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Interacción transcriptómica ectoparásito-hospedero: Modulación de la expresión génica durante la infestación de *Caligus rogercresseyi* sobre *Salmo salar.*

Tesis para optar a grado de Doctor en Ciencias con Mención en Manejo de Recursos Acuáticos Renovables

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A mi familia

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RESUMEN

La interacción parásito-hospedero comienza una vez que el parásito identifica a su hospedador, el hospedador activa su mecanismo de respuesta generando un ambiente hostil al parásito para evitar la infección. En la actualidad, los estudios transcriptómicos permiten establecer una visión global de las vías de señalización molecular que son alteradas durante la interacción parásito-hospedero. El observar los perfiles de expresión génica durante una infección en ambos actores posibilita la identificación de moléculas asociadas al proceso de interacción. Sin embargo, conocer sólo como cambia la transcripción de ciertos genes no permite explicar por completo el proceso de interacción. Los mecanismos post-transcripcionales asociados a RNA no-codificantes sugieren una participación activa de este tipo de transcritos durante la interacción parásito-hospedero, regulando la expresión de genes expresados en respuesta al parásito. El objetivo de este estudio fue identificar mediante una aproximación transcriptómica, los procesos biológicos mediadores de la interacción parásito-hospedero y evaluar como estos son modulados a nivel post-transcripcional por ARN no codificantes, utilizando como modelo de estudio la interacción entre *Caligus rogercresseyi* y salmónidos. Para ello se realizaron ensayos de infestación con *C. rogercresseyi*, en dos especies de salmónidos (salmón del Atlántico y salmón Coho), desde los cuales se tomaron muestras de piel y riñón anterior 7 y 14 días post-infección (dpi) para secuenciación de transcriptoma. Durante el periodo de infestación se observó que salmón Coho presentaba mayor nivel de resistencia al ectoparásito que salmón del Atlántico. Desde el análisis de transcriptoma fue posible identificar diferentes mecanismos de respuesta a la infestación entre salmón del Atlántico y Coho. En salmón del Atlántico se observó regulación de las vías de respuesta asociadas a receptores tipo Toll con un alto nivel de expresión *tlr22a2*. Además, se observó una respuesta inmune de tipo nutricional reflejada en los altos niveles de regulación de transportadores de hierro como *ferritina, haptoglobina*, entre otros. Adicionalmente, en *C. rogercresseyi* infectando salmón del Atlántico se observaron altos nivel de expresión de *Cr_ferritina,* sugiriendo mecanismos de competencia por hierro entre el parásito y su hospedero. Por otra parte, en salmón Coho se observaron altos niveles de respuesta pro-inflamatoria en presencia de *C. rogercresseyi*, así como aumento en la expresión de genes asociados a la vía de degradación del grupo Hemo, e incremento de los niveles de estrés oxidativo. Este hecho sugiere un ambiente desfavorable para el ectoparásito, explicando la

significativa reducción de carga de *C. rogercresseyi* en salmón Coho luego de 7 días en comparación con salmón del Atlántico. Adicionalmente, para comprender los mecanismos regulatorios de la respuesta a la infestación por *C. rogercresseyi,* se realizó un análisis de transcriptos no-codificante en peces infectados y controles. Desde los datos de secuenciación de transcriptoma se identificaron 4.140 y 2.123 lncRNAs asociados a la infestación por *C. rogercresseyi* para salmón del Atlántico y Coho, respectivamente. Luego de localizar los lncRNAs en el genoma de cada especie de salmón, se observó que los lncRNAs expresados en salmón del Atlántico durante la infestación regularían genes asociados a respuesta inmune y estrés, mientras que lo lncRNAs identificados en salmón Coho modularían genes involucrados en procesos de reparación de tejidos e interacción celular. Adicionalmente, se caracterizó el miRNoma de salmón del Atlántico infestado con *C. rogercresseyi*. Observando gran abundancia de miRNA a los 7 y 14 dpi en riñon anterior y piel, respectivamente. Las familia de los miRNA mir-21 y mir-181 fueron las más abundantes y regularían genes asociados a la respuesta a *C. rogercresseyi,* como *alas, mmp13, crr3* y *tlr22a2.* Por otra parte, desde estudios de transcriptoma de miRNA de *C. rogercresseyi* se identificó un miRNA como posible regulador de genes de respuesta inmune de salmón del Atlántico como *tlr22, ifn-g* y *cd83*, sugiriendo un mecanismo interacción parásito-hospedero modulado por miRNAs del ectoparásito. Finalmente, se evaluó una vacuna contra *C. rogercresseyi,* utilizando como antígeno la proteína ferritina clonada desde el ectoparásito. Los peces inmunizados mostraron una reducción de la carga parasitaria de Caligus de un 97 %. Además las hembras obtenidas desde los peces inmunizados presentaron deformación de sus sacos ovigeros y cambios de expresión de los genes asociados a reproducción. En el presente estudio, utilizando una aproximación transcriptómica se evidencian diferencias en los mecanismos de respuesta a *C. rogercresseyi* entre salmón del Atlántico y Coho. Estos mecanismos además, varían con respecto a lo publicado para salmones infestados por *Lepeophteirus salmonis*. Además, dentro de los mecanismos de interacción parásito/hospedero, la respuesta inmune nutricional asociada a la regulación de hierro, se presenta como una importante estrategia para combatir la presencia de *C. rogercresseyi*. Adicionalmente, se describe el posible rol de los ARNs no codificantes en el proceso de respuesta a Caligus como modulares de las vías de señalización relevantes para la respuesta del hospedador. También se plantea el posible rol de miRNA en el proceso de interacción Caligus-salmón, lo que requiere ser validado mediante ensayos

funcionales. Finalmente, este trabajo propone una vacuna con alta eficacia para el control de la Caligidosis, la cual podría ser de gran utilidad para los programas de control de Caligidosis que afecta ampliamente la industria salmonera en Chile.

ABSTRACT

The parasite-host interaction begins when the host recognice to the parasite and activate the response mechanism, generating an adverse environment to avoid the parasite infections. Actually, the transcriptome studies allow establish a global vision around the molecular pathways involved in the host-parasite interaction. Furthermore, the observation of expression profiles in both organisms during the infection allows identifying molecules associated to interaction process. However, knowing the genes involved in parasite-host interaction does not explain all transcriptome process involved during the interaction. Some transcripts known as non-coding RNA have important roles during gene expression in response to infections. Increasing genomics information around the parasite-host interaction would allow propose new strategies to parasite control. The aim of this study was identified the signaling pathways associated to parasite-host interaction and the role of non-coding RNA in their post-trancriptional modulation, using as study model, the interaction between the ectoparasite *C. rogercresseyi* and salmon. Infestation trials were conducted in Atlantic and Coho salmon with *C. rogercresseyi*, samples of skin and head kidney were collected at 7 y 14 dpi. During the infestation period differences in parasite load was observed between Atlantic and Coho salmons, showing Coho salmon as resistant specie. From transcriptome analysis differences between response mechanism of Atlantic and Coho salmon to *C. rogercresseyi* infestation were observed. Atlantic salmon response was associated to Toll-like receptor pathway, with an increase of *tlr22a2* expression levels during the infestation. Moreover, a nutritional immunity strategy was observed in Atlantic salmon with an expression increase of *ferritin* and *haptoglobin* genes. Additionally, an up regulation of *ferritin* gene was also observed in *C. rogercresseyi* infested Atlantic salmon, suggesting an iron competition between parasite and host. Coho salmon presented an upregulation of pro-inflammatory response during *C. rogercresseyi* infestation, in addition to an expression increase of Heme degradation pathways genes and increasing of ROS levels, producing a hostile environment to the parasite, which could explained the low *C. rogercresseyi* load in Coho salmon 7 dpi. Moreover, non-codings transcripts were analyzed in order to understand the mechanism involved in gene regulation. From Atlantic and Coho salmon transcriptome data were identified 4,140 and 2,123 lncRNA, respectively. The lncRNAs were localized in the genome of each salmon species for

identification of neighbor genes. Atlantic salmon neighbor genes were associated to immune and stress response. In the case of Coho salmon the processes associated to lncRNA neighbor genes were involved in cell interactions and tissue reparation. Additionally, miRNome of Atlantic salmon during *C. rogercresseyi* infestation were sequencing. High abundance of miRNA was observed at 7 and 14 dpi in head kidney and skin of Atlantic salmon, respectively. Furthermore, the most abundance miRNA families were mir-21 and mir-181. Moreover, a target genes prediction performed associated mir-181 with *alas* and *mmp13*, mir-21 to *ccr3* and mir-140-4 to tlr22. Furthermore, from miRnome of *C. rogercresseyi* Bantam miRNA is showing as putative role in Atlantic salmon immune genes regulation, with putative target genes *tlr22*, *ifn-g* and *cd83,* sugesting a parasite-host interaction mechanism regulated by ectoparasite miRNA. Finally, it was tested a vaccine formulation using a recombinant ferritin protein, characterized from *C. rogercresseyi*. The Atlantic salmon immunized with *Cr_ferritin* vaccine, showed a 97 % of parasite load reduction in compared with control group. Furthermore, in *C. rogercresseyi* females were observed phenotype changes with eggs strain shorter and egg deformation comparing with the females not exposed to salmon vaccinated. Additionally, changes in gene regulation mainly in reproduction genes were observed. These study contribute with new information about *C. rogercresseyi*-salmon interaction used transcriptome approach. Differences between Atlantic and Coho salmon response mechanisms were identified. Furthermore, these mechanims are different that were reported in salmon infested with *L. salmonis*. The nutritional immunity associated to iron regulation, appears as strategy to reduce the infestation and important parasite-host interaction mechanisms. Additionally, is describing the putative role of non-coding RNA in the process of host response to *C. rogercresseyi.* Furthermore, is proposing a miRNA role in Caligus-salmón interaction, results that require functional analysis to be validated. Finally, is developing a vaccine with high efficacy to reduce Caligus load. Tool that could be usefull for Caligus controls programs in Chile.

INTRODUCCIÓN

1. Interacción parásito-hospedero

Tanto plantas como animales se ven constantemente expuestos a parásitos. La interacción parásito-hospedero es una relación compleja donde uno de los organismos se ve beneficiado del otro. La susceptibilidad o resistencia del hospedador dependerá de la capacidad del parásito de establecerse de manera exitosa o no [1, 2]. En presencia de un parásito el hospedero presenta cambios en su comportamiento, reproducción, regulación de respuesta inmune, entre otros procesos [2]. El parásito para lograr establecerse en el hospedero debe contrarrestar los mecanismos de defensa de éste [1]. Es por esto, que los parásitos han evolucionado adquiriendo múltiples estrategias para evadir los mecanismos de defensa de sus hospederos, y de manera paralela, estos últimos han debido desarrollar estrategias para evadir a los parásitos [3]. Para lograr una infección exitosa, los parásitos deben alterar los mecanismos de defensa de sus hospedadores, secretando diversos tipos de moléculas como proteínas o péptidos. Por ejemplo, el nematodo *Meloidogynei* que parasita plantas secreta proteínas como proteasas, superóxido dismutasa y calreticulina. Similar estrategia ha sido observado en *Trichinella spiralis*, nematodo causante de triquinosis [3]. En el caso de los ectoparásitos, se sabe que estos liberan proteasas, inhibidores proteasas, catepsinas, entre otras moléculas para lograr una infestación exitosa [4].

Hasta la fecha, la mayoría de los estudios de interacción parásito-hospedero se han centrado en comprender la relación entre parásitos terrestres y sus hospederos [4, 5]. En el caso de los organismos marinos los estudios son limitados. Se sabe que en peces, condiciones como: edad, comportamiento, fisiología, condición inmunológica, cercanía a la costa, hábitos alimenticios o hábitat, pueden afectar en la capacidad infectiva del parásito [1]. Por ejemplo, el parásito, copépodo, *Lernaeocera branchialis*, al anclarse en los vasos sanguíneos de las branquias de los peces puede generar desde anemia hasta la muerte del hospedero, dependiendo de la edad de este [1]. Así también la abundancia y el daño visible de ectoparásitos puede variar dependiente del estado de desarrollo del pez, por ejemplo en *Labeo rohita*, se ha observado que en el estado pre-adulto presenta mayor carga de *Argulus siamensis,* ectoparásito, en

comparación con alevines y juveniles infestados con igual carga inicial, causando daño tisular en los pre-adultos [6]. En el caso de los copépodos ectoparásitos conocidos como piojo de mar, entre ellos *Lepeophtheirus salmonis* y *Caligus rogercresseyi,* el hábitat es un factor importante, propiciando la infestación. En este contexto, los centros de cultivos de salmónidos, en donde los peces son mantenidos en altas densidades, favorecen el asentamiento de piojos de mar en la piel de los peces sin producir la muerte del pez, pero deteriorando el estado de salud de los peces y favoreciendo de esta manera infecciones secundarias de tipo bacterianas [7-10].

2. Mecanismos moleculares involucrados en la interacción parásito-hospedero.

La interacción parásito-hospedero comienza una vez que el parásito identifica a su hospedador, por lo que conocer las moléculas que interaccionan entre el parásito y hospedero, es la clave para comprender como los parásitos logran infectar exitosamente a su hospedero. El incremento de información genómica de numerosas especies, ha sido el primer paso para comprender las funciones biológicas asociadas al proceso de interacción parásito-hospedero [11]. En el caso de los ectoparásitos, estos al alimentarse de la sangre de su hospedador, deben penetrar la piel del hospedero mediante la liberación de moléculas como proteasas. Por ejemplo, las garrapata al parasitar bovinos liberan catepsinas tipo L y serin proteasas (serpinas) e inhibidores de tripsinas [4]. En ectoparásitos marinos como *Paragnathia formica,* se ha observado que existe alta actividad de enzimas como catepsinas, las que parecen ser claves para la digestión de la sangre del hospedero [12]. Otro ejemplo, son los protozoos *Ichthyophthirius multifiliis* y *Cryptocaryon irritans,* que al adherirse a la piel de los peces secretan proteasas (serin, treonin, cisteina) y catepsina tipo L [13-15]. En relación al copépodo *L. salmonis*, se ha planteado que una de las estrategias que utiliza para lograr parasitar exitosamente a su hospedero, es mediante la secreción de moléculas que inmunodepriman al pez como: proteasas, prostagandina sintetasa E2 (PGE2) y catepsinas [16]. Por otra parte, estudios transcriptómicos realizados en *C. rogercresseyi* han demostrado cambios en los niveles de transcripción a lo largo de los distintos estadios de desarrollo del piojo de mar de genes como inhibidores de serpinas [17] y catepsinas [18].

En respuesta al proceso infectivo parásito-hospedero múltiples mecanismos de defensa son activados por el hospedero. Aun más, los diferentes niveles de susceptibilidad de un hospedero

frente a algún ectoparásito se puede deber a cambios en la fisiología del hospedador, en el caso de los peces se pueden mencionar: comportamiento del pez, cambios en la composición de la mucosa o respuesta inmune [19-21]. Dentro de ellos la respuesta inmune juega un rol primordial. En peces las barreras de defensa están compuestas por mucus, piel, branquias, enzimas digestivas y barreras inmunológicas como respuesta celular y anticuerpos [1]. El aumento e impacto económico de la acuicultura ligado al incremento de las tecnologías, la posibilidad de caracterizar líneas celulares de peces, el reporte de genomas de especies modelos como *Danio rerio, Takifugu rubripes*, entre otros; han contribuido al incremento del conocimiento relativo a la respuesta inmune de especies de relevancia económica como salmónidos, ciprínidos, entre otros [22]. Al igual que en mamíferos, en peces es posible identificar tanto el sistema inmune innato como el adaptativo. Además, varios de los mecanismos de respuesta a parásitos en mamíferos han sido observados en peces [22].

El sistema inmune innato es sin duda un mecanismo de rápida respuesta frente a la presencia de organismos patógenos. El inicio de esta respuesta esta dada por el reconocimiento de patrones moleculares asociados a patógenos (PAMPs) que son reconocidos por receptores reconocedores de patrones (PRRs), los que se pueden encontrar en las membranas celulares o endosomales e inclusive en el citoplasma [23, 24]. Dentro de los PRRs, los de mayor relevancia son los Toll-like receptors (TLR), los que se encuentran compuestos por tres dominios: dominio intracelular Toll-interleukin-1 receptor (TIR), región transmembrana y dominio extracelular. El dominio TIR es altamente conservado a lo largo de los TLR, mientras que el dominio extracelular, responsable del reconocimiento de los PAMs, se caracteriza por presentar repeticiones ricas en leucinas (LRR) y altamente variable entre diversos taxa [23, 24]. En vertebrados, hasta la fecha se han identificado cerca de 21 TLR los que se distribuyen en seis familias de TLR agrupadas de acuerdo al PAMs que reconocen, estas familias son: TLR1, TLR3, TLR4, TLR5, TLR7 y TLR11 [25]. En peces, específicamente, se han identificado alrededor de 20 TLR algunos de ellos ortólogos a mamíferos, así como también hay algunos que son específicos para peces, entre ellos: TLR13, TLR19, TLR20, TLR21, TLR22 y TLR23 [26, 27], además de una forma soluble del TLR5 identificado en *O. mykiss* [28]. En particular el TRL22, ha sido sugerido con un rol específico en la identificación de moléculas secretadas por los peces luego de ser infestados por ectoparásitos [29]. Ejemplo de

ello, es la respuesta de *L. rohita* infestados por el ectoparásito *A. siamensis,* en los que luego de la infestación se ha observado una disminución en los niveles de transcritos de *tlr22* durante los primeros 7 días de infestación, lo que se ha visto revertido luego de 15 dpi [29]. Este incremento de expresión del *tlr22*, además, se ha relacionado con altas cargas parasitarias en los peces [30]. Adicionalmente, se ha observado que entre *Catla catla* y *L. rohita,* este último más susceptible a la infestación por *A. siamensis*, existe un incremento en los niveles de transcritos de *tlr22* en la piel de la especie susceptible, ratificando la hipótesis de la participación de *tlr22* en la respuesta a la presencia de ectoparásitos [26]. Adicionalmente, en *L. rohita* se ha reportado un incremento de la expresión de *tlr*-4 y genes de respuesta inflamatoria como *il-8, il-11*, principalmente en alevines infestados por *A. siamensis* [6]. Además, se ha observado que peces expuestos a ectoparásitos como *Cryptocaryon irritans*, *Ichthyophthirius multifiliis* generan respuesta inflamatoria ligada al incremento en los niveles de expresión de genes como interleuquinas *(il)-1b, il-8, cox-2, tnf-a, lectina tipo c* [31, 32]. En el caso de salmónidos infestados por *L. salmonis* la respuesta inmune innata, de los peces, ha sido evaluada mediante cambios en los niveles de transcritos de citoquinas pro-inflamatorias como *il-1b, il-1r, il-12*, a demás de *tnf*-a y lectinas [33, 34]. Por otra parte, se ha observado que como mecanismo de respuesta inmune innata los peces incrementan sus niveles de especies reactivas de oxigeno (ROS) en respuesta al estrés generado por la presencia ectoparásitos, lo que se ve reflejado por ejemplo, en el aumento de expresión de oxido nítrico sintetasa (iNOS), la cual incrementa los niveles de ROS en *O. mykiss* infestados por *I. multifiliis* [32]. Otro ejemplo, es el incremento en los niveles de transcrito de thioredoxin en *Oncorhynchus gorbuscha* durante la infestacion con *L. salmonis* [19]. El incremento de los niveles de ROS en peces puede ser asociado al incremento de citoquinas pro-inflamatorias, ya que son estas las responsables de la producción de este tipo de moléculas [16].

En relación al sistema inmune adaptativo de los peces, al igual que en mamíferos se conforma por linfocitos T y B. Los linfocitos T son responsables del reconocimiento de antígenos, lo que es mediado por el complejo de histocompatibilidad mayor (MHC), que es activado por citoquinas. Existen dos clases de MHC: MHC I que participa en el reconocimiento de patógenos intracelulares y; MHC II que reconoce patógenos extracelulares [35]. Existen estudios que sugieren un rol de MHC II en respuesta a daños cutáneos en peces, como los

causados por la ameba que parasita branquias de los peces (*Paramoeba perurans*) [36], así también, en salmones por efecto del ectoparásito *L. salmonis* [37, 38]. Además, en zonas de la piel de *L. rohita* infestados con *A. siamensis* se ha reportado incremento en la expresión de inmunoglobulina Z al mismo tiempo que IgM disminuye en peces infestados [6].

En particular, en referencia a la respuesta de los salmónidos frente a la presencia del piojo de mar, hasta la realización de esta tesis, la mayor parte de los estudios eran relacionados con la respuesta de salmón a *L. salmonis*, especie de piojo de mar que afecta a los cultivos de salmones en el hemisferio norte. Dentro de los mecanismos de respuesta inmune asociados a la presencia de *L. salmonis* en salmones es posible mencionar: respuesta inflamatoria [33], secreción de proteasas [34], deficiencia de inmunoglobulinas [33], respuesta Th2 T [38]. La respuesta inflamatoria es la primera asociada a la presencia de piojo de mar, lo que genera variaciones en la actividad transcripcional de genes como *interleukina 1b, mmp13, mmp19, tnf-a, prostaglandinas e2 e il-8* [33, 34, 37-39]. Además, se ha evidenciado que diferentes especies de salmónidos presentan distintos grados de respuesta inmune [40, 41]. Como en salmón Coho (*Oncorhynchus kisutch*), especie que presenta una respuesta inflamatoria temprana a la presencia de *L. salmonis*, lo que hace que esta especie de salmónidos tenga mayor resistencia a la presencia del ectoparásito [37, 39].

Adicionalmente, ya que gran parte del ciclo de vida de los parásitos ocurre cuando éstos se encuentran establecidos en sus hospedadores, se ha observado que existen variaciones en la respuesta del parásito y hospedador a medida que el ciclo de vida del parásito se va desarrollando. Por ejemplo, el nematodo *Brugia malayi* al infectar el mosquito de la fiebre amarilla (*Aedes aegypti*) presenta variaciones en la dinámica de transcripción de genes que se encuentra relacionados con su desarrollo, además luego de un análisis de comparativo del hospedero infectado y sin infección, se observó que la transcripción de ciertos genes del hospedador también es influenciada por las etapas de desarrollo del parásito [42]. Otro ejemplo, es la respuesta bifásica reportada para salmones infestados con *L. salmonis* entre los días 5-10 post infección, lo que corresponde además a la etapa donde se realiza la metamorfosis de copepodito a chalimus, durante este periodo de transición se ha observado drásticos cambios de expresión en genes, entre ellos genes de respuesta inmune innata como

las lectinas las que son sobre-expresadas durante la primera etapa de la infestación principalmente el la piel del salmón, además de proteínas secretorias. Luego de los 5 dpi se observa una reducción de genes sobre-expresados en piel, y un incremento de metaloproteasa, además de comenzar una respuesta inflamatoria en el pez [34]. Adicionalmente, nuestro grupo de investigación ha observado en salmones infestados con *C. rogercresseyi* variaciones en el tipo de respuesta inmune asociado a la carga parasitaria, en el estudio se comparó la respuesta de peces infestados con 35 y 100 copepoditos por pez, observando una relación entre la carga de Caligus y la respuesta inmune. Por ejemplo, los peces con alta carga parasitaria presentaron mayores niveles de expresión de los genes *t-bet, cd83, stat4, cox-2* asociados a respuesta Th1 y otros de respuesta a estrés oxidativo como catalasa y glutatión transferasa, mientras que en aquellos peces con baja carga parasitaria se observó incremento de expresión principalmente del gen *mmp13* [43].

La variación de la abundancia del parásito en un tipo hospedero, puede ser reflejo de lo atrayente que es el hospedero para éste. Los parásitos son dependientes de la condición nutricional de su hospedero, seleccionan así aquellos que presentan mayor cantidad de recursos nutricionales para su desarrollo, supervivencia y reproducción [44, 45]. Es por esto, que se ha descrito un tipo de inmunidad denominada inmunidad nutricional, esta consiste en la limitación de nutrientes por parte del hospedero [44], lo que genera de esta forma un ambiente hostil para el desarrollo de los parásitos, generando en el parásito un incremento de su gasto energético asociado a su nutrición. Dentro de esta estrategia inmunitaria, la limitación del hierro es fundamental, debido a su rol en distintos procesos fisiológicos como la respiración, actividad enzimática, entre otros [44, 46]. En el caso de los ectoparásitos, al ser organismos hematófagos, adquieren hierro desde la sangre mediante absorción de hemoglobina la que es degradada a grupo hemo [47], e iones de hierro asociados a proteínas transportadoras de éste [47]. Por ejemplo, la estrategia de control de hierro en infecciones bacterianas ha sido bien establecida, al mismo tiempo se ha observado que las bacterias han desarrollado vías moleculares esenciales para la adquisición de hierro y su virulencia [44]. Indicios de inmunidad nutricional han sido reportados en peces infestados con el ectoparásito *L. salmonis*, observando una mayor resistencia en peces con altos niveles de regulación del grupo *hemo* [19]. Paralelamente, se ha descrito incremento en los niveles de expresión de hepcidikna,

proteína responsable de la dispersión de hierro en la célula, en peces infestados con *L. salmonis* [37].

3. Rol de los ARN no codificantes en respuesta a la presencia de patógenos.

El desarrollo de las tecnologías de secuenciación masiva ha demostrado que la mayoría de la información genética no codifica para proteínas, sino mas bien existe una gran proporción del genoma cuya información pareciera ser de tipo no funcional, lo que ha sido denominados como ARN no codificantes (*ncRNA*, en ingles). En eucariontes es posible identificar dos tipos de ncRNA: los denominados *housekeeping* ncRNAs (*ribosomal, small nuclear, small nucleolar and transfer*) y ncRNA regulatorios, dentro de estos los más abundantes son: *microRNA, piwi-interacting RNA*, *small interfering RNA*, y *long non-coding RNA* (*lncRNA*) [48, 49].

Dentro de los ncRNAs, los lncRNAs parecen tener un rol fundamental en la regulación de numerosos procesos biológicos [50]. Los lncRNAs son definidos como transcritos no mayor a 200 pares de bases con bajo potencial codificante; se pueden localizar tanto en el núcleo como en el citosol, y son transcritos de manera similar a los ARN mensajeros [51, 52]. Es posible identificar dos diferentes tipos de lncRNAs de acuerdo a su posición genómica, lncRNAs que transcriben en regiones intrónicas en sentido sense y antisense, y lncRNAs en regiones intergénicas localizadas entre exones de proteínas conocidas [53]. LncRNAs, a diferencia de las secuencias codificantes, presentan bajo nivel de conservación entre especies. De hecho, un estudio realizado entre 17 especies distintas entre ellos peces teleósteos y tetrápodos, el 70% de los lncRNA no presentaron secuencias ortólogas entre las especies, observando sólo 29 lncRNAs conservados entre peces y mamíferos [54]. En relación a la respuesta de un hospedero frente a un patógeno, se ha demostrado que los lncRNAs pueden regular las vías de respuesta inmune innata mediada por receptores tipo toll (TLR) [55] and NF-κB [56]. En peces el rol de los lncRNAs ha sido escasamente estudiado. Sin embargo, un estudio en *Larimichthys polyactis* muestra un alto número de lncRNAs expresados específicamente en presencia de poly I:C [57]. En salmón del Atlántico infectado con virus ISA se han reportado 4.967 lncRNAs diferencialmente expresados [58]. Además, un reciente estudio comparativo

entre salmón del Atlántico infectados con virus, bacterias y ectoparásitos demuestra una modulación diferencial de lncRNAs dependiente del tipo de infección [59].

Otro grupo de ncRNAs de importancia en la respuesta a patógenos son los microRNAs (miRNAs). Estas secuencias no codificantes de 22 nucleótidos aproximadamente, fueron identificados por primera vez en *Caenorhabditis elegans* [60] y actúan como reguladores de la expresión de genes [61]. Los miRNAs parecieran tener un rol fundamental en la respuesta inmune, participando en la presentación de antígenos como miR-155 y señalización de linfocitos T como miR181a [62-64], así como otros miRNAs que participarían en la diferenciación de linfocitos B y T [62, 64]. Por otra parte, en mamíferos se ha observado que la vía de respuesta inmune innata, activada por los receptores tipo Toll, no sólo es activada por una serie de proteínas sino que además intervienen miRNAs como miR-155, miR146a, miR-155 y miR-21 [63]. La información relacionada al rol de los miRNAs sobre la interacción parásito-hospedero es escasa. Sin embargo, algunos estudios recientes han reportado cambios de expresión de miRNAs como miR-184, miR-10 en células de insectos en respuesta a una infección con un virus [65]. En mosquitos afectados por el virus *west nile* (WNV) se ha observado que existe una disminución de la regulación del miR-2940, miRNA que a su vez reduce la expresión de metaloproteinasas requeridas para la replicación del virus [66]. Otro ejemplo, es el estudio realizado en los estados larvales 3 y 4 del nematodo *Angiostrongylus cantonensis*, estados especifícos que parasitan el sistema nervioso de mamíferos. En dicho estudio y luego de una secuenciación de smallRNAs de ambos estados, se identificaron diferencias en el número de miRNAs expresados en ambos estados larvales, además de identificar 26 miRNAs asociados a la respuesta inmune, los que tendrían un rol fundamental durante el proceso infectivo [67]. Con respecto a la regulación de miRNAs en peces, se ha observado variaciones de expresión de miRNAs en *Paralichthys olivaceus* durante infecciones del virus de septicemia hemorrágica (VHSV) [68]. En salmonídeos se ha observado que peces resistentes al virus de la necrosis pancreática (IPNV) presentaban un incremento en los niveles de expresión de miR-4792, sugiriendo un rol de específico de este miRNA en la respuesta a INPV en salmones [69, 70]. Además se han reportado cambios de regulación de miRNAs en peces como respuesta a infecciones bacterianas [71, 72].

4. Moléculas de interacción ectoparásito-hospedero como estrategia de control de ectoparásitos.

El principal problema para el desarrollo de nuevas formas de control de ectoparásitos, es que estos tienen baja interacción con el sistema inmune de su hospedador a diferencia de los endoparásitos. Sin embargo, el ectoparásito al estar en contacto con la sangre de su hospedador es susceptible a los anticuerpos que circulan por la sangre de este, lo que podría afectar la capacidad infestiva del parásito. Dentro de este contexto, la mejor alternativa para la generación de vacunas, es la utilización de proteínas que estén involucradas en el proceso de infestación y alimentación del parásito [4]. En especial el desarrollo de vacunas para el control de ectoparásitos marinos, como el piojo de mar surge como una alternativa interesante debido a que presentaría ventajas como: (1) mayor sustentabilidad, eliminando los residuos químicos en el agua de mar; (2) especificidad contra el parásito, evitando la muerte de otros copépodos presentes en el agua; y (3) reducida o nula generación de resistencia por parte del ectoparásito [73].

La búsqueda de moléculas blanco para el desarrollo de vacunas se ha centrado en la identificación de proteínas involucradas en los procesos de interacción parásito-hospedero donde proteasas, peptidadas e inhibidores de proteasas parecen ser candidatos para inmunizar a los hospedadores [4, 16]. Actualmente, existen reportes exitosos del uso de serpinas en el control de ectoparásitos como garrapatas [4, 74], así como el uso de proteasas en el control de ectoparásitos que afectan a las ovejas [75]. Por otra parte, recientemente un grupo de investigación desarrolló una vacuna para el control de la garrapata *Haemaphysalis longicornis,* basado en dos tipos de Ferritinas (intracelulares y secretada). Ferritina es una proteína de unión a iones de hierro, que en *H. longicornis* se ha demostrado ser esencial para reproducción y captación de hierro desde la sangre del hospedador. Luego de la identificación de los mRNAs de ambos tipos de ferritinas, las secuencias fueron clonadas, expresadas y purificadas desde *E. coli.* La proteína recombinante obtenida fue inyectada en conejos observando un incremento significativo del nivel de título de anticuerpos en aquellos animales inyectados con ferritina recombinante de tipo secretada. Adicionalmente, se evaluó el éxito de la infestación luego de tres inmunizaciones, donde si bien los autores no vieron una reducción en la carga

parasitaria entre los tres grupos, sí se reportó disminución del tamaño de las garrapatas en los conejos inmunizados con la ferritina secretada [26]. Otro caso de estudio, ha sido la búsqueda de vacunas para el control del piojo de mar, un primer acercamiento demostró que el uso de un extracto proteico de *L. salmonis* lograba reducir la cantidad de hembras con sacos ovígeros, sin embargo, no se identificó el antígeno específico [73]. Otro de los intentos ha sido la utilización de β-galactosidasa, la que mostró disminuir la fecundidad de *L. salmonis*. No obstante, ensayos posteriores no lograron replicar los resultados obtendidos durante el estudio [73]. Otra vacuna propuesta para el control de este ectoparásito es el uso de la proteína recombinante del gen denominado *my32*, ortólogo de Akirin-2 y caracterizado tanto en *L. salmonis* como *C. rogercresseyi* [76, 77]. Este gen factor de transcripcion tiene actividad en procesos de las vías de señalización de respuesta inmune innata de invertebrados a través de la vía IMD (*Immune deficiency pathway en ingles*). La proteína recombinante de my32, fue evaluada como antígeno contra Caligus, demostrando reducción de un 57% la tasa de infestación luego de 24 días en *S. salar* [78].

5. *Caligus rogercresseyi* como modelo de estudio

Caligus rogercresseyi o piojo de mar, es un ectoparásito que genera altas pérdidas en la acuicultura Chilena [80, 81], reportándose costos de US$80 millones por concepto de compra de pesticidas durante el 2013 [82]. Este ectoparásito perteneciente a la familia Caligidae, la que incluye *Lernaeocera branchialis*, *Caligus clemensi* y *Lepeophtheirus salmonis*, presenta un ciclo de vida que puede variar dependiente de la temperatura del agua de entre 26 a 46 días [83]. Su ciclo de vida consta de ocho estadios de desarrollo, los tres primeros de fase planctónica: nauplius I, II y copepoditos, este último estadío es el que reconoce y se asienta en el hospedador mediante unas antenas primarias, las que detectan estímulos químicos liberados por el pez. Una vez asentado en el hospedador se identifican los estadios larvales: chalimus (I, II, III y IV) y adultos maduros [83, 84]. Los adultos presentan dimorfismo sexual, las hembras una vez que alcanzan la madures son copuladas inmediatamente por los machos, los que en el momento de la cópula introducen espermatóforos bloqueando los ductos copulatorios de las hembras. Las hembras producen durante su vida hasta 11 generaciones de sacos ovígeros con una sola copula, los que producen en promedio 100 huevos en un período de 4 a 6 días, dependiendo de la estación del año y condiciones ambientales. Además los Caligus adultos

poseen la capacidad de moverse de manera libre sobre el huésped, siendo capaces de sobrevivir hasta 7 días libres en el mar, lo que les permite cambiar de hospedador [85].

La infestación por *C. rogercresseyi*, enfermedad conocida como Caligidosis, genera un alto nivel de estrés en los peces, generando depresión de su sistema inmune, pérdida del epitelio y necrosis tisular, lo que aumenta el riesgo de brotes de otro tipo de enfermedades infecto-contagiosas y afecta el rendimiento del cultivo [7, 80, 81, 86]. En particular *C. rogercresseyi* a diferencia de *L. salmonis,* especie que afecta a los centros de cultivos en el hemisferio norte, es capaz de parasitar peces silvestres como pejerrey, róbalo, merluza [82]. En relación a esta susceptibilidad por parte del hospedador, se ha descrito que las distintas especies de salmónidos infectadas por *L. salmonis*, presentan variaciones en la intensidad de la respuesta inmune ligada a la infestación, lo que haría algunas especies más susceptibles que otras [40, 41]. En Chile no todas las especies de salmónidos cultivadas registran igual susceptibilidad a ser infectadas por este ectoparásito, *Oncorhynchus mykiss* y *Salmon salar* han mostrado tener una altas cargas parasitarias, mientras que *O. kisutch* ha registrado bajos niveles de prevalencia de este ectoparásito [7, 80, 87].

Debido a los altos costos que presenta la industria salmonicultora por efecto de los tratamientos utilizados para el control del ectoparásito, además de los costos indirectos por ser vector que facilita otro tipo de enfermedades [88], se han implementado en Chile programas de monitoreo enfocados a mitigar la carga parasitaria en los centros de cultivo. De esta forma, encontramos los planes de control desarrollados desde 1999 por el Instituto Tecnológico del Salmón (INTESAL) y los controles generados desde 2007 por el Servicio Nacional de Pesca (SERNAPESCA). Pese a existir registros de esta enfermedad en centros de cultivo desde 1997, recién en el año 2007 la caligidos se incorporó a la lista de Enfermedades de Alto Riesgo en Chile [4]. Dentro de las medidas de tratamiento y control utilizadas se encuentran: tratamientos coordinados de barrios, terapias no farmacéuticas (dietas funcionales, ultrasonido, etc) y terapias farmacéuticas. En Chile, así como en otros paises salmonicultores que reportan infestación por piojo de mar, el método de control más utilizado es por medio de tratamientos con drogas antiparasitarias. Estas drogas alteran principalmente la transmisión de impulsos nervioso y muscular, ocasionando parálisis y/o muerte del piojo de mar. Dentro de las drogas

utilizadas destacan: avermectinas (Benzoato de emamectina), las que afectan directamente los canales de cloro-GABA; piretroides (deltametrina) que afecta los canales de Na^+ y organofosfatos (Azametifos) los que actúan sobre la actividad de acetylcholinesterase (AChE) [89]. Sin embargo, en la actualidad existen numerosos estudios que han demostrado pérdida de efectividad de estos químicos debido al desarrollo de resistencia farmacológica [90-93], lo que hace necesario desarrollar nuevos mecanismos de control para este ectoparásito.

Para desarrollar nuevos mecanismos de control de la Caligidosis, es necesario incrementar el conocimiento en torno a esta enfermedad. En los últimos años se han utilizado herramientas genómicas para incrementar el conocimiento en torno a *C. rogercresseyi* identificando genes involucrados en procesos metabólicos, ciclo de muda, sistema nervioso, reproducción, desarrollo celular, entre otros. Esta tesis doctoral busca identificar y describir los mecanismos de respuesta del hospedador y su interacción con *C. rogercresseyi*, no sólo asociados a respuesta de inmune clásicas como son la respuesta innata y adaptativa, sino también evidencia mecanismos de respuesta inmune de tipo nutricional asociada a la competencia por hierro. Por otra parte, es el primer estudio en plantear el rol de RNAs no codificantes, lncRNAs y miRNAs, en la modulación de la respuesta de salmones a la infestación con *C. rogercresseyi* y como estos podrían ser actores importantes en los procesos de interacción parástio-hospedero. Además, se plantea un nuevo método de control de este ectoparásito, mediante el desarrollo de una vacuna, basada en una proteína identificada como clave en el proceso de interacción Caligus-hospedero. Este estudio entrega información valiosa que junto con estudios funcionales permitiría el desarrollo de nuevas herramientas para el control de la Caligidosis y otras enfermedades que afectan a la industria salmonicultora.

HIPÓTESIS

H1: Los cambios en perfiles transcriptómicos durante el proceso infectivo de *C. rogercresseyi* sobre sus hospederos revelan vías de señalización molecular relevantes para la interacción ectoparásito/hospedero.

H2: La modulación transcriptómica evidenciada entre ectoparásito/hospedero se encuentra correlacionada con cambios en la expresión de transcritos no-codificantes, sugiriendo un rol clave en la respuesta inmune de peces mediado por ncRNAs.

OBJETIVOS

Objetivo General

Determinar mediante una aproximación transcriptómica los procesos biológicos mediadores de la interacción parásito-hospedero y como estos son moduladas a nivel molecular durante el proceso de infestación, utilizando como modelo de estudio la interacción *Caligus rogercresseyi*-salmónidos.

Objetivos Específicos

1. Determinar los cambios a nivel transciptómico de *Salmo salar* y *Caligus rogercresseyi* durante la interacción parásito-hospedero.
2. Caracterizar los transcritos no-codificantes que modulan a nivel post-transcripcional vías relevantes durante la interacción parásito-hospedero.
3. Evaluar a nivel experimetal un nuevo antígeno identificado desde el transcriptoma de *C. rogercresseyi* como una vacuna para el control de la caligidosis.

METODOLOGÍA

Objetivo 1. Identificar y determinar la modulación de los procesos biológicos de *Caligus rogercresseyi* y *Salmo salar* mediadores la interacción ectoparásito-hospedero.

Diseño del experimento.

En el laboratorio experimental del Laboratorio de Biotecnología y Genómica Acuicola (LBGA) – INCAR en la estación Marina de Dichato, 120 individuos de salmón del Atlántico y salmón coho de 280 g aproximadamente, fueron aclimatados por separado, en agua de mar con un fotoperiodo de 12:12 luz:noche, alimentados diariamente con dieta comercial Micro 200 (EWOS Chile). Luego de 15 días de aclimatación los peces fueron separados en estanques de 500 litros con 20 peces cada unos (6 estanques para salmón Atlántico y 6 para salmón coho). Los peces fueron aclimatados por 7 días más previo a la infestación con Caligus. El cultivo de Caligus se realizó en el laboratorio experimental en la estación Marina de Dichato. Para ello se colectaron hembras ovigeras desde un centro de cultivo comercial en Puerto Montt. Las muestras hembras fueron llevadas en agua de mar y con aireación constante hasta la estación en Dichato. Una vez ahí las hembras fueron puestas en oscuridad con temperatura constante a 12ºC para su eclosión. Las larvas eclosionadas, se mantuvieron en agua de mar filtrada con aireación constante a 12ºC y oscuridad hasta alcanzar el estado de copepodito. Para el desafío con Caligus, se redujo el volumen de los estanques hasta un 10% de su capacidad, infectando con 35 copepoditos por pez por 8 horas y posteriormente aumentando el volumen de agua hasta la capacidad máxima de los estanques (Fig. 1). Los peces fueron alimentados diariamente, mantenidos a 12ºC y con aireación constante. Se tomó muestras de riñón anterior y piel de los peces a los 0, 7, 14 días post-infestación (dpi). Para le muestreo se tomaron 10 peces los que fueron anestesiados con benzocaína y las muestras fijadas en RNA Later (Ambion) y mantenidas en -80ºC.

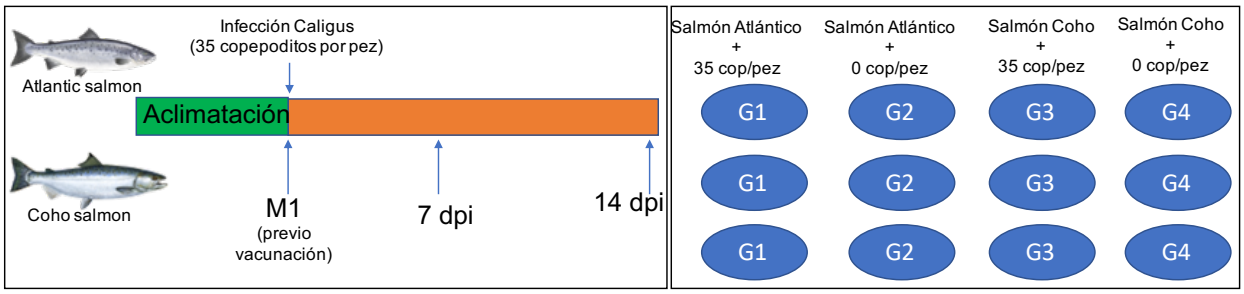

Figura 1: Diseño experimento de infestación con *C. rogercresseyi.* Fuente: Elaboración propia

Secuenciación de transcriptoma

ARN total fue extraído desde las muestras de piel y riñón anterior tomadas 0, 7 y 14 dpi utilizando Trizol Reagent, siguiendo las instrucción del fabricante. La calidad y cantidad de ARN total fueron determinados en el equipo TapeStation 2200 (Agilent Technologies Inc., Santa Clara, CA, USA) utilizando R6K Reagent Kit de acuerdo a las instrucciones del proveedor. Se seleccionaron 5 ARN por tiempo y tejido que presentaron valores de RIN > 8.0 para ser pools de muestras y la preparación de las librerías. La síntesis de librerías de ARN se realizó con el kit TruSeq RNA Sample Preparation Kit v2 (Illumina®, San Diego, CA, USA), siguiendo el protocolo propuesto para este kit. Las librerías fueron secuenciadas en corridas de 2x250 ciclos en la plataforma MiSeq (Illumina®) del LBGA.

El ensamble de las secuencias se realizó en el software CLC Genomics Workbench v9.1 software (CLC Bio, Aarhus, Denmark), por separado para cada especie de salmón. Los parámetros del ensamble fueron *overlap* of 70% y *similarity* of 0.9 para excluir secuencias paralogas [27]. El *settings* utilizado en el software fue: mismatch cost = 2, deletion cost = 3, insert cost = 3, minimum contig length = 200 base pairs, and trimming quality score = 0.05. El análisis de RNA-Seq se llevo acabo en el software CLC Genomics Workbench v9.1 software (CLC Bio, Aarhus, Denmark). Los valores de expresión fueron trabajados como TPM (transcripts per million). La distancia fue calculada a través del método de Manhattan method. Para el calculo de las variaciones de expresión, se utilizó el test estadístico de Kal`s (P = 0.0005).

Objetivo 2. Evaluar la regulación post-transcripcional (ncRNA) de aquellos genes que participan en la interacción parásito-hospedero.

Identificación de lncRNA y análisis de expresión diferencial en salmón del Atlántico y salmón Coho

A partir de los datos de trascriptoma obtenidos para salmón Atlántico y salmón Coho (obj. 1), se realizó la búsqueda de lncRNA, por separado para cada especie, de la siguiente manera: se seleccionaron todos los transcritos con un tamaño sobre 200 pb y cobertura mayor a 50; se realizó un análisis de Blastn y Blastx contra la base de datos disponible en GenBank para peces teleósteos; a aquellas secuencias que no presentaron homología con secuencias ya descritas, se les realizó una búsqueda de posibles ORF con la herramienta "*find ORF*" del software CLC Genomics Workbench 9.0, todas las secuencias que no presentaron posibles ORF fueron luego analizadas con la herramienta Coding-Potential Assessment Tool (CPAT), herramienta que determina el potencial codificante de una secuencia. Todas aquellas secuencias que pasaron el filtro de CPAT fueron denominadas como posibles lncRNA. Los análisis de expresión diferencial de los lncRNA fueron hechos en el software CLC Genomics Workbench, siguiendo los protocolos descritos en el objetivo 1.

Secuenciación y análisis de Small RNAs

A partir de aproximadamente 10 individuos de caligus en estado copepodito, chalimus I-II, chalimus III-IV y adultos caligus, así como tejido de piel y riñón de *S. salar* obtenidos desde el experimento del objetivo 1, se realizó extracción RNA total utilizando Trizol Reagent (Ambion, USA) siguiendo las instrucciones del fabricante. La integridad del RNA fue evaluado en el equipo TapeStation 2200 (Agilent Technologies Inc., Santa Clara, CA, USA) utilizando R6K Reagent Kit de acuerdo a las instrucciones del proveedor. Las muestrsa con RIN SOBRE 8.0 fueron utilizadas para la síntesis de librerías utilizando el kit TruSeq Small RNA Sample Prep Kit (Illumina®, USA). Las librerías de smallRNA fueron secuenciadas en 41 ciclos en la plataforma MiSeq (Illumina) utilizando kit de 50 ciclos (MiSeq Reagent Kit v3), en el laboratorio de Biotecnología y Genómica Acuícola, del centro INCAR. Luego de la secuenciación las lecturas de baja calidad, tamaño menor a 20 nucleótidos y que presentaron

ambigüedades fueron removidas utilizando el software CLC Genomics Workbench. Las secuencias no descartadas fueron anotadas contra la base de miRNA depositada miRBase 21.0. Finalmente, se evaluó la formación de estructuras secundarias en las secuencias precursoras de miRNA, mediante la herramienta de predicción de este tipo de estructuras del software CLC Genomics Workbench Software. Los miRNA identificados fueron utilizados como referencia para un análisis RNA-Seq utilizando el software CLC Genomic Workbench. Finalmente, se realizó una predicción de genes target utilizando como blanco aquellos genes que presentaron mayores cambios de regulación durante la infección según lo observado en el objetivo 1, utilizando para la predicción los algoritmos PITA [96], miRanda [97], and STarMir [98].

Objetivo 3. Proponer y evaluar un nuevo método de control de *C. rogercresseyi* mediante exposición de peces a un antígeno seleccionado desde los resultados de estudios transcriptómicos.

Expresión y purificación de proteínas recombinantes

Se caracterizó el gen de *Cr_ferritina* de *C. rogercresseyi*, observada como gen con altos niveles de regulación en el estado infestivo de este ectoparásito. Para la caracterización se obtuvo la secuencia completa del gen utilizando el kit SMARTer™ RACE cDNA Amplification 5' y 3' (Clontech, Mountain View, CA, USA). La secuencia caracterizada fue enviada a GenScript (www.genscript.com) en donde fue sintetizada e inserta en un vector de expresión pET-30a(+) y transformada en la cepa *E. coli BL21*(D3). Para la inducción de la proteína, a partir de este cultivo, se tomaron 50 ul y se llevaron a un matraz con 50 ml de LB/kanamicina se dejó crecer a 37ºC con agitación constante hasta alcanzar OD_{600} de entre 0.5-0.6. Al alcanzar la OD_{600} se realizó la inducción con Isopropil β-DTioglactopiranosido (IPTG) 0,1 M, evaluando dos condiciones de inducción 6 hrs a 37ºC. Luego de la inducción los cultivos fueron centrifugados a 5.000 rpm por 15 min, se eliminó el sobrenadante y los pellet fueron lavados con Tris 0.1M (pH 7,5). El pellet fue resuspendido en 3 ml de buffer de lisis (Tris-HCl 0.1 M, EDTA 0.5 M, NaCl 2 M, SDS 10%, pH7.0) y sonicado por 30 ciclos de 10 segundos con 20 segundos de descanso a un pulso de amplitud de 80% en el equipo QSONICA Modelo Q125. Posteriormente, el pellet fue centrifugado por 1 hora a 24.000 rpm,

recuperando el sobrenandante en un tubo nuevo. Finalmente, el sobrenadante fue filtrado (0,45um), y la proteína fue purificada por cromatografía de exclusión molecular. La concentración de la proteína purificada fue determinada mediante el kit Micro BCA assay (Thermo Scientific, USA). Además se corrió un gel SDS-PAGE para verificar que la proteína purificada correspondiera a *Cr_Ferritina,* usando tinción de azul de Coomassie G-250. Para análisis por Western-blot, las proteínas se transfirieron a una membrana de nitrocelulosa (Sheleicher y Schuell Bioscience) durante 1h a voltaje y amperaje contaste, detectando la proteína con una anticuerpo primario anti-hexahistidina y un anticuerpo secundario anti-ratón conjugado con peroxidasa.

Ensayo de vacunación

Se realizó un ensayo de vacunación donde se evaluó *Cr_ferritina* como vacuna en las instalaciones del LBGA-INCAR en la estación de Dichato. El ensayo de vacunación se llevó a cabo en 200 peces de 90 gr aprox., los que fueron inyectados de manera intraperitoneal con 0,1 ml de vacuna (*Cr_ferritin* 30 ug + adyuvante) y distribuidos en cuatro estanques, más cuatro estanques con grupo control inyectaos con PBS + adyuvante (Fig. 2), hasta cumplir un periodo de inmunidad de 400 unidades térmicas acumuladas (UTAs), manteniendo temperatura de 13 ±1 ºC. Luego de ese periodo los peces fueron infectados con *C. rogercresseyi* a una presión de infección de 35 copepoditos por pez. La alimentación de los peces se realizó de manera manual, diariamente con pellet de calibre 3 y 4 mm. Desde los estanques se realizó conteo de caligus luego de 25 días de infestación. Se tomaron muestras de hembras caligus y desde riñón anterior y piel, los que fueron preservados en RNA Later y almacenados a -80ºC, para los análisis moleculares posteriores.

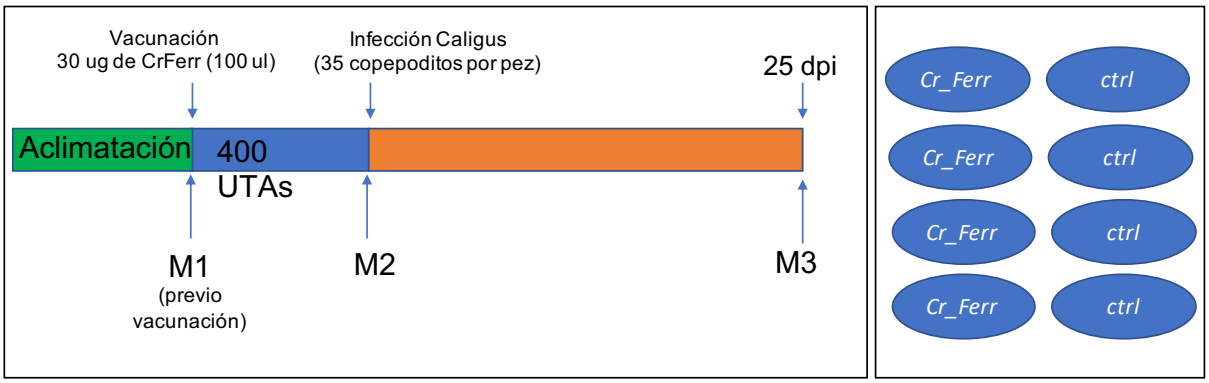

Figura 2: Diseño experimental del ensayo de vacunación. Fuente: Elaboración propia.

Evaluación del efecto de las proteínas recombinantes *C. rogercresseyi*

Se extrajo ARN total desde las muestras de hembras de caligus para síntesis de librerías de secuenciación, siguiendo los mismos protocolos descritos para las muestras de salmón en el objetivo 1. Los datos generados fueron analizados con el software CLC Genomics Workbench, siguiendo los protocolos descritos en el objetivo 1.

RESULTADOS

CAPITULO 1

Comparative immunity of *Salmo salar* and *Oncorhynchus kisutch* during infestation with the sea louse *Caligus rogercresseyi*: An enrichment transcriptome analysis

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Abstract

Caligus rogercresseyi, an ectoparasite affecting the Chilean salmon industry, can cause immunosuppression and physiological stress in farmed fish. Interestingly, coho salmon (*Oncorhynchus kisutch*) are notably resistant to infestation, whereas Atlantic salmon (*Salmo salar*) are phenotypically more susceptible to sea lice. However, comparative studies on immune responses to *C. rogercresseyi* have not been conducted. In this study, Illumina sequencing was conducted to evaluate head kidney and skin samples taken 7 and 14 days post-infestation, yielding a total of 1492 and 1522 contigs annotated to immunerelated genes for Atlantic and coho salmon, respectively. Both species evidenced an upregulation of inflammatory genes. Atlantic salmon had highly upregulated TLR22 and MHCII at 14 days postinfestation, while coho salmon had highly upregulated stat5 and il1r transcripts. Fourteen transcripts related to TH1, TH2, TLR, and macrophage responses were corroborated via RT-qPCR. Statistical analyses indicated an upregulation of *mmp13, cox2, il10, ccr3, tlr22a2*, and *tlr21* in Atlantic salmon and of ifng, *cd83, T-bet, tlr13*, and *tlr19* in coho salmon. These results suggest strong differences between the Atlantic and coho salmon immune responses, where coho salmon, the more resistant species, presented a primary TH1 response. Additionally, putative roles of TLRs in salmonids against sea lice were evidenced. This study is the first comparative transcriptome analysis that reveals species-specific immune responses in salmons infected with *C. rogercresseyi*.

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Full length article

Comparative immunity of *Salmo salar* and *Oncorhynchus kisutch* during infestation with the sea louse *Caligus rogercresseyi*: An enrichment transcriptome analysis

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ABSTRACT

Caligus rogercresseyi, an ectoparasite affecting the Chilean salmon industry, can cause immunosuppression and physiological stress in farmed fish. Interestingly, coho salmon (*Oncorhynchus kisutch*) are notably resistant to infestation, whereas Atlantic salmon (*Salmo salar*) are phenotypically more susceptible to sea lice. However, comparative studies on immune responses to *C. rogercresseyi* have not been conducted. In this study, Illumina sequencing was conducted to evaluate head kidney and skin samples taken 7 and 14 days post-infestation, yielding a total of 1492 and 1522 contigs annotated to immune-related genes for Atlantic and coho salmon, respectively. Both species evidenced an upregulation of inflammatory genes. Atlantic salmon had highly upregulated *TLR22* and *MHCII* at 14 days post-infestation, while coho salmon had highly upregulated *stat5* and *il1r* transcripts. Fourteen transcripts related to T_H1, T_H2, TLR, and macrophage responses were corroborated via RT-qPCR. Statistical analyses indicated an upregulation of *mmp13*, *cox2*, *il10*, *ccr3*, *tlr22a2*, and *tlr21* in Atlantic salmon and of *ifnγ*, *cd83*, *T-bet*, *tlr13*, and *tlr19* in coho salmon. These results suggest strong differences between the Atlantic and coho salmon immune responses, where coho salmon, the more resistant species, presented a primary T_H1 response. Additionally, putative roles of TLRs in salmonids against sea lice were evidenced. This study is the first comparative transcriptome analysis that reveals species-specific immune responses in salmons infected with *C. rogercresseyi*.

1. Introduction

Sea lice infestations, a major problem for salmon aquaculture worldwide, are primarily controlled by delousing drugs. However, the effectiveness of these drugs is limited due to pharmacological resistance and the frequency of treatments [1], ultimately causing physiological stress to fish during the production cycle [2]. In the Northern Hemisphere, the salmon louse *Lepeophtheirus salmonis* causes tissue damage and immunosuppression in infected fish [3,4]. The immune responses of Atlantic salmon (*Salmo salar*) to *L. salmonis* infestation include inflammatory responses [5], protease secretion [6], immunoglobulin deficiency [5], and T helper 2 (T_H2) responses [7]. Among these, inflammatory responses are the first type of immune response associated with sea lice infestation, specifically involving genes such as interleukin (*il*)-*1β*, matrix metalloproteinase (*mmp*) 13, *mmp19*, tumor necrosis factor α (*tnf-α*), *cox2*, prostaglandin E_2, and *il-8*, all of which are transcriptionally modulated after initial sea lice infestation [4–8]. Furthermore, different salmonid species evidence different degrees of immune responses to sea lice infestation, with some species more susceptible than others [9,10]. Early pro-inflammatory response regulation has been related to *L. salmonis* resistance, as reported in coho salmon (*Oncorhynchus kisutch*) via an increased T_H2 response [4,8].

Another immune mechanism, which has been scarcely studied in fish infested with ectoparasites, is related to the recognition of pathogen-associated molecular patterns by pattern recognition receptors. These receptors finally mediate the transcription of pro-inflammatory cytokines [11]. Among the most relevant pattern recognition receptors, the Toll-like receptors (TLRs) have been the focus of numerous vertebrate and invertebrate studies [12–17]. Nearly 20 TLRs have been identified in fish. While some are

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orthologous to mammalian TLRs, others have only been identified in fish, including TLR13, TLR19, TLR20, TLR21, TLR22, and TLR23 [11,18,19]. Additionally, some fish TLRs are paralogous and likely originated from different evolutionary events, such as gene duplication [20]. Furthermore, these receptors have reported putative roles against ectoparasites, such as TLR22 in *Catla catla* and *Labeo rohita* following infestation by the crustacean ectoparasite *Argulus siamensis* [18].

Caligus rogercresseyi is responsible for high economic losses in the Chilean salmonid aquaculture industry [21]. Atlantic salmon are most affected by *C. rogercresseyi*, while coho salmon display a more resistant phenotype to *C. rogercresseyi* infestation [22]. Moreover, the transcriptomic response in *C. rogercresseyi* was evaluated during Atlantic salmon and coho salmon infestation using 27 genes associated with the immune response, antioxidant system, and proteases [23]. Oxidative stress responses by sea lice were evidenced when infecting either host salmon species, suggesting that reactive oxygen species could modulate the sea louse antioxidant system. Furthermore, protease transcripts evidenced an upregulation of *Trypsins* and *Serpins*, particularly in Atlantic salmon as compared with coho salmon. Interestingly, sea louse *Cathepsins* and *Trypsin2* were downregulated at seven days post-infestation (dpi) in coho salmon. Overall, previous investigations suggest that Atlantic salmon strongly modulate the sea lice transcriptome at earlier infestation stages, whereas coho salmon less markedly modulate the *C. rogercresseyi* transcriptome, instead increasing transcriptional activity during the infestation process [23,24]. Despite this knowledge, the main components and transcriptome profiles of the immune response in Atlantic and coho salmon during *C. rogercresseyi* infestation remain unknown, thus limiting studies to the evaluation of blood parameters as a proxy for physiological stress [25,26].

To date, no comparative molecular studies have been carried out in salmon species with susceptible/resistant phenotypes against the sea louse *C. rogercresseyi*. Therefore, the objective of the present study was to evaluate the transcriptomic modulations of Atlantic and coho salmon immune responses to sea lice infestation using whole transcriptome sequencing. In parallel, an enrichment analysis was applied to reveal the activation of main components in immune-related pathways, including TLRs, T_H1/T_H2, and macrophages.

2. Materials and methods

2.1. Experimental design and samples

Atlantic salmon (*S. salar*, n = 120) weighing 290 ± 15 g were obtained from the Chaperano Hatchery (Multiexport Food SA) in Cochamo, Chile. Similarly, coho salmon (*O. kisutch*, n = 120) weighing 280 ± 20 g were obtained from the Philippi Center (Cermaq Chile SA) in Llaquihue, Chile. All fish were reared in brackish water (15 ppm) until smolting, after which fish were maintained in ultraviolet-treated salt water in single-pass flow-through tank systems on a 12:12 h light:dark cycle. Atlantic and coho salmon were maintained separately and fed daily with the commercial Micro 200 diet (EWOS Chile). After initial acclimatization for 15 days, individuals were randomly divided by species and in triplicate (i.e. Atlantic and coho salmon) into uninfected and infected experimental groups, containing 20 fish per tank (500 L). The salmon were acclimatized for additional 7 days and were starved at least 24 h prior to any manipulation.

Regarding *C. rogercresseyi* culture for the challenge trials, ovigerous females were collected from Atlantic salmon at a commercial farm in Puerto Montt, Chile. The egg strings were removed and placed in culture buckets supplied with flowing seawater (12 °C) and gentle aeration. The eggs were allowed to hatch and develop until the infectious copepodid stage. For the challenge, each salmon group was placed in the darkness and infected by copepodids (35 per fish) for 6 h. During the infestation trials, fish were supplemented with oxygen and fed daily. For each sampling point, 3–4 fish were taken from each tank and anaesthetized with benzocaine. Head kidney and skin from an infected area were dissected, fixed in RNAlater® (Ambion), and stored at −80 °C.

2.2. High-throughput transcriptome sequencing and de novo assembly

Head kidney and skin samples were taken from ten infected fish at 0, 7, and 14 dpi and used for MiSeq cDNA library synthesis. All samples were fixed in the RNAlater® RNA Stabilization Reagent (Ambion®, Life Technologies™, Carlsbad, CA, USA) and stored at −80 °C. Total RNA was extracted from each individual using the RiboPure™ Kit (Ambion®) following the manufacturer's instructions. Total RNA was treatment with DNAse I (Thermoscientific) at 37 °C for 30 min, according to the manufacturer's instructions. The quantity, purity, and quality of isolated RNA were measured in the TapeStation 2200 (Agilent Technologies Inc., Santa Clara, CA, USA) using the R6K Reagent Kit according to the manufacturer's instructions. Five RNA samples per tissue with RIN >8.0 were pooled and used for library preparation. Subsequently, by using RNA pools of each tissue according to species and sampling point, 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 were sequenced by the MiSeq (Illumina®) platform using sequenced runs of 2 × 251 paired-end reads at the Laboratory of Biotechnology and Aquatic Genomics, Interdisciplinary Center for Aquaculture Research (INCAR), Universidad de Concepción, Chile.

Sequence assembly was carried out using the CLC Genomics Workbench v9.1 software (CLC Bio, Aarhus, Denmark). Two *de novo* assemblies were performed using datasets from each salmon species. For this, assembling was applied with an overlap criteria of 70% and a similarity of 0.9 to exclude paralogous sequence variants [27]. The settings used were a mismatch cost = 2, deletion cost = 3, insert cost = 3, minimum contig length = 200 base pairs, and trimming quality score = 0.05. After assembly, singletons were retained in the dataset as possible representatives of low-expression transcript fragments. However, any sequence redundancy of these fragments was removed by the Duplicate Finder tool incorporated in the Geneious v8.0 software (Biomatters, Auckland, New Zealand).

2.3. Sequence annotation and RNA-seq analysis

A protein database enriched for TLR, T_H1/T_H2, and macrophage activation pathways, as available for bony fish in GenBank and UniprotKB/Swiss-Prot, was used to annotate the assembled contigs for each salmon species through tBLASTx with a cutoff E-value of 1E-10 (Table S1). The transcripts identified as immune genes were used as references for RNA-seq analyses of the head kidney and skin samples from Atlantic and coho salmon infected with sea lice. The RNA-seq settings were a minimum length fraction = 0.6 and a minimum similarity fraction (long reads) = 0.5. The expression values were set as transcripts per million (TPM), a modification of reads per kilobase of transcript per million mapped reads (i.e. RPKM) designed to be consistent across samples. More specifically, transcripts per million values are normalized by total transcript count, instead of read count, and by average read length. These normalizations allow assessments of overregulated transcripts among different groups [28]. The distance metric was calculated

with the Manhattan method, with subtraction of the mean expression level in 5–6 rounds of *k*-means clustering. Finally, a Kal's statistical analysis test was used to compare gene expression levels in terms of $\log_2$ fold-change ($P = 0.0005$; false discovery rate [FDR] corrected).

2.4. Network construction and enrichment analysis

The ClueGO + CluePedia plugins, contained within the Cytoscape v3.0.2 software, were used to identify networks and functional pathways of differentially expressed genes in response to sea lice infestation. In particular, ClueGO [29] was used to visualize an extensive dataset of functional interactions constructed from the gene ontology (GO), KEGG, and Reactome databases. For this, ClueGO first formed a binary gene-term matrix with the selected terms and corresponding associated genes. From this matrix, a term-term similarity matrix was then calculated using chance-corrected kappa statistics to determine association strength between terms. For biological networks, CluePedia [30] was used to calculate correlations for differentially expressed genes based on the following four tests: Pearson's correlation, Spearman's rank, distance correlation, and maximal information coefficient. Enrichment/depletion was calculated using the hypergeometric test with Benjamini and Hochberg FDR multiple testing correction and significance (pFDR<0.05). Additionally, complementary analyses were conducted with the ClusterMaker Cytoscape plugin [31] and the Markov Cluster algorithm to search protein-protein interaction network modules derived from tandem affinity purification/mass spectrometry.

2.5. RT-qPCR validation and statistical analysis

To corroborate RNA-seq analysis accuracy and the biological effect, RNA samples were taken from pooled fish tissues and from individual tissues to conduct RT-qPCR validations. For this, contig sequences from Atlantic and coho salmon datasets were used as templates for primer design with the Primer3 Tool [32] included in the Geneious Pro v8.1 software [33] (Table S2). For gene amplification, total RNA was isolated from the skin and head kidney using the RiboPure™ Kit (Ambion®) following the manufacturer's instructions. Purity was determined (A260/A280 ratio) with a Nanodrop ND1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and integrity was determined by agarose gel under denaturant conditions. From total RNA (200 ng/μL), cDNA was synthetized using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). The RT-qPCR runs were performed with StepOnePlus™ (Applied Biosystems®, Life Technologies™, Carlsbad, CA, USA) using the comparative ΔCt method. *Elongation factor-α* (*ef-α*), *β-tubulin*, and *18S*, three putative reference genes, were statistically analyzed by the NormFinder algorithm to assess transcriptional expression stability. Through this, *ef-α* was selected as the reference gene for normalizations. Each reaction was conducted in a volume of 10 μL using the Maxima® SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific). The amplification conditions were as follows: 95 °C for 10 min, 40 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. Finally, data from RT-qPCR analyses were screened for normality and Box-Cox transformed when necessary. To infer differences between salmon species, a factorial ANOVA was performed, and a *post-hoc* Tukey's Honest Significant Difference test was applied when differences occurred. All analyses were performed in the JMP v9.0 software (SAS Institute, Inc., Cary, NC, USA). Statistically significant differences were accepted with a $p < 0.005$. Additionally, a principal component analysis (PCA) was performed as an exploratory technique to identify underlying data structure between Atlantic and coho salmon.

2.6. Amino acid sequence analyses

To evaluate the phylogenetic relationships among Toll-like receptor family members (Table S3), protein alignments were conducted using MUSCLE, and phylogenetic trees were constructed using the neighbor-joining method with 1000 bootstrap repetitions. Both analyses were carried out in Geneious v8.1 [33]. Furthermore, deduced amino acid sequences were analyzed with the Expert Protein Analysis System (http://www.expasy.org/), and the Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de/) was used to predict the features of protein domains.

3. Results

3.1. Infestation results

Infected fish were maintained in closed tanks for 14 days after initial exposure to sea lice, and parasite counts on fish were recorded at 1, 3, 7, 10, and 14 dpi. Differences in sea lice abundances were observed between Atlantic and coho salmon groups at 1 dpi, with parasitic loads of 25 and 17.3 lice per fish, respectively. Subsequently, greater differences were found between the infected groups at 14 dpi, with roughly 21.5 lice per Atlantic salmon and 1 louse per coho salmon (Fig. S1).

3.2. Assembly and transcriptome annotation

The sequencing runs for each species yielded a total of 78,704,324 and 77,737,810 reads that assembled into 303,898 and 308,992 contigs for Atlantic and coho salmon, respectively (Table 1). All contigs were annotated using the tBLASTx algorithm against a database with genes involved in immune response, such as the TLR pathway, T_H1, T_H2, and macrophage activation. Using an E-value > 1E-10, a total of 1492 and 1522 contigs were annotated for Atlantic and coho salmon, respectively (Table S4).

The transcriptome profiles of salmon skin evidenced increasing activity from 0 to 14 dpi, particularly in Atlantic salmon as compared to coho salmon (Fig. 1A). Indeed, a similarly high transcription of immune-related genes was recorded in coho salmon at 0 and 14 dpi. Interestingly, the transcriptome profile of 7 dpi coho salmon was concentrated in some specific clusters (Fig. 1A). Moreover, Venn diagrams evidenced similar patterns of differentially expressed immune genes for both species, with notably more transcripts expressed at 7 dpi in Atlantic than coho salmon, and slight differences in coho salmon at 14 dpi (Fig. 1B and C). Regarding the transcriptome profiles for the head kidney, an inverse pattern of gene expression was observed (Fig. 2A), with high expression levels of immune genes at 14 dpi in both salmon species. However, Venn diagram analysis revealed that coho salmon presented a higher number of differentially expressed immune transcripts at 14 dpi (Fig. 2B and C).

3.3. Immune network reconstruction with macrophage and T_H1-T_H2 matrix

To investigate the functional consequence of sea lice infestation in different Atlantic and coho salmon tissues (i.e. skin and head kidney), network analyses were established via interactome analysis by using immune cluster transcripts sharing high protein-protein affinities. In particular, 72 differentially expressed genes were imported into the Cytoscape plugins ClueGO + CluePedia to examine the gene network and functional pathway probability in response to *C. rogercresseyi* infestation. Cytoscape inquiries

Table 1
Summary of Illumina sequencing for Atlantic salmon and coho salmon.

	Atlantic salmon			Coho salmon		
	Skin	Head kidney	*de novo* assembly	Skin	Head kidney	*de novo* assembly
Reads (Mb)	37.6	41.2	78.7	37	40.7	77.7
Average length (bp)	199.52	194.39	196.84	203.86	193.91	198.65
Matched (Mb)	23.88 (63.5%)	29.56 (71.7%)	65.9 (83.8%)	23.4 (63.2%)	27.8 (68.3%)	64.1 (82.5%)
Nucleotide number (Gb)	7.5	7.9	15.4	7.5	7.8	15.4
Contigs	252.614	165.171	303.898	259.389	165.879	308.992
Average length (bp)	546	596	554	542	585	547
Singletons (Mb)	13.6	11.5	12.7	13.5	12.8	13.6
Average length (bp)	203.01	198.24	196.85	207.17	191.93	199.35

revealed that a total of 133 genes built a network that linked the 72 differentially expressed genes (Fig. 3). In all, 89 GO-terms were over-represented (Bonferroni corrected $p < 0.0001$). GO enrichment of the 72 genes relevant to the salmon response were mapped by GO category, which finally resulted in a wide range of immunological processes, such as *nf-κβ* signaling, the defense response, T cell and lymphocyte activation regulations, the Jak-stat cascade, interferon gamma production, the acute phase response, hemopoiesis, cell chemotaxis, and cytokine receptor activity.

Modules with the highest number of node-node immune interactions in each tissue were selected. Subsequent analyses in the two salmon species implied functional and tissue-dependent enrichments. Interaction analysis between mRNA abundance and tissue emphasized a significant interaction between salmon species and skin-based mRNA abundances (Figs. 3 and S2; two-way ANOVA $F_{(3,\ 72)} = 2{,}716$, $p < 0.05$). Specifically, most GO categories were upregulated in Atlantic, as compared to coho, salmon at 7 dpi, and extracellular components, such as *il1-β*, *il6*, *il10*, and *ccl4*, were transcriptionally activated in Atlantic salmon during the early stages of infestation (Fig. 3A and B). Interestingly, *il22* and *ccl25* were upregulated in coho salmon as compared to Atlantic salmon at 7 dpi. Nevertheless, skin tissue gene expression profiles were similar between salmon species by 14 dpi, with the exceptions of lymphocyte activation, Jak-stat cascade, and *nf-κβ* signaling upregulations in only coho salmon (Fig. S3). Regarding head kidney mRNA abundances, significant differences were also observed between salmon species (Fig. 3-S2; two-way ANOVA $F_{(3,\ 72)} = 3065$ $p < 0.05$). In particular, 14 dpi coho salmon head kidney samples were mostly activated for the regulation of T cells and the Jak-stat cascade, whereas the main GO categories modulated in Atlantic salmon head kidney samples were lymphocyte activation, the defense response, and *nf-κβ* signaling (Fig. 3A and B).

3.4. Transcriptome enrichment analysis for TLRs, T_H1/T_H2, and macrophage activation pathway

One of the aims of this study was to evaluate candidate genes related to TLRs, T_H1/T_H2, and the macrophage activation pathway through transcriptome enrichment analysis. In general, Atlantic salmon presented a greater abundance of upregulated transcripts from 7 to 14 dpi in both analyzed tissues as compared with coho salmon (Fig. S3). Comparatively, the head kidney of Atlantic salmon presented a higher quantity of upregulated gene transcripts involving macrophage activation, T_H2, and TLR pathways at 7 dpi (Fig. S3). Nevertheless, the abundance of immune transcripts in coho salmon was decreased at 14 dpi for all evaluated genes (Fig. S3).

Additionally, changes in expression levels were estimated using TPM values at 7 and 14 dpi for skin and head kidney samples, respectively (Table S5). Atlantic salmon head kidney presented more upregulated contigs annotated as *tlr22a2*, *tlr21*, and *btk* at 7dpi, while at 14 dpi, the most-expressed contigs were annotated as *mhcII*. In turn, Atlantic salmon skin registered high expression levels of the *tnf receptor* and *mhcII* at 7 and 14 dpi, respectively. In coho salmon head kidney, the most expressed gene at 7 and 14 dpi was *tbk-1*, whereas the most highly regulated genes in coho salmon skin at 7 dpi were *iNOS*, *tnf receptor*, and *btk* and, at 14 dpi, *mhcII* and *iNOS*.

Additionally, using the fold-change values obtained from RNA-seq analysis (Table S5), a PCA analysis was performed for each genes contig annotated with the best E-value. PC1 and PC2 revealed a separation between Atlantic and coho salmon markers (Fig. 4), the pattern of which explained 79.7% of total dataset variability. In particular, the most important genes in Atlantic salmon after *C. rogercresseyi* infestation were involved in the TLR pathway and T_H2 response, involved genes such as *tlr22a2*, *tlr21*, *il1β*, *il10*, *socs5*, and *il4* (Fig. 4). In *C. rogercresseyi*-infested coho salmon, genes were principally associated with macrophage activation and the T_H1 response, including *socs1*, *il8*, and *t-bet*.

3.5. Gene cluster analysis

RNA-seq analysis was performed for immune transcripts in Atlantic and coho salmon to identify specific gene clusters in both tissues and at all sampling points. For this, a subset of 493 contigs was constructed with fold-change values at either ≥ 2 or ≤ -2, resulting in the identification of three and five main clusters for Atlantic and coho salmon, respectively (Fig. S4). Specifically, Atlantic salmon head kidney heat maps revealed that Cluster 1 was upregulated at 14 dpi, with the identifications of genes such as *tlr22*, *btk*, and *mhcII* (Table S4). Clusters 2 and 3 presented high abundances of genes involved in the TLR pathway, with most contigs annotating to *tlr22a2*. Moreover, Cluster 3 was also composed by genes involved in the T_H1 and T_H2 responses (Table S4). In turn, 218 Atlantic salmon skin tissue contigs were grouped into three clusters (Fig. S4). Cluster 1 was upregulated at 7 dpi and was comprised of contigs annotating as *mhcII*, *tlr22a2*, and *gata3*. Cluster 2 presented a high abundance of genes involved in the T_H2 response at 7 dpi. Cluster 3 was upregulated at 14 dpi, and the most abundant annotated transcripts were related to T_H1 and TLR pathway (Table S4).

Coho salmon head kidney heat maps evidenced 224 contigs in five different clusters (Fig. S4). Cluster 1 presented highly upregulated genes at 14 dpi in relation to the T_H2 response and downstream TLR pathway. Additionally, Cluster 4 was upregulated at 14 dpi and was comprised of genes related to the T_H2 response, including *il-6*, *stat5*, and *il-1r* (Table S4). In turn, five clusters were identified for the coho salmon skin. Cluster 1 presented high expression levels at 7 dpi, with genes involved in the TLR and T_H2 responses. On the other hand, Clusters 4 and 5 were upregulated at 14 dpi. Cluster 4 was comprised by genes from the T_H1 response (*cxcr3* and *il2r*) and TLR pathway (*tlr22a2*, *btk*, and *tbk-1*). Finally, Cluster 5 was mainly composed by contigs with high homology to the *btk* gene (Fig. S4).

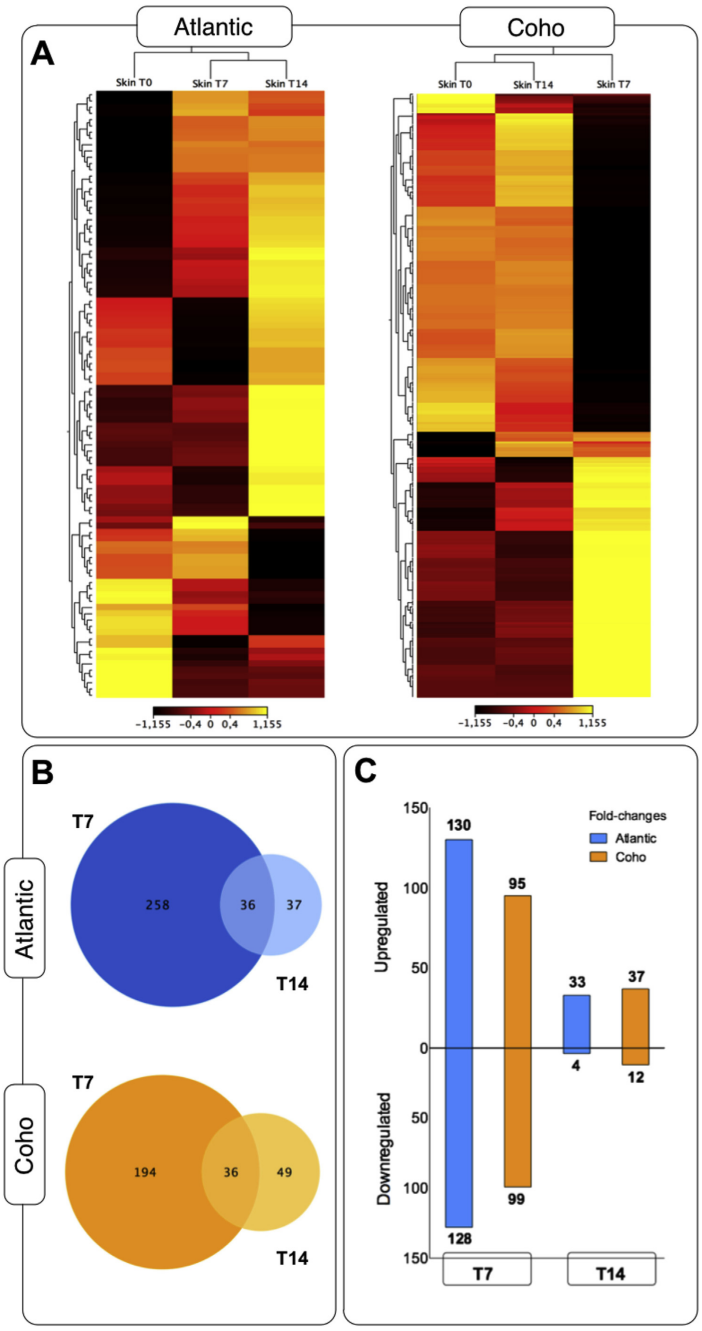

Fig. 1. Transcriptome profiles of immune-related genes in skin tissue samples from Atlantic and coho salmon infested with *Caligus rogercresseyi*. **(A)** Heatmaps of infested fish at 0, 7, and 14 days post-infestation (dpi). **(B)** Venn diagrams of differentially expressed immune-related genes (14/0 dpi and 7/0 dpi) for Atlantic and coho salmon. **(C)** Quantity of up- and downregulated immune-related genes (P-value = 0.05 and fold-change > 2) at 7 and 14 dpi for Atlantic and coho salmon.

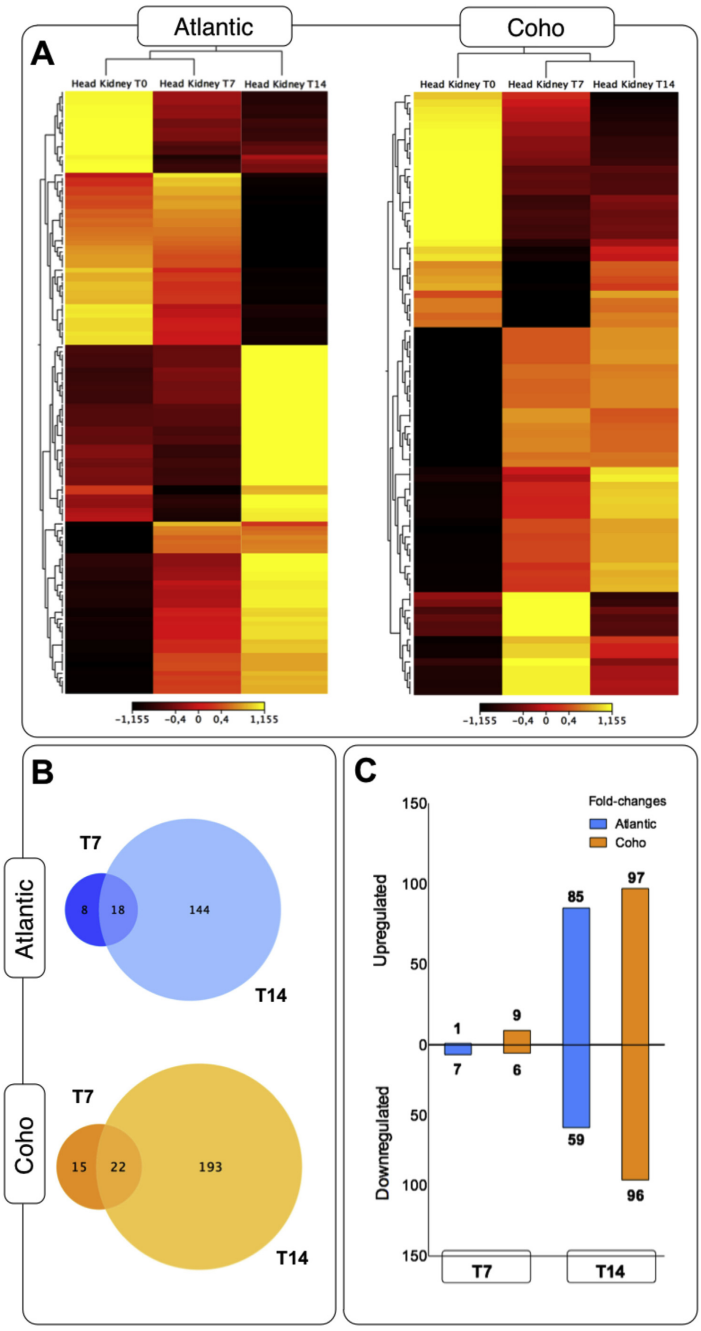

Fig. 2. Transcriptome profiles of immune-related genes in head kidney tissue from Atlantic and coho salmon infested with *Caligus rogercresseyi*. **(A)** Heatmaps of infested fish at 0, 7, and 14 days post-infestation (dpi). **(B)** Venn diagrams of differentially expressed immune-related genes (14/0 dpi and 7/0 dpi) for Atlantic and coho salmon. **(C)** Quantity of up- and downregulated immune-related genes (P-value = 0.05 and fold-change > 2) at 7 and 14 dpi for Atlantic and coho salmon.

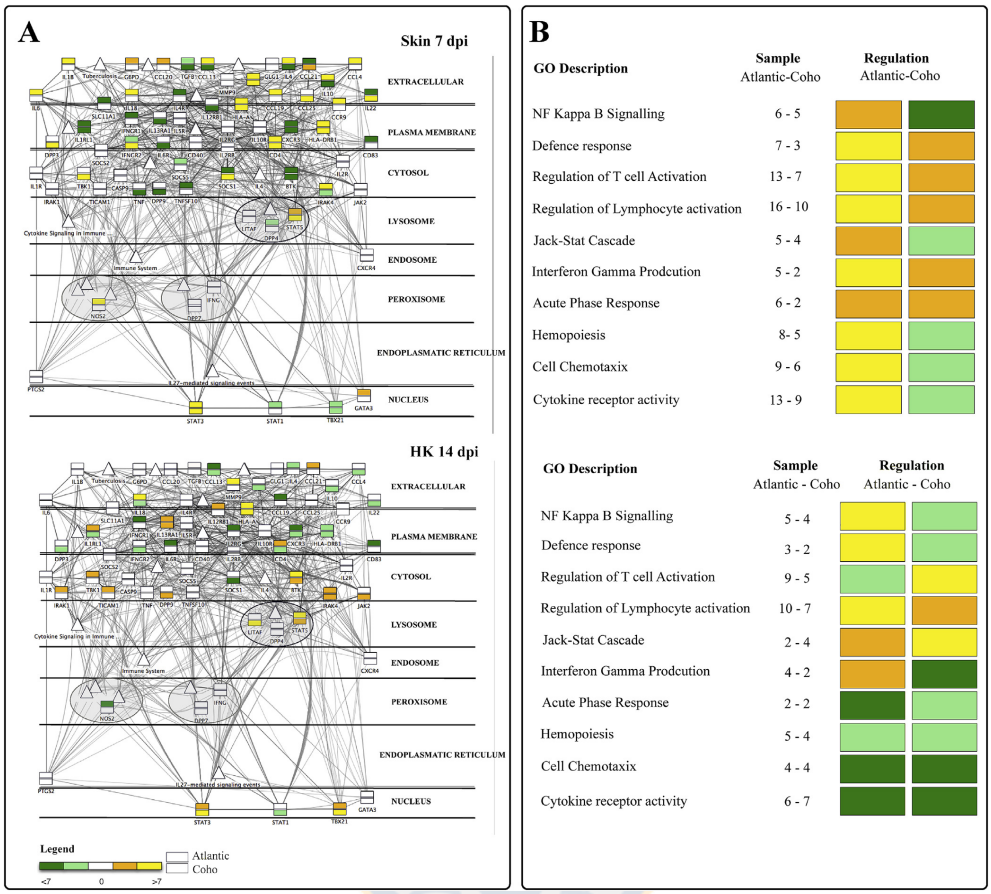

Fig. 3. Interactome network with more significant changes mapped for Atlantic and coho salmon. **(A)** Interactome modules of mRNAs expressed in the skin at 7 dpi and head kidney 14 dpi. The figure represents selected, significant protein–protein interaction network modules expressed ($P < 0.05$). **(B)** Gene ontology analysis (ClueGO + CluePedia) for each interactome-module of overexpressed GO categories ($P < 0.05$). The colored bar scale indicates relative abundances (high to low) of GO categories in dpi.

3.6. RT-qPCR analysis

To validate the RNA-seq results, 14 representative genes for the T_H1, T_H2, macrophage, and TLR responses in skin and head kidney samples were evaluated by RT-PCR for both Atlantic and coho salmon at 0, 7, and 14 dpi. In general, both species presented significant differences in the expression levels of the evaluated immune genes (Figs. 5–6). During *C. rogercresseyi* infestation in coho salmon, T_H1 genes were highly expressed in the head kidney at 7 dpi and in skin at 14 dpi (Fig. 5). Moreover, infected Atlantic salmon presented high expression levels in the head kidney and skin at 14 dpi of genes such as *cox2*, *mhcII*, *mmp13*, as well as of genes related to the T_H2 response (e.g. *il10*, *il4*, and *ccr3*) (Fig. 5). Regarding the TLR genes identified within infested salmons, *tlr13*, *tlr19*, *tlr21*, and *tlr22a2* were highly modulated in response to sea lice infestation (Fig. 6). Interestingly, *tlr21* and *tlr22a2* were mainly activated in Atlantic salmon skin and head kidney tissues at 7 dpi, whereas *tlr13* and *tlr19* were more highly activated in coho salmon head kidney and skin at 7 dpi, respectively (Fig. 6). These results suggest a putative role of TLR genes in response to sea lice infestation.

3.7. Phylogenetic analysis and molecular characterization of TLR family in Atlantic and coho salmon infested with C. rogercresseyi

From protein alignments, contigs annotated to *tlr22*a2 demonstrated an identity of 86.1% and 56.3% with sequences described for *S. salar* (CAJ80696) and *O. mykiss* (CAF31506), respectively. Regarding *tlr13*, *S. salar* evidenced 100% identity with the previously described *S. salar* sequences, but only 89.6% identity with previous *O. kisutch tlr13* sequences. Phylogenetic analysis revealed a relationship between contigs annotated as *tlr22a2* in *S. salar*, *O. kisutch*, and previously described sequences (Fig. S6A). Contigs annotated as *tlr13* were separately grouped with sequences reported for *S. salar* (NP_001133860), which was placed apart from

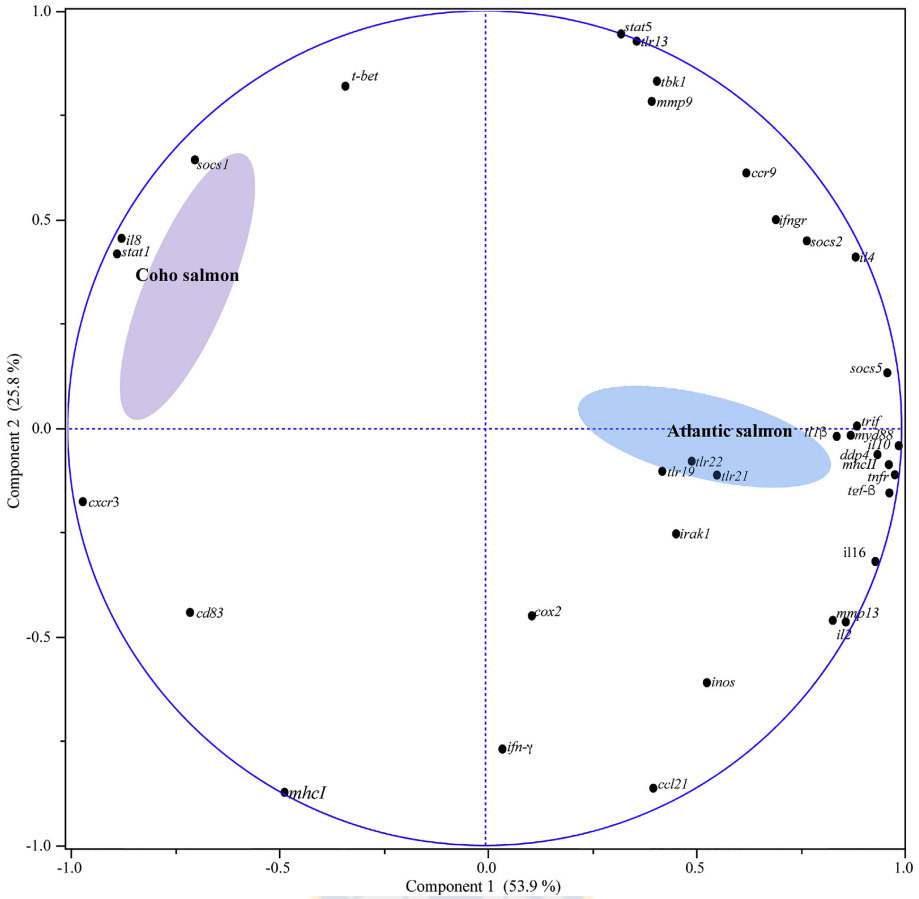

Fig. 4. Principal components analysis of RNA-seq data for genes involved in the immune responses of Atlantic and coho salmon infested by *C. rogercresseyi*.

other mammalian *tlr13* sequences (Fig. S6B). Furthermore, phylogenetic comparisons between the *tlr22a2* and *tlr13* sequences from Atlantic and coho salmon were congruent with the identified clusters for each TLR (Fig. S6C).

The type of most abundant TLR in Atlantic and coho salmon was also assessed using *in silico* data. Interestingly, the most abundant mRNA in Atlantic salmon was *tlr22a2*, and, in coho salmon, this was *tlr13* (Fig. S7). Regarding transcript abundance by tissue, *tlr22a2* and *tlr13* were the most abundant in the skin of Atlantic salmon and coho salmon, respectively. In the case of the head kidney, *tlr13* was the most abundant transcript observed in both species.

4. Discussion

Until now, studies assessing the immune response of salmonids infested by sea lice have focused on the cytokine-mediated pro-inflammatory response [5], on protease secretion [34], and on the humoral response [5,8]. However, no prior studies evaluated the response of classic innate immune pathways, such as that initiated by TLRs. Therefore, the present study used whole transcriptome sequencing on *C. rogercresseyi*-infested Atlantic and coho salmon to evaluate transcriptional changes in molecular components associated with macrophage activation and the T_H1/T_H2 responses. While these immune mechanisms are known to play important roles in the salmonids response to sea lice infestation [4,7,8], corresponding associations with specific TLR modulations during sea lice infestation had not been evaluated.

From global transcriptome analysis, Atlantic and coho salmon displayed similar tissue-specific transcription patterns, with the skin mainly modulated during early sea lice infestation stages and head kidney expressions principally modulated during late-stage parasitism. Interestingly, the acute transcriptome response of immune genes was mainly related to Atlantic, rather than coho, salmon. Specifically, the highest quantity of differentially expressed transcripts was displayed in coho salmon at 14 dpi. Considering this, together with the infestation results, Atlantic salmon appear to

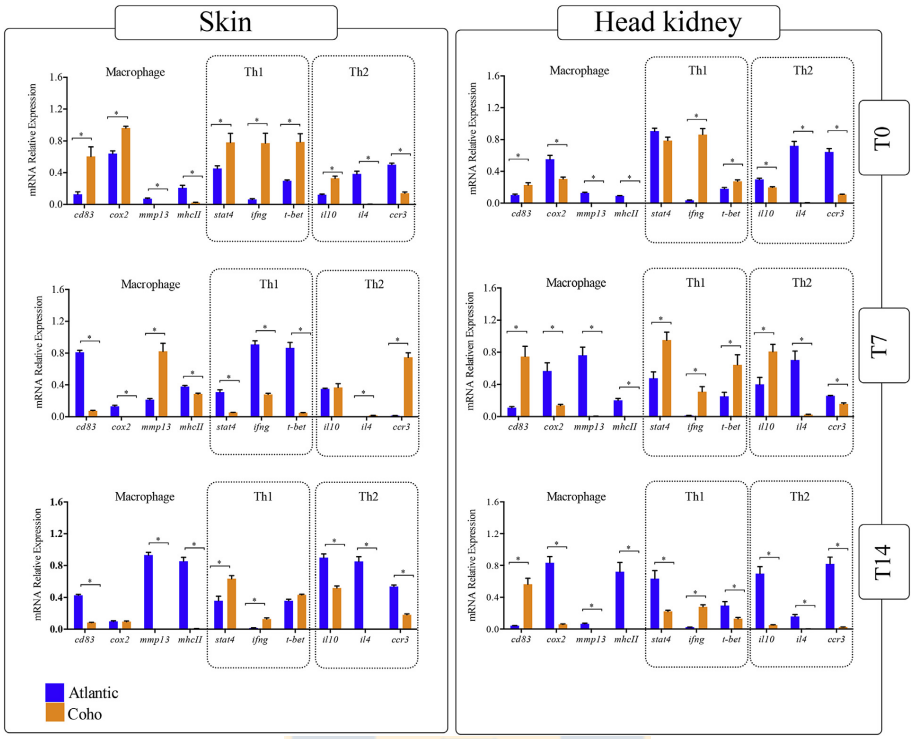

Fig. 5. RT-qPCR validation of genes involved in the pro-inflammatory response of Atlantic and coho salmon infested by *C. rogercresseyi*. **(A)** Relative transcript expressions in the head kidney and skin of Atlantic and coho salmon after infestation. **(B)** Definition of reaction norm in the head kidney and skin of Atlantic and coho salmon after infestation. Asterisks indicate significant differences between species ($P > 0.0001$).

cope with infestation via a vast repertoire of immune genes and by increasing the quantity and intensity of these during infestation. An inverse response pattern was observed in coho salmon after sea lice infestation, consequently resulting in a lower parasitic load at 14 dpi. Furthermore, a PCA analysis evidenced a clear differentiation in the immune responses of Atlantic and coho salmon infected with *C. rogercresseyi*.

It is know that coho salmon and pink salmon (*Oncorhynchus gorbuscha*) are the most resistant salmonids to *L. salmonis* infestation, specifically increasing the expressions of pro-inflammatory genes such as *il4* and *il10* [8,9]. Similar responses have been observed in resistant families of Atlantic salmon [7]. Prior reports indicate that a T_H2 immune response is generated by *L. salmonis* resistant fish [5,7,8]. Nevertheless, the T_H1 response is fundamental for protection during the early stages of *L. salmonis* infestation [4,7,8]. For example, activation of the T_H1 response by dietary immunostimulants can reduce *L. salmonis* loads in salmonids [35]. In the current study, coho salmon presented an increased T_H1 response during *C. rogercresseyi* infestation. Moreover, this salmon species, which is more resistant to *C. rogercresseyi* infestation than Atlantic salmon, presented low activity levels of the T_H2 response. The high T_H1 response in coho salmon could represent the strategy used by this resistant species to limit the amount of mucus available for ectoparasites, particularly when considering that the T_H2 response can alter mucin production to increase mucus availability [5,36,37]. Importantly, increased mucus availability would likely benefit the parasite since this substance is the primary source of food for *C. rogercresseyi*.

The T_H1 and T_H2 responses are activated after the recognition of pathogen-associated molecular patterns by TLRs. In teleosts, there are 20 different TLR types, including mammalian orthologs and TLRs only identified in fish [19,38]. Among these, *tlr22a2* is upregulated in fish infected by the ectoparasite *A. siamensis* [18]. Furthermore, research has associated *tlr22a2*, present only in fish, with the response of *L. rohita* to infestation by *A. siamensis*, with significantly increased *tlr22* expression at 15 dpi [39]. Furthermore, this increased *tlr22* expression in *L. rohita* has been directly related to increased parasite load [40]. In the present study, the skin of Atlantic salmon particularly evidenced an increased abundance and expression of *tlr22a2* transcripts during *C. rogercresseyi* infestation. This increased *tlr22a2* expression in Atlantic salmon, the more susceptible species, suggests that *tlr22a2* impacts the innate immune response during *C. rogercresseyi* infestation. Moreover, a comparative study between *L. rohita* and *C. catla*, species with different susceptibilities to *A. siamensis* supported the greater infestation-susceptibility and increased *tlr22* transcript levels of *L. rohita* as compared to *C. catla*, thus suggesting the participation of *tlr22* in the response to an ectoparasite [18].

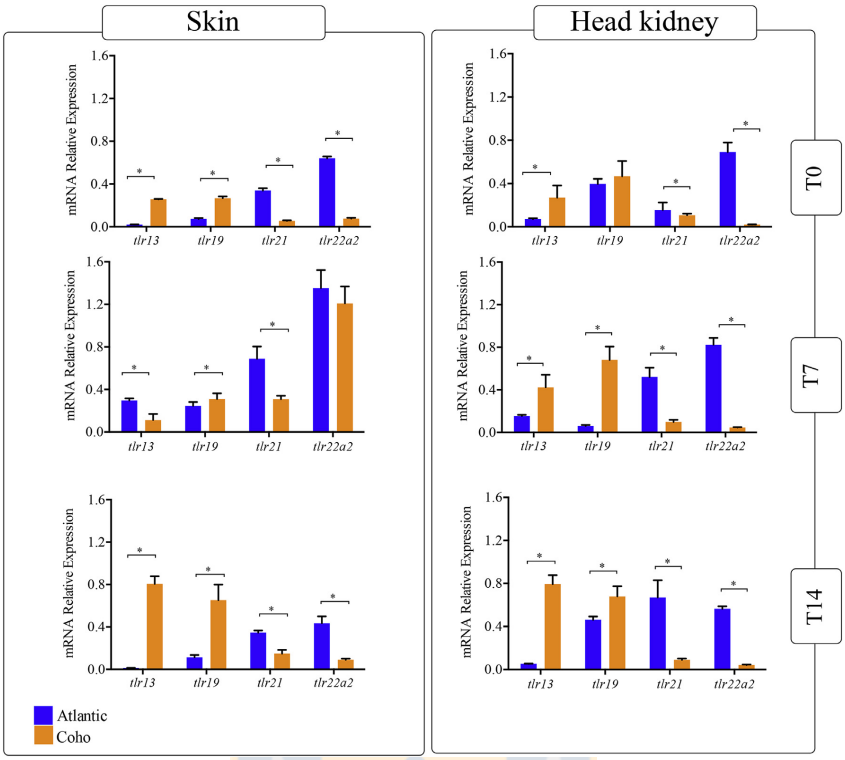

Fig. 6. Relative mRNA expression for TLR genes in Atlantic and coho salmon infested by *C. rogercresseyi*. Asterisks indicate significant differences between species ($P > 0.0001$).

The current study also found differences in the abundance and expression of *tlr13*, which was more abundant and expressed in coho salmon than in Atlantic salmon. Regarding function, *tlr13* recognizes bacterial ribosomal RNA in mammals [41,42]. Furthermore, in fish, *tlr13*, *21*, and *22* could have a common ancestor [43]. Supporting this hypothesis, Sundaram et al. [44] used phylogenetic analysis to demonstrate that the Atlantic salmon *tlr13* sequence is grouped with *tlr22* sequences described in distinct teleosts. This information, together with the presently obtained data, suggests that the abundance and overexpression of *tlr13* in *C. rogercresseyi*-infested coho salmon could be indicative of a role during the response to ectoparasite infestation.

Additionally, $MHCII^+$ cells have been observed in the tissues of fish with lesions caused by *Neoparamoeba perurans* [45], as well as in fish infested by *L. salmonis* [8]. However, *L. salmonis*-resistant and -susceptible Atlantic salmon families present a downregulation of *mhcII* [7]. The present study, in contrast to findings in salmon infected with *L. salmonis*, found an increase in the expression of *mhcII* in the skin and head kidney of Atlantic salmon infected with *C. rogercresseyi*. Considering the increased expressions of *mhcII* and *tlr22a2* in Atlantic salmon, *mhcII* appears to act as a mediator of the TLR signaling pathway following *C. rogercresseyi* infestation. This has been previously suggested by Liu et al. [46], who found a decrease of proinflammatory cytokines in MHCII-deficient mice. This decrease can be related to the interaction of MHCII with Btk, a protein that consequently stimulates molecules such as MyD88, through which TLR signaling is promoted.

Worth noting, sea lice infestations cause tissue damage requiring rapid repair to prevent additional adverse effects from osmotic changes [47]. Tissue is repaired through the stimulation of MMP in fish [48]. Among the MMPs that respond to the presence of sea lice are *mmp9* and *mmp13*, both of which have been observed in Atlantic salmon Atlantic salmon infected with *L. salmonis* [4–6]. Similar expressional behaviors were found in the present study. Specifically, Atlantic salmon infested by *C. rogercresseyi* presented increased *mmp13* expression levels in both tissues, whereas coho salmon only evidenced increased expression in the skin at 7 dpi. These results support the importance of tissue-repairing genes in fish infested by sea lice.

5. Conclusion

Previous studies have reported that Atlantic salmon infected with *L. salmonis* evidence a T_H2 immune response. The present findings complement this knowledge by revealing that highly resistant coho salmon present a strong T_H1 type response to *C. rogercresseyi* infestation. Additionally, TLRs were found to play a possible role in the immune response to sea lice. This was particularly so for *tlr22a2*, which was highly expressed in *C. rogercresseyi*-infested Atlantic salmon. Together these results indicate that host

tissue damage caused by *C. rogercresseyi* liberates molecules recognized by *tlr22a2*, thus activating the host immune response. Additionally, in coho salmon the abundance of *tlr13*, the increased expression of this receptor in the skin, and the phylogenetic similarity of *tlr22* and *tlr13* suggest a possible role of *tlr13* in the response to ectoparasites.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2016.10.046.

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CAPITULO 2

Transcription expression of immune-related genes from *Caligus rogercresseyi* evidences host-dependent patterns on Atlantic and Coho salmon

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Abstract

The transcriptomic response of the sea louse *Caligus rogercresseyi* during the infestation on Atlantic salmon (*Salmo salar*) and coho salmon (*Oncorhynchus kisutch*) was evaluated using 27 genes related to immune response, antioxidant system and secretome. Results showed early responses of TLR/IMD signaling pathway in sea lice infesting Atlantic salmon. Overall, genes associated with oxidative stress responses were upregulated in both host species. This pattern suggests that reactive oxygen species emitted by the host as a response to the infestation, could modulate the sea louse antioxidant system. Secretome-related transcripts evidenced upregulation of trypsins and serpins, mainly associated to Atlantic salmon than coho salmon. Interestingly, *cathepsins* and *trypsin2* were downregulated at 7 days post-infection (dpi) in coho salmon. The principal component analysis revealed an inverse timedependent pattern based on the different responses of *C. rogercresseyi* infecting both salmon species. Here, Atlantic salmon strongly modulates the transcriptome responses at earlier infection stages; meanwhile coho salmon reveals a less marked modulation, increasing the transcription activity during the infection process. This study evidences transcriptome differences between two salmon host species and provides pivotal knowledge towards elaborating future control strategies.

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Short communication

Transcription expression of immune-related genes from *Caligus rogercresseyi* evidences host-dependent patterns on Atlantic and coho salmon

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ABSTRACT

The transcriptomic response of the sea louse *Caligus rogercresseyi* during the infestation on Atlantic salmon (*Salmo salar*) and coho salmon (*Oncorhynchus kisutch*) was evaluated using 27 genes related to immune response, antioxidant system and secretome. Results showed early responses of TLR/IMD signaling pathway in sea lice infesting Atlantic salmon. Overall, genes associated with oxidative stress responses were upregulated in both host species. This pattern suggests that reactive oxygen species emitted by the host as a response to the infestation, could modulate the sea louse antioxidant system. Secretome-related transcripts evidenced upregulation of *trypsins* and *serpins*, mainly associated to Atlantic salmon than coho salmon. Interestingly, *cathepsins* and *trypsin2* were downregulated at 7 days post-infection (dpi) in coho salmon. The principal component analysis revealed an inverse time-dependent pattern based on the different responses of *C. rogercresseyi* infecting both salmon species. Here, Atlantic salmon strongly modulates the transcriptome responses at earlier infection stages; meanwhile coho salmon reveals a less marked modulation, increasing the transcription activity during the infection process. This study evidences transcriptome differences between two salmon host species and provides pivotal knowledge towards elaborating future control strategies.

1. Introduction

Caligidosis is a parasitic disease that affects the salmon industry caused by the ectoparasites *Lepeophtherius salmonis* in the Northern Hemisphere, and *Caligus rogercresseyi* in the Southern Hemisphere [1,2]. Infected salmons trigger a series of defense mechanisms, such as a pro-inflammatory response [3,4], and even a T_H2-type response [5,6]. In Chile, the salmonid species most affected by *C. rogercresseyi* are *Salmo salar* and *Oncorhynchus mykiss*, whereas *Oncorhynchus kisutch* presents greater resistance to infection by this copepodid [1]. *L. salmonis* has different infection patterns among salmonid species, with morphological differences of the epidermis and mucus composition accounting for varied infestation susceptibilities [7,8]. Likewise, three cell types (MHIIβ, IL1β, TNFα) are activated in coho salmon during *L. salmonis* infection [6], while in the susceptible Atlantic salmon, there is an increased expression of pro-inflammatory genes [3,4,9].

The transcriptome of *C. rogercresseyi* was recently sequenced [10], thus providing valuable molecular information for this species. Through this data, innate immune response pathways, such as the Toll-like receptor (TLR) and immune deficiency (IMD) pathways, have been molecularly characterized [11]; resulting in the identification of some genes from the reactive oxygen species (ROS) response [12] and an analysis of transcriptional changes in secretory products of *C. rogercresseyi* [13,14]. However, differences associated with the transcriptional response of *C. rogercresseyi* to each host species have not been studied. Here, the objective of the present study was to evaluate the transcriptional modulation of

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C. rogercresseyi at 1, 3, and 7 days post-infection (dpi) of Atlantic and coho salmon.

2. Materials and methods

2.1. Experimental design and sample collection

Sixty Atlantic salmon (*S. salar*) weighing 290 ± 20 g were obtained from the Chaperano Hatchery (Multiexport S.A.) in Cochamo, Chile. Likewise, sixty coho salmon (*O. kisutch*) weighing 280 g were obtained from the Phillipi Center (Cermaq Chile S.A.) close to Llanquihue, Chile. All fish were free of parasites and healthy before the infestation. All fish were reared in brackish water (15 ppm) until smolting, after which specimens were maintained in ultraviolet-treated salt water in single-pass flow-through tank systems on a 12:12 h light:dark cycle. Specimens were fed daily in proportion to 1% of total biomass. Fish were randomly divided between two experimental infected groups (*S. salar* and *O. kisutch*) in triplicate tanks (500 L), acclimated for seven days, and starved at least 24 h prior to any manipulation.

Ovigerous females of *C. rogercresseyi* were collected during Atlantic salmon harvesting at a commercial aquaculture center in Puerto Montt, Chile. After collection, the lice were transported on ice to the Fundación Chile laboratory (Chinquihe, Puerto Montt, Chile). These ovigerous females, only lice displaying attachment behavior to the collection vessel were included in the study in order to collect larvae. Their egg strings were then removed and placed in culture buckets supplied with seawater flow (12 °C) and gentle aeration. Eggs were allowed to hatch and develop until the infectious copepodid stage, at which point were harvested for fish infection. For infection, fish were placed in the dark without water flow for 2 h with a load of 35 copepodids per fish. Sea lice samples were collected from 10 fish for each group at 1, 3, and 7 dpi. All samples were fixed in the RNAlater® RNA Stabilization Reagent (Ambion, USA) and stored at −80 °C until subsequent RNA extraction.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from pools of five individuals using the TRIzol® Reagent (Ambion, USA) according to the manufacturer's instructions. The concentration and purity of RNA were determined by the Nanodrop ND-100 spectrophotometer (NanoDrop® Technologies, Inc. USA). Total RNA integrity was measured by electrophoresis on 1.2% denaturing gel stained with ethidium bromide. Following this, 400 ng of RNA were used to synthesize cDNA with the RevertAid™-H Minus Kit (Thermo Scientific, USA) according to the manufacturer's instructions.

2.3. Primer design and standardization

From contigs identified for the IMD and TLR pathways [11], primers were designed using the Geneious v7.1.3 software (Biomatters Ltd., New Zealand). Primers for ROS and secretome related genes were obtained from prior studies [12,14]. Primer standardization was performed by RT-PCR in a Veriti Thermocycler (Applied Biosystems). The RT-PCR reactions were performed in a final volume of 12.5 μl with Taq Polymerase 0.06 U/μl (Thermo Scientific, USA), 0.2 mM dNTPs, 1.5 mM MgCl2, 0.2 μg/μl BSA, and 1X buffer. Cycling conditions were as follows: 94 °C for 2 min and 30 s, 35 cycles at 94 °C for 30 s, alignment temperature °C for 30 s, and 72 °C for 45 s, with final extension at 72 °C for 5 min. After RT-PCR standardization, a dynamic range was performed for each primer set in order to determination the primer efficiency (Table 1). The dynamic range analysis was performed in Thermocycler StepOnePlus™ (Applied Biosystems, Life Technologies, USA). RT-qPCR reactions were carried out in 10 μL using the Maxima® SYBR Green/ROX master mix (Thermo Scientific, USA) with 1:5 serial dilutions. The amplification settings were 95 °C for 10 min, 40 cycles at 95 °C for 30 s, 30 s at the optimum annealing temperature for each primer (Table 1), and 72 °C for 30 s.

2.4. Expression analysis for transcripts of the TLR and IMB pathways, antioxidant system, and secretome in C. rogercresseyi during infection of Atlantic and coho salmon

Real time-qPCR analysis was performed with the cDNA of *C. rogercresseyi* individuals collected from infected Atlantic and coho salmon. Relative expression was determined by the StepOnePlus™ system (Applied Biosystems, Life Technologies, USA) using the comparative ΔCt method and *β-tubulin* as the housekeeping gene. Each reaction was performed in 10 μl using the Maxima® SYBR Green/ROX Master Mix (Thermo Scientific, USA). The stages for amplification were as follows: 95 °C for 10 min, 40 cycles at 95 °C for 30 s, 30 s at the optimum temperature for each primer (Table 1), and 72 °C for 30 s.

2.5. Data analysis

Variables were screened for normality based on the skewness and kurtosis of the respective distribution and were normalized through BoxCox transformation when normality standards were not met [15]. Fold-change differences in gene expressions of sea lice parasitizing different host species were evaluated using the Tukey HSD test for each time frame (T3/T1 and T7/T1). Significant differences between fold-changes for different hosts were accepted when $P < 0.05$. Additionally, a principal component analysis (PCA) was performed as an exploratory technique to identify underlying data structure. PCA is very useful in datasets with several variables because it reduces the original dimensions (variables) into fewer new dimensions each one defined by a linear combination of the original variables. These linear combinations are the principal components or latent variables that explain the maximum variability of the dataset as possible, and each sample will have a score for each principal component. As a final result of this analysis, differences between analyzed samples (sea lice) can be visualized by plotting the respective scores. Each principal component importance is reflected by the importance of each variable defining the component, thus PCA provides a meaningful interpretation of each principal component based on the variables that are most important in defining it. To extract the most important new dimensions (or principal components) and the variables with higher importance in defining these dimensions a set of rules were followed. The principal components were retained when their eigenvalue was higher than 1 following the Kaiser-Guttman criterion [16,17]. Based on this criterion the components that are further interpreted should account for at least the variance of a single variable (thus higher than 1). Since each principal component has a structure that is defined by the load of each variable, and the load of each variable is indicative of their importance in the component (from 0 to 1 as increasing importance) we considered the variables with more than 0.6 loading weight as very significant in representing the principal component [17] and these were used in the interpretation of the observed data structure. Data analyses were executed using the statistical software package JMP v9 (SAS Institute Inc., USA).

3. Results

Once infected, fish were kept for 14 days in closed tanks. The sea

Table 1
Primers designed for the present study.

Primer	Gene	Sequence	Tm	% Efficiency
Cr_AIP_F2	AIP	TCTTCTGCCTCACGGGACGA	62	90.78
Cr_AIP_R2		TGAGAACCCCGTGCCTGTCT		
Cr_Tollip_F2	Tollip	AGGCCCCTGCACAGCCTATT	63	92.1
Cr_Tollip_R2		GCGTTGACCGTGGCCGTTTT		
Cr_casp1_F1	Caspase	TAGAATGCCGGTGGAGCGAGA	63	92.4
Cr_casp1_R1		CGCTTACGGAGCTCAAGCCT		
Cr_Tab1_F1	TAB	TCGTCACCGTTCTCATCCCCT	62	93.9
Cr_Tab1_R1		TCCAAGACAGACTTCTGCAGGC		
Cr_Ikkβ_F1	Ikkβ	TCGACCAGAGCTCCTTGGCT	63	110
Cr_Ikkβ_R1		TGCGTGATGAGTCCGAGGCT		
Cr_dorsal_F1	Dorsal	TCGATCCCCACACCGCAAGA	60	94.9
Cr_dorsal_R1		TTAAGCTTGGGGGCCTCTGC		
Cr_akirin_F1	Akirin	ACCCTGAAGCGTCCCTTGGA	63	107.6
Cr_akirin_R1		AAGCCATGAGAGAGGAGGACG		
qCr_PHGP_F	PHGP	TGGAGCCGATGCCATTCCTCTTTTCA	66	98.87
qCr_PHGP_R		TTTTTGCTGGGCGCCAGGCGTGA		
qCr_PRX_F	PRX	TGCGGGGGCGGGCTTGGAAATCA	68°	108.8
qCr_PRX_R		TCCGCCGTCCTCGGGCAATCCTG		
qCr_CAT_F	Catalase	GGCACCCAGACGATAGCGATGAGCA	64	95.5
qCr_CAT_R		TCGCTTTCAGTCCCGGAAATCTGGT		
qCr_SOD_F	SOD	TCCACATGCAACACGCGCTCCAG	60	109.29
qCr_SOD_R		GCGTGCCCTGGTCGTACATGCCG		
qCr_GST_F	GST	GTTGGGGATGAGGCTCACGCACT	66	100.4
qCr_GST_R		GCTGGGTCGCCACCTACAGCACC		
qCr_Fer_F	Ferritin	GGAGTGACGGCCTAGAGGCACTCCG	65	91.35
qCr_Fer_R		TCCAGCCAAGTGCCGGTGAGCCA		
Cr_serpin3_F1	Serpin 3	GGGAGGATTAGGAATGGCGC	61	113.3
Cr_serpin3_R1		GACTTCTCAGGGATGGCTGG		
Cr_serpin4_F1	Serpin 4	ACAACACAGGTTTCGCATCT	58	97.2
Cr_serpin4_R1		ACGTTTGTCTCTTTCACAACCC		
Cr_serpin10_F1	Serpin 10	GGGAACTTTGACGCTCAAGTC	59	102.4
Cr_serpin10_R1		CCCATTTGAAGAAGGGAGCCT		
CRtryp-2-F2	Trypsin	CAGACCCAATGAAATCAAG	52–54	90.4
CRtryp-2-R2		ATAGAGTTCGTGGTAGATG		
CRtryp-5-F1	Trypsin	CTTTGAATTCCTCGTCCTT	54	91.1
CRtryp-5-R1		CAAAAACCAACTACGATGTC		
CRtryp-11-F1	Trypsin	CATGATGAAGACCATTTCTG	54	90.9
CRtryp-11-R1		AAAGGAGACTTGGAAAGG		
CRtryp-12-F2	Trypsin	TGTTCAGGTTGTCTCTGA	54	90.7
CRtryp-12-R2		GTTTCCTTGGACAAGTGG		
CRtryp-13-F3	Trypsin	CACTTGTCCAAGGAAACA	50	90
CRtryp-13-R3		GATCCAGTCAACGAACTC		
CRtryp-17-F1	Trypsin	AAGGAACTTGGTGACCTTTC	52	90.2
CRtryp-17-R1		CACTTGCCCAAGGAAACA		
Cr-Pstg-F	Prostaglandin	GATTCGTAATCTTCAGACAG	52	91.6
Cr-Pstg-R		CTCAGGATATCGGATCTTTT		
Cr-PhosC-F	Phospholipase C	CTATGAAAGTAATGAGGACC	54	90.6
Cr-PhosC-R		GAGTCTATCCAGAAAAACC		
Cr-eno-F	Enolase	GATATACGGGGAAGATTCAG	54	100.9
Cr-eno-R		TCCCTTGTACATTTCTCC		
qCr-CatB1F	Cathepsin	CTAATAGTATCCTGTCATCG	52	106
qCr-CatB1R		AGGTAGTTAGAAGAAGTCTC		
qCr-CatD1F	Cathepsin	CTCTTCCATCTTCCTTATAG	52	113.3
qCr-CatD1R		CAGAAGTTTGAGGTCATC		

lice that were parasitizing the fish were counted at 1, 3, 7, and 14 dpi. In Atlantic salmon, an increase in the parasite load occurred over time, reaching ~800 fixed copepodids in the experimental group infected. Inversely, coho salmon presented a drastic reduction in fixed copepodids over time, reaching ~40 in the experimental group (data not shown).

The designed primers (Table 1) were used to analyze relative expression in *C. rogercresseyi* samples collected at 1, 3, and 7 dpi from Atlantic and coho salmon. For the TLR and IMD pathways, at 3 dpi the expression levels of *caspase*, *akirin*, *apoptosis inhibitor* (*AIP*), *I kappa B kinase beta* (*IKK-β*), *dorsal,* and *TAK1 binding protein* (*TAB*) were overregulated in sea lice infecting Atlantic salmon and downregulated in those infecting coho salmon (Fig. 1A). At 7 dpi, only *akirin* showed a significant change in expression between *C. rogercresseyi* groups infecting Atlantic and coho salmon (Fig. 1A).

With respect to the antioxidant system of *C. rogercresseyi* during copepodid and chalimus stages, *phospholipid-hydroperoxide glutathione peroxidase* (*PHGP*), *peroxiredoxin-1* (*PRX*), and *ferritin* were differentially expressed between the Atlantic and coho salmon groups at both 3 and 7 dpi (Fig. 1B). In the cases of *PRX* and *ferritin*, downregulation occurred in sea lice infecting coho salmon at 3 and 7 dpi (Fig. 1B). The transcription of *SOD*, in contrast to the other observed oxidative genes, was overregulated in *C. rogercresseyi* infecting coho salmon but downregulated in those infecting Atlantic salmon (Fig. 1B).

Of the 14 genes related to the secretome of *C. rogercresseyi*, five of these (*prostaglandin-endoperoxide synthase 2* (*PTGSE2*), *Trypsin 11* (*Tryp11*), *Tryp12, Tryp13,* and *Tryp17*) had significant differences in expression at 3 dpi between Atlantic and coho salmon sea lice groups (Fig. 1C). Of these five transcripts, *PTGSE2* and *Try12* were upregulated, while *Tryp13* and *Tryp17* were downregulated in *C. rogercresseyi* infecting coho salmon at 3 dpi (Fig. 1C). At 7 dpi, 11

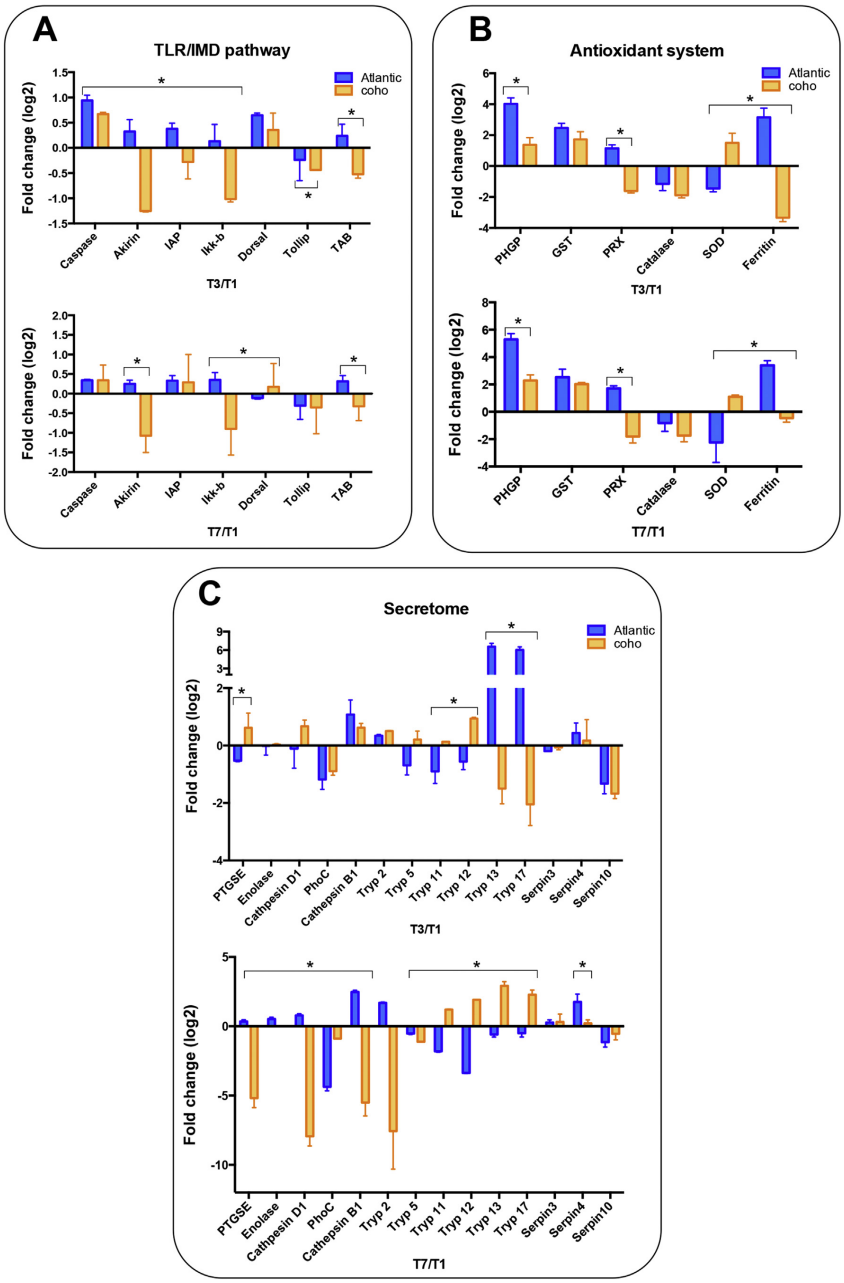

Fig. 1. Transcription expression analysis from *C. rogercresseyi* infecting Atlantic (blue bars) and coho salmon (orange bars). The comparisons were carried out between 3 and 1 days post-infection (T3/T1) and T7/T1 days post-infection. A) Immune related genes (IMD pathway), B) Antioxidant system and C) Secretome-related genes. Lines with asterisks indicate significant fold-change (log2) differences between host species (Tukey HSD test, $P < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of the evaluated genes (*PTGSE2*, *enolase*, *cathepsin D1*, *alkaline phosphatase* (*PhoC*), *cathepsin B1*, *Tryp2*, *Tryp5*, *Tryp11*, *Tryp12*, *Tryp13*, and *Tryp17*) evidenced significant transcriptional variations between infecting sea lice groups (Fig. 1C). Of these, *PTGSE1*, *cathepsin D1*, *cathepsin B1*, *Tryp2*, and *Tryp5* were downregulated at 7 dpi in sea lice infecting coho salmon (Fig. 1C). Moreover in this analysis, the genes PhoC and serpin10 did not present significant differences (Fig. 1C).

The principal component analysis revealed two main patterns of specific global scale based on the different responses from *C. rogercresseyi* when infect different hosts (Fig. 2). The correlation variability pattern between the genes expressed when infesting Atlantic salmon was prominently different than when infesting coho salmon. In addition to the global separation that defined the responses to the two hosts, an inverse time-dependent pattern was found when comparing the infestation of both fish species with different associated genes (Fig. 2A). At 1 day post-infection (T1), the response pattern of the Atlantic salmon sea lice group was loaded mainly by *Tryp12* and a low expression of *Ferritin, PRX* and IKK-β was evidenced. This was followed by an increased expression of these genes over time (T3) that ultimately changed (T7) into a response loaded mainly by the secretome-related genes *cathepsin B1* and *D1*, *Tryp2, enolase, PTGES2*; the immune-related genes *akirin, IKK-β, TAB, dorsal, AIP;* and the ROS genes *ferritin* and *PRX*. On the other hand, *C. rogercresseyi* infesting coho salmon the change in gene expression patterns was less marked. Although initial infestation period (T1), started with a higher expression of immune-related genes, this was changed at 7 dpi to downregulation, including transcripts such as *akirin*, *AIP*, *IKK-β*, *dorsal*, *TAB*, and also genes related to antioxidant system *ferritin* and *PRX* (Fig. 2B). Furthermore, the secretome genes *Serpin 3* and *10*, *PhoC*, *Tryp 1*, *11*, *12* and *13*, and *catalase* (*CAT*) did not present high differences in transcription during the infestation process on coho salmon.

4. Discussion

Previous studies in salmonids infected with the salmon louse *L. salmonis* have evidenced a fish host modulation associated to inflammatory response [3,4], as well as T_H2-type responses [6]. However, It is scarce the information at molecular level how this ectoparasite copes the fish immune system. The present study analyzes changes in genes expression related to the immune response, antioxidant system, and secretome in the sea louse *C. rogercresseyi* during the infestation process.

Transcriptional differences exist for TLR and IMD pathway genes during the developmental stages of *C. rogercresseyi* [11]. The present study evaluated the response of genes involved in the TLR and IMD innate immune pathways of *C. rogercresseyi* infecting Atlantic and coho salmon. The assessed genes presented an early response, with increased expression at 3 dpi. Those sea lice infecting Atlantic salmon had higher transcript levels in association with the IMD pathway. The transcriptional changes associated with the IMD pathway in *C. rogercresseyi* could be due to the presence of microbiota naturally colonizing the evaluated salmonids [18], and, in turn, these could liberate some pathogen associated molecular pattern (PAMP) recognized by *C. rogercresseyi*, thereby activating immune response pathways. However, the regulation of these pathways following established infection, as well as of the differences presented between sea lice infesting both host species, suggests the presence of other mechanisms or molecules liberated by the fish that would activate the immune system of *C. rogercresseyi*.

During *L. salmonis* infection, fish induces the generation of ROS, as evidenced by increased thioredoxin in the skin of pink salmon (*Oncorhynchus gorbuscha*) [19]. Furthermore, in pink salmon infected with *L. salmonis* was observed an increase of expression metalloproteinase 9 and 13 responsible of tissue reparation [20], increasing the ROS generation [21,22]. Another widely reported effect in salmonids infected by *L. salmonis* is the upregulation of pro-inflammatory cytokines and serum amyloid A genes [3,4,6,19] that are also responsible to stimulate the ROS production [23]. These antecedents in *L. salmonis* leads us to suggest that during the infection process, sea lice activate enzymes capable of responding to the increased level of reactive oxygen species. In *C. rogercresseyi*, a significant upregulation of *PHGP* and *PRX* was observed in sea lice infecting Atlantic salmon. Additionally, sea lice that infect coho salmon presented a significantly increased expression of SOD,

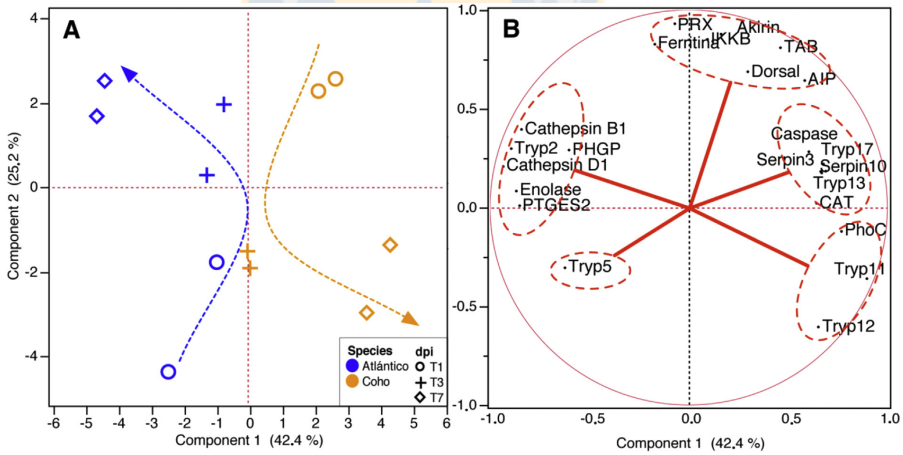

Fig. 2. Principal component analysis (PCA) of *C. rogercresseyi* genes expressed during infestation on Atlantic and Coho salmon. A) PCA grouped by species and time: Centroids of each day post-infestation (dpi) are marked with circle (T1), cross (T3) and rhombus (T7) and highlighted in blue and orange for Atlantic and in Coho salmon, respectively. B) PCA grouped by genes: Circles indicate the genes with higher correlation values during infestation in Atlantic and Coho salmon. The pattern of the first two principal components accounted for 67.6% of data set variability. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

suggesting a positive regulation of certain antioxidant enzymes as a defense mechanism of the ectoparasite to ROS. Furthermore, the antioxidant system of lice has been related to ROS production in response to delousing drugs such as pyrethroids and hydrogen peroxide [12]. In the other hand, the increased expression of genes from the antioxidant response in sea lice infecting both Atlantic and coho salmon could be associated with the molting process that this copepodid undergoes during infection. This process is known to not only increase metabolic activity, but also to elevate ROS levels [24–26]. Other genes in fish that show increased expression during infection include hepcidin-1, serotransferrin-2, and heme oxygenase, which sequester ions from the host skin so as to minimize the ions available to the parasite [19]. Linked to these observations, the present analysis of *C. rogercresseyi* demonstrated that sea lice infecting Atlantic salmon had an overregulation of *Ferritin*, a protein involved in sequestering iron ions and in regulating ROS through Fenton's reaction [27–31]. This could be indicative of a competition for ions between the parasite and host.

In relation to the proteins secreted by the parasite during infection, there is strong proteolytic activity of trypsin and chymotrypsin in the peritrophic matrix of the parasite intestine [32]. Additionally, during the digestion process of *L. salmonis* and other parasites, there is an increase of cathepsins, which in invertebrate species play an important role in the penetration of tissues, digestion, molting, and evading the immune response of hosts [33,34]. Analysis of secretome genes in *C. rogercresseyi* infecting Atlantic salmon showed increased transcripts principally for cathepsin and trypsin at 3 and 7 dpi. This observation is in line with previous studies, where there is a greater inflammatory response in coho salmon as compared to Atlantic salmon during sea lice infection [35]. These data suggest an immunosuppressive effect as a result of molecules secreted by the ectoparasite, such as trypsins, prostaglandin E_2, and cathepsins [8,23]. This was further evidenced by the pattern revealed by PCA, in which the response of sea lice to Atlantic salmon strongly increased the expression of secretome-related genes. Moreover, in *C. rogercresseyi* some cathepsins are overregulated in the copepodid stage, which could favor the infective process of this ectoparasite [13].

5. Conclusion

This study analyzed the transcriptomic responses of the sea louse *C. rogercresseyi* during the infection on Atlantic and coho salmon, evidencing host-dependent expression patterns. Results showed early responses of TLR/IMD signaling pathway in sea lice infesting Atlantic salmon. Meanwhile, genes associated with oxidative stress responses were upregulated in both host species. This pattern suggests that ROS emitted by the host could modulate the sea louse antioxidant system. Secretome-related transcripts evidenced upregulation of *trypsins* and *serpins*, mainly associated to Atlantic salmon than coho salmon. The principal component analysis revealed an inverse time-dependent pattern based on the different responses of *C. rogercresseyi* infecting both salmon species. Here, Atlantic salmon strongly modulates the transcriptome responses at earlier infection stages; meanwhile coho salmon reveals a less marked modulation, increasing the transcription activity during the infection process. This study evidences transcriptome differences between two salmon host species and provides pivotal knowledge towards elaborating future control strategies.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2015.10.022.

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CAPITULO 3

Uncovering iron regulation with species-specific transcriptome patterns in Atlantic and coho salmon during a *Caligus rogercresseyi* infestation

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Abstract

Salmon species cultured in Chile evidence different levels of susceptibility to the sea louse *Caligus rogercresseyi*. These differences have mainly been associated with specific immune responses. Moreover, iron regulation seems to be an important mechanism to confer immunity during the host infestation. This response called nutritional immunity has been described in bacterial infections, despite that no comprehensive studies involving in marine ectoparasites infestation have been reported. With this aim, we analysed the transcriptome profiles of Atlantic and coho salmon infected with *C. rogercresseyi* to evidence modulation of the iron metabolism as a proxy of nutritional immune responses. Whole transcriptome sequencing was performed in samples of skin and head kidney from Atlantic and coho salmon infected with sea lice. RNA-seq analyses revealed significant upregulation of transcripts in both salmon species at 7 and 14 dpi in skin and head kidney, respectively. However, iron regulation transcripts were differentially modulated, evidencing species-specific expression profiles. Genes related to heme degradation and iron transpor such as hepcidin, transferrin and haptoglobin were primary upregulated in Atlantic salmon; meanwhile, in coho salmon, genes associated with heme biosynthesis were strongly transcribed. In summary, Atlantic salmon, which is more susceptible to infestation, presented molecular mechanisms to deplete cellular iron availability, suggesting putative mechanisms of nutritional immunity. In contrast, resistant Coho salmon were less affected by sea lice, mainly activating pro-inflammatory mechanisms to cope with infestation.

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Uncovering iron regulation with species-specific transcriptome patterns in Atlantic and coho salmon during a *Caligus rogercresseyi* infestation

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Abstract

Salmon species cultured in Chile evidence different levels of susceptibility to the sea louse *Caligus rogercresseyi*. These differences have mainly been associated with specific immune responses. Moreover, iron regulation seems to be an important mechanism to confer immunity during the host infestation. This response called nutritional immunity has been described in bacterial infections, despite that no comprehensive studies involving in marine ectoparasites infestation have been reported. With this aim, we analysed the transcriptome profiles of Atlantic and coho salmon infected with *C. rogercresseyi* to evidence modulation of the iron metabolism as a proxy of nutritional immune responses. Whole transcriptome sequencing was performed in samples of skin and head kidney from Atlantic and coho salmon infected with sea lice. RNA-seq analyses revealed significant upregulation of transcripts in both salmon species at 7 and 14 dpi in skin and head kidney, respectively. However, iron regulation transcripts were differentially modulated, evidencing species-specific expression profiles. Genes related to heme degradation and iron transport such as *hepcidin*, *transferrin* and *haptoglobin* were primary upregulated in Atlantic salmon; meanwhile, in coho salmon, genes associated with heme biosynthesis were strongly transcribed. In summary, Atlantic salmon, which are more susceptible to infestation, presented molecular mechanisms to deplete cellular iron availability, suggesting putative mechanisms of nutritional immunity. In contrast, resistant coho salmon were less affected by sea lice, mainly activating pro-inflammatory mechanisms to cope with infestation.

Keywords: Atlantic salmon, *Caligus rogercresseyi*, coho salmon, iron metabolism, transcriptomics.

Introduction

Caligus rogercresseyi is a sea louse species responsible for significant economic losses to the Chilean salmonid aquaculture industry (Bravo, Erranz & Lagos 2009). The tissue damage inflicted by this ectoparasite induces high stress levels in hosts and, consequently, a lower performance of cultured salmons during the production cycle (Gonzalez & Carvajal 2003; Jensen *et al.* 2015). Further, it is known that sea lice infestation decreases the immune response capacity in some salmon species, mainly in Atlantic salmon (Pike & Wadsworth 1999; Fast *et al.* 2006, 2007; Wagner, Fast & Johnson 2008). In the Northern Hemisphere, *Lepeophtheirus salmonis* is the primary sea lice species affecting salmon aquaculture. Interestingly, salmonid hosts present variable susceptibility to *L. salmonis*. In particular, rainbow trout (*Oncorhynchus mykiss*), chinook salmon (*Oncorhynchus tshawytscha*), chum salmon (*Oncorhynchus keta*) and Atlantic salmon (*Salmo salar* L.) have high infestation rates, while coho salmon (*Oncorhynchus kisutch*) and pink salmon (*Oncorhynchus gorbuscha*) are notably resistant to infestation (Boxshall &

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Bravo 2000; Fast *et al.* 2002b; Braden *et al.* 2012; Jones 2013).

Differences in infestation susceptibility can occur due to physiological variations in fish hosts, including mucus composition, skin thickness, behaviour and immune response (Fast *et al.* 2002a; Ángeles Esteban 2012; Sutherland *et al.* 2014). More specifically, salmon species resistant and susceptible to *L. salmonis* infestation evidence differences in mucosal lysozyme activity and susceptible salmon show signs of cell hypertrophy (Fast *et al.* 2002b). Similarly, the immune response has reported differences among salmonid species, once again resulting in some species being more susceptible than others to sea lice infestation (Fast *et al.* 2002b; Braden *et al.* 2012; Braden, Koop & Jones 2015). Molecular studies have highlighted the various immune response mechanisms in play during *L. salmonis* infestation, such as increase in inflammatory cytokines (Skugor *et al.* 2008), protease secretion (Tadiso *et al.* 2011), immunoglobulin levels (Skugor *et al.* 2008), and T_h1 and T_h2 responses (Skugor *et al.* 2008; Braden *et al.* 2015). Braden *et al.* (2015) demonstrated differing immune responses between two susceptible species, Atlantic salmon and sockeye salmon (*Oncorhynchus nerka*), and coho salmon, an infestation-resistant species. As compared to the susceptible species, coho salmon show an increased expression of genes associated with T_h2 responses (Braden *et al.* 2015).

Another immune response mechanism observed in salmons infested by sea lice is nutritional immunity (Hood & Skaar 2012). This innate immune response limits iron availability to the parasite, thereby reducing chances for successful infestation. This immune mechanism is employed in vertebrates to prevent or reduce bacterial, parasitic and haemoparasitic infections (Toh *et al.* 2010; Hood & Skaar 2012). Furthermore, iron regulation has directly been associated as a key component of the fish immune response. For example, it is known that iron transport proteins such as hepcidin, ferroportin, lactoferrin and ferritin have important roles to cope intracellular parasites, affecting directly the iron available during pathogen infection (Johnson & Wessling-Resnick 2012). Additionally, iron homeostasis can be regulated by cytokines and acute-phase protein, modulating iron transport-related genes at transcriptional level (Weiss 2005). Indeed, hepcidin expression has been reported to be associated with the releasing of IL-6 after pattern recognition receptor activation (Nemeth *et al.* 2004). Moreover, in macrophage, iron depletion has been studied, reducing the MCHII and iNOS expression and inhibiting IFN-γ pathway (Weiss 2005). Interestingly, iron chelation affects multiple cellular pathways in T cells. Thus, the IL-12/IL-18-mediated proliferation and IFN-γ secretion are very sensitive to intracellular iron concentration (Leung *et al.* 2005).

In salmon species with resistant phenotype to *L. salmonis*, nutritional response has been evidenced (Sutherland *et al.* 2014). Indeed, resistant salmon hosts present increased heme group regulation, as reflected by a rise in *heme oxygenase* (*HO*) transcription levels. Moreover, the *hepcidin* gene, which is responsible for cellular iron availability, is upregulated in salmons during *L. salmonis* infestation, suggesting that salmon hosts limit iron availability during sea lice infestation (Braden *et al.* 2015). Worth noting, *L. salmonis* presents primarily haematophagous behaviour (Hamre *et al.* 2013), whereas the predominant Southern Hemisphere sea louse *C. rogercresseyi* primarily feeds off of fish host skin mucous (Boxshall & Bravo 2000).

In Chile, *C. rogercresseyi* mostly affects Atlantic and coho salmon cultures. Of these species, *O. kisutch* presents a greater resistance to *C. rogercresseyi* infestation (Hamilton-West *et al.* 2012). Despite being of relevance to an important economic sector, the molecular mechanisms that trigger salmonid immune responses to *C. rogercresseyi* infestation and differences in resistance between salmon species remain unknown. Currently, most molecular differences have been attributed to specific immune responses, but some authors suggest that an iron regulation mechanism may confer protection during host infestation. With this in mind, the present study analysed the transcriptomic profiles of Atlantic and coho salmon infested with *C. rogercresseyi* to determine the presence of modulations in iron metabolism occurring as a proxy for nutritional immune responses. Furthermore, possible underlying mechanisms for the regulation of iron availability in Atlantic salmon are presented and discussed as putative mechanisms that facilitate co-infections with bacterial pathogens existing in lymphoid organs/tissues related to iron storage.

Materials and methods

Experimental design and sample collection

Atlantic salmon (*S. salar*, $n = 120$) weighing 250 ± 12 g were obtained from the Chaperano Hatchery (Multiexport Foods SA). Similarly, coho salmon (*O. kisutch*, $n = 120$) weighing 280 ± 20 g were obtained from the Philippi Centre (Cermaq Chile SA). All fish were reared in brackish water (15 ppm) until smolting, after which specimens were maintained under a 12-h:12-h light:dark cycle in single-pass flow-through tanks supplied with ultraviolet-treated salt water. Atlantic and coho salmon were maintained separately and fed daily with the commercial diet (Micro 200, EWOS). After initial acclimatization for 15 days, individuals were randomly divided by species and in triplicate (i.e. Atlantic and coho salmon) into uninfected and infected experimental groups, containing 20 fish per tank (500 L). The salmon were acclimatized for additional 7 days and were starved at least 24 h prior to any manipulation.

Adult female *C. rogercresseyi* were collected during Atlantic salmon harvesting at a commercial aquaculture farm in Puerto Montt, Chile. After collection, the lice were rinsed and transported in aerated, sterile sea water (8 °C) to the experimental laboratory of Fundación Chile (Chinquihue). Only lice displaying attachment behaviour to the collection vessel were included in the study. The time between sea lice collection and infestation experiments was less than 24 h. Infestation trials were conducted in infested-group fish by placing tanks in the darkness without water flow for 2 h, with a load of 35 copepodids per fish. During the infestation trials, fish were supplemented with oxygen and fed daily. For each sampling point, 3–4 fish were taken from each tank and anaesthetized with benzocaine. Head kidney and skin from an infected area were dissected, fixed in RNAlater® (Ambion) and stored at −80 °C.

High-throughput transcriptome sequencing

Head kidney and skin samples from ten adult fish infested with *C. rogercresseyi* were used to create a cDNA library. Total RNA was extracted from each individual using the RiboPure™ Kit (Ambion®) following the manufacturer's instructions. Quantity, purity and quality of the isolated RNA were measured in the TapeStation 2200 (Agilent Technologies Inc.) using RNA ScreenTape (Agilent Technologies Inc.) according to the manufacturer's instructions. Samples with a RIN over 8.0 were pooled from each tissue according to species and sampling point and were used for library construction. Double-stranded cDNA libraries were constructed using the TruSeq RNA Sample Preparation Kit v2 (Illumina®). Two biological replicates from each sample pool were sequenced on the MiSeq (Illumina®) platform using sequenced runs of 2 × 251 paired-end reads, with sequencing performed at the Laboratory of Biotechnology and Aquatic Genomics, Interdisciplinary Center for Aquaculture Research, University of Concepción, Chile.

Sequence assembly and annotation

Sequence assembly was carried out using the CLC Genomics Workbench v9 software (CLC Bio). Four *de novo* assemblies were performed using data sets from each tissue and salmon species, with an overlap criteria of 70% and a similarity of 0.9 to exclude paralogous sequence variants (Renaut, Nolte & Bernatchez 2010). The settings used were as follows: mismatch cost = 2, deletion cost = 3, insert cost = 3, minimum contig length = 200 base pairs and trimming quality score = 0.05. After the assembly process, singletons were retained in the data set as possible representatives of low-expression transcript fragments. However, the sequence redundancy of these fragments was removed using the Duplicate Finder application included in the Geneious v8.0 software (Biomatters). Contigs were annotated by BlastX analysis using a database constructed from GenBank and UniprotKB/Swiss-Prot, with a cut-off E-value of 1E-10. Moreover, transcripts of Atlantic and coho salmon were subjected to gene ontology (GO) analysis using the Blast2GO plugins included in the CLC Genomics Workbench v9 software (CLC Bio).

RNA-seq analysis

The transcriptome database generated for each salmon species was used as a reference for different RNA-seq analyses. Using the CLC Genomic Workbench software, reads obtained from the head kidney and skin of Atlantic and coho salmon were separately mapped against all annotated

contigs. The RNA-seq settings were a minimum length fraction = 0.6 and a minimum similarity fraction (long reads) = 0.5. The expression value was set as transcripts per million reads. The distance metric was calculated with the Manhattan method, where the mean expression level from five to six rounds of k-means clustering was subtracted. Finally, a Kal's statistical analysis test was used to compare gene expression levels in terms of the log2 fold change ($P = 0.0005$; FDR corrected).

GO and pathway enrichment analyses

Transcripts that were more differentially expressed in the transcriptomes of Atlantic and coho salmon infested with *C. rogercresseyi* were analysed by the plug-in system 'ClueGO + CluePedia' (Cytoscape Software 3.0.2) to identify networks and functional pathways. ClueGO, which performs an extensive database search of functional GO, KEGG and Reactome interactions, was used for pathway enrichment analysis (Bindea *et al.* 2009). All annotated contigs were used as references for enrichment analysis. Enrichment/depletion was calculated using the hypergeometric test with Benjamini and Hochberg FDR, multiple testing correction and significance (FDR < 0.05) to identify overrepresented GO and KEGG functions. Additionally, complementary analysis was conducted with the ClusterMaker Cytoscape plug-in (Morris *et al.* 2011), using the MCL algorithm to search for protein–protein interaction network modules derived from tandem affinity purification/mass spectrometry. This approach clustered the network into modules based on enrichment (PE) score, thus indicating the strength of node associations and providing a fixed set of genes with high protein–protein affinity.

RT-qPCR analysis of heme biosynthesis/degradation, iron transport genes and antioxidant system

Contig sequences annotated for iron transport and heme metabolism from Atlantic and coho salmon data sets were used as templates for primer design with the Primer3 Tool (Rozen & Skaletsky 1999) included in the Geneious Pro v8.1 software (Drummond 2009) (Table S1). For gene amplification, total RNA was isolated from skin and head kidney samples of each species using the TRI Reagent (Invitrogen) protocol. Total RNA was treated with DNAse I (ThermoScienfic) at 37 °C for 30 min, according to the manufacturer's instructions to remove DNA contamination. Purity was determined (ratio A260/A280) on a NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific), and integrity was verified by agarose gel electrophoresis under denaturant conditions. From 200 ng μL^{-1} of total RNA, cDNA was synthetized using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Five serial dilutions started from 80 ng μl^{-1} of cDNA were used for cDNA calibration; after performing the dynamic range, the first dilution (1:5) was selected. qRT-PCR was performed on the StepOnePlus™ system (Applied Biosystems®, Life Technologies™), using the Maxima® SYBR Green/ROX qPCR Master Mix (Thermo Fish Scientific) and a final volume of 10 µL per reaction. The amplification conditions were as follows: 95 °C for 10 min, 40 cycles at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. Relative expression was calculated using the comparative $\Delta\Delta C_t$ method. *Elongation factor-α* (*EF-α*), β-*tubulin* and *18S* were selected as putative housekeeping genes and were statistically analysed with the NormFinder algorithm (Jensen & Ørntoft 2004) to assess relative expression stability. *EF-α* was selected as the best housekeeping gene for relative expression normalization.

Statistical analysis

Data were screened for normality with Shapiro–Wilk test and were Box–Cox-transformed when necessary. A two-way ANOVA was performed after qPCR analysis to infer differences between species, and a *post hoc* Tukey's honest significant difference test was applied when differences occurred. All analyses were performed in the JMP v9.0 software (SAS Institute, Inc.). *P* values <0.05 were considered statistically significant between Atlantic and coho salmon. Additionally, a principal component analysis was performed as an exploratory technique to identify underlying data structure between Atlantic and coho salmon. The analysis was separately conducted for each tissue using the relative expression values of each gene regardless time. Data analyses were performed

using the statistical software package JMP v9 (SAS Institute Inc.).

Results

Transcriptome modulation in Atlantic and coho salmon during sea lice infestation: species-specific gene expression patterns

Infested fish were maintained for 14 days in closed tanks with *C. rogercresseyi*, during which time parasite counts on fish were recorded. Atlantic salmon individuals reached maximum sea lice loads at 7 dpi (≈2000 copepodids). This value slightly decreased by 14 dpi, the end of the experimental period (Fig. 1). In contrast, *C. rogercresseyi* loads in coho salmon were near zero at 7 dpi, a condition maintained until the end of the experimental period (Fig. 1).

The sequencing runs yielded a total of 78 704 324 reads for Atlantic salmon and 77 737 810 reads for coho salmon (Table 1). From *de novo* assembly, 303 898 contigs were obtained for Atlantic salmon, and 308 992 contigs were obtained for coho salmon (Table 1). Furthermore, transcript abundances were determined for Atlantic and coho salmon in skin and head kidney samples during infestation. The highest skin transcript abundances (up- and down-regulated) were observed at 7 dpi for both Atlantic and coho salmon (Fig. 1). In turn, head kidney transcripts were more abundant for both species at 14 dpi (Fig. 1).

Using the obtained transcriptional data, the transcripts differentially expressed between species and tissues were evaluated to determine the shared/exclusive genes involved in salmonid responses to sea lice infestation. The quantity of skin transcripts shared by the assessed salmonid species decreased from 68.3% to 27.8% during the infestation trial (Fig. 2). In sharing transcripts, differences in expression levels between salmon species were observed (Table S2). Furthermore, it is possible to observe a difference in the number of transcripts regulated between Atlantic and coho salmon in both tissues evaluated. In skin at 7 dpi Atlantic salmon presented highest number of transcripts exclusive than coho salmon. These profiles change at 14 dpi where coho salmon present the double of transcripts in comparison with Atlantic salmon (Fig. 2). In head kidney samples, Atlantic salmon presented high percentage of transcripts

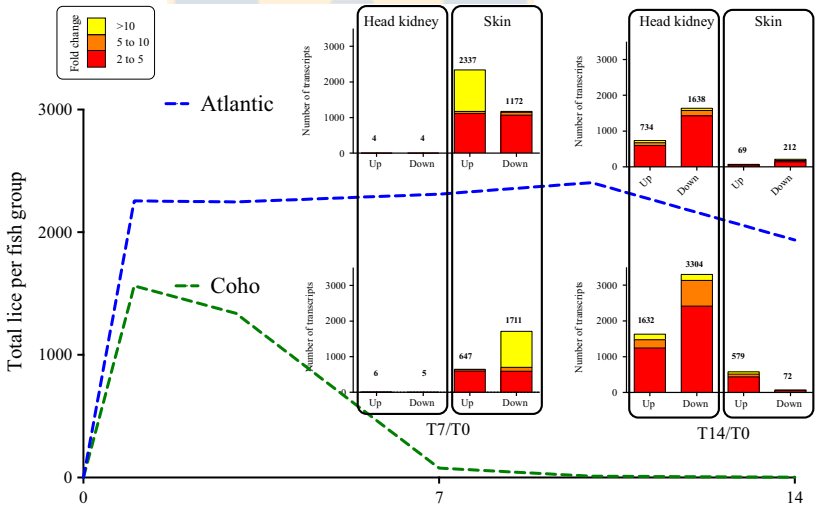

Figure 1 Infestation levels of *Caligus rogercresseyi* and transcriptional changes in Atlantic and coho salmon during 14 days of infestation. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 1 Summary of Illumina sequencing for Atlantic and coho salmon

	Atlantic salmon			Coho salmon		
	Skin	Head kidney	*De novo* assembly	Skin	Head kidney	*De novo* assembly
Reads (Mb)	37.6	41.2	78.7	37	40.7	77.7
Average length (bp)	199.52	194.39	196.84	203.86	193.91	198.65
Matched (Mb)	23.88 (63.5%)	29.56 (71.7%)	65.9 (83.8%)	23.4 (63.2%)	27.8 (68.3%)	64.1 (82.5%)
Nucleotide number (Gb)	7.5	7.9	15.4	7.5	7.8	15.4
Contigs	252.614	165.171	303.898	259.389	165.879	308.992
Average length (bp)	546	596	554	542	585	547
Singletons (Mb)	13.6	11.5	12.7	13.5	12.8	13.6
Average length (bp)	203.01	198.24	196.85	207.17	191.93	199.35

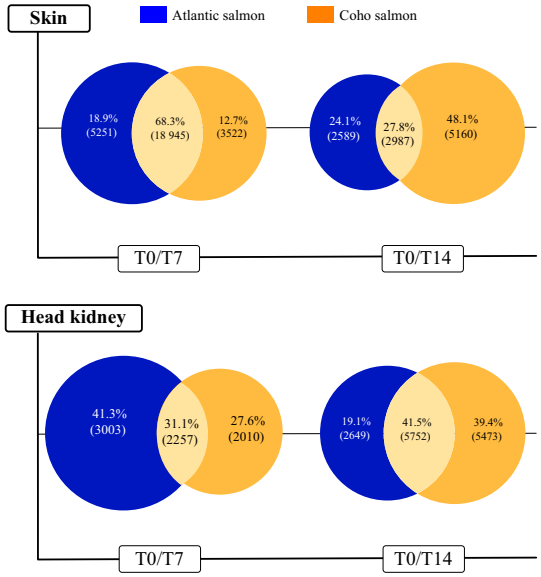

Figure 2 Venn diagram representation of the percentage of transcripts differentially regulated (shared and exclusive) in Atlantic and coho salmon infested with *Caligus rogercresseyi* at 7 and 14 dpi. [Colour figure can be viewed at wileyonlinelibrary.com]

exclusive at 7 dpi with 3003 transcripts, whereas coho salmon has 2010 transcripts. Similar to that observed in skin, in head kidney samples of coho salmon, the number of transcripts exclusive at 14 dpi was two times more than Atlantic salmon.

RNA-seq analysis was conducted by separate to evaluate the modulation at transcriptome level of Atlantic and coho salmon during the infestation with sea lice. From heat map representation, similar expression patterns in skin and head kidney of each salmon species during infestation were observed (Figs 3a & 4a, Table S3). Specifically, skin samples registered greater transcriptome modulation with 16 299 and 12 902 transcripts differently regulated at 7 dpi in Atlantic and coho salmon, respectively (Fig. 3b), whereas in head kidney samples, less transcripts were modulated compared with skin samples. Moreover, most transcripts were differently expressed at 14 dpi,

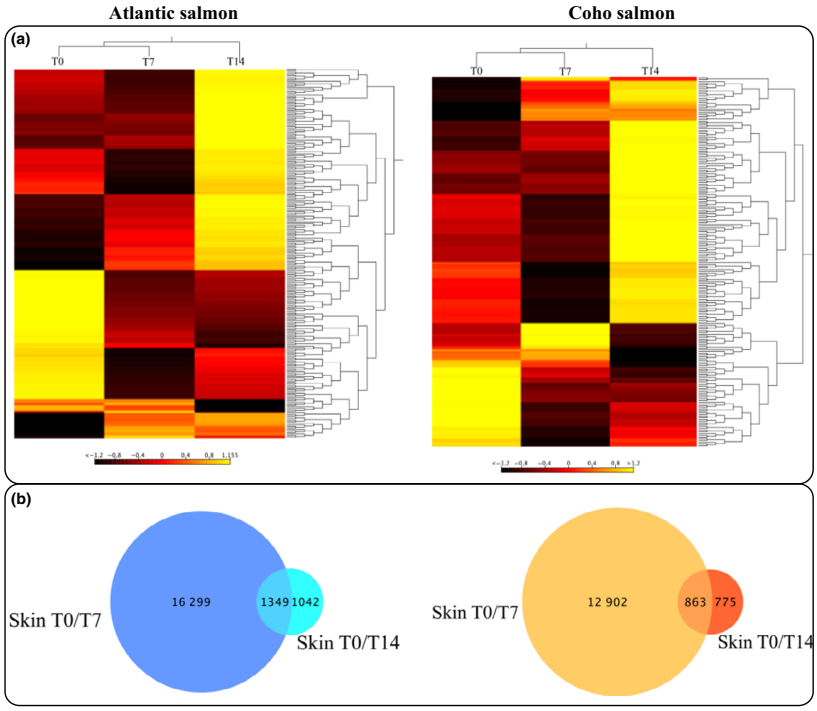

Figure 3 RNA-Seq analysis of transcripts from Atlantic and coho salmon skin at 7 and 14 dpi with *Caligus rogercresseyi*. (a) Heat map representation for skin of Atlantic and coho salmon. (b) Venn diagram for transcripts differentially regulated at 7 and 14 dpi. Expression levels were measured in TPM from normalized values. [Colour figure can be viewed at wileyonlinelibrary.com]

with 3471 and 5966 transcripts in Atlantic and coho salmon, respectively (Fig. 4b).

GO enrichment analysis: iron metabolism during sea lice infestation

On GO terms at level of salmon species and tissues infected with sea lice, comparisons among biological process (BP) and molecular functions (MF) were carried out. For skin at 7 dpi, the most annotated BPs were primarily associated with Atlantic salmon, accounting 62% of GO terms; meanwhile, at 14 dpi, the coho salmon presented 59.3%. Among the annotated terms identified, metabolic and cellular process, regulation of BP and single-organism cellular process were modulated following lice infestation. Interestingly, some GO terms related to stress and immune response were mainly regulated in infected Atlantic salmons (Fig. 5). With respect to MFs, the GO term patterns evidenced a high regulation of protein binding, organic cyclic compound binding and ion binding in both salmon species. However, at early infestation stages, Atlantic salmon showed the major number of transcripts modulated with 64.4% of GO terms. In contrast, the coho salmon significantly increases the GO terms at 14 dpi, accounting 59.3% in skin tissue (Fig. 5). Additionally, the same analysis was carried out for head kidney in Atlantic and coho salmon (Fig. 6), where the patterns observed were congruent with the previous results for skins infected with sea lice. However, a major number of the transcripts in the four main BPs categories

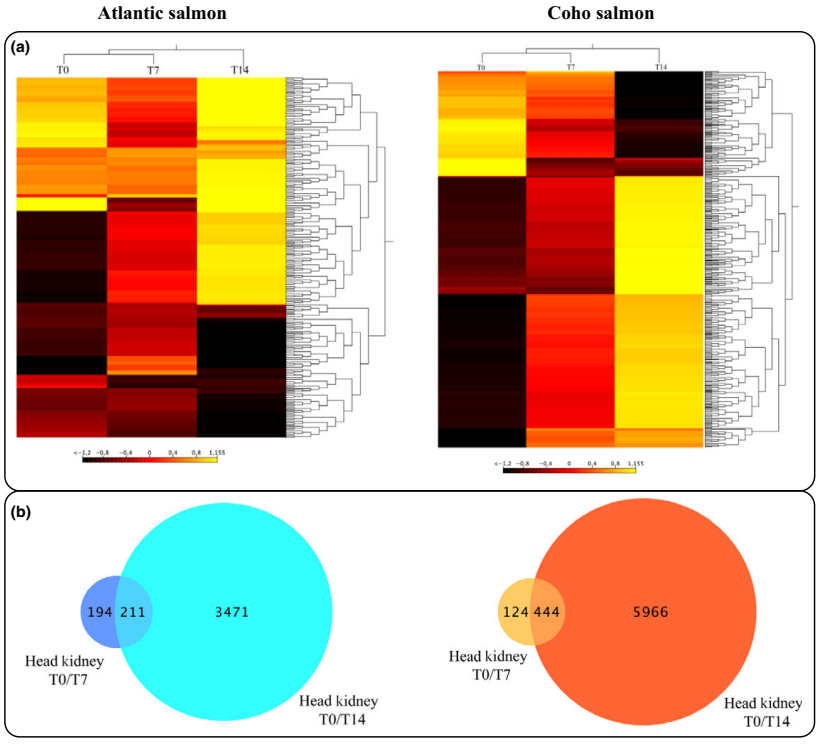

Figure 4 RNA-Seq analysis of transcripts from Atlantic and coho salmon head kidney at 7 and 14 dpi with *Caligus rogercresseyi*. (a) Heat map representation for head kidney of Atlantic and coho salmon. (b) Venn diagram for transcripts differentially regulated at 7 and 14 dpi. Expression levels were measured in TPM from normalized values. [Colour figure can be viewed at wileyonlinelibrary.com]

belonged tower found in coho salmon at 14 dpi; meanwhile, for MF, a strong modulation of ion and protein binding was associated with coho salmon during the entire infestation period studied, accounting 51.5% and 70.3% for 7 and 14 dpi, respectively (Fig. 6). In turn, these results evidence putative regulation of ion binding processes at functional level in response to lice infection, unravelling differences between salmon species. Herein, enrichment analyses from the most differently expressed contigs were compared between infected Atlantic and coho salmon with ClueGO and CluePedia Cytoscape plug-ins (Bindea *et al.* 2009; Bindea, Galon & Mlecnik 2013). Following filtering process, four main functional categories were observed: *binding*, *purine nucleotide*, *ion binding* and *signalling by RHO ATPase* (Fig. 7, Table S4). Here, high abundance of GO terms for transition metal ion binding, ion binding, cation binding, zing binding and metallopeptidase activity was primarily annotated (Table S4). Interestingly, at 7 dpi, the most GO terms related to ion binding were clustered in skin with down-regulation of transcripts associated with metal ion binding (73/146) and transition metal ion binding (56/103) in infected Atlantic salmon (Fig. 7). It is important to note that the transition metal ion binding GO terms is primary comprised of genes

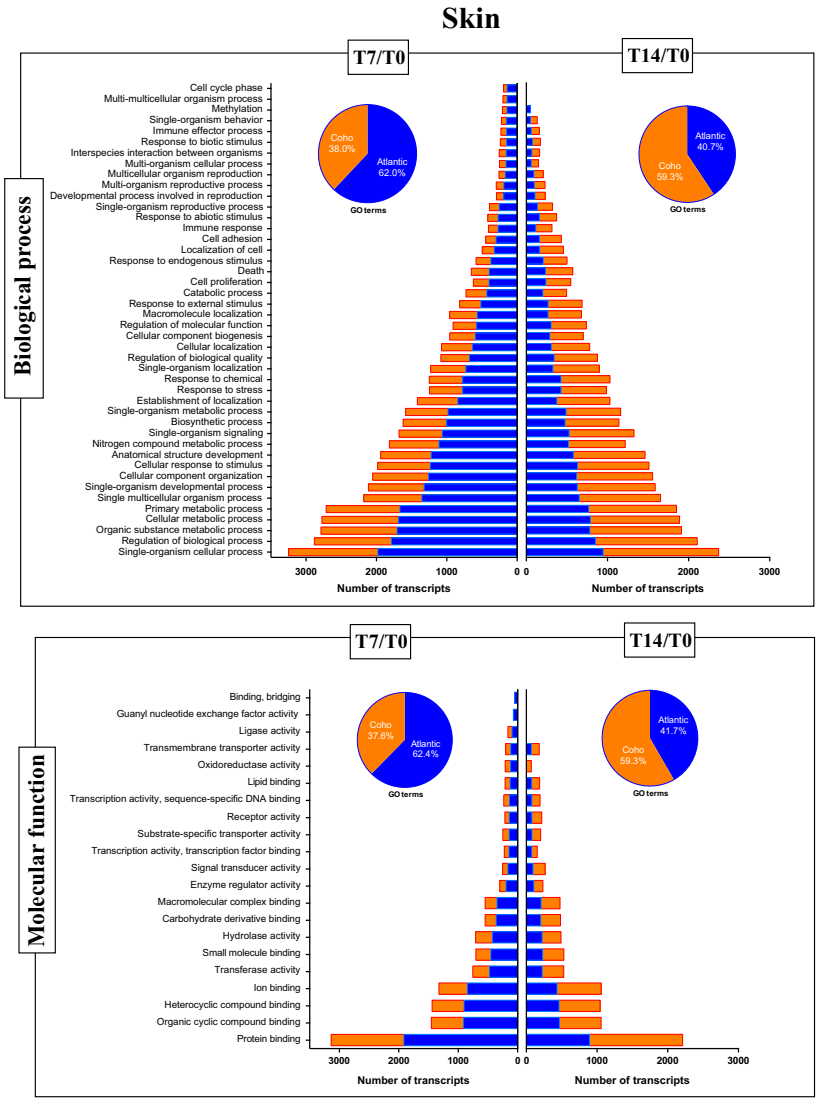

Figure 5 Gene ontology analysis of transcripts differentially regulated in skin of Atlantic and coho salmon infested with *Caligus rogercresseyi*. [Colour figure can be viewed at wileyonlinelibrary.com]

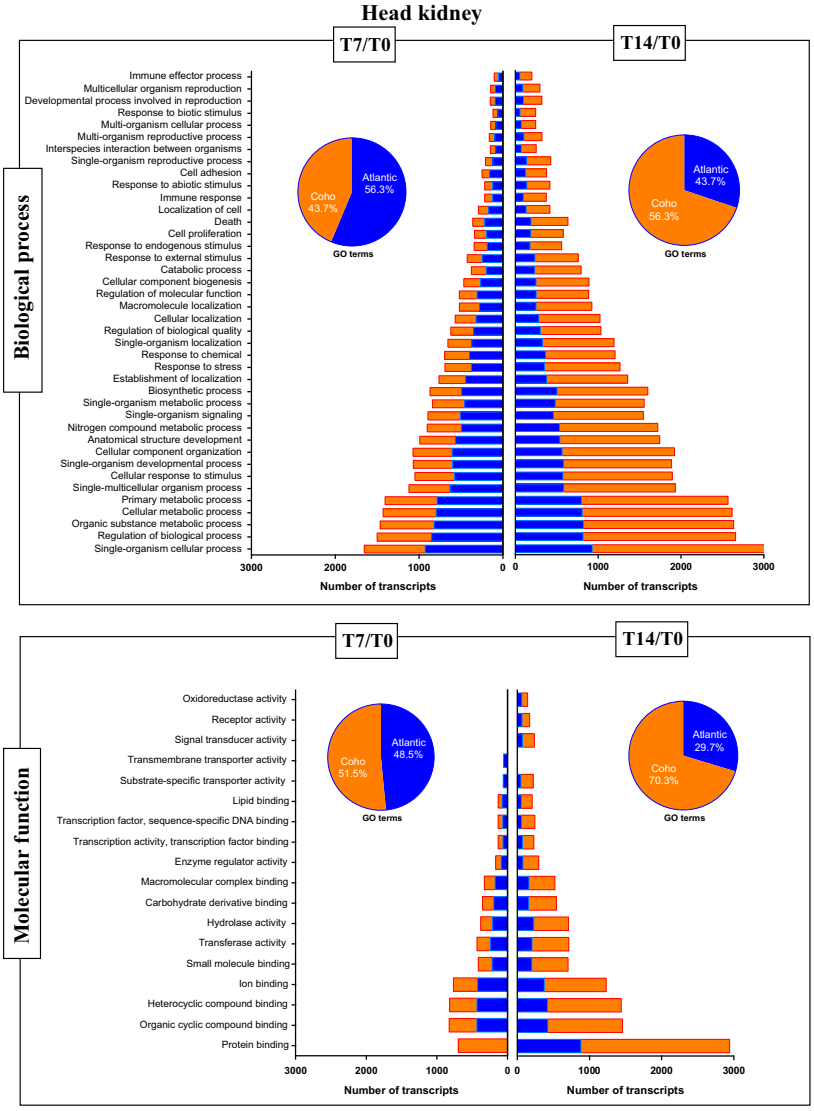

Figure 6 Gene ontology analysis of transcripts differentially regulated in head kidney of Atlantic and coho salmon infested with *Caligus rogercresseyi*. [Colour figure can be viewed at wileyonlinelibrary.com]

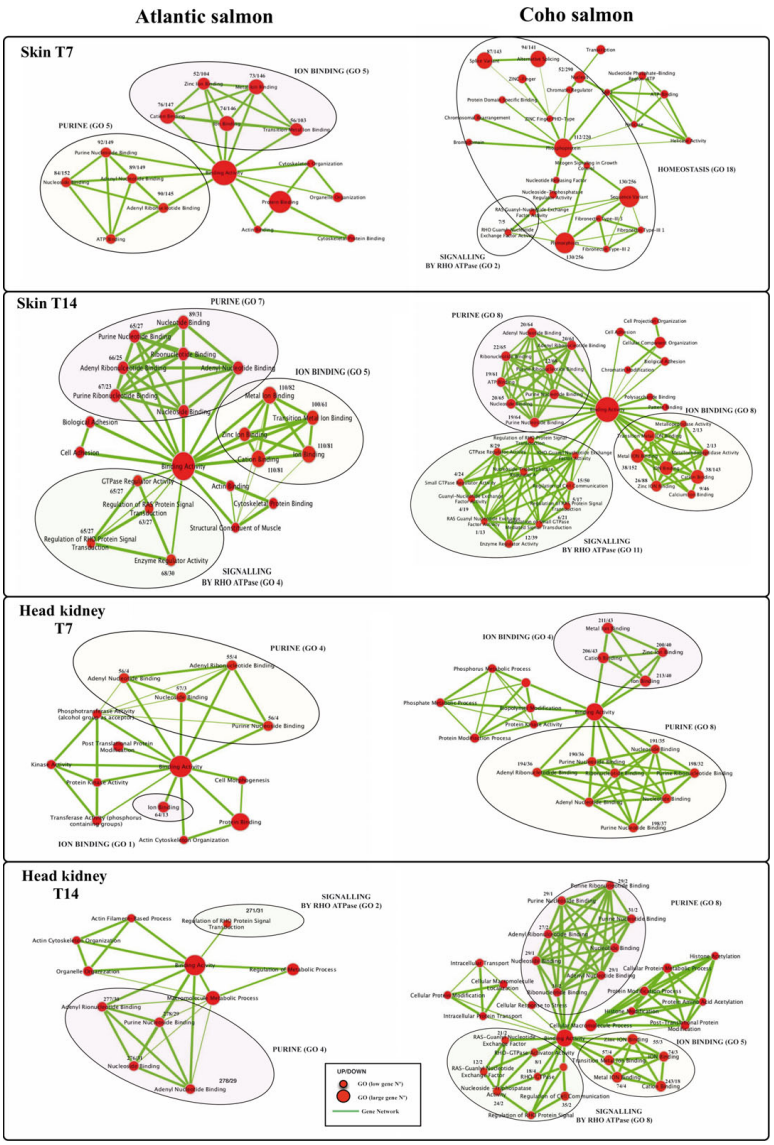

Figure 7 Gene ontology enrichment analysis of transcripts involved in binding, purine nucleotide, ion binding and signalling by RHO ATPase in skin and head kidney of Atlantic and coho salmon during infestation with *Caligus rogercresseyi*. [Colour figure can be viewed at wileyonlinelibrary.com]

associated with ferric ion binding, iron chaperone activity and ferrous ion binding, among others. In contrast, post-infection coho salmon did not evidence modulation for ion binding proteins. Furthermore, infected skin showed strong interactions among GO terms at 14 dpi. However, an opposite pattern was observed between the up- and downregulated genes with respect to ion binding proteins at 7 dpi, where upregulation of genes associated with metal ion binding (110/82) and transition metal ion binding (100/61) the most expressed in Atlantic salmon in comparison with Coho salmon (Fig. 7). Regarding the head kidney transcriptome responses in infected fish, and specifically with the ion binding process, strong modulation was observed at 7 and 14 dpi in coho salmon; meanwhile, infected Atlantic salmon evidenced less responses to the GO terms evaluated (Fig. 7). Indeed, at early infestation with sea lice, the binding activity was only represented by a single GO term of ion binding. Overall, the results evidence that coho salmon, the most resistant species to the lice infection, displays a strong upregulation of proteins related to ion regulation such as metal ion binding, ion binding and cation binding that in turn are involved in iron metabolism.

RT-qPCR analysis of iron transport and heme biosynthesis

While transcriptome data analysis revealed variations in immune system and iron regulation processes, molecular data for these processes during sea lice infestation remained lacking. Considering the RNA-seq results and high quantity of representative GO terms obtained in association with ion binding, subsequent RT-qPCR analysis was performed for candidate genes involved in the heme pathway, iron transport and the antioxidant system, thereby validating *in silico* findings (Fig. 8). Regarding heme biosynthesis/degradation, *aminolevulinic acid synthase* (*ALAs*) and *coproporphyrinogenase* (*CPBGo*), genes with an important role in heme biosynthesis process, were mainly upregulated during sea lice infestation in coho salmon skin and head kidney samples (Fig. 8). Heme degradation was activated in Atlantic salmon through *biliverdin reductase* (*BLVr*) in both analysed tissues, while the other important gene in heme degradation process, *HO*, was downregulated. Regarding iron transport genes, in both tissue analysed for coho salmon was observed an up-regulation at 7 dpi (Fig. 8), specically in ferritin, iron regularoty protein 2 (IRP2). In contrast, infested Atlantic salmon displayed an inverse pattern, with a higher transcription of iron transport genes at 14 dpi. Furthermore, both salmon species exhibited similar antioxidant system expression patterns during *C. rogercresseyi* infestation; specifically, *glutathione S-transferase* (*GST*) was predominantly modulated in coho salmon head kidney at 7 and 14 dpi (Fig. 8b).

Principal component analysis revealed notable differentiation in gene regulation between Atlantic and coho salmon skin and head kidney samples (Fig. 8a, b). In salmon skin, genes involved in iron transport, such as *hepcidin, transferrin* and *haptoglobin,* were largely transcribed in Atlantic salmon, whereas genes associated with heme biosynthesis, such as *aminolevulinic acid dismutase*, *CPBGo* and *ALAs,* were regulated in coho salmon (Fig. 8a). Similarly, from head kidney sample analysis, it was observed that genes related to iron transport as *haptoglobin, hepcidin* and *transferrin receptor* were more expressed in Atlantic salmon, while coho salmon displayed a regulation of genes associated with heme biosynthesis (e.g. *ALAs*) and degradation (e.g. *HO*) (Fig. 8b).

Discussion

This study used RNA-seq analysis to uncover transcriptomic differences between Atlantic and coho salmon subjected to a 14-day infestation trial with *C. rogercresseyi*. Transcriptome profiles revealed similar patterns of transcript abundances in both salmon species, with a high number of transcripts differentially expressed in skin and head kidney at 7 and 14 dpi, respectively. Using an enrichment approach, differences were observed in BPs and MF related to iron regulation. Pivotal information was obtained that allows for understanding mechanisms involved in the immune responses of Atlantic and coho salmon infested with *C. rogercresseyi*.

Microarray and qPCR analyses of salmon infested with *L. salmonis* had shown differences in the immune mechanism used by resistant and susceptible species (Sutherland *et al.* 2014; Braden *et al.* 2015; Holm *et al.* 2015). Moreover, an early regulation of pro-inflammatory responses occurs in *L. salmonis*-resistant species (Fast *et al.*

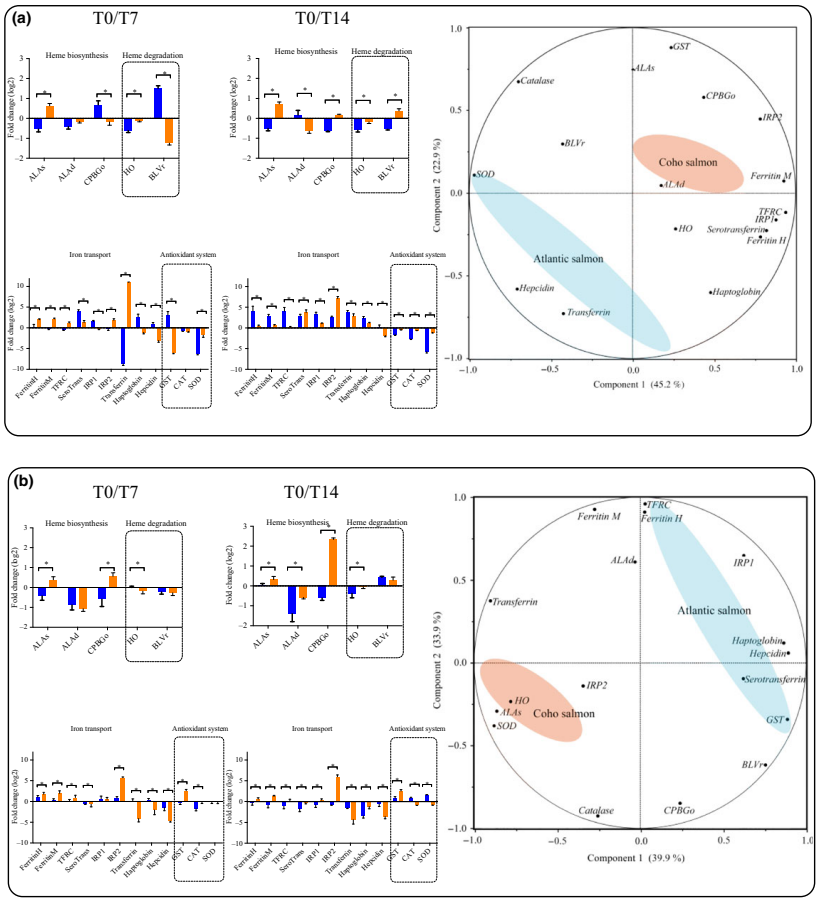

Figure 8 RT-qPCR analysis of genes related to iron regulation in Atlantic (blue) and coho (orange) salmon. (a) Gene expression in skin of Atlantic and coho salmon and principal component analysis (PCA) analysis for fold change expression values. (b) Gene expression in head kidney of Atlantic and coho salmon and PCA analysis for fold change expression values. Asterisk indicates significant differences between salmon species ($P < 0.001$). [Colour figure can be viewed at wileyonlinelibrary.com]

2006; Braden *et al.* 2012). Related to this, a cohabitation experiment with chum, Atlantic and pink salmon revealed increased pro-inflammatory cytokine *IL-1*β expression in pink salmon, a species with known infestation resistance (Sutherland *et al.* 2014). In this study, the global transcriptome analysis of infested Atlantic and coho salmon identified differences in the regulation of genes associated with the pro-inflammatory response, with high regulation of these genes in coho salmon, species with resistant phenotype, during *C. rogercresseyi* infestation.

GO enrichment analysis of Atlantic and coho salmon transcriptomes after infestation with *C. rogercresseyi* led to the identification of a high number of contigs clustered as ion binding GO

terms. Furthermore, RNA-seq analysis revealed changes in the regulation of iron transport genes. These results, together with those obtained by Sutherland *et al.* (2014) regarding increased heme degradation in resistant salmon species, led to subsequent analyses focusing on differences in iron regulation genes. Vertebrates use iron regulation as a strategy of nutritional immunity to reduce parasite success during infestation (Toh *et al.* 2010; Hood & Skaar 2012). Moreover, previous studies in salmon infested with *L. salmonis* have reported high *hepcidin* regulation (Sutherland *et al.* 2014; Braden *et al.* 2015), *HO* upregulation and *haemoglobin subunit* downregulation in resistant species, suggesting that salmon infested with sea lice use a strategy of nutritional immunity to reduce the availability of heme (Sutherland *et al.* 2014). Supporting this, in the current study, RNA-seq analysis of Atlantic and coho salmon infested with *C. rogercresseyi* showed an upregulation of the *haemoglobin subunit* in both species. Moreover, in contrast to previous studies in salmon infested with *L. salmonis* (Sutherland *et al.* 2014), coho salmon infested with *C. rogercresseyi* showed strong upregulation of heme biosynthesis (e.g. *ALAs* and *CPBGo*) and downregulation of heme degradation genes (e.g. *HO).* In turn, Atlantic salmon displayed upregulation of *BLVr* in skin at 7 dpi, suggesting that heme degradation processes increase in infested Atlantic salmon. In this regard, a recent study reported a decrease in Atlantic salmon haemoglobin during *C. rogercresseyi* infestation (González *et al.* 2016), suggesting a strategy of Atlantic salmon to reduce heme available during *C. rogercresseyi* infestation.

Additionally, iron homeostasis is not exclusively regulated by heme biosynthesis; other important proteins involved in this process are *hepcidin*, *ferritin*, *transferrin*, *haptoglobin* and *IRP* (Shi & Camus 2006; Gkouvatsos, Papanikolaou & Pantopoulos 2012). The heme degradation process releases iron molecules that induce iron depletion such as *transferrin*, *ferritin* and *haptoglobin* (Cassat & Skaar 2013). From our expression analysis in skin, it was observed that skin of Atlantic salmon infested by *C. rogercresseyi* presented positive regulation of *BLVr* (heme degradation gene) at the same time that increase the regulation of transferrin. Furthermore, iron levels are controlled by IRP (Cassat & Skaar 2013); in this study, *IRP1* was higher regulated in Atlantic salmon and not in coho salmon, suggesting an increase in iron molecules available in susceptible specie. Related to *hepcidin* gene, a downregulation of this gene has been linked with an anaemia condition in catfish and zebrafish (Fraenkel *et al.* 2005; Hu *et al.* 2007). Furthermore, *L. salmonis*-resistant salmon species, as compared to susceptible species, showed upregulation of *hepcidin* expression during lice infestation (Sutherland *et al.* 2014; Braden *et al.* 2015). In the present study, *hepcidin* was upregulated in Atlantic salmon skin during *C. rogercresseyi* infestation. This upregulation of *hepcidin* can be linked to an increase in the haematocrit, as has been previously reported for Atlantic salmon infested with *C. rogercresseyi* (González *et al.* 2016). Furthermore, our results suggest an increase in iron availability in Atlantic salmon during *C. rogercresseyi* infestation, a process regulated with high levels of iron transport genes.

Otherwise, reactive oxygen species (ROS) increase in ectoparasite-infested fish, inducing the expression of antioxidant proteins such as superoxide dismutase and catalase. For example, *Epinephelus coioides* infested with the ectoparasite *Cryptocaryon irritans* present increased superoxide dismutase activity under high parasite loads (Yin *et al.* 2014). An increase in *superoxide dismutase* and *catalase* transcript expression occurred in Atlantic salmon at 14 dpi, when *C. rogercresseyi* loads were high as compared to coho salmon, was recorded. Furthermore, *GST* was upregulated in coho salmon, and the expression increase in this gene was also accompanied by increased expression of the *ALAs* and *CBPGo* genes in the resistant species, which participate in heme biosynthesis, process associated with an increase in ROS (Drummond 2009).

Overall, the present results suggest that salmon infested with sea lice modulate iron metabolism, a process that occurs during bacterial infection (Wooldridge & Williams 1993; Krewulak & Vogel 2008; Skaar 2010). Accordingly, iron sequestration, or limiting the iron available for bacterial grow, is one of the first lines of host defence against pathogens (Skaar 2010; Cherayil 2011). Iron regulation in fish has been postulated as a key factor in terms of Atlantic salmon resistance/susceptibility to the intracellular bacterium *Piscirickettsia salmonis*, given that resistant families can decrease cellular iron content during infection (Pulgar *et al.* 2015). Furthermore, genes associated with intracellular iron accumulation, such as *haptoglobin*, *hepcidin-1, transferrin receptor* and the *ferritin middle chain,* have also been reported as being significantly modulated.

Although haptoglobin is not directly involved in iron uptake, it binds free haemoglobin in bony fish (Wicher & Fries 2006), thus preventing oxidative activity and, primarily, preventing pathogen iron acquisition from free haemoglobin (Hood & Skaar 2012). Similarly, iron metabolism in salmon infected by either sea lice or bacteria could be modulated by genes related to iron regulation, such as *hepcidin*, *haptoglobin*, *ferritins* and heme biosynthesis/degradation pathways. Future studies will address understanding how iron modulation could promote or not synergic mechanisms of co-infection between ectoparasites and pathogenic bacteria, which would affect salmon survival during the production cycle.

In conclusion, RNA-seq analyses revealed differences in the molecular mechanisms of Atlantic and coho salmon during infestation with the ectoparasite *C. rogercresseyi*. Atlantic salmon evidenced a nutritional immunity response, strongly modulating heme biosynthesis/degradation pathways and iron transport proteins. In contrast, coho salmon did not apparently regulate heme availability during *C. rogercresseyi* infestation.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Primers list.

Table S2. Contigs annotated with more expression differences between Atlantic and coho salmon.

Table S3. Contigs annotated from Veen diagram analysis.

Table S4. Specific enriched GO terms.

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CAPITULO 4

Iron metabolism modulation in Atlantic salmon infested with the sea lice *Lepeophtheirus salmonis* and *Caligus rogercresseyi*: A matter of nutritional immunity?

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Abstract

Sea lice are copepodid ectoparasites that produce high economic losses and environmental issues, thus impacting the salmon aquaculture worldwide. Atlantic salmon (*Salmo salar*) from Northern and Southern Hemispheres are primarily parasitized by *Lepeophtheirus salmonis* and *Caligus rogercresseyi*, respectively. To cope *L. salmonis* infestation, studies suggest that Atlantic salmon can restrict iron availability as a mechanism of nutritional immunity. However, no molecular studies of iron regulation from salmonids infected with *C. rogercresseyi* have been reported. The aim of this study was to determine if there are differences in the regulation of iron metabolism in Atlantic salmon infested with L. salmonis or *C. rogercresseyi*. For comparisons, skin and head kidney were profiled using qPCR of 15 genes related to iron regulation in Atlantic salmons infected with each sea louse species in Norway and Chile, respectively. Prior to infestation, no significant differences were observed between fish group. However, genes involved in iron transport and Heme biosynthesis were highly upregulated in Atlantic salmon infested with *L. salmonis*. Interestingly, hepcidin and Heme oxygenase, a component of the Heme degradation pathway, were upregulated during *C. rogercresseyi* infestation. Oxidative stress related genes were also evaluated, showing higher transcription activity in the head kidney than in the skin of Atlantic salmon infested with L. salmonis. These comparative results suggest pathogen-specific responses in infected Atlantic salmon, where iron metabolism is primarily regulated during the infestation with *L. salmonis* than *C. rogercresseyi*. Feeding behavior, for instance haematophagy, of the infesting sea lice species in relation to iron modulation is discussed.

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Short communication

Iron metabolism modulation in Atlantic salmon infested with the sea lice *Lepeophtheirus salmonis* and *Caligus rogercresseyi*: A matter of nutritional immunity?

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ABSTRACT

Sea lice are copepodid ectoparasites that produce high economic losses and environmental issues, thus impacting the salmon aquaculture worldwide. Atlantic salmon (*Salmo salar*) from Northern and Southern Hemispheres are primarily parasitized by *Lepeophtheirus salmonis* and *Caligus rogercresseyi*, respectively. To cope *L. salmonis* infestation, studies suggest that Atlantic salmon can restrict iron availability as a mechanism of nutritional immunity. However, no molecular studies of iron regulation from salmonids infected with *C. rogercresseyi* have been reported. The aim of this study was to determine if there are differences in the regulation of iron metabolism in Atlantic salmon infested with *L. salmonis* or *C. rogercresseyi*. For comparisons, skin and head kidney were profiled using qPCR of 15 genes related to iron regulation in Atlantic salmons infected with each sea louse species in Norway and Chile, respectively. Prior to infestation, no significant differences were observed between fish group. However, genes involved in iron transport and Heme biosynthesis were highly upregulated in Atlantic salmon infested with *L. salmonis*. Interestingly, *hepcidin* and *Heme oxygenase*, a component of the Heme degradation pathway, were upregulated during *C. rogercresseyi* infestation. Oxidative stress related genes were also evaluated, showing higher transcription activity in the head kidney than in the skin of Atlantic salmon infested with *L. salmonis*. These comparative results suggest pathogen-specific responses in infected Atlantic salmon, where iron metabolism is primarily regulated during the infestation with *L. salmonis* than *C. rogercresseyi*. Feeding behavior, for instance haematophagy, of the infesting sea lice species in relation to iron modulation is discussed.

1. Introduction

Iron (Fe) is one of the primary nutrients acquired by parasites from their hosts, and the ability of hosts to limit iron availability is an innate immune response mechanism termed nutritional immunity [1,2]. Iron is an important element for organisms as it is a co-factor for numerous enzymes, playing important roles at the physiological and molecular levels [1]. Free iron can be found in organisms at low concentrations and as part of the Heme group. This group consists of a Fe^{+2} molecule that has a high affinity for blood oxygen, which allows this molecule to move throughout an organism [1,2]. Parasites cannot biosynthesize Heme, and this nutritional requirement must be obtained from the host [3].

Regarding fish ectoparasites, sea lice are responsible for significant economic losses in the salmon industry worldwide. The most relevant and prevalent sea lice species affecting salmonid farming are *Lepeophtheirus salmonis* in the Northern Hemisphere and *Caligus rogercresseyi* in the Southern Hemisphere [4]. However, there are no comparative studies between the biology and infection mechanisms of these species. One important aspect worth comparing is how each ectoparasite feeds. As a hematophagous species, *L. salmonis* feeds from host blood during the juvenile and adult stages [5]. In contrast, *C. rogercresseyi* primarily feeds from the skin mucous of fish hosts [6]. Further concerning nutrition, there is evidence that sea lice and their salmonid hosts compete for iron. Adult *L. salmonis* deprived of blood show a significant decrease in the expression of the iron regulatory proteins (IRP) 1 and 2, which are responsible for modulating the abundance of iron transport and

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storage proteins [7]. In turn, seven days after infestation *C. rogercresseyi* shows increased *ferritin* expression, a gene whose function has been associated with iron sequestering at the cellular level and with controlling oxidative stress through Fenton's reaction [8].

The objective of the present study was to evaluate the transcriptional changes in genes associated with the regulation of iron metabolism in Atlantic salmon infested by *L. salmonis* or *C. rogercresseyi*. Overall, the results suggest pathogen-specific responses in infected Atlantic salmon, with iron metabolism being more regulated during infestation with *L. salmonis* than with *C. rogercresseyi*. Discussion is provided on the possible implication of iron regulation as a key factor in depressing the immune system of cultivated fish during processes of bacterial/ectoparasite co-infection.

2. Materials and methods

2.1. Experimental design and samples

Atlantic salmon (*S. salar*) from Norway and Chile were used to conduct infestation challenges with *L. salmonis* and *C. rogercresseyi*, respectively. Since current sanitary regulations in these countries prohibit the transport of sea lice, performing challenge assays in a single country and group of fish was not possible. Therefore, to minimize the effect of different fish groups, Atlantic salmon were obtained from the same international farming company and were within the same weight (150 ± 15 g) and SFR% (1.8) ranges. All fish were reared in brackish water (15 ppm) until smolting, after which they were maintained in ultraviolet-treated saltwater in single-pass, flow-through tank systems on a 12:12 h light:dark cycle. Atlantic salmon were fed daily with a commercial diet (EWOS, Micro200) and acclimated for 15 days prior to the challenges. Then, individuals were randomly divided among 500 L tanks (60 fish per tank, in triplicate), acclimated for seven days, and starved at least 24 h prior to any manipulation.

Sea lice (*L. salmonis* and *C. rogercresseyi*) used in the trials were collected, respectively, from Oltesvik in Norway and Aquainnovo in Chile. These sea lice populations were propagated and maintained on Atlantic salmon hosts kept in laboratory-based sea lice cultivation systems at the Ewos Innovation's test facilities respectively located in Dirdal, Norway and Aquainnovo, Chile. Lice and host fish were held in circular flow through tanks (500 L), and egg-bearing females were collected from fish anaesthetized with benzocaine (Sigma; 20% w/v, 50 mg/L). The egg strings were allowed to hatch and reach the infective copepodid stage while maintained for 14 days at 9 °C.

2.2. Sampling and RNA extraction

Head kidney and skin samples were collected from ten fish per tank before infection and 7 and 8 days post infestation (dpi) for fish infested by *C. rogercresseyi* and *L salmonis*, respectively. All samples were collected following the same sampling protocol. Briefly, small portions of head kidney tissue (4 mm^3) were taken per fish, and skin tissue samples were taken from areas where sea lice were attached. Then, all the tissues were fixed in the RNAlater® RNA Stabilization Reagent (Ambion®, Life Technologies™, Carlsbad, CA, USA) and stored at −80 °C until RNA extraction. The collected samples were incubated and homogenized with the TRIzol® Reagent (Ambion®, Life Technologies™, Carlsbad, CA, USA) according to the manufacturer's instructions, and RNA concentration and purity were determined by the Nanodrop ND-100 spectrophotometer (NanoDrop® Technologies, Inc., Wilmington, DE, USA). Total RNA integrity was measured by electrophoresis on 1.2% denaturing gel stained with ethidium bromide.

Table 1
Primer list for RT-qPCR analysis.

Gene	Primer Name	Sequence 5′-3′	Tm	Efficiencies (%)
EF	ELF1a-F2	CCCCATCCCGCCCCACAGA	58	98
	ELF1a-R2	GGGTTCCAGTCTCCACACGGC		
FerritinH	Ss_FerritinH_F2	TCTGAACACAACGACCCACA	60	93
	Ss_FerritinH_R2	GTCAAACAGGTACTCGGCCA		
FerritinM	SS_FerritinM_F1	TATCACCACGATTGCGAAGC	60	103
	SS_FerritinM_R1	CTCGTCGCTGTTCTCCTTGA		
Trans_Recp	Ss_Transf_Recp_F2	CAGAGTCTGTAGCAGTGCCA	60	87
	Ss_Transf_Recp_R2	AGTGAGAAGCCAAACCGTGT		
IRP1	Ok_IRP1_F1	TGCTCTGAACAACGAAGGGA	60	106
	Ok_IRP1_R1	GCGTTCCAGCGTTCATTCAC		
IRP2	Ss_IR2_F2	GTTCAATTCATACGGCGCCC	60	83
	Ss_IR2_R2	GCCTCAAACACGTCCAGAGT		
GST	Ont_GST-F	CTCTGCTCCAGTTGCCTGGAT	60	108
	Ont_GST-R	GTTGCCATTATTGGGCAGTTTCT		
CAT	Om_CAT_F1	GGTTCAGACCCTACTCAACAA	60	102
	Om_CAT_R1	GGTGGAAGTTAAGGCATCAC		
SOD	Om_SOD_F1	CCGTATTCTTTGAGCAGGAG	54	94
	Om_SOD_R1	AGCCGTTGGTGTTGTCTC		
Aminolevulinic synthase	SS_ALAs_F	GGTAGGATGCCTGCTGACTG	63	101
	SS_ALAs_R	CCCCAAGCCTGTTTTGCTGA		
Aminolevulinic dehydratase	SS_ALAd_F	CCACTCGCCCATCCATCATA	59	97
	SS_ALAd_R	ACACCTCACATGGACACTGT		
Heme Oxygenase	SS_HO_F	TCCTCCTCCTCCAGCTCTATG	61	98
	SS_HO_R	ACAAGTCCTTGGCCGCATTA		
Coproporphyrinogen_oxidase	SS_CPOX_F	CTGCGTCTCCATCAGCA	60	91
	SS_CPOX_R	GCATCGGTGATCCCCAAAAC		
Biliverdin reductase	SS_BLVr_F	AAACAGATCCACCAGCCAGG	59	105
	SS_BLVr_R	ACAGCCGAGTTTAAGCAGCT		
Hepcidin	Om_Hep_F	GCTGTTCCTTTCTCCGAGGTGC	60	89
	Om_Hep_R	GTGACAGCAGTTGCAGCACCA		
Haptoglobin	Ss_haptoglobin_F1	GGCATGTAGGCAGAGAGCTT	58	95
	Ss_haptoglobin_R1	GGAGAATGTGTGCTTTGGGG		

2.3. RT-qPCR analysis

To evaluate the modulation of iron metabolism in Atlantic salmon infested with sea lice, 15 genes related to iron transport, Heme biosynthesis/degradation, and oxidative stress were analyzed. Specific primers were designed with the Primer3 Tool [9] included in the Geneious Pro v8.1 software (Biomatters Limited, Auckland, New Zealand) [10] and using sequences deposited in the NCBI database (Table 1). Complementary DNA was synthetized from total RNA (200 ng/µl) using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific™, Waltham, MA, USA). The RT-qPCR runs were performed with StepOnePlus™ (Applied Biosystems®, Life Technologies™, Carlsbad, CA, USA) using the comparative ΔCt method. Putative reference genes such as

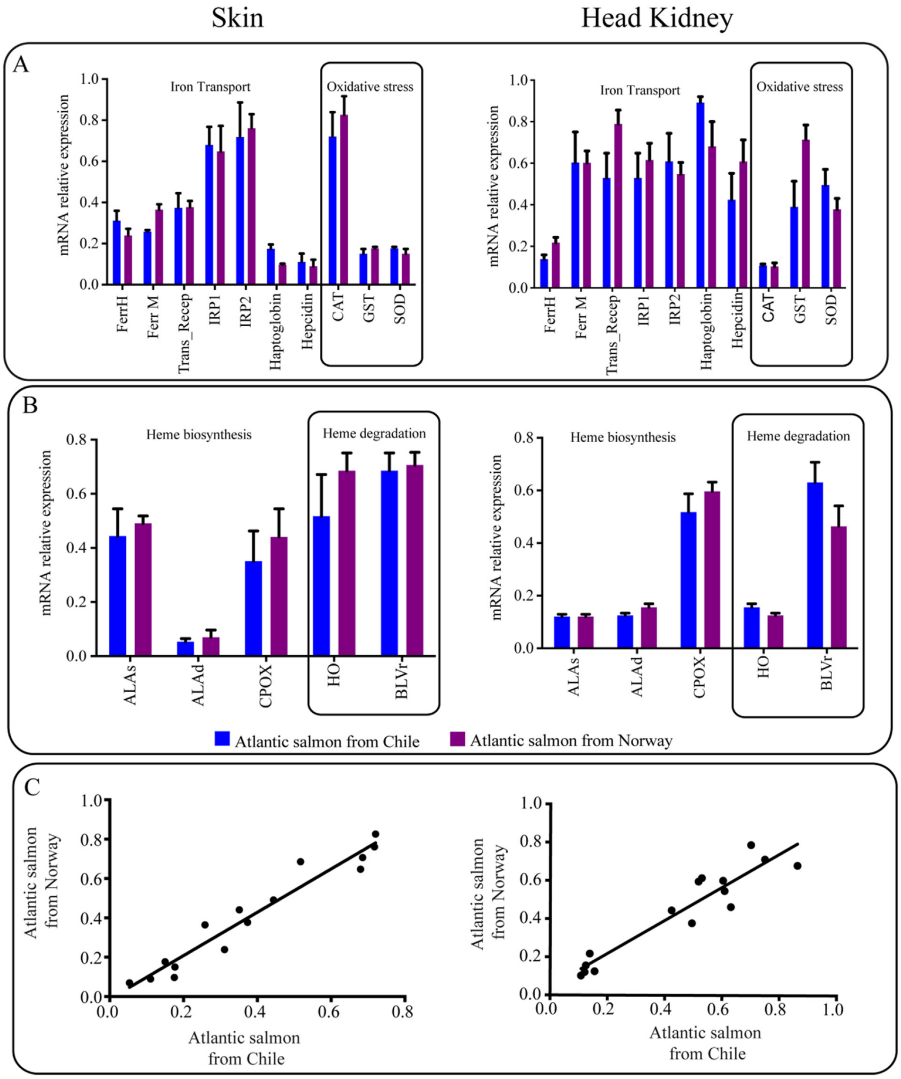

Fig. 1. Evaluation of basal expression levels for genes involved in iron transport in skin and head kidney of Atlantic salmon from Chile and Norway. A: mRNA relative expression irons transports and oxidative response genes. B: mRNA relative expression for Heme biosynthesis genes. C: Pearson correlation for genes evaluated in samples from Norway and Chile, for skin and head kidney, respectively ($p < 0.0001$).

Elongation factor-α (EF-α), β-tubulin, and *18S* were statistically analyzed by the NormFinder algorithm to assess transcriptional expression stability. Through this, *EF-α* was selected for gene normalization. Each reaction was conducted in a final volume of 10 μL using the Maxima® SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). PCR amplification conditions were as follows: 95 °C for 10 min, 40 cycles at 95 °C for 30 s, annealing T °C for 30 s, and 72 °C for 30 s.

2.4. Statistical analysis

Variables were screened for normality based on the skewness and kurtosis of the respective distribution and were normalized through BoxCox transformation when normality standards were not met [11]. Fold-change differences in gene expressions of Atlantic salmon were evaluated using the Tukey HSD test for T8/T0 in the case of *L. salmonis* infestation and T7/T0 for *C. rogercresseyi* infestation. Significant differences between fold-changes for different hosts were accepted when $P < 0.05$.

3. Results and discussion

As parasites cannot produce Heme groups [3], higher organisms have developed a nutritional immunity-like response mechanism that restricts pathogen access to iron or Heme groups, thereby restricting pathogen development [1]. This type of response is likely a strategy used by salmonids infested by *L. salmonis* [12,13]. As a hematophagous species, *L. salmonis* might obtain iron from the host blood, possibly triggering a host response to limit free iron. Considering this context, it is worth investigating how fish regulate iron availability during infestation with the apparently non- or facultative hematophagous *C. rogercresseyi*.

Before comparing the effects of iron metabolism in Atlantic salmon during infection with *L. salmonis* or *C. rogercresseyi*, the basal profiles of the Chilean and Norwegian Atlantic salmon groups were evaluated. No significant differences were observed in gene expressions related to iron transport, Heme biosynthesis/degradation, or the antioxidant system (Fig. 1). A Pearson's correlation analysis of relative gene expressions evidenced a high association between basal profile values in the analyzed Chilean and Norwegian Atlantic salmon tissues (Fig. 1). The correlation coefficients were $R^2 = 0.940$ and 0.886 for the skin and head kidney, respectively, and a p value < 0.0001 was obtained for each correlation.

Variations in gene expressions were determined in Atlantic salmon infested by *L. salmonis* and *C. rogercresseyi*. Furthermore, most of the evaluated genes were upregulated in the skin and head kidney of Atlantic salmon infested by *L. salmonis* (Fig. 2). One important gene in iron transport is *transferrin and ferritin*, which

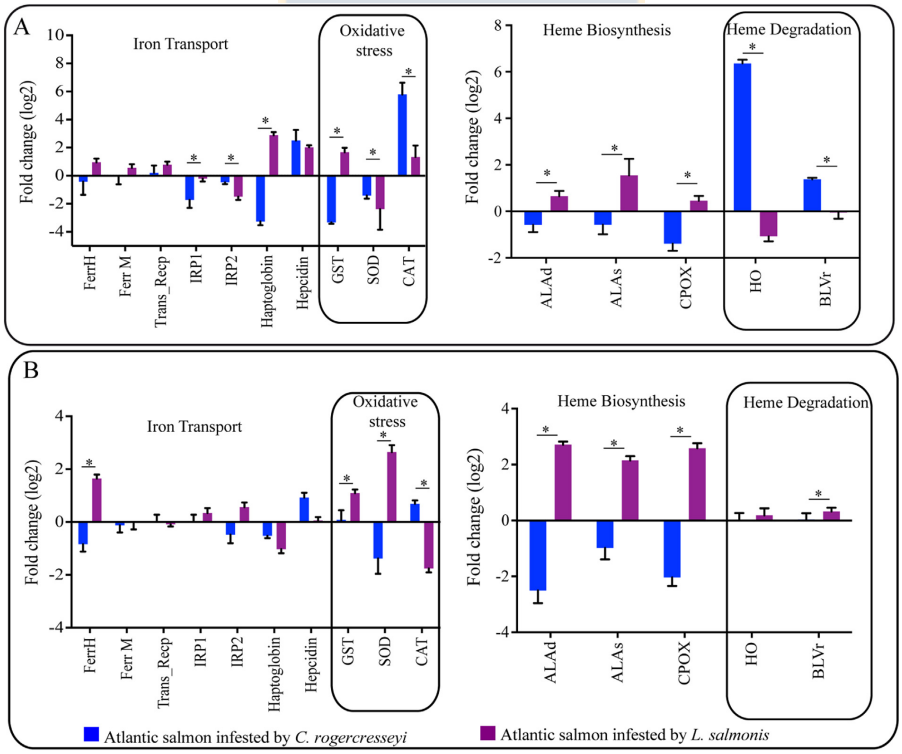

Fig. 2. Gene expression profile for genes involved in iron transport for Atlantic salmon infested by *C. rogercresseyi* and *L. salmonis*. A) Fold changes (log_2) for genes evaluated in skin of Atlantic salmon. B) Fold changes (log_2) for genes evaluated in head kidney of Atlantic salmon. Asterisk indicated significant differences between groups ($p < 0.0001$).

limits extracellular iron from pathogens [14]. Evaluation of the *transferrin receptor and ferritin* revealed an upregulation of this receptor in the skin of Atlantic salmon infested by *L. salmonis* (Fig. 2A), suggesting an increase in transferrin activity. Similar expression patterns were reported in salmonid species susceptible to *L. salmonis* infestation, indicating a nutritional immunity response [12]. Another important gene in the regulation of intracellular iron is *hepcidin* [15,16], which has been found overexpressed in salmon species with a high resistance to *L. salmonis* [12,13]. In this study, *hepcidin* was overexpressed in the head kidney of Atlantic salmon infested by *C. rogercresseyi* (Fig. 2B), suggesting an iron restriction strategy similar to that reported in fish infested by *L. salmonis* [12].

The majority of hematophagous organisms acquire Heme directly from hosts [3,17]. In this study, only host infested by *L. salmonis* presented an increased expression of genes related to Heme group biosynthesis in both evaluated tissues, such as *aminolevulinic acid dehydrogenase* (*ALAd*), *aminolevulinic acid synthase* (*ALAs*), and *coproporphyrinogenase* (*CPOX*) (Fig. 2). On the other hand, Heme biosynthesis is associated with an increase in reactive oxygen species [3], as reflected in the present study by upregulation of *glutathione S-transferase* (GST) and *superoxide dismutase* (SOD) in the head kidney of fish infested by *L. salmonis* (Fig. 2B). Notably, there was transcriptional activity for genes involved in the Heme degradation pathway; *HO* and *biliverdin reductase* (*BLVr*) were significantly upregulated in the skin of Atlantic salmon infested by *C. rogercresseyi* (Fig. 2A). Therefore, it is possible that the increased rate of Heme biosynthesis in salmon infested by *L. salmonis* as compared to salmon infested by *C. rogercresseyi* is associated with the hematophagous behavior of this Northern Hemisphere sea louse. Overall, principal component analysis (PCA) showed that iron metabolism in Atlantic salmon is strongly modulated during infestation with *L. salmonis*, evidencing a high transcriptional expression of genes related to the Heme biosynthesis pathway, iron transport, and the antioxidant system (Fig. 3).

Regarding the feeding behavior of *C. rogercresseyi*, previous

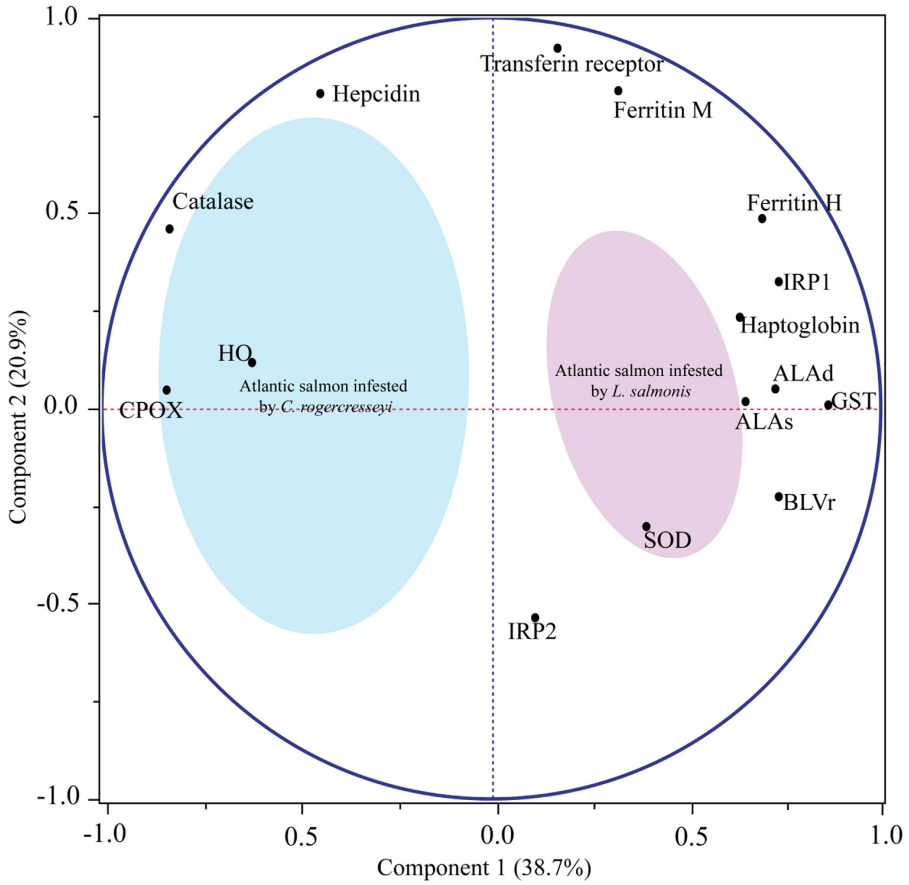

Fig. 3. PCA analys for RT-qPCR data for genes involved in iron transport for Atlantic salmon infested by *C. rogercresseyi* and *L. salmonis*.

studies have reported that this sea louse species primarily feeds from the skin mucous of fish hosts [6]. However, recent observations (pers. comm.) note an increasing number of female adults with apparently hematophagous behavior (Supplementary material 1). This finding suggests that *C. rogercresseyi* adults display facultative hematophagous feeding behavior during Atlantic salmon infestation. However, no scientific evidence exists yet to support if *C. rogercresseyi* absorbs the Heme group as an iron source or, alternatively, acquires iron indirectly from the fish host.

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2016.11.045.

Additionally, species such as *S. salar, Oncorhynchus keta*, and *Salmo trutta* show decreased levels of hematocrit when infested with *L. salmonis* [18,19]. Reduced hematocrit levels are related to infection intensity, in addition to being indicative of microcytic anemia induced by parasite-feeding skin wounds and blood loss. Therefore, increased Heme biosynthesis activity suggests a compensatory response of the host to reduced hematocrit levels produced by the hematophagous parasite. Interestingly, studies performed during *C. rogercresseyi* infestation of *S. salar* and *Eleginops maclovinus* demonstrate similar host responses to reduced hematocrit and hemoglobin concentrations [20,21]. Nevertheless, the results of the present study did not evidence a correlation between *C. rogercresseyi* infestation and Heme biosynthesis in Atlantic salmon. This finding suggests that the response of Atlantic salmon to *C. rogercresseyi* infection is to positively regulate the Heme degradation pathway, thus limiting the cellular availability of iron. Together, these results support that infected Atlantic salmon display pathogen-specific responses, differentially regulating iron availability against *C. rogercresseyi* or *L. salmonis* infestation.

Recognized limitations of this study are that infestation trials were not carried out at the same location and that the different fish groups could have distinct genetic backgrounds. However, the transport of *L. salmonis* or *C. rogercresseyi* between Chile and Norway is prohibited by regulations. Nevertheless, considering the extensive damage caused within the salmon industry by sea lice infestations, the present study still provides pivotal comparative information for host/sea lice interactions. The present analyses found fish infested by *L. salmonis* to present greater regulatory changes for genes associated with iron transport, supporting the use of nutritional immunity as a defense mechanism in Atlantic salmon in response to this ectoparasite. Additionally, the present results suggest that *L. salmonis*, as a hematophagous organism, sequesters Heme from Atlantic salmon, in turn increasing the expression of Heme biosynthesis genes in the host to maintain Heme group homeostasis. In contrast, Atlantic salmon infested by *C. rogercresseyi* did not evidence an upregulation of genes related to Heme biosynthesis, suggesting that parasitic feeding by *C. rogercresseyi* on fish skin does not cause microcytic anemia. Interestingly, the Heme degradation pathway was strongly activated in response to sea lice infestation, a process that could, in turn, decrease iron availability as a mechanism of nutritional immunity.

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CAPITULO 5

Comparative analysis of long non-coding RNAs in Atlantic and Coho salmon reveals divergent transcriptome responses associated with immunity and tissue repair during sea lice infestation.

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Abastract

The increasing capacity of transcriptomic analysis by high throughput sequencing has highlighted the presence of a large proportion of transcripts that do not encode proteins. In particular, long non-coding RNAs (lncRNAs) are sequences with low coding potential and conservation among species. Moreover, cumulative evidence has revealed important roles in post-transcriptional gene modulation in several taxa. In fish, the role of lncRNAs has been scarcely studied and even less so during the immune response against sea lice. In the present study we mined for lncRNAs in Atlantic salmon (*Salmo salar*) and Coho salmon (*Oncorhynkus kisutch*), which are affected by the sea louse *Caligus rogercresseyi*, evaluating the degree of sequence conservation between these two fish species and their putative roles during the infection process. Herein, Atlantic and Coho salmon were infected with 35 lice/fish and evaluated after 7 and 14 days post-infestation (dpi). For RNA sequencing, samples from skin and head kidney were collected. A total of 5658/4140 and 3678/2123 lncRNAs were identified in uninfected/infected Atlantic and Coho salmon transcriptomes, respectively. Species-specific transcription patterns were observed in exclusive lncRNAs according to the tissue analyzed. Furthermore, neighbor gene GO enrichment analysis of the top 100 highly regulated lncRNAs in Atlantic salmon showed that lncRNAs were localized near genes related to the immune response. On the other hand, in coho salmon the highly regulated lncRNAs were localized near genes involved in tissue repair processes. This study revealed high regulation of lncRNAs closely localized to immune and tissue repair-related genes in Atlantic and coho salmon, respectively, suggesting putative roles for lncRNAs in salmon against sea lice infestation.

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Comparative analysis of long non-coding RNAs in Atlantic and Coho salmon reveals divergent transcriptome responses associated with immunity and tissue repair during sea lice infestation

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C. rogercresseyi

ABSTRACT

The increasing capacity of transcriptomic analysis by high throughput sequencing has highlighted the presence of a large proportion of transcripts that do not encode proteins. In particular, long non-coding RNAs (lncRNAs) are sequences with low coding potential and conservation among species. Moreover, cumulative evidence has revealed important roles in post-transcriptional gene modulation in several taxa. In fish, the role of lncRNAs has been scarcely studied and even less so during the immune response against sea lice. In the present study we mined for lncRNAs in Atlantic salmon (*Salmo salar*) and Coho salmon (*Oncorhynkus kisutch*), which are affected by the sea louse *Caligus rogercresseyi*, evaluating the degree of sequence conservation between these two fish species and their putative roles during the infection process. Herein, Atlantic and Coho salmon were infected with 35 lice/fish and evaluated after 7 and 14 days post-infestation (dpi). For RNA sequencing, samples from skin and head kidney were collected. A total of 5658/4140 and 3678/2123 lncRNAs were identified in uninfected/infected Atlantic and Coho salmon transcriptomes, respectively. Species-specific transcription patterns were observed in exclusive lncRNAs according to the tissue analyzed. Furthermore, neighbor gene GO enrichment analysis of the top 100 highly regulated lncRNAs in Atlantic salmon showed that lncRNAs were localized near genes related to the immune response. On the other hand, in Coho salmon the highly regulated lncRNAs were localized near genes involved in tissue repair processes. This study revealed high regulation of lncRNAs closely localized to immune and tissue repair-related genes in Atlantic and Coho salmon, respectively, suggesting putative roles for lncRNAs in salmon against sea lice infestation.

1. Introduction

The increasing capacity of next generation sequencing has highlighted the presence of transcripts that do not encode proteins, denominated noncoding RNA (ncRNA). Among the ncRNA transcribed in eukaryotes, two types have been identified; housekeeping ncRNAs (ribosomal, small nuclear, small nucleolar and transfer) and regulatory ncRNAs. Among them, microRNA, piwi-interacting RNA, small interfering RNA, and long non-coding RNA (lncRNA) represent the most abundant ncRNAs (Johnsson et al., 2014; Tang et al., 2017). In particular, lncRNAs are defined as transcripts with a length higher than 200 base pairs and low coding potential. LncRNA can be located in nuclear or cytosolic fractions, can be polyadenylated or not and are transcribed in a similar way to mRNA (Cech and Steitz, 2014; Ponting et al., 2009). It is possible to observe two different classes according to their genomic position, lncRNA transcribed from intron regions in sense or antisense orientations, and lncRNA transcribed from intergenic regions located between known proteins (Mallory and Shkumatava, 2015).

LncRNAs, in contrast to coding sequences, present low degree of sequence conservation among species. In fact, from a study performed in 17 different species it was observed that more than 70% of lncRNAs have no corresponding orthologs, and less than 100 lncRNA sequences have a common ancestor between teleost fish and tetrapod (Hezroni et al., 2015). Where, the authors report only 29 lncRNA conserved between fish and mammals (Hezroni et al., 2015). In addition, lncRNA conservation between species does not only depend on sequence conservation, such as orthologous sequences of coding genes, lncRNA conservation can be given by its sequence, structure, function and syntenic loci (Diederichs, 2014; Johnsson et al., 2014).

Each cell type as a specific LncRNAs are expressed in a cell-specific way, suggesting that each cell type has a specific lncRNA repertoire involved in cell identity and function. In the case of immune cell

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Table 1
RNA sequencing statistics for Atlantic and coho salmon transcriptomes.

	Atlantic salmon			Coho salmon		
	skin	head kidney	*de novo* assembly	skin	head kidney	*de novo* assembly
Reads (Mb)	37.6	41.2	78.7	37	40.7	77.7
Average length (bp)	199.52	194.39	196.84	203.86	193.91	198.65
Matched (Mb)	23.88 (63.5%)	29.56 (71.7%)	65.9 (83.8%)	23.4 (63.2%)	27.8 (68.3%)	64.1 (82.5%)
Nucleotide number (Gb)	7.5	7.9	15.4	7.5	7.8	15.4
Contigs	252,614	165,171	303,898	259,389	165,879	308,992
Average length (bp)	546	596	554	542	585	547
Singletons (Mb)	13.6	11.5	12.7	13.5	12.8	13.6
Average length (bp)	203.01	198.24	196.85	207.17	191.93	199.35

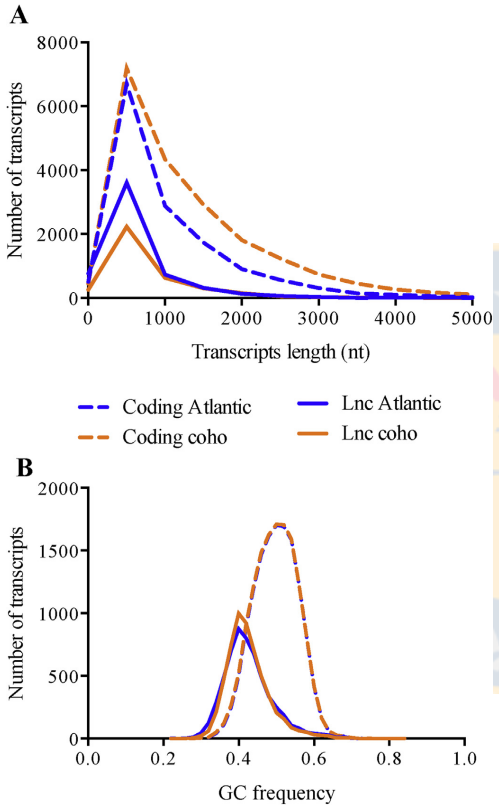

Fig. 1. Features of predicted lncRNAs from Atlantic and coho salmon transcriptomes. A) Length distribution for predicted lncRNAs and coding transcripts. B) Comparison of GC content between lncRNAs and coding transcripts.

development and differentiation, several studies reported a unique lncRNA expression profile, indicating a role of lncRNA in the immune system (Aune and Spurlock, 2016). Indeed, lncRNAs are specifically expressed in T cell linages of Th1, Th2 and Th17 responses in humans (Spurlock et al., 2015). In mammal lncRNA are responsible of myeloid and dendritic cell development as lncRNA denominated Morribid, HOTAIRM1 or lnc-DCs which participated in DC cell differentiation and regulation of STAT3 in presence of antigen (Atianand et al., 2017). Moreover, lncRNAs play important roles during pathogens infestation, for instance, a down regulation of lncRNA NRAV reduce the capacity of virus replication, working as immune component (Atianand et al., 2017). Furthermore, lncRNAs have been demonstrated to possess a regulatory role in important innate immune pathways, such as toll-like receptors (TLR) (Carpenter et al., 2013) and NF-κB (Zhou et al., 2016). The role of lncRNAs in the fish immune response has been poorly studied. However, a transcriptomic study in yellow croaker showed a high number of lncRNAs specifically expressed in fish exposed to polyinosinic-polycytidylic acid (poly I:C). (Jiang et al., 2016). Furthermore, lncRNAs annotated with immune functions were highly expressed in spleen and the upregulated lncRNAs evidenced to be activators of Toll-like receptors signals pathway. Additionally, through RNA-Seq analysis of Atlantic salmon infected with the ISA virus, 4967 putative lncRNAs were differently regulated during viral infection with a positive correlation between the lncRNA abundance and fish ISAv load, showing a putative immune role for lncRNA in Atlantic salmon (Boltaña et al., 2016). Differences in lncRNAs abundance were observed between ISAv target genes, showing a tissue-specific distribution of lncRNA during the infestation (Boltaña et al., 2016). With respect to fish bacterial infection, a study performing in Atlantic salmon infected with *Piscirickettsia salmonis* evidenced a similar expression profiles between coding RNA and lncRNA, suggesting a putative role of lncRNA in fish immune response (Valenzuela-Miranda and Gallardo-Escárate, 2016). This study revealed a positive regulation between lncRNA and neighbor genes associated to *P. salmonis* infection such as *hepcidin, clathrin and haptoglobin* (Valenzuela-Miranda and Gallardo-Escárate, 2016). Furthermore, a recent comparative study among Atlantic salmon infected with virus, bacterial and ectoparasite pathogens demonstrated high modulation of lncRNAs with immune-related genes (Tarifeño-Saldivia et al., 2017). The authors reported high number of lncRNA differently expressed in skin of Atlantic salmon infected with sea lice compared with fish infected with ISAv and *P. salmonis* (Tarifeño-Saldivia et al., 2017). However, comparative studies with respect to the conservation of lncRNAs among salmon species and their expression patterns have not yet been conducted. Herein, we characterized lncRNAs from Atlantic and coho salmon transcriptomes stimulated by the sea louse *C. rogercresseyi*, the most detrimental ectoparasite that affects the Chilean salmon industry. This ectoparasite species can infect both Atlantic and coho salmon, however, different degrees of susceptibility and immune response mechanisms have been reported. In our group, previous transcriptome studies have suggested differences between Atlantic and Coho salmon, for instance, infected Atlantic salmon increases the transcription levels of the TLR22 gene and deplete the cellular iron availability, while coho salmon displays pro-inflammatory mechanisms in response to the infestation (Valenzuela-Muñoz et al., 2017). Furthermore, differences in iron regulation has been observed between both salmon species infected with *C. rogercresseyi*, suggesting high regulation of heme degradation and iron transport genes in Atlantic salmon comparing with Coho salmon (Valenzuela-Muñoz et al., 2017). Due the differences in the responses mechanisms of Atlantic and Coho salmon to *C. rogercresseyi* infestation, we aimed to explore the

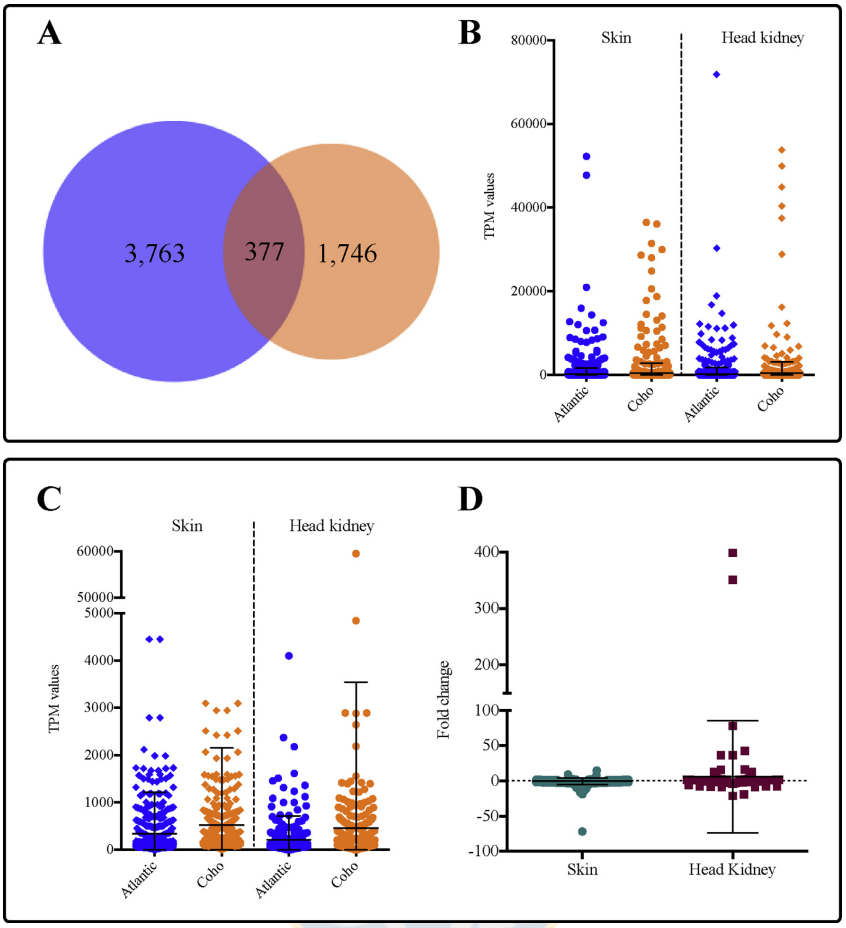

Fig. 2. Features of predicted lncRNAs expressed constitutively in Atlantic and coho salmon. A) Veen diagram representation of lncRNAs sharing and exclusive constitutively presented in both salmon species. B) TPM values profile for exclusive lncRNAs in skin and head kidney of Atlantic and coho salmon. C) TPM values profile of sharing lncRNAs in skin and head kidney of Atlantic and coho salmon. D) Fold-change values of sharing lncRNAs between skin and head kidney of Atlantic and coho salmon.

conservation of lncRNAs between Atlantic and coho salmon and evaluate their modulation during sea lice infestation. Moreover, we investigated the correlation between both immune response processes previously described for both salmon species. Furthermore, neighbor gene analysis and co-expression pattern assessments were conducted to evidence putative regulatory functions that could explain the molecular mechanisms involved during the salmon immune response against sea lice.

2. Materials and methods

2.1. Experimental design

The experimental trials were previously reported by Valenzuela-Muñoz et al. (2016). Briefly, one hundred and twenty Atlantic salmon and Coho salmon were fed daily with a commercial diet. After acclimatization of fifteen days, individuals were randomly divided and placed in triplicate in tanks (500 L) and starved for at least 24 h prior to any manipulation. For infections, the experimental tanks were placed in the dark without water flow for 2 h with a load of 35 copepodids per fish. Samples of the head kidney and skin from infected area without skeletal muscle were collected from 10 fish per tank before infection and at 7 and 14 days post infestation (dpi). All samples were fixed in the RNAlater® RNA Stabilization Reagent (Ambion®, Life Technologies™, Carlsbad, CA, USA) and stored at −80 °C until subsequent RNA extraction.

2.2. LncRNA mining

Using raw data generated from RNA sequencing previously

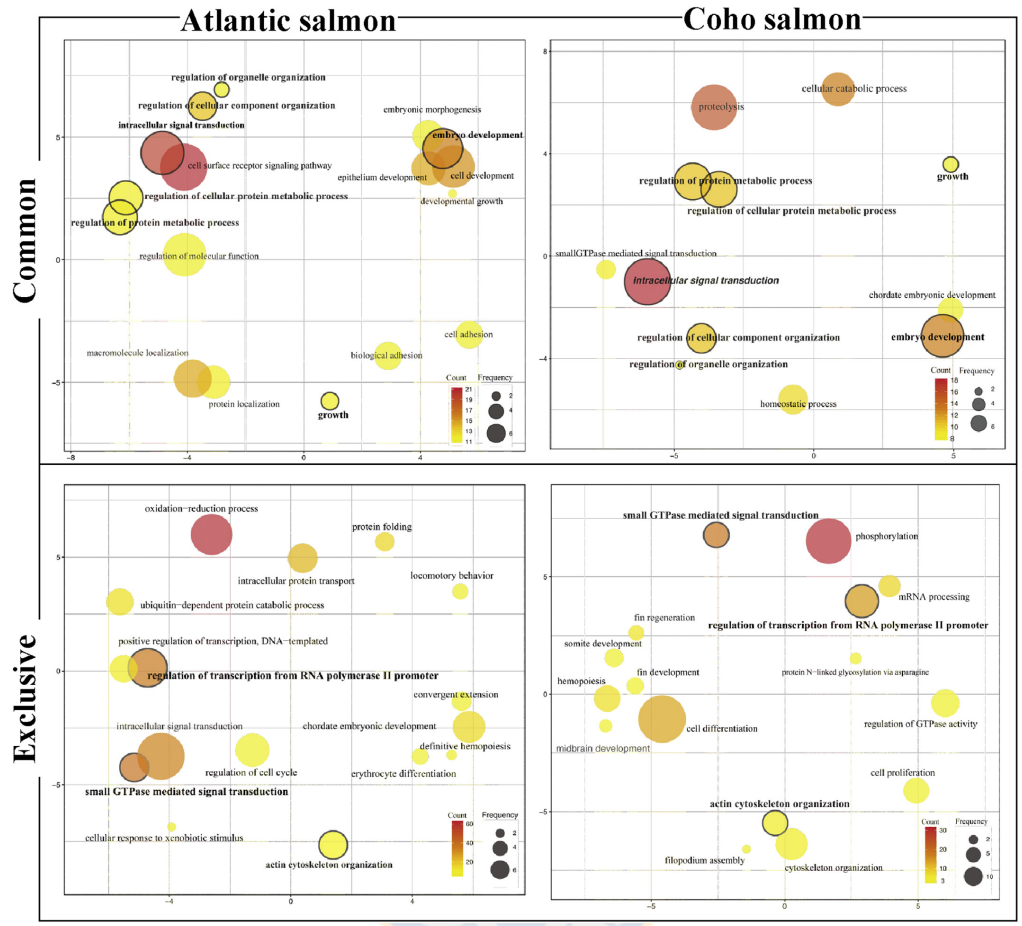

Fig. 3. Gene Ontology (GO) enrichment for LncRNAs expressed constitutively in Atlantic and coho salmon. The graphs represent the GO enrichment for lncRNAs sharing and exclusives.

performed by Valenzuela-Muñoz et al. (2016), lncRNA sequences were mined using the pipeline described by Tarifeño-Saldivia et al. (2017). Briefly, Atlantic and Coho salmon transcriptome assemblies were filtered by the average coverage < 50 and Blastx analysis was performed in selected sequences, discarding all sequences with positive Blast-hits (BlastX, e-value < 1E-05) against NCBI Genbank and UniProt database. Open reading frames were predicted from the remainder contigs and all contigs with putative ORF > 200 bp were discarded. Finally, the free Coding Potential Assessment Tool (CPAT) (http://lilab.research.bcm.edu/cpat/) was used to discard sequences with coding potential. Furthermore, lncRNA sequence conservation between Atlantic and Coho salmon lncRNAs and those reported for other two teleost fish species - rainbow trout (Al-Tobasei et al., 2016; Núñez-Acuña et al., 2017) and zebrafish (Dhiman et al., 2015) - was performed by Blastn analysis considering all lncRNAs with an e-value < 1E-50 or similar.

2.3. Genome mapping and pathway analysis

To gain a better understanding of the genomic context of lncRNAs in both species, transcripts were mapped against the last version of the Atlantic salmon genome (https://www.ncbi.nlm.nih.gov/genome/369) (Lien et al., 2016) and against the Coho salmon linkage groups (https://www.ncbi.nlm.nih.gov/genome/13127). Thus, lncRNAs were mapped using the following parameters: length fraction = 0.8, similarity fraction = 0.8 and mismatch, insertion and deletion cost of 2, 3 and 3, respectively. Later, any coding gene flanking up to 10,000 bases from any annotated lncRNA was identified and extracted for further analysis. Thus, GO enrichment analysis (Huang et al., 2009) was conducted to identify the most represented biological processes among protein coding genes proximal to lncRNAs. The results were finally plotted using the REVIGO platform (Supek et al., 2011), R and CYTOSCAPE software (Shannon et al., 2003).

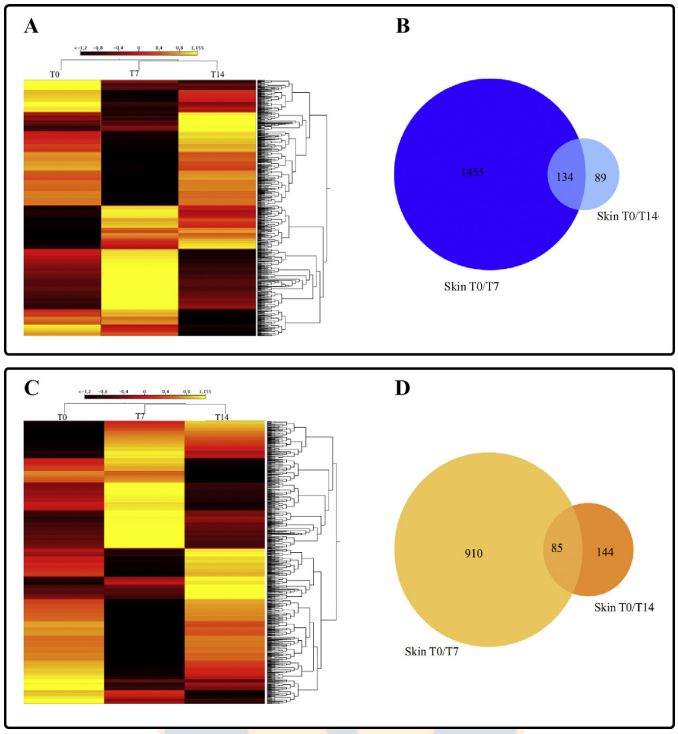

Fig. 4. Differentially expressed lncRNAs in response to *Caligus rogercresseyi* infestation. A-C) Hierarchical clustering of expressed transcripts (TPM values) for skin (A) Atlantic salmon and (C) coho salmon. B-D) Venn diagrams representing the significantly expressed LncRNA (B) Atlantic salmon skin, (D) coho salmon skin (Fold-change 4, p-value 0.01).

2.4. RNA-Seq analysis

To test differentially expressed transcripts, RNA-Seq was set with a minimum length fraction = 0.6 and a minimum similarity fraction (long reads) = 0.5. The expression value was set as Transcripts per Million model (TPM). The distance metric was calculated with the Manhattan method, with the mean expression level in 5–6 rounds of k-means clustering subtracted. Finally, a Kal's statistical analysis test was used to compare gene expression levels in terms of the log2 fold change (P = 0.0005; FDR corrected). To identify co-expression patterns among the fifty lncRNA more up and down regulated (absolute fold change value > 4) at 7 dpi and 14 dpi in Atlantic and Coho salmon skin and head kidney tissue and neighbor genes and modulated during sea lice infestation (Valenzuela-Muñoz et al., 2016, 2017), Pearson correlation coefficients were estimated between expression values (TPM) of lncRNA and genes and plotted using the Corrplot library in R (https://cran.r-project.org/web/packages/corrplot/index.html).

2.5. RT-qPCR validation and statistical analyses

Quantitative real-time PCR was used to validate the transcription activity of selected lncRNAs. Primer sets were designed with the Primer3 Tool (Rozen and Skaletsky, 2000) included in the Geneious Pro software (Supplementary Table 1). To evaluate gene transcription, from five individuals of Atlantic and Coho salmon skin and head kidney tissues, total RNA were isolated from each group using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and manufacturer protocol. Purity was measured using a Nanodrop ND-1000 spectrophotometer (ThermoFisher 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 Supplementary Table 1); after performing the dynamic range, the first dilution (1:5) was selected. RT-qPCR was performed on the StepOnePlusTM system (Applied Biosystems, Life Technologies™) using the Power SYBR Green PCR Master Mix (Applied Biosystems, USA) and a final volume of 10 μl per reaction. The qPCR validation was performed by triplicate using in five individuals per tissue of Atlantic and Coho salmon. 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). Relative expression was calculated using the comparative ΔΔCt method. Elongation factor-α (EF-α), β-tubulin and 18S were selected as putative normalizer genes and were statistically analyzed with the NormFinder algorithm (Jensen and Ørntoft, 2004) to assess relative expression stability. EF-α was selected as the best normalizer gene for relative expression evaluation. 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 $p < 0.05$.

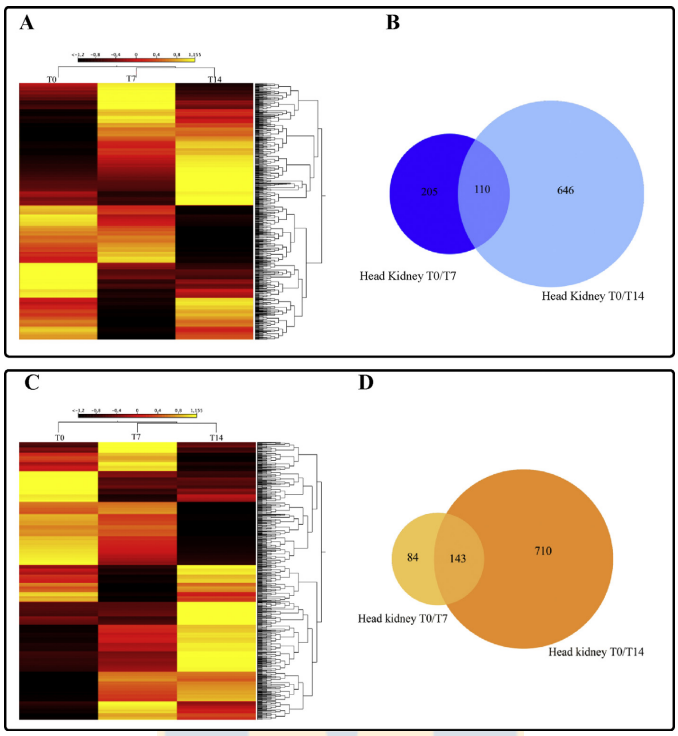

Fig. 5. LncRNAs differentially expressed in response to *Caligus rogercresseyi* infestation. A-C) Hierarchical clustering of expressed transcripts (TPM values) for head kidney (A) Atlantic salmon and (C) coho salmon. B-D) Venn diagrams representing the significantly expressed LncRNA (B) Atlantic salmon head kidney, (D) coho salmon head kidney (Fold-change 4, p-value 0.01).

3. Results

3.1. Sequence assembly and LncRNA identification

From the total assembly, 303,898 and 308,992 contigs were obtained for Atlantic and Coho salmon, respectively (Valenzuela-Muñoz et al., 2016). From these contigs, 5658 lncRNA and 13,967 coding sequences for Atlantic salmon and 3678 lncRNA and 19,857 coding sequences for Coho salmon were identified (Table 1). The lncRNA identified in both salmon species presented a length distribution between 200 and 2000 nt, compared with coding sequences that presented lengths between 200 and 5000 nt (Fig. 1A). Moreover, lncRNAs showed lower GC frequency than coding sequences (Fig. 1B).

3.2. LncRNA comparative analysis between Atlantic and Coho salmon transcriptomes

Before lice infestation a total of 4140 and 2123 lncRNAs were identified for Atlantic salmon and Coho salmon, where 3763 and 1746 evidenced exclusive transcription profiles, respectively (Fig. 2A). Furthermore, exclusive lncRNAs of Atlantic and Coho salmon presented similar lncRNA abundance in skin and head kidney tissues (Fig. 2B). Moreover, from homology sequence analysis 377 lncRNAs were identified as common lncRNA sequences between both salmon species (Table S2). Common lncRNAs displayed similar expression patterns in both tissues analyzed in Atlantic and Coho salmon, showing higher abundance of lncRNAs in skin compared with head kidney (Fig. 2C). From differential expression analysis of common lncRNAs between Atlantic and Coho salmon, skin and head kidney tissue non-significant expression changes were observed (Fig. 2D). Common lncRNAs of Atlantic and Coho salmon were validated by RT-qPCR analysis, evidencing similar transcription expression between Atlantic and Coho salmon before infestation and in congruency with the *in-silico* expression values (Fig. S3A).

Additionally, in order to determinate sequence conservation of lncRNAs between fish species, Blastn analysis was performed using the lncRNAs identified for Atlantic and Coho salmon in the present study and lncRNAs reported for rainbow trout (Al-Tobasei et al., 2016; Núñez-Acuña et al., 2017) and zebrafish (Dhiman et al., 2015). Overall, low levels of sequence conservation (less that than 2%) was observed among Atlantic salmon, Coho salmon, rainbow trout and zebrafish (Fig. S1). Notably, Coho salmon exhibited 895 common lncRNAs with rainbow trout with a percentage of identity between 83.9 and 100%, meanwhile between Atlantic and Coho salmon 374 lncRNAs were annotated with 82.4–100% of sequence identity. Additionally, Atlantic salmon shared 723 lncRNAs with rainbow trout with 77.30–100% of sequence identity (Fig. S1).

Moreover, differences in abundance of lncRNAs were observed in skin and head kidney tissue in both salmon species. For instance, Atlantic salmon skin and head kidney presented 730 and 1237 lncRNAs, respectively, sharing 2174 lncRNA sequences that presented similar transcription levels (Fig. S2 A-B). For Coho salmon 654 and 445

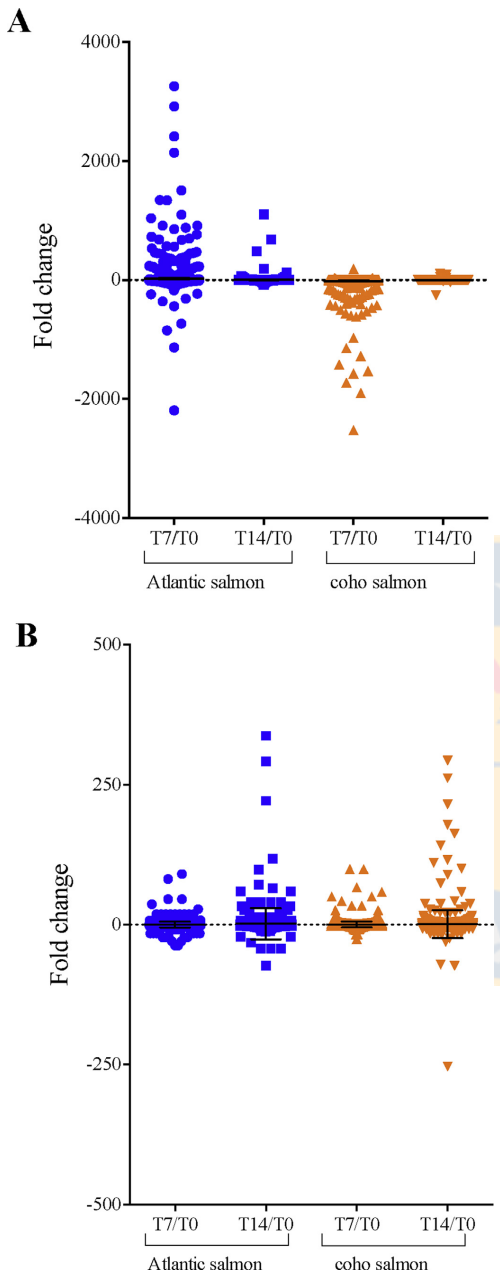

Fig. 6. LncRNA transcription expressions in Atlantic and Coho salmon after *C. rogercresseyi* infestation. A) Fold-change variation in skin of Atlantic and coho salmon at 7 and 14 dpi. B) Fold-change variation in head kidney of Atlantic and coho salmon at 7 and 14 dpi.

lncRNAs were identified exclusively in skin and head kidney tissue, respectively. Furthermore, both tissues shared 1024 lncRNAs with similar TPM values observed for Atlantic salmon (Fig. S2 C-D). Finally, lncRNAs identified for Atlantic and Coho salmon were mapped to the Atlantic salmon genome and Coho salmon scaffolds, respectively. Notably, common lncRNAs between Atlantic and Coho salmon revealed similar patterns of gene ontology categories with respect to neighbor genes, where key biological processes such as cell surface receptor signaling pathways, intracellular signal transduction, protein metabolism and cell development were mainly annotated (Fig. 3). Neighbor genes of exclusive lncRNAs of Atlantic salmon were mainly related to oxidation-reduction processes, protein transport, cell cycle regulation, hematopoiesis and intracellular signal transduction (Fig. 3). Meanwhile, exclusive lncRNAs from Coho salmon were co-located with genes involved in biological processes such as cell differentiation, phosphorylation, cell proliferation, cytoskeleton organization and regulation of GTPase activity (Fig. 3).

3.3. LncRNA expression profile during lice infestation

RNA-Seq analysis was conducted for lncRNAs identified in Atlantic and Coho salmon during *C. rogercresseyi* infestation. Here, heatmap representation evidenced high number of lncRNAs upregulated in skin tissue of both infected salmon species after 7 days of *C. rogercresseyi* infestation (Fig. 4A, C). Moreover, the number of lncRNAs from Atlantic and Coho salmon skin tissue was higher at 7 dpi, compared with the lncRNAs expressed at 14 dpi (Fig. 4B, D). However, 1455 lncRNAs were differentially expressed in Atlantic salmon skin at 7 dpi, compared with 910 lncRNAs at the same day post-infection in Coho salmon skin. In contrast, RNA-Seq analysis evidenced that in head kidney for both salmon species the lncRNA profiling was mainly related at the end of the infection process (Fig. 5). Notably, we estimated that the differences between the number of lncRNAs differentially expressed at 7 and 14 dpi was roughly 10-fold. From transcription expression analysis, we observed a high number of lncRNAs upregulated in Atlantic salmon skin at 7 dpi, compared with Coho salmon (Fig. 6A). Moreover, at 14 dpi skin tissue of Atlantic salmon showed more lncRNAs expression changes than lncRNA from Coho salmon skin (Fig. 6A). In head kidney tissue, Atlantic and Coho salmon showed similar differential expression profiles at 7 and 14 dpi, with high number of transcripts differently modulated at 14 dpi (Fig. 6B). Additionally, RT-qPCR analysis was performed using five individuals per sample to validate the lncRNAs transcription patterns observed. Here, similar expression profiles between TPM values and relative expression values during the *C. rogercresseyi* infestation process were observed (Fig. S3B-C).

3.4. Putative lncRNA target gene regulation during lice infestation

One hundred lncRNAs that were up- and down-regulated in Atlantic and Coho salmon skin and head kidney tissues were mapped to the reference salmon genomes available at NCBI. The lncRNAs were selected from the fifty more up and down-regulated transcripts (absolute fold change value > 4) at 7 dpi and 14 dpi in Atlantic and Coho salmon. For Atlantic salmon skin, we observed that highly-regulated lncRNAs were neighbor genes related to processes such as the inflammatory response, response to other organisms and organophosphate metabolism (Fig. 7). For head kidney in Atlantic salmon, highly regulated lncRNAs were associated with genes involved in regulation of response to stress, regulation of the immune response, development and muscle cell proliferation, among others (Fig. 7). The putative relationships of lncRNAs with neighbor genes were assessed by differential expression analysis for genes and lncRNAs during lice infestation. Herein, Atlantic salmon skin and head kidney evidenced a negative correlation between genes related to apoptosis and TLR-7, and a positive correlation with genes related to immunoglobulin production (Table 2). With respect to Coho salmon, lncRNAs identified in skin

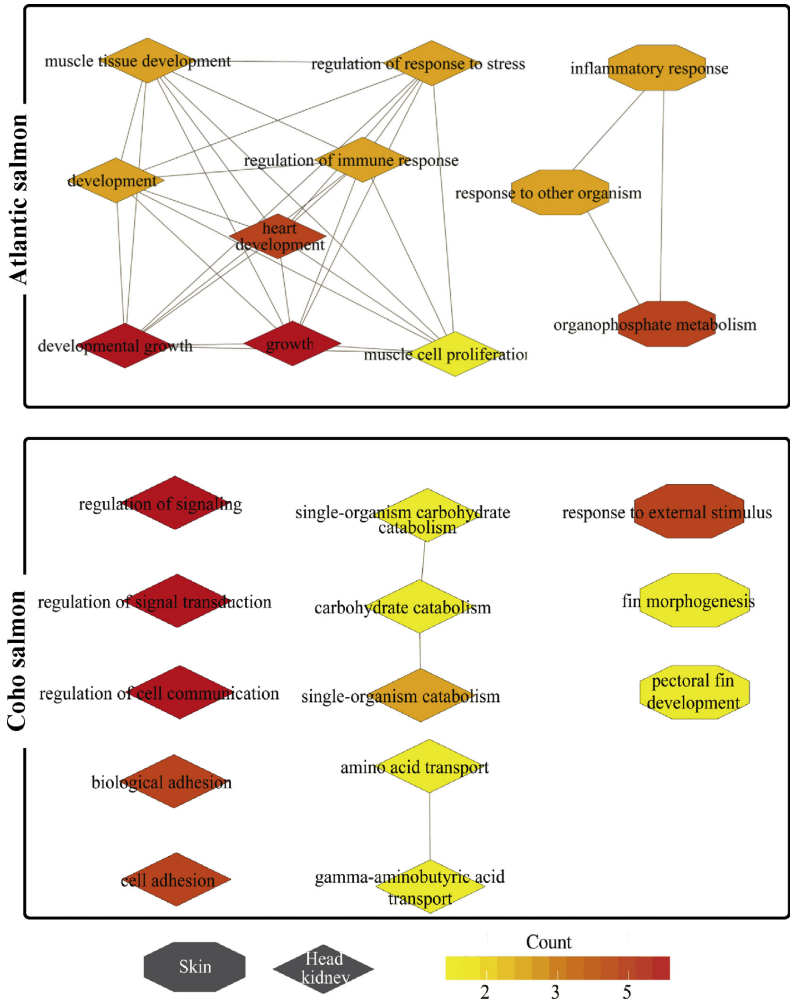

Fig. 7. GO enrichment analysis for exclusive lncRNAs annotated in Atlantic and coho salmon transcriptomes highly regulated during *C. rogercresseyi* infestation.

revealed a high correlation with nearest-neighbor genes associated with response to external stimuli, fin morphogenesis and pectoral fin development (Fig. 7). For Coho salmon head kidney, a high number of genes associated with regulation of signaling, regulation of signal transduction processes, regulation of cell communication and cell adhesion were co-located with highly regulated lncRNAs (Fig. 7). Notably, for Coho salmon genes such as collagen, tetraspanin, mucin 5AC and trichohyalin were putatively correlated with lncRNAs co-located nearby. Moreover, genes such as myosin and microtubule proteins were negatively correlated, suggesting a decrease in gene expression (Table 2).

Co-expression analysis between lncRNAs identified and genes related to the immune response and iron homeostasis.

Correlation matrix by Pearson analysis was conducted among highly regulated lncRNAs (fifty more up and downregulated lncRNAs with absolute fold change value > 4 at 7 dpi and 14 dpi in Atlantic and Coho salmon) and genes previously reported with key roles during the immune responses of Atlantic and Coho salmon against lice infestation (Valenzuela-Muñoz et al., 2016, 2017). Herein, genes evaluated were related to macrophage activation, Th1/Th2 responses and TLRs. In the skin of Atlantic salmon five lncRNA clusters were identified (Fig. 8A). Cluster 1 mainly showed a positive correlation with the panel genes selected (macrophage, Th2, TLR response and iron homeostasis, Table S5). Cluster 2 and 4 in Atlantic salmon skin showed a positive correlation with Th1 response and iron homeostasis genes, further these two clusters showed negative correlations with TLR pathway genes (Fig. 8A, Table S5). With respect to Coho salmon skin, four clusters of lncRNAs were identified with a lower level of positive correlation between

Table 2
Fold-change values for genes near to lncRNAs highly regulated in Atlantic and coho salmon after lice infestation. The fold changes were estimated by DEG analysis between tissue samples before infestation vs tissue samples 7 and 14 dpi.

Atlantic salmon skin						
Gene ID	**T0/T7**	**T0/T14**	**Lnc**	**T0/T7**	**T0/T14**
LOC106572953_uncharacterized protein LOC106572953 isoform X1	↑ 15.05	↑ 0.00	SsLnc_0012986	↑ 2.20	↑ 4.11
LOC106564629_glial fibrillary acidic protein-like	↑ 10.23	↑ 7.57	SsLnc_0005882	↑ 4.12	↓ -17.85
LOC106564934_nicotinate-nucleotide pyrophosphorylase [carboxylating]-like isoform X1	↑ 10.23	↑ 0.00	SsLnc_0015577	↓ -10.33	↑ 3.31
LOC106573461_apoptosis-associated speck-like protein containing a CARD	↑ 5.07	↑ 1.75	SsLnc_0074719	↓ -6.57	↑ 1.01
LOC106586984_high choriolytic enzyme 1-like	↑ 4.77	↑ 3.17	SsLnc_0033488	↑ 14.84	↓ -9.42
LOC106566827_histone-lysine N-methyltransferase SUV39H1-like	↑ 2.28	↑ 3.26	SsLnc_0050434	↑ 7.82	↑ 5.72
LOC106603057_lipocalin-like	↑ 2.18	↓ -1.13	SsLnc_0138996	↑ 1.33	↓ -1.37
LOC106580961_high affinity immunoglobulin gamma Fc receptor I-like isoform X2	↑ 2.17	↑ 1.19	SsLnc_0026331	↑ 24.68	↑ 3.31
LOC106601960_rho GDP-dissociation inhibitor 2-like	↑ 1.87	↑ 2.51	SsLnc_0050538	↓ -10.33	↑ 1.71
LOC106585832_tetraspanin-3-like	↑ 1.84	↑ 1.00	SsLnc_0097953	↑ 1.50	↑ 2.51
LOC106564627_protein RRP5 homolog	↑ 1.81	↑ 3.06	SsLnc_0005882	↑ 4.12	↓ -17.85
LOC106574207_dermatopontin-like	↑ 1.69	↑ 1.21	SsLnc_0071648	↓ -1102.88	↑ 1.53
LOC106587764_keratin, type I cytoskeletal 13-like isoform X3	↑ 1.67	↑ 1.09	SsLnc_0072604	↓ -1.48	↓ -1.97
LOC106609457_mitogen-activated protein kinase 12-like	↑ 1.51	↓ -1.24	SsLnc_0038751	↑ 5.81	↑ 1.76
LOC106603060_zinc finger Y-chromosomal protein 1-like isoform X2	↑ 1.19	↑ 1.70	SsLnc_0138996	↑ 1.33	↓ -1.37
LOC106564216_adhesion G protein-coupled receptor E2-like	↑ 1.15	↑ 1.08	SsLnc_0065491	↓ -10.33	↓ -9.42
LOC106602516_ubiquitin-like modifier-activating enzyme 6	↑ 1.13	↑ 2.00	SsLnc_0211437	↓ -1.93	↓ -1.43
LOC106573462_uncharacterized protein LOC106573462	↑ 1.07	↓ -1.26	SsLnc_0074719	↓ -6.57	↑ 1.01
LOC106568019_cytochrome c oxidase subunit 7B, mitochondrial-like isoform X1	↑ 1.01	↑ 1.04	SsLnc_0009648	↑ 9.23	↓ -1.10
LOC106560581_prothymosin alpha-A-like isoform X1	↓ -1.07	↓ -1.06	SsLnc_0004300	↑ 1.30	↑ 1.06
LOC106564853_eosinophil peroxidase-like	↓ -1.09	↑ 1.30	SsLnc_0113142	↓ -10.33	↓ -9.42
LOC106575435_Ig kappa chain V-V region MOPC 21-like	↓ -1.14	↑ 1.05	SsLnc_0180279	↓ -1.25	↑ 1.71
LOC106566973_keratin, type II cytoskeletal 8-like	↓ -1.18	↓ -1.03	SsLnc_0252932	↓ -1.10	↓ -1.34
nnmt_Nicotinamide N-methyltransferase	↓ -1.18	↑ 1.02	SsLnc_0057546	↓ -10.33	↓ -9.42
LOC106589785_glycerophosphodiester phosphodiesterase 1-like	↓ -1.21	↑ 1.64	SsLnc_0038648	↑ 3.75	↑ 1.75
LOC106564961_keratin, type I cytoskeletal 13	↓ -1.32	↓ -1.50	SsLnc_0153966	↑ 1.55	↓ -1.65
LOC106567903_solute carrier organic anion transporter family member 2B1-like	↓ -1.36	↓ -1.03	SsLnc_0006463	↓ -8.90	↓ -1.17
LOC106612590_kinesin light chain 1-like	↓ -1.50	↑ 1.02	SsLnc_0036052	↓ -460.04	↓ -1.36
LOC106609456_mitogen-activated protein kinase 11-like	↓ -1.63	↓ -1.20	SsLnc_0038751	↑ 5.81	↑ 1.76
fam195a_protein FAM195A	↓ -1.68	↑ 1.23	SsLnc_0038648	↑ 3.75	↑ 1.75
LOC106561721_cytochrome c oxidase subunit 5A, mitochondrial-like	↓ -1.86	↑ 1.39	SsLnc_0197493	↓ -10.33	↑ 1.71
LOC106573640_zinc finger protein 319-like isoform X2	↓ -1.94	↓ -1.33	SsLnc_0042417	↓ -10.33	↑ 3.31
LOC106566828_probable G-protein coupled receptor 173	↓ -2.47	↓ -2.06	SsLnc_0050434	↑ 7.82	↑ 5.72
LOC106575429_Ig kappa chain V-III region NG9-like	↓ -2.50	↓ -1.92	SsLnc_0180279	↓ -1.25	↑ 1.71
LOC106589632_collagen alpha-1(I) chain-like	↓ -2.68	↓ -1.13	SsLnc_0251880	↓ -1.52	↓ -1.75
LOC106604645_CD97 antigen-like	↓ -3.04	↑ 1.61	SsLnc_0040302	↑ 4.31	↑ 8.12
LOC100286406_collagen alpha-1(I) chain	↓ -3.14	↓ -1.13	SsLnc_0252090	↓ -1.90	↓ -1.70
LOC100380654_periostin	↓ -4.11	↓ -1.31	SsLnc_0057546	↓ -10.33	↓ -9.42
LOC106561725_uncharacterized protein LOC106561725	↓ -4.66	↓ -3.86	SsLnc_0197493	↓ -10.33	↑ 1.71
LOC106600855_uncharacterized protein LOC106600855 isoform X2	↓ -6.15	↓ -2.72	SsLnc_0055868	↑ 20.46	↑ 3.31
mlc-2_myosin regulatory light chain 2	↓ -9.37	↓ -1.64	SsLnc_0127667	↓ -6.21	↓ -2.25
LOC106580960_inner ear-specific collagen-like	↓ -14.95	↓ -10.94	SsLnc_0026331	↑ 24.68	↑ 3.31
LOC106586812_toll-like receptor 7	↓ -14.95	↓ -1.37	SsLnc_0104663	↓ -1.04	↑ 1.27
LOC106586812_toll-like receptor 7	↓ -14.95	↓ -1.37	SsLnc_0153719	↓ -1.13	↑ 1.19
LOC106585833_histone H1-like	↓ -54.85	↑ 1.05	SsLnc_0097953	↑ 1.50	↑ 2.51
LOC106588693_adenylate cyclase type 2-like isoform X3	↓ -54.85	↓ -5.10	SsLnc_0112159	↓ -1.25	↑ 4.92
ccdc113_coiled-coil domain-containing protein 113 isoform X1	↓ -68.15	↓ -6.34	SsLnc_0042417	↓ -10.33	↑ 3.31

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Table 2 (*continued*)

Coho salmon skin						
Gene ID	**T0/T7**	**T0/T14**		**Lnc**	**T0/T7**	**T0/T14**
apmap_adipocyte plasma membrane-associated protein isoform X1	2.01	1.36		OkLnc_0000917	1.30	6.49
cds43_	0.18	1.02		OkLnc_0029693	2.49	2.06
crbn_protein cereblon	1.78	2.57		OkLnc_0040986	4.08	3.17
LOC109864961_O-acetyl-ADP-ribose deacetylase MACROD1-like	1.73	2.57		OkLnc_0017161	-11.18	1.56
LOC109865057_hepatocyte cell adhesion molecule-like isoform X2	1.07	-3.81		OkLnc_0025493	-21.34	-1.23
LOC109865749_ankyrin repeat domain-containing protein 1-like isoform X1	0.16	1.06		OkLnc_0236473	1.16	1.35
LOC109865796_keratin, type I cytoskeletal 13	1.78	3.45		OkLnc_0148374	1.40	1.02
LOC109865796_CD276 antigen homolog isoform X2	1.39	3.45		OkLnc_0148374	1.40	1.02
LOC109866401_uncharacterized protein LOC109866401 isoform X1	-2.50	9.36		OkLnc_0129319	1.29	14.41
LOC109867438_CD209 antigen-like protein C isoform X2	2.06	10.49		OkLnc_0022997	2.87	-1.53
LOC109869059_prostaglandin E synthase 3-like	1.23	1.38		OkLnc_0077804	6.09	5.02
LOC109870807_guanylate-binding protein 1-like isoform X1	1.96	7.15		OkLnc_0002517	1.44	1.14
LOC109870807_guanylate-binding protein 1-like isoform X1	0.02	7.15		OkLnc_0002517	1.44	1.14
LOC109872986_equilibrative nucleoside transporter 2-like	1.51	2.42		OkLnc_0039615	6.09	4.53
LOC109873212_protein FAM161A-like isoform X1	-23.57	-27.27		OkLnc_0039615	6.09	4.53
LOC109873219_protein FAM57B-like	2.26	-14.21		OkLnc_0020003	1.42	-1.23
LOC109874752_mucin-5B-like	1.00	-1.22		OkLnc_0256620	-25.64	-1.54
LOC109874752_mucin-5B-like	0.35	-1.22		OkLnc_0256620	-25.64	-1.54
LOC109875169_tetraspanin-3-like	-2.18	1.56		OkLnc_0108036	-1.45	2.55
LOC109875178_histone H1-like	2.26	3.54		OkLnc_0108036	-1.45	2.55
LOC109888425_ferritin, heavy subunit	0.21	1.57		OkLnc_0040566	1.29	1.56
LOC109888768_cystatin-F-like	0.18	3.08		OkLnc_0000917	1.30	6.49
LOC109889785_protein FAM45A-like	0.20	1.37		OkLnc_0000802	1.89	5.52
LOC109890947_uncharacterized protein	3.17	2.33		OkLnc_0057755	1.34	-1.23
LOC109890948_CXXC-type zinc finger protein 1-like isoform X2	0.09	-2.10		OkLnc_0057755	1.34	-1.23
LOC109891086_Krueppel-like factor 15	1.84	1.17		OkLnc_0185332	1.43	-1.01
LOC109891951_serine/threonine-protein kinase N1-like	1.33	1.58		OkLnc_0007274	1.89	2.55
LOC109892204_troponin T, slow skeletal muscle-like	-21.59	-16.80		OkLnc_0041243	-11.18	-12.66
LOC109892867_roundabout homolog 1-like	1.59	1.17		OkLnc_0248324	1.29	1.56
LOC109894726_complement component C1q receptor-like	0.78	1.56		OkLnc_0064106	-1.06	1.17
LOC109895228_mucin-5AC-like	0.12	-1.68		OkLnc_0114260	2.69	-1.13
LOC109895229_mucin-5AC-like	0.28	-1.43		OkLnc_0114260	2.69	-1.13
LOC109895656_proliferation-associated protein 2G4-like isoform X1	-2.51	-6.56		OkLnc_0040986	4.08	3.17
LOC109897271_uncharacterized protein	1.64	0.00		OkLnc_0252403	-1.71	-1.43
LOC109898318_collagen alpha-1(I) chain-like	1.12	-1.46		OkLnc_0080590	-1.21	-1.60
LOC109900334_uncharacterized protein	0.00	6.25		OkLnc_0014512	5.02	1.08
LOC109900663_collagen alpha-1(X) chain-like	1.63	-4.99		OkLnc_0273949	1.91	-4.48
LOC109900664_5'-nucleotidase domain-containing protein 1-like	-3.58	-5.96		OkLnc_0273949	1.91	-4.48
LOC109900795_apolipoprotein Eb-like	0.31	0.00		OkLnc_0053749	-1.54	-1.21
LOC109901049_SPARC-like protein 1	0.27	-1.59		OkLnc_0014512	5.02	1.08
LOC109901894_poliovirus receptor-like isoform X1	1.16	2.04		OkLnc_0212235	5.49	1.56
LOC109902563_zinc finger protein 692-like isoform X2	0.80	5.01		OkLnc_0026025	-2.77	-1.23
LOC109903380_collagen alpha-2(I) chain isoform X1	1.03	0.00		OkLnc_0222212	-1.14	-1.47
LOC109903644_trichohyalin-like	7.01	2.67		OkLnc_0018293	-11.18	-12.66
LOC109904463_trichohyalin-like	3.63	1.33		OkLnc_0018293	-11.18	-12.66
LOC109905667_LIM domain kinase 1-like	0.18	1.14		OkLnc_0087162	2.49	1.07
LOC109905827_mucin-5AC-like	1.21	0.00		OkLnc_0024402	-21.34	1.59
LOC109905828_oligodendrocyte-myelin glycoprotein-like	-80.07	1.28		OkLnc_0024402	-21.34	1.59
LOC109905834_serine/threonine-protein kinase NLK-like	-2.66	0.00		OkLnc_0044905	1.46	-1.11
LOC109906723_apolipoprotein C-I-like	0.00	0.00		OkLnc_0053749	-1.54	-1.21
LOC109906820_leukotriene B4 receptor 1-like	0.01	1.17		OkLnc_0020888	-1.21	2.13
LOC109907025_N-fatty-acyl-amino acid synthase/hydrolase	0.98	-1.61		OkLnc_0211017	-1.02	-1.42
LOC109908135_lamina-associated polypeptide 2, isoforms beta/gamma-like	13.65	0.00		OkLnc_0211017	-1.02	-1.42
LOC109908354_intermediate filament protein ON3-like isoform X2	1.00	0.00		OkLnc_0253082	-2.37	-1.30
st8sia4_CMP-N-acetylneuraminate-poly-alpha-2,8-sialyltransferase isoform X2	1.59	0.00		OkLnc_0005343	3.41	3.71
tmsb4x_thymosin beta-4 isoform X2	-23.57	-3.81		OkLnc_0082387	-1.18	1.25

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Table 2 (*continued*)

Coho salmon skin						
Gene ID	**T0/T7**	**T0/T14**		**Lnc**	**T0/T7**	**T0/T14**
apmap_adipocyte plasma membrane-associated protein isoform X1	2.01	1.36		OkLnc_0000917	1.30	6.49
cds43_	0.18	1.02		OkLnc_0029693	2.49	2.06
crbn_protein cereblon	1.78	2.57		OkLnc_0040986	4.08	3.17
LOC109864961_O-acetyl-ADP-ribose deacetylase MACROD1-like	1.73	2.57		OkLnc_0017161	-11.18	1.56
LOC109865057_hepatocyte cell adhesion molecule-like isoform X2	1.07	-3.81		OkLnc_0025493	-21.34	-1.23
LOC109865749_ankyrin repeat domain-containing protein 1-like isoform X1	0.16	1.06		OkLnc_0236473	1.16	1.35
LOC109865796_keratin, type I cytoskeletal 13	1.78	3.45		OkLnc_0148374	1.40	1.02
LOC109865796_CD276 antigen homolog isoform X2	1.39	3.45		OkLnc_0148374	1.40	1.02
LOC109866401_uncharacterized protein LOC109866401 isoform X1	-2.50	9.36		OkLnc_0129319	1.29	14.41
LOC109867438_CD209 antigen-like protein C isoform X2	2.06	10.49		OkLnc_0022997	2.87	-1.53
LOC109869059_prostaglandin E synthase 3-like	1.23	1.38		OkLnc_0077804	6.09	5.02
LOC109870807_guanylate-binding protein 1-like isoform X1	1.96	7.15		OkLnc_0002517	1.44	1.14
LOC109870807_guanylate-binding protein 1-like isoform X1	0.02	7.15		OkLnc_0002517	1.44	1.14
LOC109872986_equilibrative nucleoside transporter 2-like	1.51	2.42		OkLnc_0039615	6.09	4.53
LOC109873212_protein FAM161A-like isoform X1	-23.57	-27.27		OkLnc_0039615	6.09	4.53
LOC109873219_protein FAM57B-like	2.26	-14.21		OkLnc_0020003	1.42	-1.23
LOC109874752_mucin-5B-like	1.00	-1.22		OkLnc_0256620	-25.64	-1.54
LOC109874752_mucin-5B-like	0.35	-1.22		OkLnc_0256620	-25.64	-1.54
LOC109875169_tetraspanin-3-like	-2.18	1.56		OkLnc_0108036	-1.45	2.55
LOC109875178_histone H1-like	2.26	3.54		OkLnc_0108036	-1.45	2.55
LOC109888425_ferritin, heavy subunit	0.21	1.57		OkLnc_0040566	1.29	1.56
LOC109888768_cystatin-F-like	0.18	3.08		OkLnc_0000917	1.30	6.49
LOC109889785_protein FAM45A-like	0.20	1.37		OkLnc_0000802	1.89	5.52
LOC109890947_uncharacterized protein	3.17	2.33		OkLnc_0057755	1.34	-1.23
LOC109890948_CXXC-type zinc finger protein 1-like isoform X2	0.09	-2.10		OkLnc_0057755	1.34	-1.23
LOC109891086_Krueppel-like factor 15	1.84	1.17		OkLnc_0185332	1.43	-1.01
LOC109891951_serine/threonine-protein kinase N1-like	1.33	1.58		OkLnc_0007274	1.89	2.55
LOC109892204_troponin T, slow skeletal muscle-like	-21.59	-16.80		OkLnc_0041243	-11.18	-12.66
LOC109892867_roundabout homolog 1-like	1.59	1.17		OkLnc_0248324	1.29	1.56
LOC109894726_complement component C1q receptor-like	0.78	1.56		OkLnc_0064106	-1.06	1.17
LOC109895228_mucin-5AC-like	0.12	-1.68		OkLnc_0114260	2.69	-1.13
LOC109895229_mucin-5AC-like	0.28	-1.43		OkLnc_0114260	2.69	-1.13
LOC109895656_proliferation-associated protein 2G4-like isoform X1	-2.51	-6.56		OkLnc_0040986	4.08	3.17
LOC109897271_uncharacterized protein	1.64	0.00		OkLnc_0252403	-1.71	-1.43
LOC109898318_collagen alpha-1(I) chain-like	1.12	-1.46		OkLnc_0080590	-1.21	-1.60
LOC109900334_uncharacterized protein	0.00	6.25		OkLnc_0014512	5.02	1.08
LOC109900663_collagen alpha-1(X) chain-like	1.63	-4.99		OkLnc_0273949	1.91	-4.48
LOC109900664_5'-nucleotidase domain-containing protein 1-like	-3.58	-5.96		OkLnc_0273949	1.91	-4.48
LOC109900795_apolipoprotein Eb-like	0.31	0.00		OkLnc_0053749	-1.54	-1.21
LOC109901049_SPARC-like protein 1	0.27	-1.59		OkLnc_0014512	5.02	1.08
LOC109901894_poliovirus receptor-like isoform X1	1.16	2.04		OkLnc_0212235	5.49	1.56
LOC109902563_zinc finger protein 692-like isoform X2	0.80	5.01		OkLnc_0026025	-2.77	-1.23
LOC109903380_collagen alpha-2(I) chain isoform X1	1.03	0.00		OkLnc_0222212	-1.14	-1.47
LOC109903644_trichohyalin-like	7.01	2.67		OkLnc_0018293	-11.18	-12.66
LOC109904463_trichohyalin-like	3.63	1.33		OkLnc_0018293	-11.18	-12.66
LOC109905667_LIM domain kinase 1-like	0.18	1.14		OkLnc_0087162	2.49	1.07
LOC109905827_mucin-5AC-like	1.21	0.00		OkLnc_0024402	-21.34	1.59
LOC109905828_oligodendrocyte-myelin glycoprotein-like	-80.07	1.28		OkLnc_0024402	-21.34	1.59
LOC109905834_serine/threonine-protein kinase NLK-like	-2.66	0.00		OkLnc_0044905	1.46	-1.11
LOC109906723_apolipoprotein C-I-like	0.00	0.00		OkLnc_0053749	-1.54	-1.21
LOC109906820_leukotriene B4 receptor 1-like	0.01	1.17		OkLnc_0020888	-1.21	2.13
LOC109907025_N-fatty-acyl-amino acid synthase/hydrolase	0.98	-1.61		OkLnc_0211017	-1.02	-1.42
LOC109908135_lamina-associated polypeptide 2, isoforms beta/gamma-like	13.65	0.00		OkLnc_0211017	-1.02	-1.42
LOC109908354_intermediate filament protein ON3-like isoform X2	1.00	0.00		OkLnc_0253082	-2.37	-1.30
st8sia4_CMP-N-acetylneuraminate-poly-alpha-2,8-sialyltransferase isoform X2	1.59	0.00		OkLnc_0005343	3.41	3.71
tmsb4x_thymosin beta-4 isoform X2	-23.57	-3.81		OkLnc_0082387	-1.18	1.25

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Table 2 (*continued*)

Coho salmon head kidney						
Gene ID	**T0/T7**	**T0/T14**	**Lnc**	**T0/T7**	**T0/T14**
kpna4_twist-related protein 2-like	↑ 0.01	↑ 2.06	OkLnc_0114250	↓ -1.26	↓ -1.05
LOC109865796_histone deacetylase 7-like	↑ 0.08	↑ 2.64	OkLnc_0148374	↑ 1.09	↑ 1.19
LOC109866401_sodium- and chloride-dependent GABA transporter 2-like	↓ -4.15	↑ 4.04	OkLnc_0129319	↑ 8.69	↑ 24.94
LOC109867975_glyceraldehyde-3-phosphate dehydrogenase	↑ 1.45	↑ 3.60	OkLnc_0108346	↑ 1.54	↑ 8.59
LOC109868179_transmembrane protein 147-like isoform X1	↑ 0.73	↑ 2.83	OkLnc_0108346	↑ 1.54	↑ 8.59
LOC109868267_transmembrane protein 147-like isoform X1	↑ 0.58	↓ -1.26	OkLnc_0120631	↑ 2.25	↓ -2.39
LOC109868899_myosin light chain 1, skeletal muscle isoform-like	↑ 0.24	↑ 1.28	OkLnc_0001904	↑ 1.09	↓ -1.62
LOC109868900_microtubule-associated protein 2-like	↑ 0.61	↑ 2.28	OkLnc_0001904	↑ 1.09	↓ -1.62
LOC109869233_E3 ubiquitin-protein ligase Midline-1-like	↑ 0.04	↑ 1.83	OkLnc_0162612	↑ 5.83	↑ 2.55
LOC109870178_tetraspanin-3-like	↓ -7.34	↑ 0.00	OkLnc_0069981	↓ -9.09	↓ -15.90
LOC109870179_histone H1-like	↑ 22.89	↑ 44.54	OkLnc_0069981	↓ -9.09	↓ -15.90
LOC109870807_coactosin-like protein	↓ -3.18	↑ 3.73	OkLnc_0002517	↑ 1.13	↑ 1.12
LOC109870914_stromal cell-derived factor 1-like	↓ -7.34	↑ 0.00	OkLnc_0074106	↓ -1.24	↓ -1.30
LOC109871491_uncharacterized protein LOC109890947 isoform X4	↑ 11.29	↓ -1.22	OkLnc_0086486	↓ -1.21	↑ 1.84
LOC109871551_Krueppel-like factor 15	↑ 0.00	↑ 5.19	OkLnc_0108717	↑ 1.55	↑ 2.98
LOC109871636_TRAF3-interacting JNK-activating modulator-like isoform X1	↓ -2.12	↓ -1.87	OkLnc_0001564	↑ 1.02	↓ -1.21
LOC109871769_interferon regulatory factor 6-like isoform X1	↓ -2.02	↑ 8.90	OkLnc_0108717	↑ 1.55	↑ 2.98
LOC109871786_guanylyl cyclase-activating protein 1-like	↑ 0.02	↑ 3.15	OkLnc_0086486	↓ -1.21	↑ 1.84
LOC109872825_unconventional myosin-Vb-like	↑ 0.00	↓ -1.81	OkLnc_0149077	↓ -1.21	↑ 1.13
LOC109872826_roundabout homolog 1-like	↑ 0.43	↑ 0.00	OkLnc_0149077	↓ -1.21	↑ 1.13
LOC109874600_sodium- and chloride-dependent GABA transporter 2-like isoform X2	↓ -7.34	↑ 5.19	OkLnc_0069534	↓ -1.26	↓ -1.05
LOC109875169_heat shock cognate 70 kDa protein	↑ 1.58	↑ 1.53	OkLnc_0108036	↓ -2.18	↑ 1.22
LOC109875178_cytohesin-3-like	↑ 2.66	↓ -1.75	OkLnc_0108036	↓ -2.18	↑ 1.22
LOC109889293_peroxidasin homolog	↑ 0.22	↓ -1.01	OkLnc_0002739	↑ 1.30	↑ 1.16
LOC109890947_titin-like	↓ -2.44	↑ 7.63	OkLnc_0057755	↑ 1.04	↓ -1.02
LOC109891086_coatomer subunit zeta-1-like isoform X5	↑ 0.32	↑ 2.89	OkLnc_0185332	↑ 1.27	↑ 1.75
LOC109891119_nuclear factor erythroid 2-related factor 1-like	↑ 0.47	↑ 0.00	OkLnc_0105958	↑ 4.40	↑ 6.81
LOC109891120_aspartyl/asparaginyl beta-hydroxylase-like isoform X4	↑ 2.18	↑ 3.41	OkLnc_0105958	↑ 4.40	↑ 6.81
LOC109891563_integrin beta-1-like isoform X1	↑ 1.92	↓ -1.22	OkLnc_0105958	↑ 4.40	↑ 6.81
LOC109891737_ras-related protein Rab-18	↓ -7.34	↑ 0.00	OkLnc_0120642	↓ -9.09	↓ -2.39
LOC109892484_focal adhesion kinase 1 isoform X11	↑ 3.76	↑ 13.93	OkLnc_0067587	↑ 1.18	↓ -1.71
LOC109892867_60S acidic ribosomal protein P1-like	↑ 21.49	↑ 1.09	OkLnc_0248324	↓ -1.03	↓ -73.18
LOC109892873_ly6/PLAUR domain-containing protein 2-like	↑ 1.14	↑ 1.40	OkLnc_0001462	↓ -1.13	↓ -1.48
LOC109892874_cleft lip and palate transmembrane protein 1-like protein	↑ 21.49	↓ -40.15	OkLnc_0001462	↓ -1.13	↓ -1.48
LOC109893568_tail-anchored protein insertion receptor WRB-like	↑ 0.12	↑ 1.91	OkLnc_0079683	↑ 1.54	↑ 4.68
LOC109893786_non-histone chromosomal protein HMG-14B-like	↑ 0.00	↑ 5.19	OkLnc_0101406	↓ -2.32	↑ 1.32
LOC109894964_bromodomain and WD repeat-containing protein 3-like isoform X1	↑ 0.02	↑ 1.47	OkLnc_0140021	↑ 1.93	↑ 1.32
LOC109898324_serine protease HTRA3-like isoform X2	↑ 2.13	↓ -3.50	OkLnc_0037153	↓ -1.24	↑ 1.87
LOC109899309_proteasome subunit beta type-7-like	↓ -7.34	↑ 0.00	OkLnc_0080810	↓ -1.24	↑ 1.69
LOC109899550_proteasome subunit beta type-8-like	↓ -2.01	↓ -1.61	OkLnc_0069754	↑ 1.54	↑ 1.84
LOC109903424_class I histocompatibility antigen, F10 alpha chain-like	↑ 1.24	↓ -2.65	OkLnc_0004385	↓ -1.01	↓ -2.72
LOC109903425_nascent polypeptide-associated complex subunit alpha ,muscle-specific forr	↑ 0.74	↑ 1.45	OkLnc_0004385	↓ -1.01	↓ -2.72
LOC109905777_N-fatty-acyl-amino acid synthase/hydrolase	↑ 1.02	↑ 1.10	OkLnc_0005529	↓ -1.09	↓ -1.38
LOC109905779_lamina-associated polypeptide 2, isoforms beta/gamma-like	↑ 1.25	↓ -1.22	OkLnc_0005529	↓ -1.09	↓ -1.38
LOC109905780_importin subunit alpha-3	↑ 0.62	↑ 0.00	OkLnc_0005529	↓ -1.09	↓ -1.38
LOC109906323_protein phosphatase 1L-like	↑ 2.70	↓ -3.44	OkLnc_0088005	↓ -9.09	↓ -15.90
LOC109907025_CD276 antigen homolog isoform X2	↑ 1.10	↑ 1.19	OkLnc_0211017	↓ -1.01	↓ -1.21
LOC109907308_uncharacterized protein LOC109866401 isoform X1	↑ 0.01	↓ -1.16	OkLnc_0010190	↑ 1.01	↓ -1.78
LOC109907317_PDZ and LIM domain protein 5-like	↑ 1.19	↑ 1.07	OkLnc_0010190	↑ 1.01	↓ -1.78
LOC109907335_PDZ and LIM domain protein 5-like	↑ 5.27	↓ -79.07	OkLnc_0010190	↑ 1.01	↓ -1.78
LOC109907525_chromobox protein homolog 5-like	↑ 0.51	↑ 1.72	OkLnc_0001049	↑ 1.21	↓ -1.23
LOC109907528_heterogeneous nuclear ribonucleoprotein A1-like isoform X4	↑ 0.70	↑ 1.53	OkLnc_0001049	↑ 1.21	↓ -1.23
LOC109907529_transcription factor NF-E2 45 kDa subunit	↑ 0.24	↑ 1.71	OkLnc_0001049	↑ 1.21	↓ -1.23
LOC109907789_adenosine deaminase-like	↑ 3.76	↑ 44.54	OkLnc_0086973	↓ -3.43	↑ 1.64
LOC109908135_parvalbumin, thymic-like	↑ 21.49	↑ 0.00	OkLnc_0211017	↓ -1.01	↓ -1.21
LOC109909072_parvalbumin-2-like	↓ -26.60	↑ 31.42	OkLnc_0114250	↓ -1.26	↓ -1.05
nfe2_parvalbumin beta 2	↑ 1.72	↑ 0.00	OkLnc_0001904	↑ 1.09	↓ -1.62
ptk2_integrin alpha-6-like isoform X1	↓ -7.34	↑ 0.00	OkLnc_0004385	↓ -1.01	↓ -2.72
tmsb4x_glypican-5-like	↑ 21.49	↓ -2.36	OkLnc_0082387	↓ -1.06	↑ 1.02

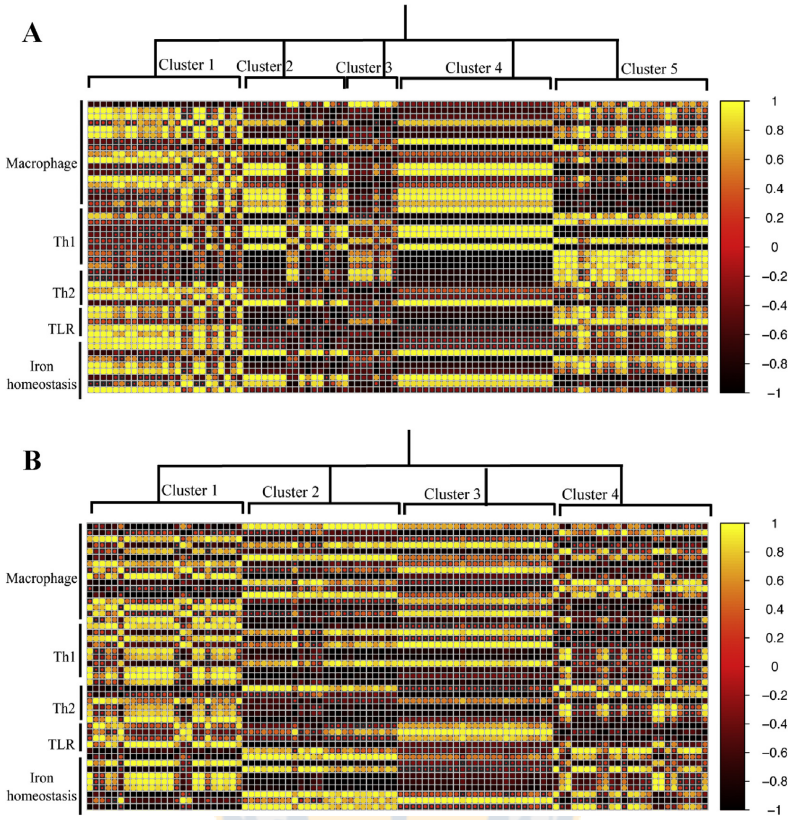

Fig. 8. Correlation matrix between highly regulated exclusive-lncRNA and immune response/iron homeostasis genes in skin tissue of Atlantic salmon (A) and coho salmon (B). Color scale represents the correlation values; the values in the scale indicate the Person correlation values. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

lncRNAs and the panel selected genes, compared with Atlantic salmon skin. Moreover, in skin tissue it was not possible to link lncRNA clusters with a particular gene group (Fig. 8B). From Atlantic salmon head kidney correlation analysis, four clusters were identified (Fig. 9A). Cluster 1, showed positive correlations between highly regulated lncRNAs with macrophages, Th1, TLR and iron homeostasis genes (Fig. 9A, Table S5). For cluster 3, a positive correlation expression was observed between lncRNAs highly regulated in head kidney of Atlantic salmon and genes associated with iron homeostasis in contrast with lncRNA from cluster 4 which presented negative correlation with the same gene group (Fig. 9A, Table S5). On the other hand, cluster 4 showed high correlations between lncRNAs and genes associated with the immune response (Fig. 9A). In Coho salmon head kidney, four clusters of lncRNAs were identified (Fig. 9B). Cluster 1 and 4 showed positive correlations between highly regulated lncRNAs and genes associated with macrophage response and iron homeostasis; further, negative correlation between Th1, Th2 and TLR genes and lncRNA from cluster 1 and 4 were observed (Fig. 9B). Moreover, positive correlations were evidenced at cluster 2 and 3 (Fig. 9B, Table S5).

4. Discussion

While the capacity of high-throughput sequencing platforms has continuously increased in parallel with data quality, transcriptome studies have revealed that the major proportion of sequences yielded are transcripts that lack coding potential or do not evidence similarity with any known protein. Here, the big challenge is how to attribute functional roles to transcripts that by nature are not canonically functional. Novel molecular tools and bioinformatic analyses in functional genomics have supported the putative role of non-coding RNAs as key regulators of several biological processes. However, the bottleneck to conduct comparative studies among species is the low conservation level of lncRNAs through the different taxa. This point, mostly in non-model organisms, makes the functional annotation of lncRNAs and their experimental validation difficult. In fish, lncRNAs have been poorly studied, even more so there conservation degree. In a study performed in 17 species, including four fish species, the number of common lncRNAs between humans and their last ancestor was higher than the number of lncRNAs shared between the bony fish species analyzed (Hezroni et al., 2015). From the sequence homology analysis performed in this study between Atlantic salmon, Coho salmon, rainbow trout and zebrafish, the number of similar lncRNA sequences was less than 2.1%.

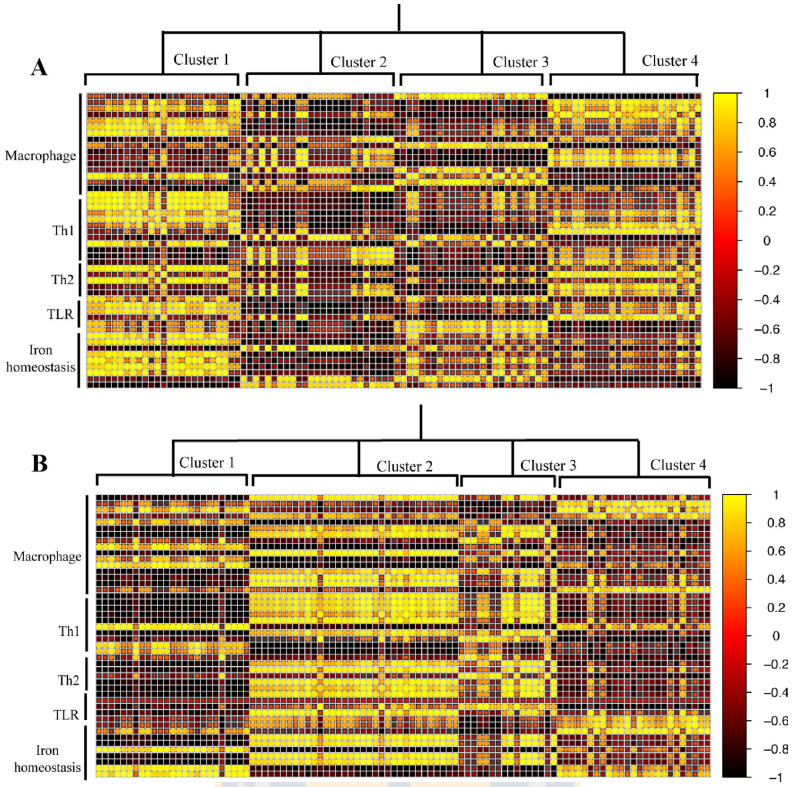

Fig. 9. Correlation matrix between highly regulated exclusive-lncRNA and immune response/iron homeostasis genes in head kidney tissue of Atlantic salmon (A) and coho salmon (B). Color scale represents the correlation values; the values in the scale indicate the Person correlation values. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Interestingly, coho salmon and rainbow trout shared higher numbers of lncRNAs, which could be explained by the phylogenetic closeness of these two fish species (Stearley and Smith, 1993).

Due to the role of lncRNAs as modulators of different biological processes, they are transcribed in a tissue-specific manner, similarly to coding genes (Aune and Spurlock, 2016; Ponting et al., 2009). In fish as well as in mammals, differences in expression levels of annotated lncRNAs have been observed between tissues, making it possible to identify exclusive lncRNAs (Al-Tobasei et al., 2016; Kaushik et al., 2013; Necsulea et al., 2014). For instance, in adult zebra fish 342 from 429 putative lncRNAs were transcribed in more than one assessed tissue (heart, liver, muscle, brain and blood) and just 77 lncRNAs showed tissue-specific transcriptional patterns (Kaushik et al., 2013). Interestingly, 54,503 putative lncRNAs have been reported in rainbow trout, where 5.4% (2935) are said to be tissue-specific and near 5.9% are expressed across all evaluated tissues (testis, brain, gill, head kidney, skin, spleen, muscle, among others) (Al-Tobasei et al., 2016). In our study, from the transcriptome sequencing of skin and head kidney from Atlantic and Coho salmon, exclusive lncRNAs were identified for each tissue. We observed more abundance of exclusive lncRNAs in head kidney from Atlantic and Coho salmon compared with skin tissue. Interestingly, in rainbow trout head kidney tissue has been reported as a tissue with more exclusive lncRNAs (Al-Tobasei et al., 2016).

LncRNAs play important roles in complex biological processes and participate in the modulation of several innate and adaptive immune pathways (Aune and Spurlock, 2016). For instance, in fish lncRNA modulation has been observed during pathogen infestation (Boltaña et al., 2016; Jiang et al., 2016; Tarifeño-Saldivia et al., 2017). For Atlantic salmon infected with the ISA virus, a panel of lncRNAs were reported to be upregulated in response to viral infection (Boltaña et al., 2016). The large yellow croaker — *Larimichtys crocea* — infected with *Vibrio anguillarum* evidenced upregulation of lncRNAs neighbored to immune-related coding genes (Jiang et al., 2016). Furthermore, pathogen-specific lncRNA regulation has been observed between Atlantic salmon infected with bacterial, viral and ectoparasite agents, suggesting pivotal roles for lncRNAs in salmon immunity (Tarifeño-Saldivia et al., 2017). Furthermore, Tarifeño-Saldivia et al. (2017), reported 438 lncRNAs differently transcribed in Atlantic salmon after 15 days of *C. rogercresseyi* infestation. In the current study, we observed 756 lncRNAs differently modulated in head kidney at 14 dpi. Homology analysis between these 438 and 756 lncRNAs, revealed that only 21 lncRNAs showed a sequence identity of 92.31–100% (Table S3). Furthermore, from Tarifeño-Saldivia et al. (2017), the lncRNAs regulated during the sea lice infestation showed to be closely located to genes related to transcriptional regulation and cell migration. On the other hand, the lncRNAs identified in this study during the *C. rogercresseyi* infestation are neighbor genes associated to stress response, regulation of the immune response, development and muscle cell proliferation. Besides the cumulative evidence regarding the

roles of lncRNAs in fish immunity, comparative studies at the transcriptional and sequence levels have not yet been conducted. The present study represents the first comparative analysis of salmon lncRNAs in response to sea lice infestation. Until now this response has been characterized through innate and adaptive immune signaling pathways (Braden et al., 2012, 2015; Valenzuela-Muñoz et al., 2016), iron homeostasis and nutritional immunity (Valenzuela-Muñoz and Gallardo-Escárate, 2017; Valenzuela-Muñoz et al., 2017). Moreover, transcriptional differences during sea lice infestation have been reported among salmon species (Valenzuela-Muñoz et al., 2016). For instance, Atlantic and Coho salmon infected with *C. rogercresseyi* show highly regulated coding genes at 7 dpi in skin tissue and 14 dpi in the case of head kidney tissue (Valenzuela-Muñoz et al., 2016, 2017). Similar modulations were observed in skin and head kidney lncRNAs identified in the present study. Furthermore, for Atlantic salmon infected with *C. rogercresseyi* the immune strategy was associated with TLR22 response (Valenzuela-Muñoz et al., 2016) and iron homeostasis mechanisms (Valenzuela-Muñoz et al., 2017). In the present study we observed high regulation of lncRNAs neighbored to coding genes associated with biological processes such as stress, the immune response, and the inflammatory response, among others. On other hand, lncRNAs highly modulated in Coho salmon apparently regulate the expression of coding genes associated with processes such as cell adhesion, response to external stimuli and the regulation of cell communication. Furthermore, highly regulated lncRNAs suggest a negative modulation of ferritin gene transcription, which was observed to be downregulated in Coho salmon during *C. rogercresseyi* infestation (Valenzuela-Muñoz et al., 2017).

5. Conclusion

This study is the first to identify lncRNA sequence conservation between Atlantic and coho salmon and suggests putative roles for lncRNAs in these fish during *C. rogercresseyi* infestation. Furthermore, transcription profiling of lncRNAs at 7 and 14 dpi with *C. rogercresseyi* was similar to expression patterns previously reported for coding genes in these salmon species against sea lice infestation. Neighbor gene analysis with respect to highly-regulated lncRNAs revealed a strong immune response in Atlantic salmon, while for Coho salmon the same analysis was associated with tissue reparation and cell adhesion, suggesting divergent functional roles for lncRNAs in response to sea lice infestation.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.dci.2018.05.016.

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CAPITULO 6

Modulation of Atlantic salmon miRNome response to sea louse infestation

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MicroRNAs are non-coding RNA that plays a crucial role in post-transcriptional regulation and immune system regulation. On other hand, sea lice are prevalent parasites that affect salmon farming, generating different degrees of immune suppression depending on the salmon and sea louse species. *Caligus rogercresseyi* for example, which affects the salmon industry in Chile, decreases Th1 response, macrophage activation, TLR-mediated response and iron regulation in infected fish. In this study, we explore Atlantic salmon miRNome during infestation by C. rogercresseyi. Using small RNA sequencing, we annotated 1718 miRNAs for skin and head kidney from infected Atlantic salmon. The most abundant families identified were mir-10, mir-21, mir-30, mir-181 and let7. Significant differences were found between tissue, with 1404 annotated miRNA in head kidney and 529 in skin. Differential analysis of transcript expression indicated that at an early stage of infestation miRNA expression was higher in head kidney than in skin tissue, revealing tissue-specific expression patterns. In parallel, miRNA target prediction using 3UTRs from highly regulated immune-related genes and iron metabolism showed that mir-140-4 and mir-181a- 2-5 modulate the expression of TLR22 and Aminolevulinic acid synthase, respectively. This study contributes knowledge about the immune response of Atlantic salmon during infestation with sea lice.

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Modulation of Atlantic salmon miRNome response to sea louse infestation

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ABSTRACT

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1. Introduction

Advances in recent years in sequencing technologies have enabled the identification of a large number of untranslated transcripts, termed non-coding RNAs. Although non-coding RNAs do not encode proteins, they do play an important role in key biological processes in vertebrate species (Bartel, 2009; Bushati and Cohen, 2007). Their significance is mainly evidenced in what are termed microRNAs (miRNAs), which are small highly conserved RNA sequences of 21–24 nucleotides (Bartel, 2004) that play a crucial role in post-transcriptional regulation, binding to target mRNAs in 3′UTR and repressing translation to proteins (Bartel, 2004, 2009). Some miRNAs display tissue-specific patterns, strongly regulating biological processes like development, growth, cell division, metabolism and apoptosis (Bi et al., 2009; Judice et al., 2016; Lindsay, 2008; Sonkoly et al., 2008).

With respect to immune response, miRNAs play critical roles in the adaptive and innate system, participating in hematopoiesis activation in response to pathogenic microorganisms (Davidson-Moncada et al., 2010; Lu and Liston, 2009; O'Connell et al., 2012). They have also been reported as key components in T cell differentiation, modulating the inflammatory response and activating toll-like receptor pathways in macrophages (Lu and Liston, 2009; O'Connell et al., 2012). For instance, miR-181 is preferentially expressed in B-lymphoid cells in mouse bone marrow, and its ectopic expression in hematopoietic stem/progenitor cells results in an increased fraction of B-lineage cells (Chen et al., 2004). Regarding innate response, the presence of lipopolysaccharide induces the expression of miR-155 and miR146, which in turn inhibits the TLR pathway to avoid excessive inflammatory response (Lu and Liston, 2009; Sonkoly et al., 2008; Taganov et al., 2006). Several studies have reported host modulation of miRNAs during infection by pathogens. For example, significant changes occur in the miRNA profiles of the crab *Eriocheir sinensis* during infection by the motile bacterium *Spiroplasma eriocheiris* (Ou et al., 2012). Differences have also been observed in miRNA expression levels of the flounder *Paralichthys olivaceus* during infection by the viral hemorrhagic septicemia virus (VHSV) (Najib et al., 2016). A high degree of miRNA regulation associated with innate immune response was observed in the Chinese tongue sole *Cynoglossus semilaevis* infected with the bacteria *Vibrio anguillarum* (Sha et al., 2014).

The Atlantic salmon is an important economic resource for aquaculture endangered by prevalent pathogens that have caused significant economic losses Norway, Chile and Canada. The role of miRNAs is critical to understanding transcriptome modulation in fish biology. The miRNA repertoire of Atlantic salmon has been reported (Andreassen et al., 2013; Barozai, 2012; Bekaert et al., 2013) and is mainly associated with toxicological stressors (Kure et al., 2013). The Chilean aquaculture industry has been severely affected by the sea louse *Caligus rogercresseyi*, which feeds on mucus and blood on the fish epidermis, causing tissue damage and immunosuppression and allowing infection by other prevalent pathogens (Fast, 2014; González and Carvajal, 2003). Recent studies have demonstrated that Atlantic salmon infested with *C. rogercresseyi* have specific immune responses involving TLR pathway signaling, Th1 response and nutritional immunity (Valenzuela-Muñoz et al., 2016). Our goal in this study was to explore the Atlantic salmon miRNome during infestation by *C. rogercresseyi* and identify putative target genes associated with differentially expressed miRNAs, which can represent valuable information for developing therapeutic strategies to control sea lice.

2. Materials and methods

2.1. Experimental design and sample collection

Atlantic salmon (*S. salar*, n = 60) weighing 250 ± 12 g were obtained from the Chaperano Hatchery (Multiexport Food SA) in Cochamo, Chile. All fish were reared in brackish water (15 ppm) until smolting, after which specimens were maintained under a 12:12 h light:dark cycle in single-pass flow-through tanks supplied with ultraviolet-treated salt water. The salmon were fed daily with a commercial diet (Micro 200, EWOS). After initial acclimatization for 15 days, individuals were randomly divided into three tanks with 20 fish per tank (500 L). The salmon were starved at least 24 h prior to any manipulation.

Adult female *C. rogercresseyi* were collected during Atlantic salmon harvesting at a commercial aquaculture farm in Puerto Montt, Chile. After collection, the lice were rinsed and transported in aerated, sterile seawater (8 °C) to the experimental laboratory of the Marine Biological Station, University of Concepción, Dichato, Chile. Their egg strings were then removed and placed in culture buckets supplied with a seawater flow at 12 °C and gentle aeration. The eggs were allowed to hatch and develop until the infectious copepodid stage, at which point they were used to infest fish. The culture was carried out according to Bravo (2010). For infestation, the experimental tanks were placed in darkness without water flow for 6 h and with a load of 35 copepodids per fish. During the infestation, fish were supplemented with oxygen. Samples were taken before infestation and at 7 and 14 days post-infestation (dpi). Three to four fish were taken from each tank (ten fish per sampling) and anaesthetized with benzocaine. Head kidney and skin from an infected area (area with sea lice attached) were dissected, fixed in RNA Later (Ambion) and stored at −80 °C.

2.2. Illumina sequencing of small RNAs

Head kidney and skin samples from ten adult fish infested with *C. rogercresseyi* were used for small RNA library synthesis. Total RNA was extracted from each individual using the Trizol Reagent (Ambion®) following the manufacturer's instructions. Quantity, purity, and quality of the isolated RNA were measured in the TapeStation 2200 (Agilent Technologies Inc., CA, USA) using RNA ScreenTape (Agilent Technologies Inc., CA, USA) according to the manufacturer's instructions. Samples (n = 5) with a RIN over 8.0 were pooled for each tissue and sampling point for library construction. Small RNA libraries were constructed using the TruSeq Small RNA Library Preparation Kit (Illumina®, CA, USA). Three biological replicates from each sample pool were sequenced on the MiSeq (Illumina®) platform using 41 cycles at the Laboratory of Biotechnology and Aquatic Genomics, Interdisciplinary Center for Aquaculture Research (INCAR), University of Concepción, Chile.

2.3. miRNAs annotation in Atlantic salmon

Low-quality reads from the Illumina sequencing data, reads with a quality score of less than 0.05 on the Phred scale, with a short length, or with three or more ambiguous nucleotides, were strictly removed using CLC Genomics Workbench Software (Version 10.01, CLC Bio, Denmark). Any cleaned sequences matching metazoan mRNA, rRNA, tRNA, snRNA, snoRNA, repeat sequences, or other ncRNAs deposited in the NCBI databases (http://www.ncbi.nlm.nih.gov/), RFam (http://rfam.janelia.org/), or Repbase (http://www.girinst.org/repbase/) were discarded. The remaining transcripts were then counted to generate a single small

RNA list. These sequences were aligned against pre-miRNA and mature miRNA (5′ and 3′) sequences listed for *Salmo salar, Danio rerio, Cyprinus carpio, Fugu rubripes* comprised in miRBase 21 (Kozomara and Griffiths-Jones, 2011, 2014). Putative miRNA sequences were checked for secondary fold-back structure predictions using CLC Genomics Workbench Software with default folding conditions.

2.4. RNA-seq analysis of Atlantic salmon during C. rogercresseyi infestation

The miRNAs identified were used as references for RNA-seq analyses through different sample points for each tissue. The RNA-seq settings were a minimum length fraction = 0.6 and a minimum similarity fraction (long reads) = 0.5. The expression values were set as transcripts per million (TPM), a modification of reads per kilobase of transcript per million mapped reads (i.e. RPKM), designed to be consistent across samples. More specifically, transcript per-million values were normalized by the total transcript count (instead of by the read count) and average read length. The normalizations allowed for assessing overregulated transcripts among different groups (Wagner et al., 2012). The distance metric was calculated with the Manhattan method, with the mean expression level subtracted in 5–6 rounds of *k*-mean clustering. Finally, a Kal's statistical analysis test was used to compare gene expression levels in terms of $\log_2$ fold-change ($P = 0.0005$; false discovery rate [FDR] corrected). In order to identify co-expression patterns among miRNAs and mRNAs differentially expressed in Atlantic salmon during *C. rogercresseyi* infestation, Pearson correlation coefficient were estimated for each tissue and plotted using the Corrplot library in R (https://cran.r-project.org/).

2.5. Prediction of genes targeted by miRNAs from C. rogercresseyi

Three computational target prediction algorithms were used to predict the genes targeted by miRNAs: PITA (Kertesz et al., 2007), miRanda (John et al., 2004), and STarMir (Rennie et al., 2014). The datasets used were the assembled immune-related and iron modulation gene sequences reported by Valenzuela-Muñoz et al. (2016) for Atlantic salmon. STarMir was used to search for miRNA seed matches (nucleotides 2–8 from the 5′ end of miRNA) in the 3′UTR sequences, and miRanda and PITA were used to match entire miRNA sequences. The STarMir parameters was set at free energy < −15 kcal/mol and a score >50. The results predicted by the two algorithms were combined, and the overlaps were calculated to identify putative target genes in Atlantic salmon. Finally, *in silico* transcription expression analysis was conducted using five immune-related genes (*TLR12, MMP13, COX2, IFNg, CCR3*), three iron transport genes (*haptoglobin, ferritin H, IRP2*), three heme biosynthesis-related genes (*ALAs, HO, BLVr*) and their putative miRNAs identified in the Atlantic salmon miRNome.

2.6. Real-time quantification of miRNAs and their putative target genes

Mir-21-2, mir-10b-4-3, let7C-1 were analyzed in combination with *CCR3*, *HO* and *Ferritin* genes to determine the transcription expression of miRNAs and their putative target genes. Briefly, total RNA (including miRNAs and mRNAs) from both tissue types and all sampling points was isolated using TRI Reagent® (InvitrogenTM, Carlsbad, CA, USA), according to the manufacturer's protocol. The purity was determined (A260/A280 ratio) with a Nanodrop ND1000 spectrophotometer (Thermo Fisher Scientific, Copenhagen, Denmark), and the integrity was determined by agarose gel under denaturant conditions. The cDNAs were synthesized for mRNA and miRNA expression analysis using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Glen Burnie, Maryland, USA) and the miScript II RT Kit (Qiagen Inc., USA) in a 20 μl reaction, respectively. An RT-minus negative control reaction with all the components for the RT reaction (except the Reverse Transcriptase enzyme) was carried out for each sample to control genomic DNA contamination. The RT-qPCR runs were performed in triplicates for each sample using the StepOnePlus™ (Applied Biosystems®, Life Technologies, USA). To evaluate the transcriptional level, comparative $2^{-\Delta\Delta Ct}$ method was applied according Livak and Schmittgen (2001) using Elongation factor alpha (Valenzuela-Muñoz and Gallardo-Escárate, 2017) and Ssa-mir-455–5p (Johansen and Andreassen, 2014) as reference transcripts for mRNAs and miRNAs, respectively. Reactions were conducted with a volume of 10 μL using the Maxima® SYBR Green/ROX qPCR Master Mix (Thermo Scientific, USA) and miScript SYBR Green PCR Kit (Qiagen, USA) for mRNA and miRNAs, respectively. The amplification cycle was as follows: 95 °C for 10 min, 40 cycles at 95 °C for 15s, and 60 °C for 1 min, followed by a disassociation curve under the same conditions. The efficiency of the primers was calculated and reported according MIQE guidelines (Bustin et al., 2009). Finally, all data was checked for normality using the Shapiro-Wilk test. Data not meeting this criteria were normalized through BoxCox transformation (Westfall and Henning, 2013). Statistical differences in expression data were evaluated using a one-way ANOVA (analysis of variance), followed by Tukey's multiple comparison tests using the JMP v9 software (SAS Institute Inc., USA). Statistically significant differences were accepted with a $p < 0.05$.

3. Results

3.1. Identification of miRNA in Atlantic salmon

After adapter trimming, 12,986,381 and 18,068,288 reads were obtained respectively from Atlantic salmon skin and head kidney. In total, 62,182 small RNA clean reads were identified from skin and head kidney (Table 1), which were annotated using sequences available in miRBase 21 (http://www.mirbase.org) for *Salmo salar, Danio rerio, Cyprinus carpio, Ictalurus punctatus* and *Fugu rubripes*, yielding a total of 1718 annotated miRNAs. The average length for all annotated miRNAs was 21–23 nucleotide (Fig. 1A). Several isoforms or isomiRs of mature 5and mature 3′ precursors were found, the most abundant variants being mapped to mature 5′ and mature 3' sequences (Fig. 1B). miRNAs were classified into several families, the most abundant in salmon infested with sea lice being mir-10, mir-181 and mir-21, mir-30 and let7 (Fig. 1C). The analysis of skin and head kidney tissue revealed significant differences between them in the number of annotated miRNAs, with a total of 529 and 1404 transcripts, respectively (Table 1). Moreover, comparative sequence analysis identified 486 miRNAs shared between the two types of tissue, while 43 and 918 were identified as only in skin and head kidney, respectively (Fig. 1D). Among the miRNAs only in skin tissue, the most abundant families were mir-203, mir-499, mir-148, while the most abundant in head kidney were mir-731, mir-202,

Table 1
Summary of small RNA sequencing in Atlantic salmon infected with *Caligus rogercresseyi.*

Tissue	Skin	Head kidney	Total
Number of reads	21,213,927	29,865,990	51,079,917
Average length (nt)	41	41	41
Number of reads after trimmed	12,986,381	18,068,288	31,054,669
Average length after trimming (nt)	21	21	21
Number of small RNA annotated	529	1404	1718

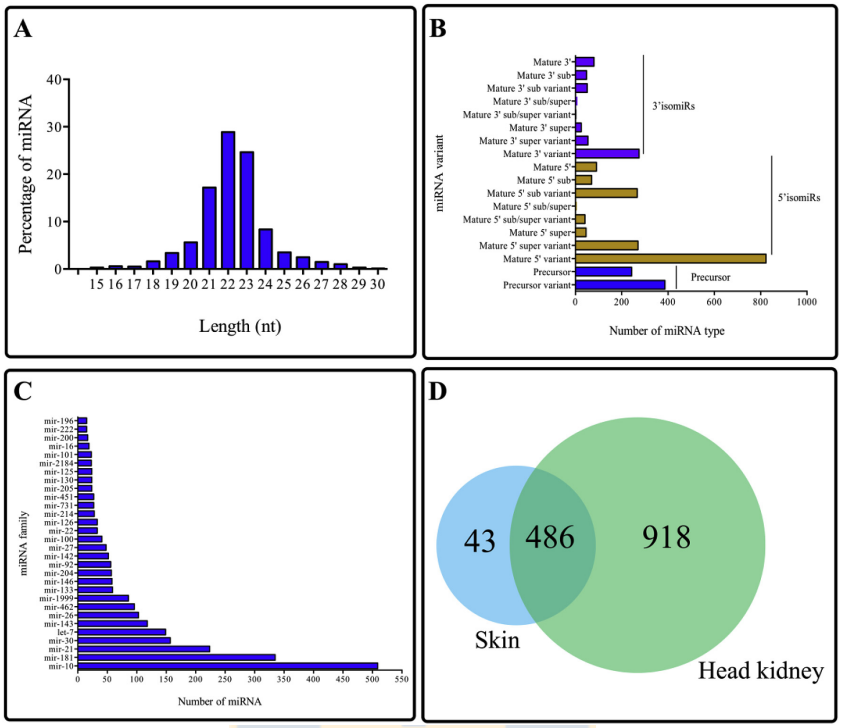

Fig. 1. Identification and annotation of Atlantic salmon miRNAs using sRNA-sequencing. A) Size distribution of Atlantic salmon miRNAs after trimming and mapping against miRBase 21, C) Number of isomiRs 5′- 3and precursor variants identified in the Atlantic salmon transcriptome, D) Venn diagram of the number of miRNA annotated for Atlantic salmon skin and head kidney.

and mir-144 (Table S1). Some miRNA families were like mir-196, mir-133, mir-99 and mir206 were related only to skin tissue, while mir-1388, mir-223a and mir-551 families were only associated with head kidney (Table S1).

3.2. Modulation of miRNA profiles in Atlantic salmon tissue during sea louse infestation

Using the 1718 miRNAs identified in Atlantic salmon, RNA-seq analysis compared changes in miRNA expression in skin and head kidney during the sea louse infestation. Heat-map representation showed different expression profiles of miRNAs over the course of infestation (Fig. 2). Clustering analysis using Euclidian distances showed families of miRNAs that were differentially expressed between tissues and among post-infection days (Table S2). Seven clusters were identified in skin tissue from the infected area (Fig. 2A). A large number of miRNAs of the mir-10, mir-21, mir-181 and mir-462 families were observed in these clusters (Fig. 2B). The expression patterns in the clusters differed during *C. rogercresseyi* infestation, for instance, clusters 1 and 4 were highly regulated before infestation and 14 dpi; cluster 2, 3 and 7 were highly regulated before infestation. Moreover, cluster 5 was upregulated at 14 dpi, showing an enrichment of mir-143, mir-199 and mir-2184 families (Fig. 2B). 2Nine clusters were detected in head kidney, two of them, cluster 1 and 8, with high expression values at 14 dpi, while cluster 3 and 5 were also upregulated at 14 dpi (Fig. 2C). Cluster 2–7 showed low expression at 7 dpi. Furthermore, the most enriched miRNA families were mir-26, mir-21, mir-181, mir-10 and mir-462 (Fig. 2C and D).

Statistical analyses were conducted with skin and head kidney of infected Atlantic salmon to detect differentially expressed miRNAs (Fig. 3). Thirteen miRNAs differently regulated at 14pdi in skin and seventy-one miRNA were differently regulated 7 dpi in head kidney (Fig. 3, Table S3). Notably, miRNome of Atlantic salmon skin 7 and 9 miRNAs exclusively expressed at 7 dpi and 14 dpi (Fig. 3A), while 69 and 3 miRNAs from head kidney tissue were highly regulated during the sea louse infestation. Two miRNAs were regulated at 7 and 14 dpi in head kidney (Fig. 3B). Analysis of the correlation expression of miRNAs revealed a high degree of correlation among specific miRNAs (Fig. 4). Pairwise comparison revealed a conspicuous clusters with correlation values > 0.8 (Table S4). Skin transcriptome showed low a number of significant correlations among the tested miRNAs, where the most abundant transcripts, mir-462, mir-21 and mir-181, had positive correlations (Fig. 4A). In contrast, a large number of miRNAs were significantly expressed in head kidney (Fig. 4B). For instance, mir-142 and mir-181 families showed positive correlations with let-7, mir-10a, mir-126a, mir-130a, mir-143, mir-30b and mir-30c families

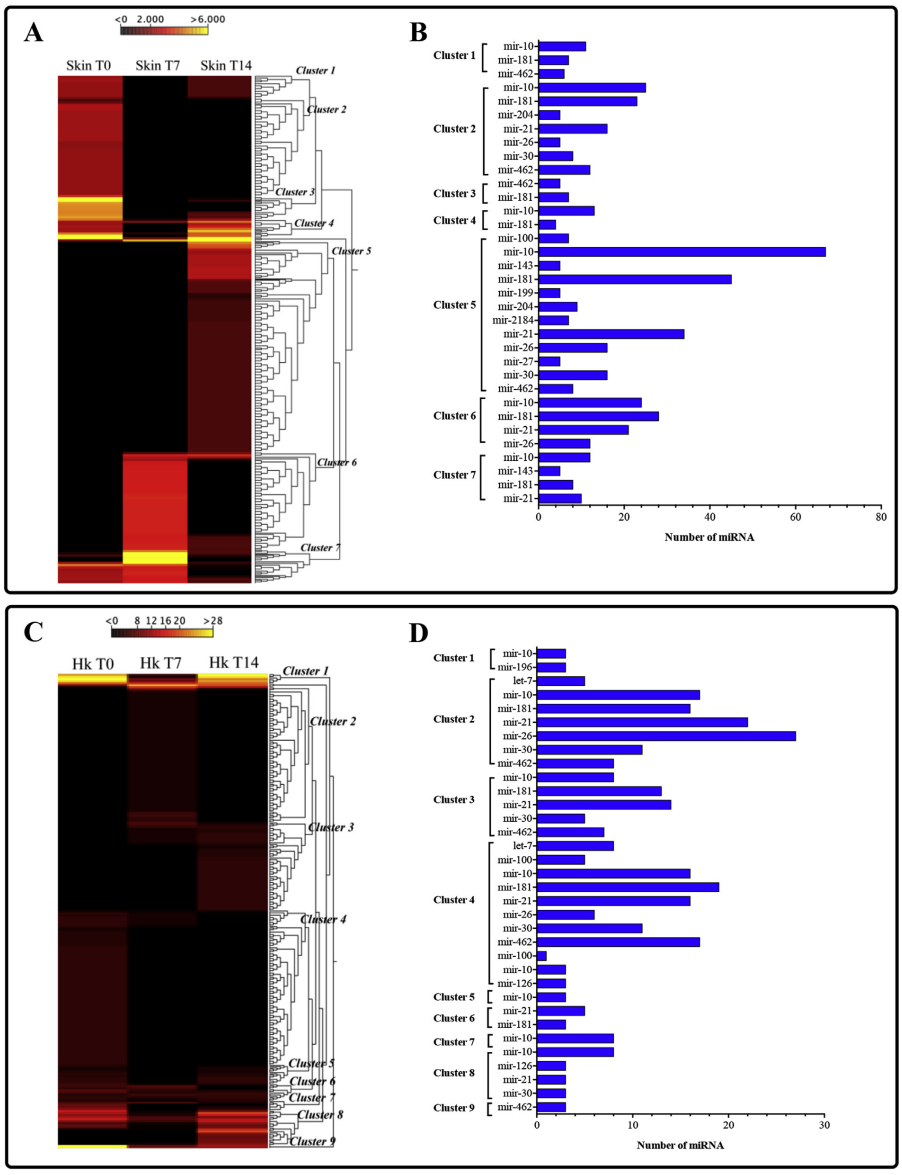

Fig. 2. Transcriptome profiles of miRNAs for skin and head kidney of Atlantic salmon infested with *C. rogercresseyi*. A: Clustering analysis for skin during sea lice infestation. B: Graphical representation for more abundant miRNA founding in skin by cluster. C: Clustering analysis for head kidney during sea lice infestation. D: Graphical representation for more abundant miRNA founding in head kidney by cluster.

(Fig. 4B). Correlation analysis was also conducted to evaluate co-expression patterns among miRNAs and mRNAs highly expressed during the *C. rogercresseyi* infection. Notably, genes related to iron regulation and immune response correlated positively with the miRNAs analyzed previously (Fig. 1S).

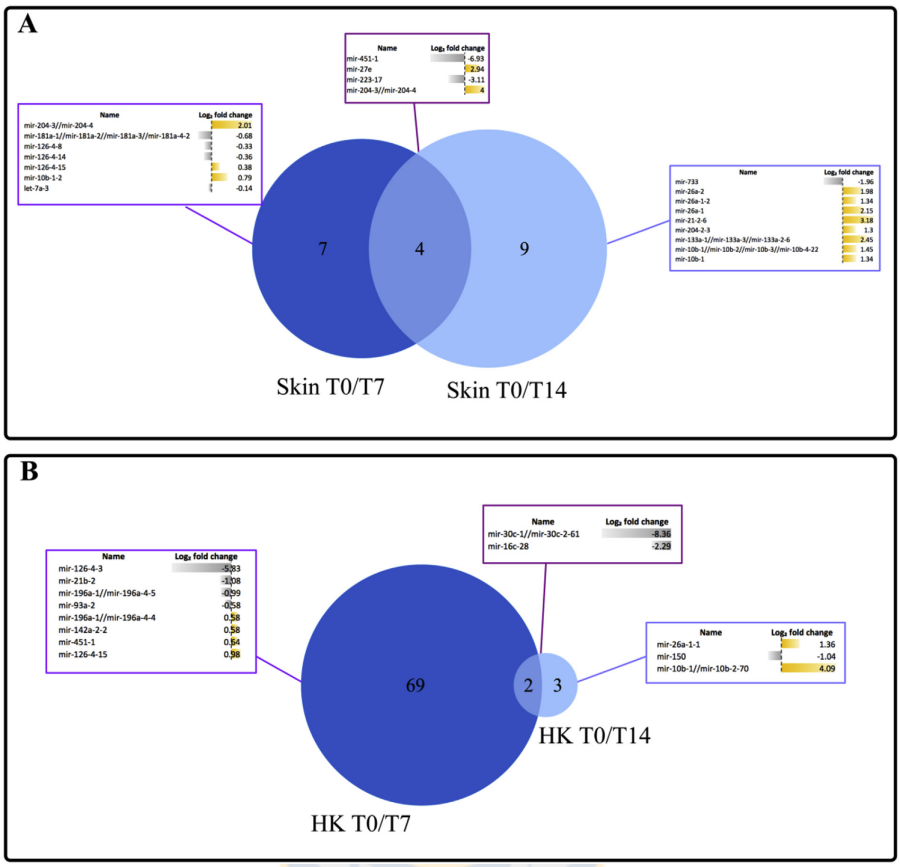

Fig. 3. Veen diagrams of differentially expressed miRNAs among infestation process in Atlantic salmon. A) Comparison in Atlantic salmon skin T0 vs. T7, and T0 vs. T14, respectively, and B) Comparison in Atlantic salmon head kidney T0 vs. T7, and T0 vs. T14. Each box shows the miRNAs exclusives and shared for each pairwise comparison. Red and green bars represent the fold-changes values, up and downregulated, respectively (p-value 0.05 and fold change |1|). * The eight top regulated miRNA for T0vs T7 in head kidney samples were include in the figure. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Atlantic salmon miRNome and immune response against sea lice

Five immune-related genes (*TLR12, MMP13, COX2, IFNg, CCR3*), three iron transport genes (*haptoglobin, ferritin H, IRP2*) and three heme biosynthesis-related genes (*ALAs, HO, BLVr*) were evaluated as putative target genes to determine the role of miRNAs in Atlantic salmon in response to *C. rogercresseyi* infestation. These genes have evidenced high modulation in Atlantic salmon in response to sea louse infestation. Using target gene prediction analyses to determine the binding sites, we found low levels of free energy in mir-140-4, mir-181a-5-2, mir-10b-19, mir-126-03, mir-21-2 and 3′UTRs of the immune genes *TLR12, MMP13, COX2, IFNg* and *CCR3*, respectively (Fig. 5A). RT-qPCR analysis was applied using the fold-change values of immune-related genes from skin and head kidney to evaluate the miRNA expression with their putative target genes. Overall, there is an inverse relationship between miRNA expression and target genes during sea louse infestation (Fig. 5C–D), suggesting putative modulation of these immune-related genes mediated through miRNAs. Furthermore, a key role of iron regulation has been reported in Atlantic salmon in response to sea louse infestation and particularly related to nutritional immunity (Valenzuela-Muñoz and Gallardo-Escárate, 2017). Low levels of duplex-free energy were estimated from bioinformatic target gene prediction for mir-1338, let-7c-1, mir-124a-1, mir-181a-2-5, mir-10b-4-3 and let7d-1 binding to *haptoglobin, ferritin H, IRP2, ALAs, HO* and *BLVr* genes (Fig. 6A). Expression analysis of these miRNAs and their putative iron regulation-related genes showed inverse transcription patterns in tissue infected with sea lice (Fig. 6C–D). However, the transcription analysis evidenced tissue-specific patterns in the miRNA/mRNA expression paterns, suggesting that the molecular mechanism of miRNAs is influenced at the same time by the transcriptional activity expressed during the sea lice infestation. Finally, RT-qPCR corroborated the *in silico* results of this study.

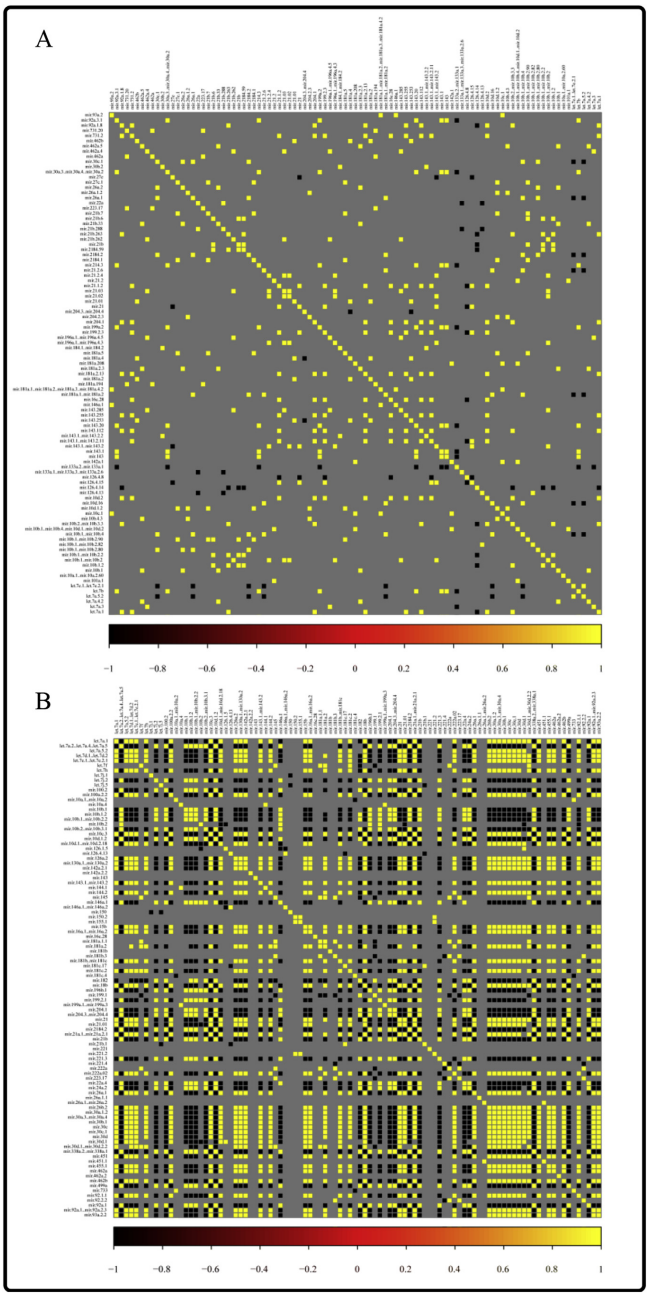

Fig. 4. Correlation plot matrix between miRNA differently expressed in skin (A) and head kidney (B) of Atlantic salmon infested with *C. rogercresseyi*. Color scale represents the correlation values. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

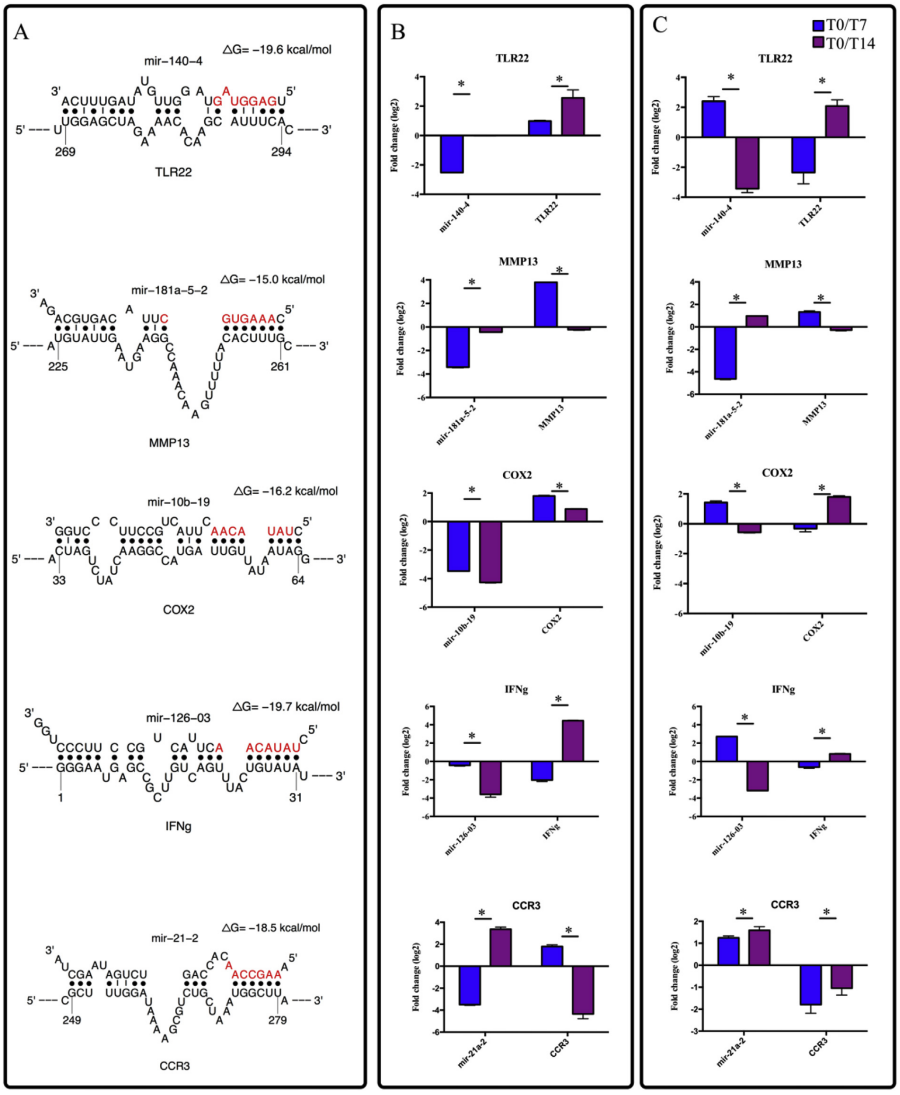

Fig. 5. miRNA target prediction for immune-related genes in Atlantic salmon infested with *C. rogercresseyi*. A) Schematic representation of immune-related mRNAs and predicted pairing of miRNAs on 3′ UTRs, and B) Atlantic salmon skin fold changes by *in silico* analysis for miRNA and fold change profiling by RT-qPCR of their putative predict target genes. C) Atlantic salmon head kidney fold changes by *in silico* analysis for miRNA and fold change profiling by RT-qPCR of their putative predict target genes. (* indicated significant differences between sample points $p < 0.05$).

No similarities were observed in expression patterns between the *in silico* and RT-qPCR analyses in skin tissue (Fig. 7). However, RT-qPCR analysis showed distinct expression patterns between miRNA and target genes in Atlantic salmon skin, as was expected (Fig. 7). A high level of correspondence was observed between TPM and the relative expression values of head kidney miRNA, and an opposite expression pattern was observed with its target gene (Fig. 7).

4. Discussion

The increasing use in recent years of new generation sequencing

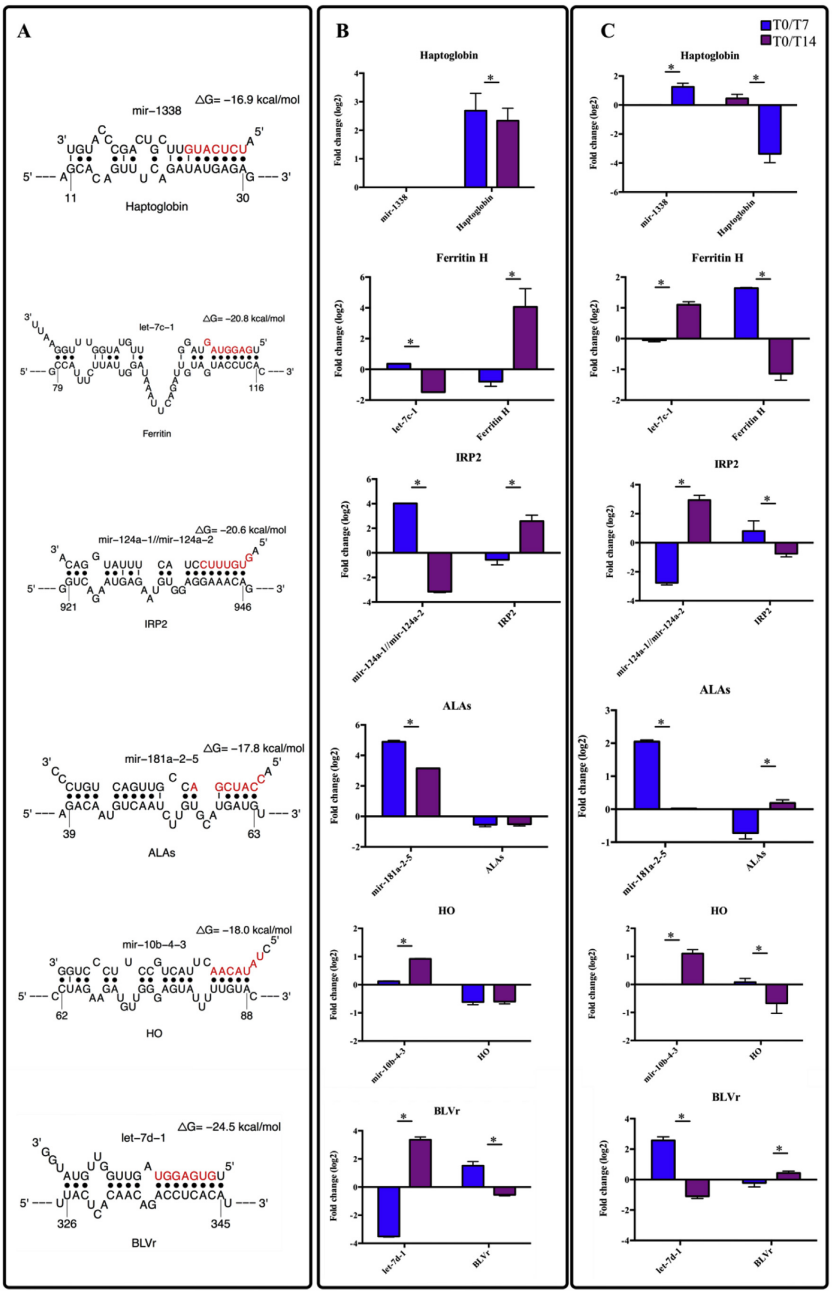

Fig. 6. miRNA target prediction for iron transport genes and heme biosynthesis/degradation pathways in Atlantic salmon infested with *C. rogercresseyi*. A) Schematic representation of iron modulation-related mRNAs of and predicted pairing of miRNAs on 3′ UTRs, B) In silico fold changes values of Atlantic salmon skin miRNA and fold change profiling by RT-qPCR of their putative predict target genes. C) In silico fold changes values of Atlantic salmon head kidney miRNA and fold change profiling by RT-qPCR of their putative predict target genes. (* indicated significant differences between sample points $p < 0.05$).

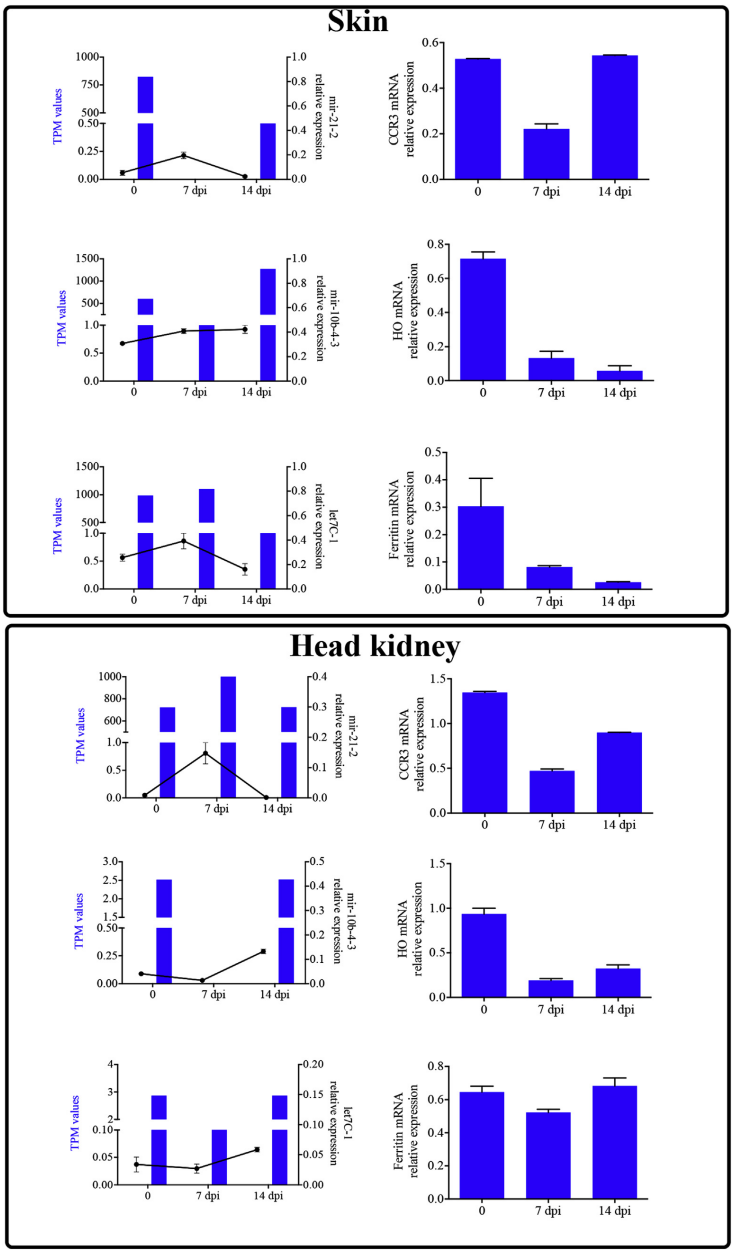

Fig. 7. RT-qPCR validation of miRNAs and putative target genes identified in Atlantic salmon transcriptome infected with *C. rogercresseyi*.

in animal biology has improved our understanding of the molecular mechanisms that modulate transcriptomes related to key biological processes. For instance, non-coding RNA and miRNAs have emerged as pivotal regulators of the immune response, playing important roles during host/pathogen interactions (Bi et al., 2009; Hussain and Asgari, 2014; Lindsay, 2008). To the best of our knowledge, this study is the first to apply small RNA sequencing to identify miRNA expression profiles in Atlantic salmon during sea louse infestation and to evaluate putative miRNA target genes related to the immune response against *C. rogercresseyi*. A total of 62,182 small RNAs were identified from small RNA libraries constructed from skin and head kidney tissue of infected fish. Here, using miRBase we annotated 1718 transcripts with a high percentage of small RNAs annotated to miRNAs previously reported for *Salmo salar*. As in similar studies of other fish species, the most abundant transcripts belonging to mir-181, mir-21 and let-7 families were identified (Bekaert et al., 2013; Farlora et al., 2015; Salem et al., 2010; Sha et al., 2014).

MiRNAs are highly conserved among vertebrates and invertebrates. They have the capacity to regulate more than one target gene, making it possible to modulate the expression of several genes in different tissues (Lagos-Quintana et al., 2002; Wang et al., 2016). For instance, comparison of expression levels in samples from different Atlantic salmon tissue evidenced tissue-specific expression differences in three conserved and one novel miRNA. Here, Ssa-miR 736 was only detected in heart tissue, while two other clustered miRNAs (ssa-miR 212 and132) seems to have higher expression levels in brain tissue. Differential expression analysis using DESeq suggests that Ssa-miR 8163 is enriched in liver tissue and putatively regulates the transferrin gene (Andreassen et al., 2013). In the present study, a higher number of miRNAs were annotated in head kidney than in skin tissue. Some miRNAs were only identified in tissue infected with sea lice. For example, mir-196, which has previously only been reported in the head kidney tissue of rainbow trout (Salem et al., 2010), was only expressed in our study in Atlantic salmon skin. We found miRNAs that are exclusive to head kidney, such as mir-1388 and mir-155. These transcripts have been described as critical in erythropoiesis in Antarctic fish, modulating genes involved in the TGF-b pathway (Xu et al., 2015). In turn, the expression of mir-1388 and mir-155 in Atlantic salmon head kidney indicate conserved functions related to blood cell lineages.

Changes in miRNA expression levels were detected in different tissue and at different sampling times during infestation with sea lice. For example, *Cynoglossus semilaevis* infected with *Vibrio anguillarum* showed variation in the expression levels of mir-142−5p, mir-223 and mir-181a in spleen, head kidney, intestine and liver tissues, suggesting that bacterial infestation modulate miRNA expression differently, depending on the target tissue of infection (Gong et al., 2015). Recent studies with tilapia have shown differences in expression profiles of miRNAs after 72 h of *Streptococcus agalactiae* infection, with downregulation of mir-214, mir-155 and mir-29 at early infection stages (Wang et al., 2016). Similarly, *Paralichthys olivaceus* infected with VHSV revealed higher expression levels of mir-146a and mir-155 at 48 h post-infection (Najib et al., 2016). Our results show differences in miRNA expression patterns between skin and head kidney of Atlantic salmon after *C. rogercresseyi* infestation. A significant number of highly regulated miRNAs was detected in head kidney at 7 dpi, contrasting with the skin tissue miRNA expression profile. Keeping in mind that the function of miRNAs is to interfere with the transcription activity of their target genes (Bartel, 2004, 2009) and the large number of highly regulated coding transcripts in skin and head kidney at 7 and 14 dpi, respectively (Valenzuela-Muñoz et al., 2016), we hypothesize that the high number of miRNA regulated in head kidney at 7 dpi are turning-off the coding-transcripts in Atlantic salmon at early stages of sea louse infestation. Our results indicate that the skin transcriptome, as the target tissue for this ectoparasite, requires downregulation by miRNAs to trigger the immune response against sea lice.

RNA-seq analysis of Atlantic salmon infested with *C. rogercresseyi* evidenced exclusive miRNA clusters during infestation. Notably, all the clusters have been reported as conservative miRNAs across vertebrate species. One is mir-181, which regulates hematopoiesis and T-lymphocyte maturation (Xiao and Rajewsky, 2009). Mir-181 and mir-21 in humans activates inflammatory cytokines like STAT-3 and IL6 in the presence of cancer (Iliopoulos et al., 2010). Mir-10 is another highly abundant miRNA that regulates *Hox* genes (Woltering and Durston, 2008). Mir-10 has been described as highly regulated in coelomocytes of sea cumbers with skin ulcers (Sun et al., 2016). Mir-462 was also present in all the clusters we identified for Atlantic salmon skin and head kidney. Mir-462 has been observed highly modulated in fish during viral (Bela-ong et al., 2013) and bacterial infections (Gong et al., 2015), which suggests that immune regulation in Atlantic salmon is modulated through conserved miRNAs independent of the parasite. Indeed, miRNAs can regulate lymphocyte differentiation, monocyte proliferation, inflammatory response, and toll-like receptor signalling, among other immune processes (Baltimore et al., 2008; Lindsay, 2008; Lu and Liston, 2009; O'Neill et al., 2011). The response of Atlantic salmon to *C. rogercresseyi* infestation has been associated with immune aspects like Th1-type response, toll like receptor activation (TLR22a) and nutritional immunity related to iron availability (Boltaña et al., 2016; Valenzuela-Muñoz et al., 2016; Valenzuela-Muñoz and Gallardo-Escárate, 2017). MiRNAs have the capacity to reduce the RNA stability by imperfect binding to the 3′UTR region of target genes (Lindsay, 2008). Gene target prediction was conducted to associate this feature with the immune response in Atlantic salmon and the analyzed miRNAs. The high level of expression of mir-140 in head kidney at 7 dpi was correlated with downregulation of the *TLR22* gene. This cell receptor is involved in fish response to sea louse infestation (Panda et al., 2014; Valenzuela-Muñoz et al., 2016). Atlantic salmon infected with *Lepeophtheirus salmonis* have been described as having a Th2 type immune response. However, this type of response has been not observed in Atlantic salmon infected with *C. rogercresseyi* (Valenzuela-Muñoz et al., 2016). Furthermore, in our analysis, we identified significantly upregulated miRNA that targets the CCR3 gene involved in Th2 response. With respect to nutritional immunity, heme degradation increases in Atlantic salmon infected with *C. rogercresseyi* (Valenzuela-Muñoz and Gallardo-Escárate, 2017). In the present study, we observed high levels of mir-181a-2-5 expression, which seems to modulate the ALAs gene involved in heme biosynthesis, suggesting that mir-181a-2-5 allows free iron to increase as a consequence of heme degradation.

5. Conclusion

This is the first study to relate miRNA profiles of Atlantic salmon to an ectoparasite species like the sea louse *C. rogercresseyi*. We report differences in miRNA expression signatures during infestation, with a high number of miRNAs regulated in head kidney in early infestation. Furthermore, gene target prediction analysis revealed strong relationships between miRNA expression and candidate genes involved in immune system response and iron regulation. However, further functional analyses are required to validate their molecular role during sea louse infection. The present work offers a valuable resource that, combined with functional studies, will foster the development of novel therapeutic tools in fish aquaculture.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.dci.2017.07.009.

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CAPITULO 7

Desarrollo de un nuevo método de control de Caligidosis*:* inmunización de peces con ferritina recombinante caracterizada desde *C. rogercresseyi*.

Para el control de la caligidosis a nivel mundial se utilizan un amplio número de productos químicos, los cuales pueden ser administrados a los peces de forma oral, parenteral o tópicamente. Actualmente en Chile, se utilizan productos como el Teflubenzuron (Calicide®), Azamethiphos (Salmosan®), Peróxido de Hidrógeno, Deltametrina (Alphamax) y Diflubenzuron. Hasta el 2006, el único tratamiento autorizado por las autoridades Chilenas fue el Benzoato de Emamectina (EMB), el cual se aplicó desde 1999 para el control de la Caligidosis, generando posteriormente pérdida de sensibilidad del ectoparásito frente a EMB [99]. En este contexto, la resistencia frente a tratamientos químicos es común en organismos parásitos. Por ejemplo, en Escocia se demostró resistencia a tratamientos con Aquagard (dicholorvos) en *L. salmonis*, el cual fue posteriormente reemplazado por el peróxido de hidrógeno [100]. Es por ello que se han buscado nuevas alternativas para el control del piojo de mar, entre ellas, el desarrollo de vacunas, estrategia que ha sido adoptada desde vacunas generadas para ectoparásitos terrestres. Una de las moléculas que ha sido propuestas para ser utilizada como vacuna es la proteína denominada my32, ortólogo con Akirin-2, caracterizado en *C. rogercresseyi* [78]. Este gen tiene actividad en varios procesos de transcripción como en diferentes vías de señalización durante procesos inmunes (vías de Tool-like receptors, tumor necrosis factor, interleuquinas). La efectividad del gen my32, como molécula para el control de ectoparásitos ha sido evidenciada mediante ensayos de ARN de interferencia, observando reducción del número de copépodos en grupos tratados con el dsRNA de my32. Además, de reportar bajas en las cargas parasitarias de peces vacunados con la proteína recombinante de my32 [101], pese a lo exitoso que ha sido en pruebas de laboratorio, este antígeno aun no se encuentra comercialmente disponible para ser utilizado en el control de la Caligidosis. En Chile durante el 2014, Tecnovax firma biofarmacéutica Anasac recibe autorización del Servicio Agrícola Ganadero (SAG) para la utilización de la vacuna cuyo principio activo de esta vacuna es una antígeno subunitario sintético conjugado con una “mega” proteína de tipo Keyhole limpet hemocyanin (KLH), vacuna que cuenta con patente en Noruega, Canadá,

Reino Unido, Nueva Zelanda. La vacuna de Tecnovax conocida como Providean Aquatec Sea Lice ®, reduce la carga de juveniles y adultos de caligus, induciendo anticuerpos en el mucus y suero de los peces luego de 300 días de inyección y reporta hasta un 73% de éxito en la reducción de la carga parasitaria (http://www.aquatecsealice.com/providean.html). Así también, la empresa Centrovet ha desarrollado una vacuna de aplicación oral e inyectable, que generaría alteraciones en diferentes procesos fisiológicos y reproducción del ectoparásito, demostrado tener una eficiencia de 30-40% en pruebas de campo. En este capitulo se muestran resultados obtenidos ensayos de vacunación utilizando como antígeno *Cr_ferritina* caracterizada previamente en *C. rogercresseyi*.

Resultados

Preparación de vacuna y evaluación de eficacia

Desde la cepa de *E. coli* BL21 transformada con el plásmido de expresión, se indujo la proteína *Cr_ferritina*, obteniendo una proteína de tamaño cercano a los 20 kDa, en concentración cercana a 437,6 μg/ml. Una vez obtenida la proteína recombinante, se realizó la formulación de la vacuna en relación adyuvante/antígeno de 70/30, utilizando el adyuvante comercial MONTANIDE^{TM} ISA 761VG.

Para el ensayo de vacunación se trabajó con dos grupos experimentales, uno de ellos inyectados de manera intraperitoneal con 30 μg de *Cr_ferritina* y el segundo grupo control inyectado con PBS. Cumplidas 400 unidades térmicas acumuladas (UTAs), ambos grupos de peces fueron infectados con una carga inicial de 35 copepoditos por pez. Previo a la infestación ambos grupos de peces mostraron un crecimiento normal, con un índice de Fulton de 0,8 ± 0,1, sin daños visibles por efecto de la vacuna. Luego de 25 días de infestación, cuando los Caligus llegaron a estado adulto, se hizo recuento de Caligus adultos, observando una tasa de reducción de Caligus de un 97% en el grupo vacunado con *Cr_ferritina* (Fig. 3)

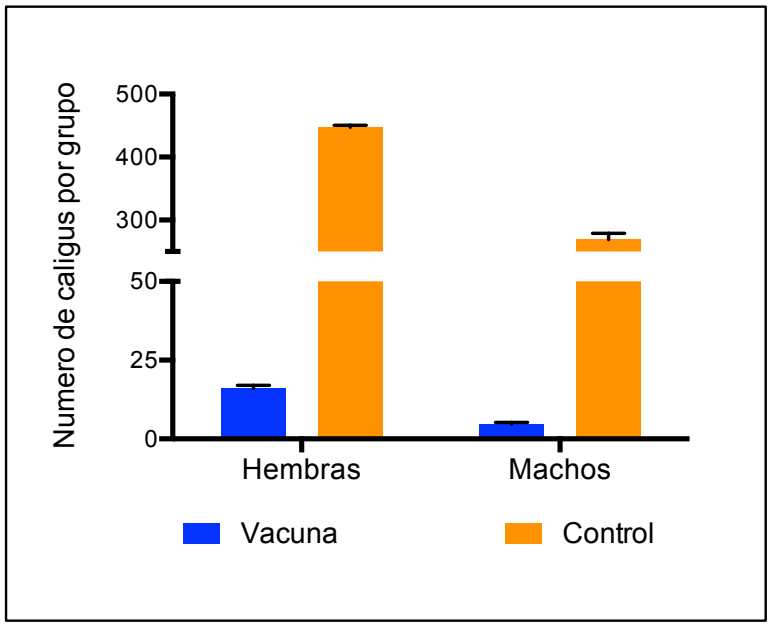

Figura 3. Carga de Caligus adultos en peces vacunados con *Cr_ferritina*. Fuente: Elaboración propia

Cambios fenotípicos y transcriptómicos en hembras de *C. rogercresseyi* expuestas a peces vacunados

Luego de 25 dias de enfestación, se recolectaron hembras de *C. rogercresseyi* registrando marcadas diferencias fenotípicas, desde ambos grupos de peces inyectados. Las hembras provenientes de peces vacunados con *Cr_ferritina* presentaron reducción del largo de sus sacos ovigeros y deformación de sus huevos, mientras que las hembras obtenidas desde peces del grupo control presentaron sacos ovigeros de largo normal y sin deformidad (Fig. 4).

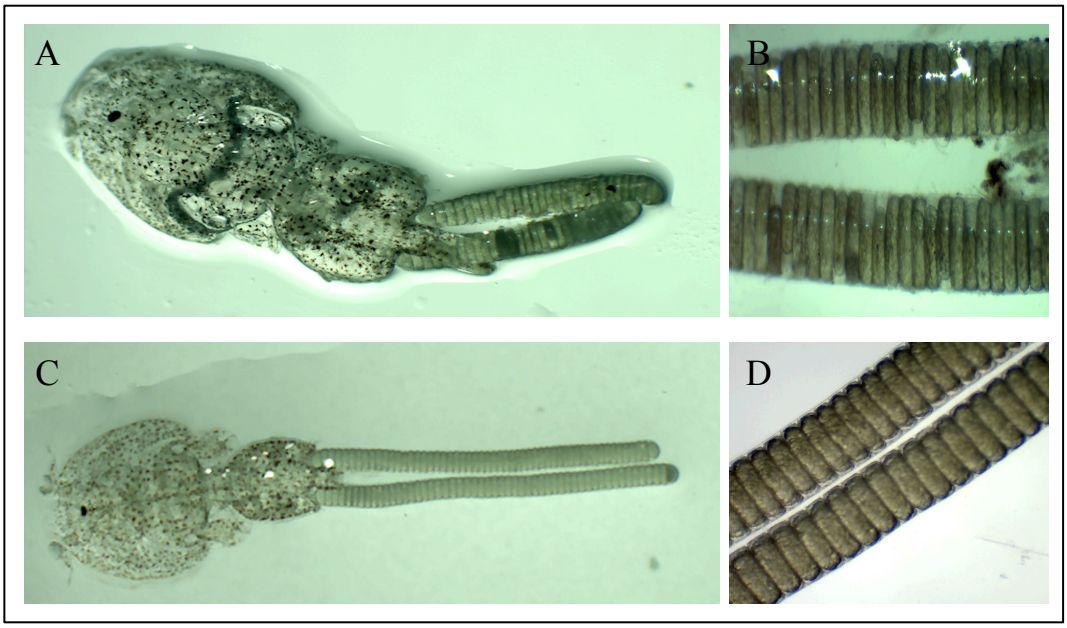

Figura 4. Hembras de Caligus obtenidas desde peces vacunados. (A) obtenidos desde peces vacuandos con *Cr_ferritina,* (B) acercamiento a los sacos ovigeros de hembras obtenidas desde peces vacunados con *Cr_ferritina*, (C) hembras obtenidas desde peces del grupo control, (D) sacos ovigeros de hembras obtenidas desde peces control. Fuente: Elaboración propia

Adicionalmente, a partir de las hembras recolectadas se realizó extracción de ARN total y secuenciación de transcriptoma. Desde la secuenciación de cada grupos se obtuvieron 13 M y 19 M de lecturas para las hembras obtenidas desde los peces vacunados y grupo control, respectivamente, las que generaron 53.488 contigs. De manera de eliminar el efecto del adyuvante, para los análisis de RNA-Seq y expresión diferencial se incluyo un grupo de datos de transcriptoma obtenido desde hembras peces no vacunados, datos previamente obtenidos y publicados por el grupo de laboratorio [95]. Del análisis de RNA-Seq entre hembras obtenidas de peces vacunados con *Cr_ferritina,* grupo control y peces no vacunados, se observaron variaciones de expresión transcritos analizados. De manera específica, la representación del heatmap generado a partir de los datos de expresión fue posible observar que las hembras de Caligus obtenidas desde peces vacunados (*Cr_ferritina* y control) se agrupan en un cluster distinto a las hembras colectadas desde peces no vacunados (Fig. 5A). A partir de un análisis de expresión diferencial se observaron diferencias en la cantidad de transcritos sobre-expresados y negativamente regulados, con límite de cambio de expresión mayor a valor absoluto de 4, registrando gran número de transcritos regulados de manera negativa en las

hembras recolectadas desde peces vacunados con *Cr_ferritina,* en comparación con el grupo control (Fig. 5B).

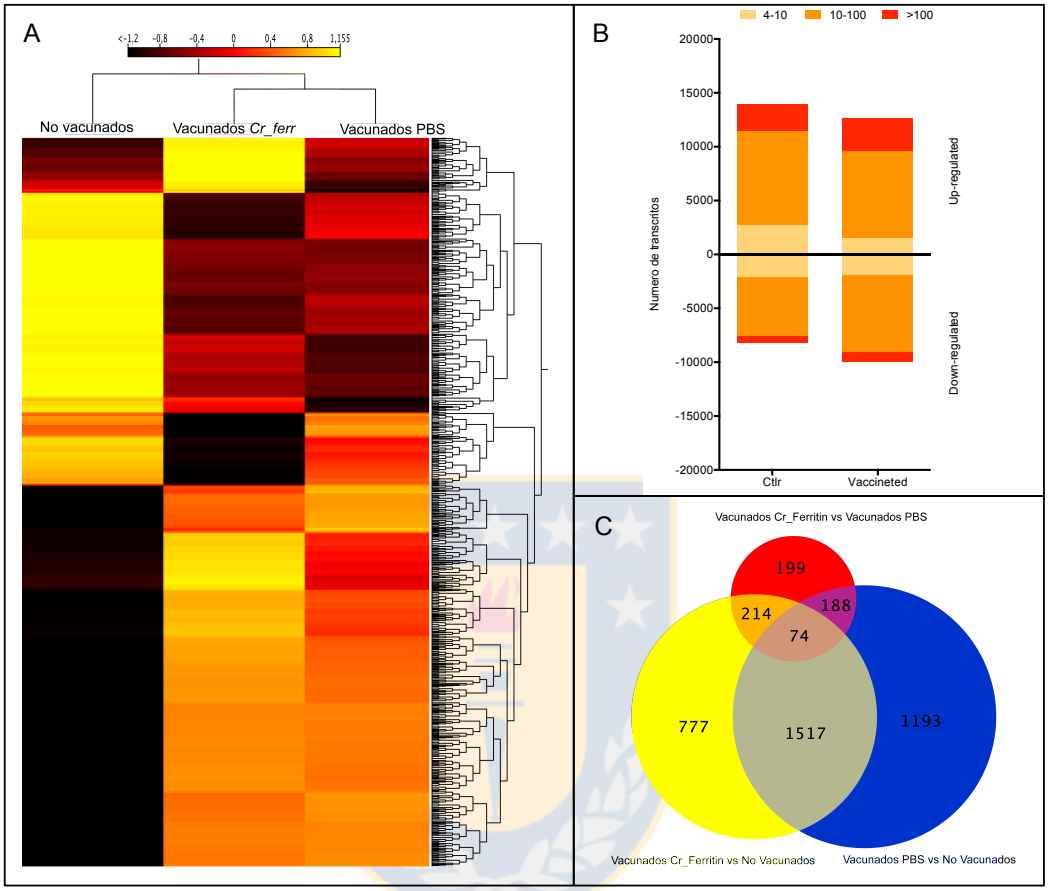

Figura 5: Análisis de transcriptoma de hembras de *C. rogercreseyi* obtenidas desde peces vacunados. (A) representación heatmap de los cambios de expresión de transcritos entre hembras obtenidas desde peces vacunados. (B) Número de transcritos expresados de manera positiva y negativa en hembras de peces vacunados y control utilizando como referencia hembras obtenidas desde peces no vacunados. (C) Análisis de expresión diferencial representado en diagrama de Venn para transcritos expresados en hembras de *C. rogercresseyi* obtenidas desde peces vacunados y grupo control. Fuente: Elaboración propia

A partir de los datos de expresión diferencial, es posible observar que 777 trascritos expresados de manera exclusiva en las hembras de Caligus obtenidas desde peces vacunados con *Cr_ferritina* (Fig. 5C). Adicional a estos transcritos, se identifican 214 transcritos más, que son expresados en este grupo de hembras, sin contemplar el efecto del adyuvante (Fig. 5C). De la anotación de los transcritos que fueron expresados exclusivamente en hembras

colectadas desde peces vacunados con *Cr_ferritina*, fue posible observar gran abundancia de genes anotados en procesos biológicos como biosíntesis de serinas, respuesta a estrés oxidativo, regulación de procesos catabolicos, crecimiento, desarrollo embrionario, ovogénesis, metabolismo de ecdisona y transporte de transferrina (Fig. 6). Dentro de estos transcritos anotados y expresados exclusivamente en hembras obtenidas desde peces vacunados, se observó una sobreexpresión de genes como *serine proteinasas* y *tripsinas*, mientras que los genes *vitelogeninas, y subunidades de colagenasa* se muestran regulados negativamente (Tabla 1). Además, desde los transcritos que son expresados tanto en hembras del grupo vacunado como en el grupo control, se observaron diferencias en los niveles de expresión del gen *ferritina,* expresándose de manera negariva en aquellas hembras provenientes de peces vacuandos con *Cr_ferritina*.

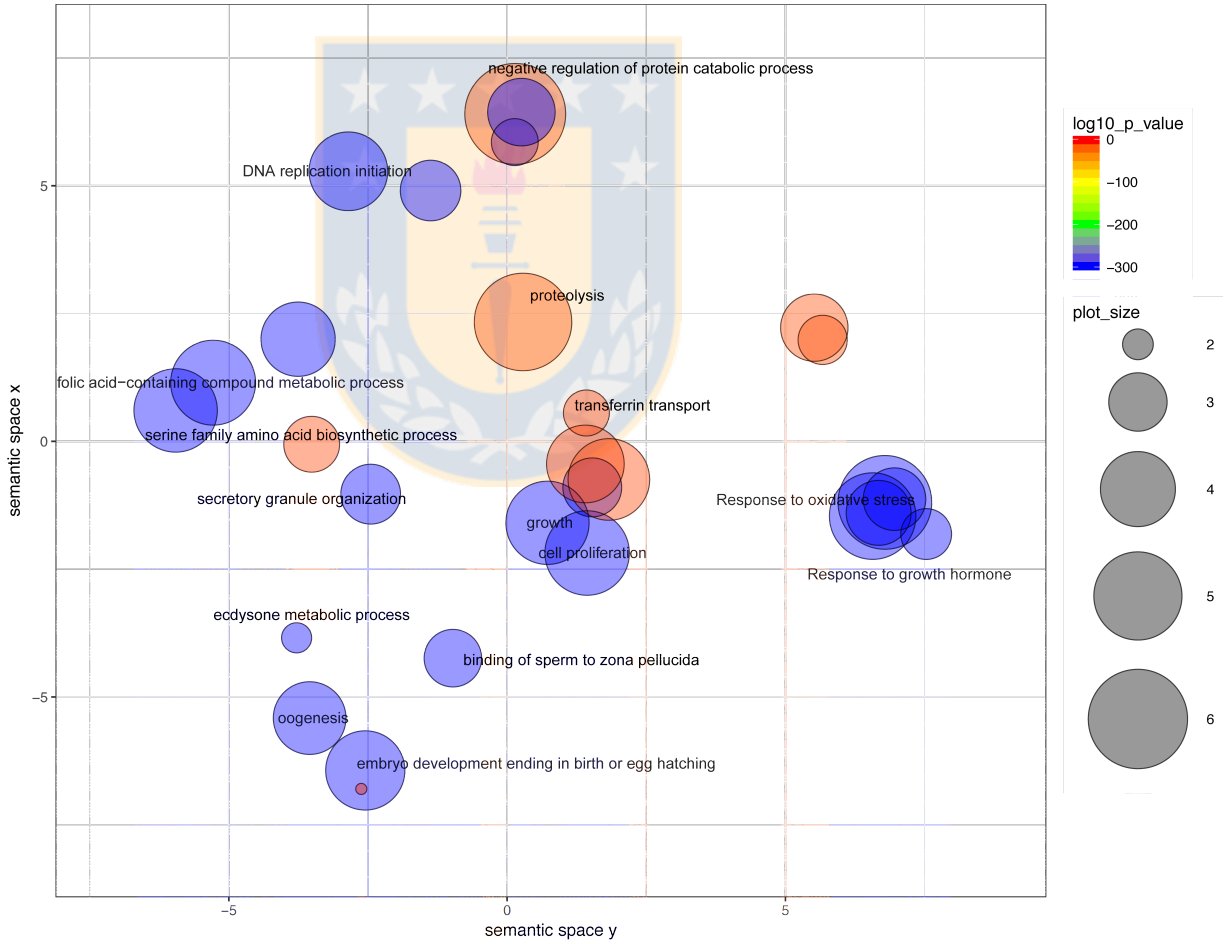

Figura 6: Análisis de enriquecimiento de transcritos expresados de manera exclusiva en hembra de Caligus obtenidas desde peces vacunados con *Cr_ferritina*. Fuente: Elaboración propia.

Tabla 1. Anotacion de transcritos exclusivos de hembras de *C. rogercresseyi*, expresados por efecto de la vacuna *Cr_ferritina* y grupo control.

Vacuandos Cr_ferritin	**Anotación**	**Fold change sin vacuna vs Cr_ferritin**	
Contig0005911	Serine proteinase stubblelike	↑35.901	
Contig0002867	Serine protease homolog 21	↑15.506	
Contig0004160	Serine proteinase stubblelike	↑26.064	
Contig0015667	Vitelline membrane protein	↓-4.337	
Contig0019994	Vitellogenin-like protein	↓-14.674	
Contig0017714	Vitellogenin-like protein	↓-8.456	
Contig0024274	Vitellogenin-like protein	↓-10.257	
Contig0004985	Putative cuticle protein	↓-36.533	
Contig0006494	Collagen alpha1(II) chainlike	↓-4.849	
Contig0021327	Collagen alpha1(XI) chainlike	↓-4.256	
Contig0000923	Putative trypsin-like serine protease	↑6.305	
Contig0015945	Putative trypsin-like serine protease	↑4.178	
Contig0025201	Putative trypsin-like serine protease	↑8.929	
Contig0036766	Trypsin type 6	↓-70.077	
Contig0020436	Heat shock protein hsp20.8	↑9.206	
Contig0007472	Hsp90 co-chaperone Cdc37	↓-14.033	
Vacunados Control	**Anotación**		**Fold change sin vacuna vs PBS**
Contig0036327	Vitellogenin 2		↓-59.941
Contig0003229	Tolloidlike protein 2like		↓-5.090
Contig0003230	Tolloidlike protein 2like		↓-4.128
Contig0004540	Trypsin type 4, partial		↓-4.590
Contig0019886	Cationic trypsin3like		↓-7.004
Contig0003736	Trypsin type 5, partial		↓-33.316
Contig0021535	Serine proteinase stubblelike		↓-5.938
Contig0015285	Serine/threonine kinase 11		↑4.574
Contig0013116	Putative cuticle protein		↓-64.836
Contig0013584	Larval/pupal cuticle protein H1C-like		↑4.023
Contig0015202	Matrix metallopeptidase 9		↓-9.858
Compartidos	**Anotación**		
Contig0049464	Ferritin, lower subunitlike	↓-1.137	↑14.715
Contig0021705	Putative transferrin 2	↑2.414	↑1.318

Fuente: Elaboración propia

DISCUSION

Mecanismos biológicos modulados durante la infestación por *C. rogercresseyi*

El creciente interés en el desarrollo de la acuicultura a nivel mundial ha hecho necesario incrementar los esfuerzos para comprender los mecanismos de defensa en peces frente a la presencia de infecciones. En las últimas décadas debido al explosivo avance de las tecnologías de secuenciación, ha incrementado sustancialmente la cantidad de información genómica de teleósteos y de la mano de esto el conocer las vías de respuesta inmune de peces [22]. En general, los organismos teleósteos presentan un sistema inmune similar al que presentan los mamíferos con elementos de respuesta inmune innata y adaptativa. En particular, el conocimiento en torno a los mecanismos de respuesta inmune de peces en presencia de copépodos es escaso. Se puede mencionar como ejemplo la respuesta de salmón del Atlántico a la presencia de *L. salmonis*, donde los mecanismos de respuesta inmune se han asociado a variación en los niveles de citoquinas pro-inflamatorias [33], secreción de proteasas [16], y respuesta humoral [33, 37]. Por ejemplo, se ha reportado incremento de IL4/13 y IL10 en familias de salmón de Atlántico resistentes a *L. salmonis*, al igual que especies de salmones como salmón rosado y salmón Coho que presentan menor susceptibilidad a ser infestados por este ectoparásito, sugiriendo una respuesta tipo Th2 a la presencia de *L. salmonis* [37-39]. En el presente estudio se demuestra que los mecanismos de respuesta de los salmónidos infestados por *C. rogercresseyi* varían en relación a lo previamente reportado para salmones infestados con *L. salmonis* [19, 33, 34, 38, 40]. Se observó que salmón del Atlántico presenta mayores niveles de susceptibilidad a *C. rogercresseyi* en comparación con salmón Coho, lo que además genera variaciones en los tipos de respuesta inmune de ambas especies a la presencia del ectoparásito. Salmón del Atlántico responde a la presencia de *C. rogercresseyi* mediante la activación de un receptor tipo toll (TRL), *tlr22a2*, presente sólo en peces [27]. Este TLR ha sido asociado en otras especies de peces infestados por ectoparásitos, como uno de los primeros mecanismos de respuesta inmune activados. Por ejemplo, se ha reportado una sobre-regulación de *tlr22a2* en *L. rohita* infestados por el ectoparásito *A. siamensis* [26, 29]. En *L. rohita,* además se ha observado una relación directa entre los niveles de expresión de este *tlr22a2* y la cantidad de *A. simensis* en los peces [30]. Adicionalmente, salmón Coho, no

presenta un incremento significativo de la expresión de *trl22a2,* contrariamente sí se observó incremento en la expresión de *tlr13*, TLR que en peces tiene un antecesor común con TLR22 [102]. De esta forma, la sobreexpresión del *tlr13* en salmón Coho podría ser un indicativo de que esta especie de salmón responde vía TLR a la presencia de *C. rogercresseyi*, similarmente a salmón del Atlántico con el *tlr22a2*. Por otra parte, los peces que presentan menor susceptibilidad a la infestación con *L. salmonis* despliegan una respuesta inmune de tipo Th2 [33, 37, 38], en este estudio se observó que la especie menos susceptible a la presencia de *C. rogercresseyi*, salmón Coho, presenta baja expresión de genes asociados a la respuesta Th2. Sin embargo, como respuesta temprana a la presencia de *C. rogercresseyi* en salmón Coho se observa un incremento en los niveles de expresión de genes asociados a respuesta Th1. Se plantea la hipótesis de que la baja expresión de genes Th2 en salmón Coho podría ser una estrategia para evitar la sobreproducción de mucus en los peces, principal fuente de alimento de *C. rogercresseyi*, ya que se ha descrito que la respuesta Th2 incrementa la sercrecion de mucus mediante la producción de mucina [33, 103, 104].

Adicionalmente a la búsqueda de transcritos asociados a respuesta inmune innata y adaptativa, desde un enriquecimiento GO de los datos de transcriptoma, se observó una gran cantidad de transcritos asociados a unión de iones en salmón Atlántico y Coho luego de ser infestados por *C. rogercresseyi*. Los mecanismos de regulación de la disponibilidad de iones (Fe, Mn, Zn) en el hospedador, han sido descritos como una estrategia inmunitaria conocida como inmunidad nutricional [44]. Este tipo de respuesta reduce la disponibilidad de nutrientes que son obtenidos por el parásito desde su hospedero afectando en el éxito de una infección. Dentro de los nutrientes de mayor relevancia en esta estrategia inmunitaria se encuentra el hierro, el que, se ha observado afecta la modulación de citoquinas y proteínas de fase aguda [105]. Por ejemplo, el incremento de hepcidina, proteína reguladora de hierro intracelular, ha sido asociado a la secreción de IL-6 luego de la activación de receptores reconocedores de patrones (PPRs) durante infecciones bacterianas [106]. En macrófagos, se ha observado que la limitación de hierro reduce a la vez la expresión de MHCII, iNOs e inhibe la vía de señalización IFN-g [105]. Así también, en peces con menor susceptibilidad al ectoparásito *L. salmonis,* se ha observado un incremento de moléculas reguladoras del grupo *hemo*, reflejado en el incremento de los niveles de expresión de *heme oxigenasa* [19]. Además, en peces

resistentes a *L. salmonis* se ha observado incremento de los niveles de expresión de *hepcidina* [37], gen responsable de regular la disponibilidad de hierro a nivel celular, lo que sugiere una estrategia del hospedero a la limitación de hierro en la sangre, principal alimento de estos ectoparásitos. Desde los datos de secuenciación de piel y riñón anterior de salmón Atlántico y Coho infestando *C. rogercresseyi*, se observaron variaciones en los niveles de expresión de genes asociados a regulación de hierro. Por ejemplo, una sobreregulación de *subunidad de hemoglobina* en ambas especies estudiadas. En particular en salmón Coho, contrario a lo descrito para especies resistentes a *L. salmonis* [19], se observa una disminución en la expresión de *heme oxigenasa* asociada a la degradación del grupo Hemo y un incremento en la expresión de genes de biosíntesis de este grupo como acido aminolevulinico sintetasa y coproporphyrinogenasa (*Alas* y *CPBGo*). Mientras que en salmón del Atlántico se observa un incremento en la expresión de *BLVr,* gen asociado a la degradación del grupo Hemo, en piel luego de siete días de infestación. Este resultado, asociado a lo reportado por Gonzáles *et al.* (2016) [107], donde se observa una reducción de hemoglobina en salmón del Atlántico infestados por *C. rogercresseyi,* sugiere una reducción del grupo Hemo como fuente de hierro, como una estrategia para combatir la infestación por *C. rogercresseyi*. La homeostasis del hierro no esta dada sólo por la regulación del grupo Hemo, existen además proteínas encargadas de reducir el hierro circulante como consecuencia de la degradación del grupo Hemo [46]. Entre estas proteínas se pueden mencionar *hepcidina, haptoglobina, ferritina, transferrina*. En este estudio el aumento de expresión de *BLVr* se ve acompañado por el aumento de *transferrina* en salmón del Atlántico luego de la infestación con *C. rogercresseyi*. En esta misma especie de salmónido, se han registrado incrementos en los niveles de expresión de *hepcidina*, gen que además ha sido asociado a estados anémicos en peces [108, 109]. Lo que se asocia a los aumentos hematocritos observados en salmón del Atlántico durante la infestación con *C. rogercresseyi* [107]. El incremento en los mecanismos de regulación de hierro, biosíntesis/degradación del grupo Hemo, así como de genes transportadores de hierro, observada en salmón del Atlántico infestado con *C. rogercresseyi*, sugiere la existencia de una respuesta nutricional en esta especie de salmón.

Para confirmar que la estrategia de regular las concentraciones de hierro en el hospedador, por efecto de la infestación por *C. rogercresseyi* se realizó un estudio entre piel y riñón anterior de

salmón del Atlántico infestados por *L. salmonis* y *C. rogercresseyi*. Mediante análisis de qPCR se observaron diferencias de expresión entre genes asociados a biosíntesis/degradación del grupo Hemo así como de genes transportadores de hierro y respuesta a estrés oxidativo. Entre las diferencias observadas se puede destacar que los peces infestados por *C. rogercresseyi* mostraron un incremento en la expresión de *hepcidina*, la que como ya se ha mencionado es altamente expresada salmones resistentes a *L. salmonis* [37]. Además, los peces infestados con *C. rogercresseyi* mostraron aumento en la expresión de genes asociados a la degradación de grupo Hemo. Mientras que los genes asociados a biosíntesis del grupo Hemo, *ferritina y receptor de transferrina* se encontraron altamente expresados en los peces infestados con *L. salmonis*. Además, el incremento de la biosíntesis de Hemo se asocia a un incremento en los niveles de estrés oxidativo [110], lo que se refleja en los peces infestados con *L. salmonis* mediante el incremento de la expresión de *glutatión S-transferasa* (*GST*) y *superoxido dismutasa* (*SOD*). Con este análisis es posible confirmar que existe un mecanismo de respuesta nutricional en salmón del Atlántico infestados por piojo de mar. Según los resultados, la estrategia nutricional para responder a *L. salmonis* y *C. rogercresseyi* varía en la misma especie de salmónidos, siendo de mayor relevancia en la respuesta a *L. salmonis*, el que es un organismo hematófago [110], mientras que *C. rogercresseyi,* es un organismos hematófago facultativo.

Desde que reconoce al hospedero y durante toda su etapa de parasitismo *C. rogercresseyi* debe combatir los mecanismos de rechazo de su hospedero. Para conocer los mecanismos moleculares que utiliza este ectoparásito para su defensa, desde datos transcriptómicos publicados para los distintos estadios de desarrollo de *C. rogercresseyi* [95] se han caracterizado las vías de respuesta inmune innata IMD y TLR [76], genes asociados al control de especies reactivas de oxigeno (ROS) [111] y productos secretores como serpinas y tripsinas [17]. A partir de esta información, se decidió evaluar si existían variaciones en la respuesta de *C. rogercresseyi* infestando salmón del Atlántico y Coho, recolectando Caligus durante 1, 3 y 7 dpi. Mediante análisis de RT-qPCR se observaron diferencias de expresión de los genes evaluados (IMD/TLR, ROS, *serpinas*, *tripsinas*) entre los Caligus infestando salmón del Atlántico y salmón Coho. En los Caligus infestando ambas especies de salmones se observó incremento de la expresión de genes asociados al control de estrés oxidativo, lo que puede ser

utilizado por los Caligus para responder a la gran cantidad especies reactivas de oxigeno (ROS) que se genera en los peces como consecuencia de la infestación, lo que ha sido observado en peces con bajos niveles de susceptibilidad a la infestación por *L. salmonis* [19]. Además, el incremento de ROS en los peces se ha ligado al aumento de la expresión de citoquinas [88] y metaloproteasas [40] liberadas durante la infestación. Los Caligus provenientes de salmón del Atlántico presentaron un incremento significativo en la expresión de *ferritina*, como consecuencia del incremento de ROS que debe ser controlado por la reacción de Fenton catalizada por *ferritina*. Adicionalmente, es necesario recordar que *ferritina* también se encuentra altamente expresada en salmón del Atlántico durante la infestación, por lo que se puede sugerir que tanto el hospedero como el ectoparásito compiten por el hierro libre para lo cual sobre-expresan *ferritina*.

Desde los análisis de transcriptoma de salmón del Atlántico y Coho junto con los datos de expresión de genes de *C. rogercresseyi* expresados durante la infestación, se plantea la siguiente hipótesis de interacción entre Caligus y salmónidos: la infestación de *C. rogercresseyi* induce en los salmónidos una respuesta inmune mediada por citoquinas proinflamatorias y/o receptores tipo Toll, además, de incrementar la regulación de los mecanismos de homeostasis de hierro, lo que a la vez induce incremento de estrés oxidativo. Por su lado, *C. rogercresseyi* responde incrementando la expresión de genes de respuesta a estrés oxidativo, junto con aumentar la expresión de ferritina, como una posible estrategia para competir por hierro libre desde su hospedero (Fig. 7).

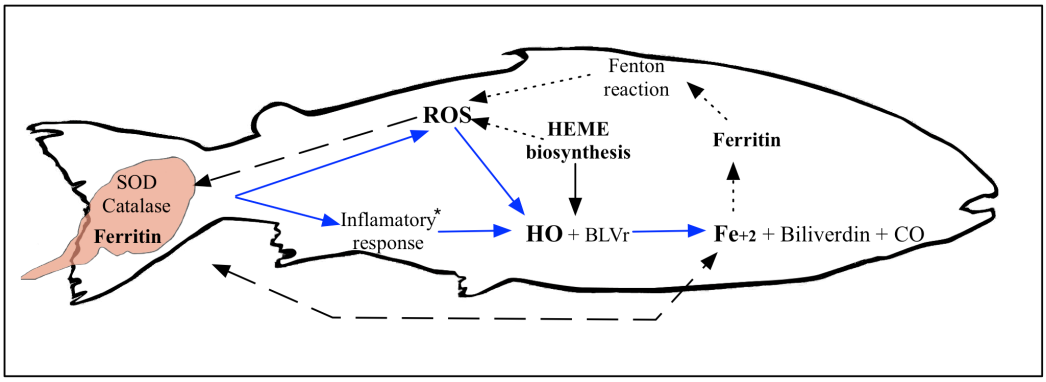

Figura 7. Modelo de interaccion propuesto ectoparásito-hospedero, para *C. rogercresseyi* y salmón. Fuente: Elaboración propia

Rol de transcritos no codificantes durante la infestación por *C. rogercresseyi*

Si bien en este estudio se observó gran modulación de transcritos codificantes durante la infestación por *C. rogercresseyi*, identificando diferencias de respuesta entre hospederos, además de diferencias con lo previamente publicado para peces infestados por *L. salmonis*. Es necesario comprender el rol de la fracción de transcritos que no fueron asociados a genes conocidos y sin potencial codificante. Dentro de los ncRNAs se encuentra un grupo que actúan como reguladores de la expresión de RNA mensajeros, destacandosé los miRNAs y lncRNAs [48, 49]. Los lncRNAs son secuencias de largo superior a 200 nucleótidos, transcritos de forma similar a los RNA codificantes [51, 52]. El estudio de los lncRNAs es relativamente nuevo, los estudios genómicos han demostrado que en mamíferos así como otros vertebrados el número de RNAs que son traducidos a proteína es cercano a 20.000 y debido a las diferencias en los tamaños genómicos de los organismos, se ha planteado que los lncRNAs han ido incrementando durante el proceso de evolución, por lo que los organismos con mecanismos más complejos han adquirido mayor cantidad de lncRNAs, como mecanismo de ayuda para el control de los procesos biológicos [112]. Los estudios en torno los lncRNAs reportan que existe una proporción que se expresan de manera célula-específica, e incluso en células del sistema inmune [112, 113]. Por ejemplo, en pez cebra 342 lncRNA son transcritos en más de un tejido y sólo 77 lncRNAs son expresados de manera específica en un sólo tejido [114]. Otro ejemplo, es en trucha arcoíris donde se ha observado que de 2.935 lncRNAs, cerca del 5.2% de lncRNAs identificados en esta especie, se expresan de manera tejido específica. En el presente estudio se identificaron lncRNAs desde piel y riñón anterior de salmón del Atlántico y Coho, observando mayor abundancia de lncRNAs exclusivos en riñón anterior de ambas especies en comparación con los lncRNAs exclusivos identificados en piel, similar a lo reportado para trucha arcoíris [115].

Los lncRNAs juegan un rol importante en la modulación de procesos biológicos, se ha demostrado que incluso participan en procesos de regulación de vías del sistema inmune como la vía de señalización Toll [55] y NF-kB [56]. En peces, el rol de los lncRNAs como reguladores de la respuesta inmune ha sido escasamente estudiada. Sin embargo, algunos estudios realizados en torno a patologías en peces han demostrado, por ejemplo, en *Larimichthys polyactis* luego de una exposición a *Vibrio anguillarum*, un alto número de

lncRNAs sobreregulados como respuesta a la infección. Adicionalmente, los autores sugieren que estos lncRNAs estarían modulando genes cercanos asociados a respuesta inmune [57]. Otro estudio transcriptómico, realizado en salmón del Atlántico infectados con el virus ISA, identificó 4.967 lncRNAs diferencialmente expresados como una correlación positiva entre la carga de ISAv y la abundancia de lncRNA expresados [58]. También se han descrito lncRNAs en salmones infectados por patógenos bacterianos como *Piscirickettsia salmonis,* evidenciando que los lncRNA modulados durante la infección, estarían directamente asociados a la regulación de genes involucrados en el proceso de respuesta a esta bacteria como lo son genes de endocitosis y homeostasis de hierro [116]. Aun más, un estudio comparativo en salmón del Atlántico expuestos a tres distintos patógenos, virus, bacteria y ectoparásito demuestran que los lncRNAs se regulan de manera específica en respuesta a cada tipo de infección [59]. En este estudio, realizado en salmón del Atlántico y Coho expuestos a *C. rogercresseyi*, se observó que la abundancia de lncRNAs expresados durante el tiempo de infestación es similar a la observada para transcritos codificantes. En ambas especies de salmón se observó un alto número de lncRNAs expresados a los 7 dpi y 14 dpi en piel y riñón anterior, respectivamente. Los lncRNAs expresados en salmón Coho en presencia de *C. rogercresseyi* se localizaron cerca de genes asociados a procesos como respuesta a estímulos externos, reparación de tejidos y comunicación celular. Por otro lado, los lncRNAs identificados en salmón del Atlántico expresados durante la infestación por *C. rogercresseyi*, fueron localizados dentro del genoma cerca de genes asociados a procesos biológicos como estrés, respuesta inmune e inflamatoria. Previamente, Tarifeño-Saldivia *et al.* [59] reportaron 439 lncRNAs expresados en riñón anterior de salmón del Atlántico luego de 15 días de infestación con *C. rogercresseyi*, donde los genes cercanos a los lncRNAs asociados a la infestación con *C. rogercresseyi* fueron genes asociados a procesos de regulación transcripcional. Al realizar un análisis de homología entre los lncRNAs reportados en este estudio y los reportados por Tarifeño-Saldivia *et al.* [59], sólo 21 de los lncRNAs mostraron similitud. De la identificación y análisis de expresión de los lncRNAs en salmones infestados por *C. rogercresseyi,* es posible establecer que los lncRNAs jugan un rol importante en el mecanismos de respuesta a este ectoparásito, sin embargo, se requiere profundizar en los estudios mediante análisis funcionales para identificar los genes que son directamente modulados por los lncRNAs identificados en este estudio.

Adicionalmente, en busca de identificar ncRNAs asociados a la respuesta a *C. rogercresseyi* se secuenciaron librerías de smallRNAs desde piel y riñón anterior de salmón del Atlántico luego de 7 y 14 días iniciada la infestación. Dentro de los smallRNA destacan los microRNAs (miRNA) que son pequeñas secuencias de RNA de no más de 22 nucleótidos, son capaces de unirse a un RNA mensajero, principalmente en su extremo 3' UTR y bloquear la traducción de un gen [117, 118]. Al igual que los lncRNAs, los miRNAs son expresados de manera tejido-especifica y son capaces de regular procesos biológicos como desarrollo, crecimiento, metabolismo, apoptosis, etc [119-121]. En lo que respecta a la respuesta inmune, los miRNAs participan tanto en la regulación de respuesta inmune innata y adaptativa, como diferenciadores de células T, moduladores de respuesta inflamatoria y activación de receptores tipo Toll [62, 63, 122, 123]. Este estudio, es el primero en identificar miRNAs en salmón del Atlántico durante una infestación por ectoparásitos como *C. rogercresseyi* y posibles genes de respuesta inmune que pudiesen ser blanco de miRNAs como respuesta a la infestación. Se logró anotar 1.718 miRNAs desde piel y riñón anterior de salmón del Atlántico, con alta abundancia de las familias de miRNA denominas como mir-21, mir-181 y let-7. Familias que han demostraron ser abundantes en otras especies de peces como trucha y lenguado [71, 124, 125]. En este estudio se observaron diferencias en el número de miRNAs identificados en piel y riñón anterior, con mayor abundancia de miRNAs en riñón anterior, incluso se identificaron miRNAs expresados sólo en este tejido como mir-1388 y mir-155, descritos previamente como claves en el proceso de regulación de eritropoyesis en pez antártico [126]. En otros peces se ha observado de igual manera variaciones en los patrones de expresión y abundancia de miRNAs entre tejidos como respuesta a un patógeno. Por ejemplo, en *Cynoglosus semilaevis* infectados con *V. anguillarum* se reportaron variaciones de expresión de mir-142-5p, mir-223, mir-181 entre los tejidos baso, riñón anterior e hígado [127]. Además, de diferencias de expresión de miRNAs entre tejidos también se han observado variaciones de expresión durante el periodo de infección, por ejemplo en tilapias, mir-214, mir-155, mir-29 son regulados de manera negativa durante las primeras horas de infección por *Streptococcus agaactiae* [72]. En este estudio, a diferencia de lo observado en los datos transcriptómicos (RNA codificantes y lncRNAs) se vio una gran abundancia de miRNAs expresados en piel de salmón del atlántico 14 dpi y en riñón anterior 7 dpi, además este último tejido presentó mayor abundancia de miRNAs anotados que el tejido de la piel. Dado que el rol de los miRNA es

interferir en la actividad post-transcripcional de genes [117, 118], se puede inferir que la gran abundancia de miRNAs a los siete días después de la infestación en riñón anterior, mantiene niveles bajos de trascripción de genes relacionados a respuesta inmune en salmón del Atlántico.

Los miRNAs con mayores niveles de expresión durante la infestación fueron utilizados para la búsqueda de genes blanco, los que fueron seleccionados de acuerdo a lo observado en los estudios de transcriptoma, los que demostraron que la respuesta inmune de salmón del Atlántico a la presencia de *C. rogercresseyi* esta dada por una respuesta tipo Th1, activación de la via toll por el TLR22a2 y mecanismos de inmunidad nutricional. Como resultado de la predicción se observó, que el mir-140 altamente expresado en riñón anterior 7 dpi, tiene como target el gen *tlr22a2*, el que además se encuentra regulado de forma negativa en ese tejido a los 7 dpi. Otro miRNAs que parece influir en la respuesta de salmón del Atlántico a *C. rogercresseyi* es el mir-21, el que presenta como target el gen *CCR3* asociado a respuesta Th2, la que se ha descrito de gran relevancia en la respuesta de salmones resistentes a *L. salmonis* [37]. Adicionalmente y asociado a la regulación de hierro en el hospedero, también se identificó el mir-181a-2-5 como posiblemente regulador de ALAs, gen asociado a la biosíntesis del grupo Hemo, proceso el cual se observó inhibido en salmón del Atlántico infestados con *C. rogercresseyi.*

Existen estudios que demuestran que los miRNAs no sólo son reguladores de procesos biológicos en los organismos, si no que también pueden participar en los procesos de interacción parásito-hospedero. En insectos afectados por virus se ha observado que los virus son capaces de liberan miRNAs que pueden regular la expresión de genes de su hospedero para establecer con éxito la infección [61]. Otro ejemplo, en el mosquito *A. aegypti* al estar infectada por la bacteria *Wolbachia* sobre expresa el miRNAs denominado aae-miR2940-5p, el que a su vez inhibe el gen AsDnmt2 que facilita la replicación del virus del dengue, protegiendo de esta forma al mosquito del virus [128]. Para *C. rogercresseyi* nuestro grupo de investigación ha caracterizado los diferentes miRNAs expresados durante los distintos estadios de desarrollo de este ectoparásito [129]. Dentro del perfil de miRNAs caracterizados para *C. rogercresseyi*, el miRNA denominado Bantam, se encuentra en gran abundancia y altamente

expresado en la etapa infectiva de copepodito. Esto sugiere que este miRNA es de gran importancia para el éxito de la infección. Teniendo esto en cuenta junto con la hipótesis de que los miRNAs pudiesen participar en la interacción parásito-hospedero, es que se realizó un análisis de predicción entre los genes de salmón del Atlántico *trl22a2, cd83* y IFNg y el miRNA Bantam, observando alto potencial de regulación de estos genes por Bantam. Sin duda para establecer si el miRNA Bantam expresado en *C. rogercresseyi* interactúa con los genes de respuesta inmune de salmón del Atlántico es necesario realizar estudios funcionales, sin embargo, este estudio entrega indicios de mecanismos de interacción parásito-hospedero que no han sido reportados para ectoparásitos.

Desarrollo de una nueva herramienta para el control de *C. rogercresseyi*

El incrementado de la información genómica ha permitido ampliar el conocimiento en torno a los distintos mecanismos asociados a procesos de interacción parásito-hospedero, permitiendo identificar nuevos antígenos para el desarrollo de vacunas y control de ectoparásitos. En la actualidad los ectoparásitos marinos como *Caligus rogercresseyi*, son tratados mediante el uso de antiparasitarios como emamectina, piretroides y organofosforados, los que a medida que son utilizados pierden eficacia debido a la aparición de resistencia farmacológica [130, 131]. Este hecho enfatiza la necesidad de investigar y desarrollar nuevas estrategias de control de la Caligidosis. Debido a que la mayoría de los ectoparásitos se alimentan desde la sangre de su hospederos, éstos están expuestos a componentes del sistema inmune del hospedador, lo que hace de las vacunas una alternativa para el control de ectoparásitos [4]. Por ejemplo, desde un estudio de interacción parásito-hospedero en *Rhipicephalus (Boophilus) microplus*, se identificaron y testearon como antígenos las proteínas Q38, SILK y Subolesin, reportando una disminución entre 60 y 75% la infestación. Adicionalmente, se evidenció una correlación positiva entre el título de anticuerpo específico para cada antígeno y la reducción de la carga parasitaria, además de una reducción en la generación de nuevos huevos [132]. Otro ejemplo, son las vacunas que han sido propuestas para el control de *L. salmonis,* entre ellas la proteínas tripsina, secretada por el copépodo en el momento de alimentarse desde su hospedero. El uso de tripsina recombinante como vacuna contra *L. salmonis* ha mostrado una disminución del 20% en la carga parasitaria luego de seis semanas de ser administrada, afectando principalmente, a los estadios copepoditos y chalimus [133, 134]. Otro antígeno que ha sido

evaluado en *L. salmonis* es vitelogenina, donde los peces vacunados con la proteína recombinante presentaron una reducida prevalencia de hembra y escaso daño tisular [134]. Akirin, factor nuclear involucrado en la respuesta inmune innata, es otro de los antígenos que ha sido propuesto para el control de la Caligidosis. En salmones del Atlántico vacunados con la recombinante de akirin se ha reportado un 57% de reducción de la carga parasitaria luego de 24 días de infestación, además de observar un incremento en los niveles de IgM en los peces inmunizados [77, 79]. Otra alternativa de antígeno para el control de extoparásitos, que ha sido testaeada el ectoparásito *Haemaphysalis longicornis*, es ferritina. En *Haemaphysalis longicornis* se ha reportado que existen dos isoformas de ferritina que tienen incidencia en el proceso reproductivo de este ectoparásito, expresándose en ovoductos y superficie de ovocidos. Se ha observado que el bloqueo de estas isoformas de ferritina generan muerte del ectoparásito, los que no logran generar huevos, además de reducir los niveles de expresión de vitelogenina [47, 135]. En este estudio, debido a la importancia que presenta la regulación del hierro en la interacción caligus-salmón, y el efecto del bloqueo de ferritina en *Haemaphysalis longicornis*, se decidio probar una vacuna en base a ferritina identificada en *C. rogercresseyi*. La evaluación de la vacuna se llevo acabo en salmón del Atlántico los que fueron inmunizados por 30 días, y luego infestados con *C. rogercresseyi.* Posteriormente y luego de 25 días de infestación, se observó una alta eficacia de nuestra vacuna reduciendo en un 97 % la carga parasitaria. Además, se observó daño en los sacos ovígeros de las hembras de Caligus recolectadas desde peces vacunados con *Cr_ferritina*, las que mostraron una disminución en la expresión de vitelogenina, proteína esencial para el desarrollo de los huevos. Similar resultado fue observado en conejos inmunizados con ferritina de *Haemaphysalis longicornis*, los que presentaron una reducción en la carga parasitaria, además de reducción de la viabilidad de los huevos [135]. Los resultados de este estudio en torno al uso de ferritina como antígeno para el control de *C. rogercresseyi*, demuestran que esta proteína es una candidato potencial para la evaluación clínica de una vacuna basada en *Cr-ferritina.* De esta forma, el presente estudio se plantea como una excelente alternativa para el control de la Caligidosis al reducir la carga parasitaria en los peces y afectar el proceso de fecundación de las hembras de *C. rogercresseyi.*

CONCLUSIONES

En relación a la primera hipótesis planteada en torno a cambios de perfiles transcriptómcos en vías de señalización molecular relevantes para el proceso de interación parásito/hospedero. Se establecieron diferencias en los mecanismos de respuesta de dos especies de salmónidos a la presencia de *C. rogercresseyi.* Además, se demuestra que existen diferencias en los mecanismos de respuesta inmune de salmónidos infestados con *C. rogercresseyi,* a las previamente reportadas en salmones infestados por *L. salmonis*. Del análisis de los mecanismos inmunes asociados a la infestación por *C. rogercresseyi*, se observó por primera vez para esta enfermedad, procesos de regulación asociados a la respuesta inmune innata mediada por vías de señalización reguladas por receptores tipo Toll, en salmón del Atlántico. Mientras que para salmón Coho los mecanismos de respuesta inmune que son escencialmente regulados están asociados a respuesta pro-inflamatoria. Adicionalmente, se observó que existen mecanismos de respuesta inmune nutricional vinculados a la infestación por este ectoparásito. Estos mecanismos de respuesta inmune nutricional estarían dados principalmente por la competencia por hierro entre salmón del Atlántico y Caligus, lo que es reflejado en el incremento de los niveles de expresión de ferritina tanto en el ectoparásito como en su hospedero.

En relación a la segunda hipótesis que planteaba el rol de los RNA no codificantes en el proceso de interacción parásito-hospedero, por primera este estudio demuestra cambios de expresión de lncRNA y miRNA identificados en salmón durante el proceso infestivo con *C. rogercresseyi*. Se sugiere que en salmón del Atlántico los lncRNA expresados durante la infestación tendrían un rol fundamental en la regulación de la respuesta inmune durante la infestación con *C. rogercresseyi*. Mientras que en salmón Coho los lncRNA mayormente regulados durante la infestación con *C. rogercresseyi*, se regularian procesos de regeneración tisular. Aun más, se obtuvo el miRNoma de salmón del Atlántico infestado por *C. rogercresseyi*, observando gran abundancia de miRNA de las familias mir-21 y mir-181, las que además tendrían como blanco de regulación genes como *tlr22a2* e *INF-g*. Adicionalmente, desde el miRNoma de *C. rogercresseyi* se identificó un miRNA altamente expresado durante el proceso de infestación, que sería capaz de regular la expresión de genes del hospedero como *tlr22a2* e *INF-g*. Lo que es un indicio de la existencia de mecanismos de interacción

parásito/hospedero regulada por miRNA. Sin embargo, aun es necesario realizar algunas pruebas funcionales que permitan sustentar este posible mecanismo de interacción entre *C. rogercresseyi* y su hospedero.

Finalmente, en base a las observaciones de competencia por el hierro entre *C. rogercresseyi* y salmón del Atlántico asociado al incremento de la expresión de ferritina. El estudio presenta una nueva herramienta para el control de la Caligidosis, como es el desarrollo de una vacuna recombinante en base a la proteína ferritina, caracterizada desde *C. rogercresseyi,* en donde peces inmunizados con esta proteína presentan un porcentaje de reducción de la carga parasitaria de un 97 %, además de un efecto sobre el proceso reproductivo de Caligus, al generar hembras con sacos ovigeros deformes.

Este estudio explica las principales vías de señalización molecular que se ven involucradas en el proceso de interacción *C. rogercresseyi*-salmonidos. Es el primer estudio en incorporar identificación y análisis de RNA no codificantes como moduladores de la respuesta del hospedador. Además, deja abierta la interrogante en relación a como los miRNA del ectoparásito podrían intervenir en la respuesta del hospedero, como un mecanismo de interacción similar a lo descrito en algunos insectos. Estos resultados junto con estudios funcionales permitiría el desarrollo de nuevas herramientas para el control de la Caligidosis y otras enfermedades que afectan a la industria salmonicultora.

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