoreception is critical for marine ectoparasites such as sea lice to identify and locate salmonid
31 hosts. The molecular receptors that the parasite employs to detect host-specific chemical stimuli
32 from hosts (kairomones) have not been well characterized. In the present study, the sea louse
33 *Ionotropic receptor 25a* (*IR25a*) was evaluated as a chemical receptor for a specific kairomone
34 isolated from the Atlantic salmon host. This was achieved by synthetizing RNA interference
35 (RNAi) oligonucleotides by *in vitro* transcription and then exposing sea lice larvae (nauplii) to
36 RNAi by soaking overnight. Silencing of the *IR25*a gene was confirmed by qPCR in experimental
37 groups of knock-down copepodids (dsIR25a). Swimming behavior in the silenced group was
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39 peptide, which has been identified as a host-specific kairomone. The dsIR25a group decreased
40 expression levels of the corresponding silenced receptors and also decreased their activity level
41 (swimming speed) by about 25% after activation by the kairomone. Since the swimming activity
42 of sea lice copepodids is associated with the activation of the chemosensory system, these results
43 indicate that the *L. salmonis* chemosensory perception system was not fully activated due to gene
44 silencing. This demonstrates the role of ionotropic receptor 25a during host recognition by sea lice,
45 information that could provide a basis for developing a method for sea lice control by disrupting
46 host recognition.

47

48

49

50 *Keywords:* Salmon louse*, IR25a,* olfactory receptor, host recognition, RNA interference.

51

52

53 **1. Introduction**

54 Myriad chemicals are released by marine organisms and form the basis for complex chemically
55 mediated interactions between conspecifics, predators and prey, and parasites and their hosts (Hay,
56 2009; Lindsey, Lasker, 1974; Steiger, Schmitt, Scaefer, 2011). In marine parasitic copepods, such
57 as Lepeophtheirus salmonis (hereafter referred to as salmon lice), the non-feeding, free-living early
58 life stages must find a suitable host before their energy reserves are exhausted. To do this, the
59 salmon lice rely on a diverse suite of sensory mechanism of which chemical communication is a
60 crucial component (Fields, Skiftesvik, Browman, 2017; Fields, Weissburg, Browman, 2007;
61 Heuschele, Selander, 2014; Mordue Luntz, Birkett, 2009). The detection of chemical signals
62 (semiochemicals, kairomones, pheromones) relies on the presence of membrane bound receptors
63 to which chemical stimulants bind and, in turn, activate sensory transduction pathways.

64 In this study, we investigate an ionotropic (IR) chemoreceptor which, when excited, leads
65 to elevated gene expression that varies depending on the concentration of host-derived chemical
66 compounds and decreases expression in the presence of semiochemical-blocking compounds
67 (Núñez-Acuña, Vera-Bizama, Boltaña, Hawes, Marambio, Wadsworth, Gallardo-Escárate, 2016).
68 This receptor initiates transduction in the ionotropic receptor 25a gene (IR25a), which is involved
69 in the response of sea lice (*Caligus rogercresseyi*) to the presence of the antimicrobial peptide
70 cathelicidin-2 (Cath-2). Cath-2 is a small peptide produced by salmon (the host fish) (Núñez-
71 Acuña, Marambio, Valenzuela, Wadsworth, Gallardo-Escárate, 2016) that has been identified as a
72 molecular fingerprint that sea lice use as a kairomone during the host-recognition process
73 (Komisarczuk, Grotmol, Nilsen, 2017). Using gene silencing techniques targeting the IR25a gene,
74 we investigated the disruption of this chemosensory pathway as a mechanism to diminish the
75 efficacy of host detection by the salmon lice. This has both inherent interest in understanding the
76 connection between parasites and hosts, as well as practical significance since sea lice parasites are

77 prevalent pests in marine aquaculture, particularly of salmonids, and cause significant economic
78 losses through damage to the fish and the cost of pest control (Costello, 2009; Igboeli, Burka, Fast,
79 2014; Torrissen, Jones, Asche, Guttormsen, Skilbrei, Nilsen, Horsberg, Jackson, 2013).
80

81 **2. Materials and methods**

82 *2.1. Sea lice culture*

83 Gravid *Lepeophtheirus salmonis* females were collected from an experimental salmon farm located
84 at the Austevoll Research Station of the Institute of Marine Research (IMR), Norway. Gravid
85 females of Lepeophtheirus salmonis were collected from an experimental salmon farm located at
86 the Austevoll Research Station of the Institute of Marine Research (IMR), Norway. Egg strings
87 were separated from the female using a scalpel and placed in a hatching container (100 cm in
88 diameter fitted with a 100 um sieve on the bottom). The egg chambers were suspended in a running
89 seawater bath (20 L min-1) at 8°C under a 14:10 hr light:dark photoperiod. Sieves were checked
90 daily for the presence of hatched nauplii. Unhatched egg strings were transferred to a new sieve,
91 which was suspended in the water bath. Sieves containing newly hatched nauplii were labelled
92 with the date to generate cohorts of lice of the same age. Larvae were observed under a microscope
93 to evaluate development until the copepodite stage was reached. Groups of copepodids of the same
94 age were kept separately for further analyses.
95

96 *2.2. Ionotropic receptor 25a gene expression after kairomone exposure*

97 Groups of 150 copepodids were exposed to different concentrations of the Atlantic salmon Cath-2
98 peptide: 7, 70 and 700 ppb in a 2 L glass tank containing filtered seawater for 5 minutes at 8º C.
99 The Cath-2 peptide was obtained by chemical synthesis (Núñez-Acuña, Marambio, Valenzuela,
100 Wadsworth, Gallardo-Escárate, 2016). After incubation, animals were filtered with a kitasato

vacuum, transferred to a cryogenic tube with 1 mL of RNA Later solution (Ambion, USA) and stored at -80º C. Total RNA was extracted from each group using Trizol (Invitrogen, USA), following the manufacturer's instructions. RNA concentrations were measured with a NanoDrop spectrometer (NanoDrop Technologies Inc., USA) and its integrity assessed by electrophoresis in a MOPS-agarose gel under denaturing conditions. From 200 ng/µL of RNAs, cDNAs synthesis reactions were performed by using the RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific, USA). Quantitative PCR reactions were conducted from cDNAs in a StepOne Plus qPCR system (LifeTechnologies, USA) in 10 µL reactions including holding stage at 95º C for 2 min, 40 cycles of 95º C for 15 s, 60º C for 60 s and a melting curve from 60º to 95º C. Master mix for *IR25a* and endogenous control genes were performed using the PowerUp™ SYBR® Green Master Mix kit (Applied Biosystems, Thermo Fisher Scientific, USA). The endogenous control corresponded to the elongation factor-1 (EF1) gene (Frost, Nilsen, 2003). Quantification of gene expression was conducted by the $\Delta\Delta C_T$ method, applying a t-test for statistical analyses with a p-value < 0.05 considered as significant.

### *2.3. IR25a gene silencing using RNAi treatment*

A specific 678 base pair (bp) region of the *IR25a* was amplified using the following primers: Fwd-GGAGTGTGGAGAGCATTCAG and Rev- CGATGCAGTATCCCGTGTAG. T7 promoter sequence was added to the oligonucleotides (TAATACGACTCACTATAGGGAGA). In vitro dsRNA synthesis was conducted applying the MEGAscript™ T7 Transcription Kit (Ambion, Thermo Fisher Scientific). Obtained dsRNA was measured with a NanoDrop spectrometer. RNAi treatments were performed according to Eichner et al. (2014). Groups of 150 copepodids were subdivided into 25 per experimental group and exposed to 2 µg of RNAi using small chambers at

124 a concentration of 20 ng/µL of seawater. Exposure to RNAi was conducted for 16 h. Two control
125 groups were used: negative control (without RNAi oligonucleotides) and animals exposed to a non-
126 related RNAi oligonucleotide, corresponding to the green fluorescent protein (GFP) gene. To
127 confirm RNAi treatment efficacy, qPCR reactions to measure gene expression levels of *IR25a* were
128 conducted on all of the experimental groups using the same methodology as described above.
129

130 *2.4. Swimming behavior analyses of dsIR25a copepodids*

131 Swimming behavior was evaluated using silhouette video photography (SVP) (Browman, St-
132 Pierre, Skiftesvik, Racca, 2003; Fields, Skiftesvik, Browman, 2017). All of the experimental
133 groups (dsIR25a, dsGFP control and no-RNAi control) – with 150 salmon lice copepodids in each
134 - were incubated in a 2 L glass tank with water containing Cath-2 as described above. A fourth
135 group was added consisted of copepodids that were not incubated with the Cath-2 peptide. A
136 flashing light from a 1000 W Xenon arc lamp (Oriel Instrument, USA) was projected from above
137 to induce a swimming response (Fields, Skiftesvik, Browman, 2017): 16 cycles of light/darkness,
138 produced by ON:OFF cycles of the lamp of 13:47 s. Video sequences were recorded using
139 StreamPix software (v 5.0, Norpix Inc., Canada) and swimming response data was extracted from
140 the first 10s after the beginning of the light stimulus (ON) in each of the 16 cycles. SVP image
141 sequences were analysed by measuring % activity (% of animals responding to the signal) and
142 swimming speed (velocity of swimming hops in response to the light stimulus) (Fields, Skiftesvik,
143 Browman, 2017). Frame-by frame analysis (MANTRACK software, JASCO Scientific, Canada)
144 was used to characterize the response of the lice to the light signal. Statistical differences were
145 evaluated by applying one-way ANOVA followed by Tukey's post-hoc test.
146

147 **3. Results**

148 *3.1. Gene transcription analyses*

149 Copepodids exposed to different concentrations of the Atlantic salmon Cath-2 peptide exhibited
150 significant differences relative to the control group (Fig. 1a). Higher expression differences were
151 found at 7 and 70 ppb ($p < 0.001$), although there were also differences in 700 ppb ($p < 0.01$) with
152 respect to the control group.

153 After RNAi treatment, qPCR analyses confirmed that the silencing assays with nauplii were
154 successful (Fig. 1b). Expression of *IR25a* was significantly lower in the dsIR25a group after
155 exposure to Cath-2 compared to the no-RNAi ($p < 0.01$) and dsGFP negative control groups.
156 However, there were also differences between the control groups; reduced expression levels were
157 also found in the dsGFP group with respect to the no-RNAi negative control ($p < 0.05$).

158

159 *3.2. Swimming behavior of the dsIR25a treated group*

160 RNAi treatment was associated with a reduction in the swimming activity of lice after being
161 exposed to Cath-2 and light signals (Fig. 2). The number of copepodids that responded to the signal
162 decreased by 17% in treated animals with respect to the control ($p < 0.05$). Treated animals
163 decreased to the same level as animals that were not incubated with Cath-2. There were no
164 significant differences between the dsGFP and no-RNAi control groups (Fig. 2a). Furthermore,
165 there was a significant reduction in swimming velocity of dsIR25a with respect to controls (18
166 mm/s slower, $p < 0.05$). The group with no Cath-2 in the water was 31 mm/s slower than control
167 ($p < 0.001$). There were no differences with the dsGFP group with respect to the control (Fig. 2b).

168

169 **4. Discussion**

170 The small peptide Cath-2, found in the mucus of salmon, activates a chemosensory transduction
171 pathway (IR25a) in sea lice (Núñez-Acuña, Marambio, Valenzuela, Wadsworth, Gallardo-
172 Escárate, 2016). Salmon lice showed a concentration-dependent increase in swimming speed in the
173 presence of Cath-2. Silencing the *IR25a* gene altered host-seeking behavior in *L. salmonis.,*
174 resulting in a pronounced reduction in the number of animals responding to the light stimulus and
175 a decrease in their average swimming speed. These observations support the conclusion that Cath-2
176 plays a direct role in the detection and identification of the host fish.

177 The expression profile of IR genes in the salmon louse species *L. salmonis* is strongly
178 correlated with chemical signals in seawater (Núñez-Acuña, Marambio, Valenzuela, Wadsworth,
179 Gallardo-Escárate, 2016). In addition, expression patterns of IR genes were located in the antennae,
180 which is where chemosensory organs are located (Komisarczuk, Grotmol, Nilsen, 2017). These
181 observations are consistent with the expression patterns obtained in this study and provide strong
182 evidence that *IR25a* is a membrane bound receptor that is part of the louse chemosensory system.

183 The IR25a gene is a member of the class of IR proteins involved in chemoreception (Croset,
184 Rytz, Cummins, Budd, Brawand, Kaessmann, Gibson, Benton, 2010). In invertebrates, IRs bind to
185 chemical signals in the environment using specific binding domains (Benton, 2015; Benton,
186 Vannice, Gomez-Diaz, Vosshall, 2009). These structural features were also found in the olfactory
187 organs of the spiny lobster (Corey, Bobkov, Ukhanov, Ache, 2013) and in the sea louse C.
188 rogercresseyi, indicating a high degree of conservation among Arthropoda (Núñez-Acuña,
189 Valenzuela-Muñoz, Marambio, Wadsworth, Gallardo-Escárate, 2014).

190 The results obtained in the present study support the hypothesis that the *IR25a* gene is
191 involved in host-recognition through a peptide released from salmon skin in response to stress or
192 pathogen infection. Further experiments will be conducted to evaluate if disrupting the expression

193 of this gene decreases the attachment rate of copepodids on salmon skin, which would be a novel
194 alternative method to control sea lice infestation in salmon farms.

195

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202 Norway).

203

204 **Conflict of interest statement**

205 The authors declare no conflicts of interest.

206

207

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269

270 **Figure legends**

271 **Figure 1**. Methodological approach used for the RNAi treatment of salmon lice *Lepeophtheirus*
272 *salmonis* copepodids to silence the *IR25a* gene. **A**: Gene expression levels of *IR25a* in copepodids
273 exposed to the cathelicidin-2 peptide. Salmon lice were exposed for 20 minutes to the peptide at 7,
274 70 and 700 ppb and gene expression levels were measured through qPCR. The control group
275 consisted of animals incubated in seawater for 20 minutes but without peptide. Each group included
276 150 animals, and exposure trials were conducted in triplicate. *B-tubulin* gene was used as
277 endogenous control. **: p –value < 0.01, ***: p –value < 0.001. **B**: Experimental design and
278 efficacy of the gene silencing experiment. Double-stranded RNAi was designed to be
279 complementary to *IR25a*, and was used for incubation in salmon lice larvae for 16 h at nauplius
280 stage II. When the animals reached the copepodid stage, *IR25a* gene expression levels were
281 measured by qPCR. The control group was not incubated with any oligonucleotide, the dsIR25a
282 group was incubated with RNAi silencing *IR25a*, and the dsGFP group was a second control
283 incubated with a non-related oligonucleotide (*GFP* gene). *: p –value < 0.05, **: p –value < 0.01.
284

285 **Figure 2.** Swimming behavior of *Lepeophtheirus salmonis* copepodids after RNAi treatment and
286 incubation with the cathelicidin-2 peptide. Groups of 150 animals from RNAi treatments - Control,
287 dsGFP and dsIR25a - were incubated with the cathelicidin-2 peptide for 20 minutes and their
288 swimming behavior was observed using silhouette video photography. A negative control group
289 was also conducted (lice without peptide in the water). **A**: Percentage of salmon lice copepodids
290 that responded to the light signal. **B**: Swimming velocity of copepodids in response to the light
291 stimulus. Statistical differences relative to the control group are identified as * p –value < 0.05,
292 *** p –value < 0.001.
293

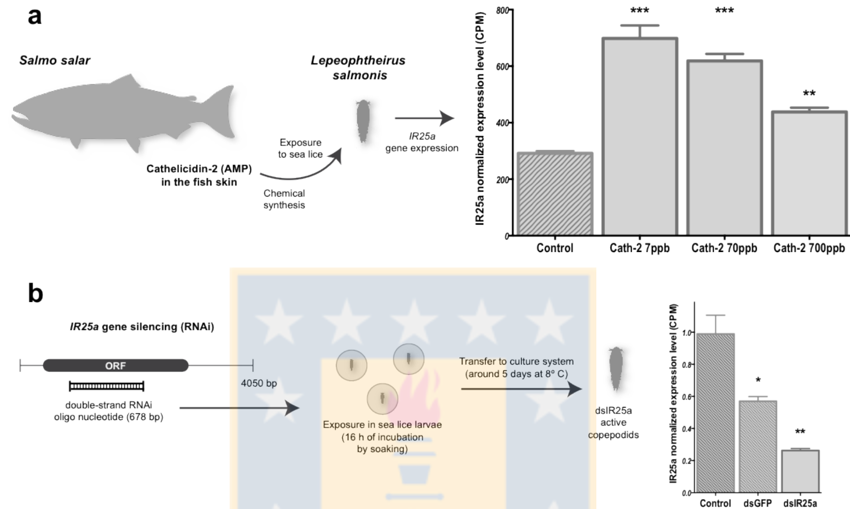


**Figure 1**

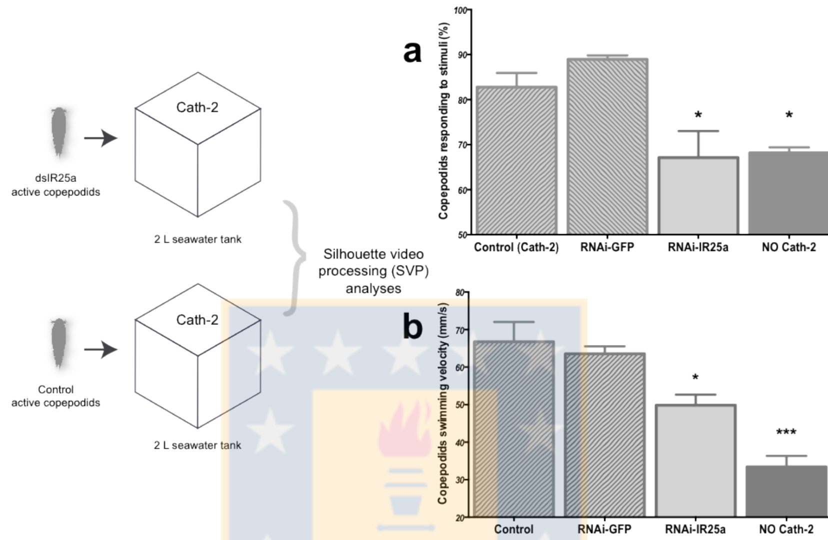


**Figure 2**

# 5. DISCUSIÓN

## Identificación receptores quimiosensoriales en *C. rogercresseyi*

Los resultados obtenidos en el Capítulo I muestran un conjunto de genes homólogos a receptores ionotrópicos (IRs) en el piojo de mar *Caligus rogercresseyi*. Esta fue la primera vez que se publica este tipo de receptores en una especie de copépodo, y la primera vez que se evalúa sus niveles de expresión en los distintos estados desarrollo en cualquier especie de invertebrado marino. Se sugiere su función relacionada con el sistema quimiosensorial de la especie, dado que estos genes presentaron una estructura altamente similar a las reportadas para insectos, con conocida función olfativa (Benton et al. 2009). Adicionalmente, estos genes tuvieron un perfil de expresión altamente relacionado con la etapa infectiva del parásito (*IR25a*), o bien a los machos adultos (receptores de kainato), quienes tienen un comportamiento de buscar a la hembra para el apareamiento (Ingvarsdóttir et al. 2002b) (Fig 6 – Capítulo I). Estos receptores generalmente actúan en conjunto con proteínas de enlace, que en el caso de los insectos se conocen como *odorant binding proteins* (OBP), las cuales no fueron encontradas en este estudio (Leal 2013). Sin embargo, esto no excluye todo tipo de proteínas de enlace, ya que existen otros tipos que pudieran interactuar con receptores quimiosensoriales, como son las *chemosensory proteins* (CSP), las cuales se encuentran en diversas especies de artrópodos (Vieira and Rozas 2011). Los resultados obtenidos respecto a la identificación de estos genes son concordantes con estudios recientes en *Lepeophtheirus salmonis*, otra especie de piojo de mar, donde se identificó que estos genes estaban principalmente asociados a la expresión génica de las anténulas del parásito, las cuales son estructuras con función quimiosensorial (Komisarczuk et al. 2017). En este caso, los genes descritos para ambas especies son altamente similares entre sí en cuanto a la estructura de la secuencia nucleotídica.

Una vez que los estímulos son detectados por los receptores quimiosensoriales, se inicia una compleja respuesta celular para transducir la señal química y activar las respuestas necesarias en el organismo. Esta transducción de señales es dependiente de una serie de proteínas que se activan o desactivan en función de impulsos nerviosos que se gatillan a partir

de la actividad iniciada por los receptores quimiosensoriales (Frank and Friedrich 2015; Laurent 2002). Dentro de los genes que se activan para permitir esta actividad sináptica se incluyen rutas genéticas importantes a nivel neuronal como son las vías glutamatergicas, gabaergicas, entre otras. De ellas participan como receptores de membrana receptores ionótropicos de glutamato (IGluR, similares a IRs) y metabotrópicos (mGluR), siendo ambos receptores asociados a proteínas G (GPCRs), y teniendo implicancia en el reconocimiento de señales derivado de la comunicación química entre organismos (Xiao et al. 2006). Los resultados presentados en el Capítulo II muestran que estos genes están altamente ligados a procesos neuronales en *C. rogercresseyi*, y que la presencia de drogas altera estocásticamente su expresión. Esto indica que la actividad neuronal y la capacidad quimiosensorial de este parásito están estrechamente ligadas, y reciben fuertes impacto por el uso de drogas antiparasitarias en la industria salmonera. Uno de los genes del sistema quimiosensorial más afectados por la aplicación de drogas fue la enzima glutamato-amonio ligasa (GLNS), la cual corresponde a una proteína multifuncional que está asociada a fenotipos resistentes a drogas (Stäubert et al. 2015). Esta enzima cataliza la conversión del neurotransmisor glutamato y amonio a glutamina, la cual juega un rol crucial en la activación de los GPCRs iniciando la cascada de señalización química del sistema quimiosensorial (Suárez et al. 2002). Adicionalmente, la actividad de GLNS está relacionada directamente con la percepción quimiosensorial, específicamente olfativa, en artrópodos terrestres (Sinakevitch et al. 2010). En conjunto, estos antecedentes sugieren que el proceso de percepción quimiosensorial del piojo de mar está relacionado con complejas rutas genéticas que incluyen rutas clave de la actividad neuronal, y que puede ser afectado por diversos tipos de moléculas presentes en el medio marino.

**Efecto de compuestos enmascarantes sobre la actividad quimiosensorial del piojo de mar *C. rogercresseyi***

Los resultados expuestos en el Capítulo III indican que existe una expresión diferencial de genes relacionados con el sistema quimiosensorial de *C. rogercresseyi*, dependiendo del tipo de hospedero que está infectando el parásito. En una escala temporal, los resultaron reflejaron que existe una relación entre la expresión de IRs en el parásito y la dinámica de la carga parasitaria en salmónidos infectados (Figs. 1-2 del Capítulo III). En este estudio se utilizó *S. salar* y *O. kisutch*, donde los últimos son una especie descrita como resistente a las infecciones por piojo de mar (Fast et al. 2002). En general, se observó mayor expresión de IRs en los parásitos afectando a la especie susceptible, lo cual, dado a las evidencias y resultados mencionados anteriormente, es indicativo de un mayor éxito de la infección. Adicionalmente, se observa que los patrones de expresión de estos genes varían al agregar aditivos a las dietas de los salmones infectados, que en este caso fueron compuestos enmascarantes para confundir al parásito en el proceso de reconocimiento de hospedero, e inmunoestimulantes para fortalecer la defensa inmune innata del hospedero (Figs. 3-4 del Capítulo III), los cuales tienen efecto validado en peces salmónidos (Núñez-Acuña et al. 2015). La disminución de la expresión de IRs en parásitos infectando a *S. salar* alimentado con dietas con enmascarantes indica que el éxito de infestación se ve reducido vía disrupción de la capacidad quimiosensorial de la especie. A la vez también confirma lo ya visto en el Capítulo I, dando más robustez a la hipótesis de que estos genes se relacionan con el proceso de identificación de hospedero en *C. rogercresseyi* (Núñez-Acuña et al. 2014; Núñez-Acuña et al. 2016). Es importante señalar que los copepoditos evaluados corresponden a aquellos que volvieron a infectar a los peces a pesar de la aplicación del compuesto enmascarante (sobrevivientes). Por el contrario, existe un aumento de la expresión de estos genes en parásitos que infectan peces alimentados con el suplemento inmunoestimulante, especialmente en la especie susceptible. Esto sugiere que a pesar de que esta dieta suplementada favorece la actividad de defensa del hospedero, produce algo, quizás como efecto secundario, que favorece el reconocimiento de hospedero por parte del piojo de mar. Por esta razón se evaluó en mayor profundidad aquellos genes que están siendo producidos por el hospedero durante la infección en los órganos blanco de la infección.

**Rol de péptidos antimicrobianos de salmones hospedero sobre el sistema quimiosensorial del piojo de mar**

En peces salmónidos, péptidos y polipéptidos dentro de los cuales se encuentran los AMPs, han sido descrito como importantes mecanismos de defensa que se expresan en órganos y tejidos que generalmente están expuestos a infecciones. Estas moléculas son secretadas mediante el mucus y en el caso de salmones a través de la piel (Noga et al. 2011; Rajanbabu and Chen 2011). Es importante señalar que el ectoparásito *C. rogercresseyi* se adhiere a la piel de salmones y comienza a alimentarse del mucus del pez hospedero (González and Carvajal 2003), por lo que se sugiere que existe contacto directo entre AMPs y el piojo de mar. Se ha reportado en otras especies de peces (lubinas), que sus AMPs pueden entrar en contacto con ectoparásitos que los infectan en los tejidos blanco (Colorni et al. 2008). Los perfiles de expresión de AMPs en hospederos mostraron que se expresan más temprano en *O. kisutch* frente a la infección con piojo de mar, lo cual es congruente con la hipótesis de que esta especie es resistente dada a una rápida y efectiva activación de los mecanismos de defensa (Sutherland et al. 2014). Los resultados del Capítulo IV muestran que uno de los AMPs producidos por los salmónidos, cathelicidina-2 (Cath-2), promueve el desarrollo del filamento frontal de *C. rogercresseyi* y activa la expresión de IRs, así como genes relacionados con la formación de la cutícula. El filamento frontal es una estructura altamente asociada con el éxito de la infección por este parásito, por lo que el estímulo de su desarrollo indica que se está favoreciendo la adhesión del piojo sobre la piel del salmón (Pike and Wadsworth 1999). Respecto de los genes sobre-expresados por Cath-2 en *Caligus*, aquellos que codifican para proteínas de la cutícula tienen múltiples funciones en artrópodos. Si bien estos genes son constitutivos a las estructuras que forman la cutícula en artrópodos, favoreciendo mudas y otros procesos asociados al desarrollo de los organismos, han sido relacionados con la sensibilidad a pesticidas (Vannini et al. 2014). Lo anterior, básicamente porque el grosor de la cutícula impide que penetren de buena forma las drogas utilizadas para el control de pestes como algunas especies de insectos (Lin et al. 2012). Esto también se describió en el piojo de mar *L. salmonis*, donde cepas resistentes a la droga benzoato de emamectina presentan altos niveles de genes codificantes para proteínas de cutícula (Carmichael et al. 2013). En el caso de *C. rogercresseyi*, estos genes también tuvieron patrones de expresión específico en piojos

tratados con las drogas azametifos y deltametrina (Chávez-Mardones et al. 2016). Si bien el diseño experimental del Capítulo IV no incluyó bioensayos con drogas antiparasitarias, los resultados obtenidas, donde se asocia fuertemente los patrones de expresión de genes relacionado con el desarrollo de la cutícula, con receptores quimiosensoriales y con el grado de desarrollo del filamento frontal, indican que estas proteínas podrían tener funciones desconocidas. Sin embargo, nuevos análisis son requeridos para probar esta hipótesis.

Los resultados obtenidos en el Capítulo V son consistentes en demostrar que Cath-2 tiene una actividad atractante sobre piojos de mar, especialmente en su etapa infectiva. Esto según los análisis neurofisiológicos (incremento de la actividad de quimiorreceptores) y conductual (incremento de conducta infectiva en el nado), sumado a nuevos análisis moleculares realizados por secuenciación masiva, donde se observa un incremento de los niveles de expresión de genes quimiosensoriales (Figs. 2,3 y 5 del Capítulo V). Estos resultados son congruentes con el resto de los capítulos, y la hipótesis de que este péptido es una kairomona para piojo de mar se robustece dada las características de Cath-2: su peso molecular, estructura tridimensional, naturaleza química y concentración cumple con todos los criterios para poder ser detectable por estos parásitos (Fields et al. 2007). Por otra parte, análisis estadísticos complejos, como lo es la prueba de random-walk, permitió establecer que el movimiento de copepoditos fue direccionado por la presencia de los estímulos aplicados, incluyendo Cath-2. Esto es congruente con lo reportado en otro crustáceo, *Daphnia pulex*, el cual tiene comportamiento de nado direccionado por presencia de estímulos que genera atractancia (Dodson et al. 1995). Los resultados de los análisis transcriptómicos también son congruentes con los capítulos anteriores. En primer lugar, existe una activación global de la respuesta transcriptómica de los copepoditos expuestos a Cath-2, lo cual es congruente con otras especies de artrópodos (terrestres) que muestran una amplia activación a nivel de transcriptomas en presencia de kairomonas específicas (Eyres et al. 2017). Además, varios de los genes sobre-expresados en los copepoditos correspondieron a genes quimiosensoriales y relacionados con la actividad sináptica de la especie, lo cual es congruente con otros estudios similares en crustáceos del género *Daphnia* (Miyakawa et al. 2010; Schwarzenberger et al. 2009). Tomando en consideración los resultados fisiológicos, genómicos, conductuales, y sumados a los ensayos morfológicos previamente descritos, se infiere que el péptido

antimicrobiano Cath-2 actúa como kairomona que atrae piojos de mar, favoreciendo las infecciones por este parásito.

## Silenciamiento de genes quimiosensoriales en piojo de mar

Respecto del ensayo de silenciamiento de la expresión génica, se utilizó el gen *IR25a*, puesto que se sugiere que es el mejor candidato para probar su funcionalidad en el reconocimiento de hospedero del piojo de mar. Esto porque los niveles de expresión de este gen aumentan progresivamente en copepoditos en función de la concentración del péptido Cath-2, descrito ya como kairomona para el parásito, disuelto en agua de mar (Fig. 1A del Capítulo VI). El método de aplicación de RNA interferente en este capítulo fue exposición directa de larvas de piojo nadando en pequeños volúmenes de agua de mar en presencia del oligonucleótido correspondiente a la concentración deseada (Eichner et al. 2014). Esto permitió efectivamente silenciar el gen, aunque también con una caída en la expresión de IR25a, menos significativa en uno de los controles (el oligo no relacionado), lo que sugiere que el método de incubación con el interferente podría ser optimizado. Futuros estudios buscarán nuevos métodos para asegurar una buena eficacia en el silenciamiento génica con una especificidad óptima. Esto no es trivial dado el pequeño tamaño de las larvas en estado copepodito, la cual es de alrededor de 100 µm (Pike and Wadsworth 1999). De todas maneras, el ensayo dio un buen resultado, mostrando que los copepoditos que no pudieron expresar el gen *IR25a* redujeron su conducta de nado asociada a la infestación de peces hospedero. Esto indica que este gen está estrechamente relacionado con el sistema quimiosensorial de la especie, y con los mecanismos de reconocimiento de hospedero en el medio marino. Este gen es uno de los más conservados de la familia de IRs (Croset et al. 2010), y a la vez un receptor que se une directamente a señales químicas en el ambiente dada la presencia de ciertos dominios conservados que le confieren tal propiedad (Benton 2015). Estos dominios fueron primeramente encontrados en un organismo marino en la langosta *Panulirus argus* (Corey et al. 2013), los cuales estructuralmente son muy similares a los encontrados en *C. rogercresseyi* en este estudio. Al ser éste un buen candidato para entender este proceso en *Caligus*, nuevos estudios estarán orientados a tratar de disociar la interacción de *IR25a* con kairomonas

específicas para el parásito, como lo es Cath-2, como vía de control de las infecciones ocasionadas por este copépodo marino.

**Comentarios finales**

La principal fortaleza de este estudio doctoral se basa en la consistencia entre los resultados obtenidos a partir de diferentes aproximaciones metodológicas, incluyendo la fisiología, morfología, pruebas conductuales y análisis transcriptómicos. De todas ellas se infiere que el piojo de mar es capaz de detectar el péptido antimicrobiano Cath-2 como señal química para identificar un hospedero correcto. Adicionalmente, se ha identificado genes clave en este proceso como son los receptores ionotrópicos y otros genes relacionados con el sistema quimiosensorial de *C. rogercresseyi*. Esto no sólo ha permitido la publicación de 6 artículos en revista de alto impacto en el área, sino que también de notas de prensa, presentaciones en jornadas con el sector salmonero, pasantías en el extranjero, entre otras actividades. Producto de esto, no se puede dar por finalizado este estudio con esta tesis doctoral, sino que, por el contrario, se abren nuevas oportunidades de investigación en el corto y mediano plazo. Estas investigaciones estarán orientadas a la implementación de nuevos métodos de control de la caligidosis en Chile, así como también del entendimiento de los mecanismos adaptativos y evolutivos que permiten el reconocimiento de hospedero en *C. rogercresseyi*.

# 6. CONCLUSIONES

1. La conservación de los genes que codifican para receptores ionotrópicos (IRs) y metabotrópicos (mGluRs), respecto a homólogos encontrados en otros artrópodos (insectos), así como sus patrones de expresión asociados a la etapa infectiva del parásito, sugieren el rol de estos genes en el proceso de reconocimiento de hospedero.
2. Una vez que se activen receptores como los ionotrópicos o metabotrópicos de *C. rogercresseyi*, se activan consigo importantes cascadas de señales genéticas que permiten la transducción de señales químicas identificadas en el ambiente marino.
3. Los compuestos enmascarantes son capaces de alterar la capacidad quimiosensorial de los parásitos. Por otro lado, la relación que existe entre estos resultados con la disminución de cargas parasitarias, sugiere que una buena manera de evaluar el efecto de estos compuestos es a través de la medición de los niveles de expresión de genes quimiosensoriales.
4. El péptido antimicrobiano Cath-2 tiene propiedades atractantes sobre piojo de mar, y por ende se puede considerar como una kairomona que favorece el reconocimiento de hospedero.
5. Dado los resultados obtenidos en el capítulo referido a la aplicación de RNA interferentes de IR25a, es posible sugerir que nuevos métodos de control del piojo de mar podrían estar basados en interferencia génica de genes quimiosensoriales, ya que se reduce su capacidad infectiva.
6. El principal aporte de esta investigación fue dilucidar los mecanismos moleculares asociados a la identificación de hospedero en el piojo de mar. La descripción de este sistema permite el diseño de nuevos métodos para controlar infecciones asociadas al piojo de mar. Estos pueden estar basados tanto en la utilización de interferencia génica, como también en el uso de enmascarantes u otros métodos preventivos como trampas de semioquímicos; los cuales podrían asegurar ser eficaces sin provocar resistencia en los parásitos, y no causarían el mismo impacto que los pesticidas en el medio marino.
7. Nuevas preguntas emergen a partir de los resultados obtenidos en este estudio doctoral, especialmente en relación a los mecanismos evolutivos involucrados en el sistema quimiosensorial del piojo de mar. Al día de hoy son desconocidos los mecanismos de

adaptación del parásito que le permiten favorecer el reconocimiento de hospedero mediante la detección de nuevas kairomonas en el medio marino. El hecho de que estos parásitos sean capaces de detectar un péptido antimicrobiano como kairomona, los cuales normalmente deben funcionar como una primera línea de defensa de los hospederos, sugieren que estos mecanismos evolutivos existen, pero aun no han sido descritos.

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