Centro de Investigación en Alimentación y Desarrollo, A.C.

INTERACCIONES MOLECULARES ENTRE EL PARÁSITO *Rhabdosynochus viridisi* Y SU HOSPEDERO *Centropomus viridis*

Por:

Marian Mirabent Casals

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Los miembros del comité designado para la revisión de la tesis de Marian Mirabent Casals la han encontrado satisfactoria y recomiendan que sea aceptada como requisito parcial para obtener el grado de Doctora en Ciencias.

Dra. Alejandra García Gasca
Directora de Tesis

Dr. Francisco Neptalí Morales Serna
Co-director de tesis

Dr. Juan Manuel Martínez Brown
Integrante del comité de tesis

Dr. Anaguiven Avalos Soriano
Integrante del comité de tesis

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DEDICATORIA

"…al Rey eterno, inmortal, invisible, al único y sabio Dios, sea honor y gloria
por los siglos de los siglos…"

A mi esposo Jorge Luis Batista Roche por ser mi gran amigo y colaborador en Cristo.
Juntos por la fe (certeza de lo que esperamos y convicción en lo que no vemos)
alcanzamos las promesas.

A mis padres Magaly Casals Hung y Jordi Mirabent Ávila,
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y disfrutar al mismo tiempo de la paternidad y la maternidad

A toda nuestra familia y amigos por su apoyo incondicional

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RESUMEN

El robalo blanco del Pacífico *Centropomus viridis* es una especie de pez adecuada para la maricultura en jaulas. Se ha documentado que el parásito monogeneo *Rabdosynochus viridisi* es el agente causal de la mortalidad de reproductores y juveniles de robalo en el noroeste de México. El objetivo general de este trabajo fue dilucidar las principales estrategias de penetración, fijación y modulación de la respuesta inmune que emplea el parásito *R. viridisi* para establecerse en el epitelio branquial del robalo. Con el uso de herramientas bioinformáticas se identificaron y anotaron funcionalmente las proteínas secretadas al medio extracelular (secretoma) por el parásito. Se identificaron posibles proteínas multifuncionales y proteínas *Venom Allergen-Like*, cuya homología de secuencia sugiere su participación en estrategias de evasión inmune. Se realizó un experimento de infección para evaluar la expresión de genes del sistema inmune en branquias de robalos. Las muestras de branquias se enviaron a Azenta (USA) para la secuenciación de ARN. Se usó el algoritmo Seq2Fun para la obtención de perfiles funcionales de los datos transcriptómicos sin genoma de referencia. ExpressAnalyst se utilizó para determinar la expresión diferencial de genes, realizar los análisis estadísticos y visualizar los resultados. Se encontraron un total de 20,106 transcritos y 1,430 (7%) resultaron ser genes expresados diferencialmente (GEDs) entre el grupo infectado y el control. De ellos, 860 genes estuvieron sub-regulados y 570 sobre-regulados. Aunque las citocinas proinflamatorias permanecieron sin cambios en las branquias infectadas de *C. viridis* y el factor de crecimiento transformante β (*tgfβ*) se reguló negativamente, los ligandos de interleucina-17 *il17d* e *il17a/f1* se sobreexpresaron, lo que indica que la infección con *R. viridisi* promueve la inmunidad similar a Th17. En este estudio también se detectó la sobreexpresión de marcadores de actividad de células B plasmáticas, como genes similares a cadenas ligeras de inmunoglobulina y la cadena ligera sustituta 3 de células pre-B v-set (*vpreb3*). Se discuten las posibles implicaciones de los GEDs relacionadas con el desequilibrio iónico, la adaptación a la hipoxia, la hemostasia y la inmunidad. En conjunto, los resultados sugieren que la infección con *R. viridisi* mantiene un microambiente antinflamatorio en las branquias de *C. viridis*, y promueve la expresión de genes asociados con la proliferación de células B plasmáticas.

Palabras clave: secretoma, Monogenea, RNA-seq, inmunidad

ABSTRACT

The Pacific white snook *Centropomus viridis* is a fish species suitable for cage mariculture. The monogenean parasite *Rabdosynochus viridisi* has been documented to be the causal agent of mortality of snook broodstock and juveniles in northwest Mexico. The general objective of this work was to elucidate the main strategies of penetration, fixation and modulation of the immune response used by *R. viridisi* to establish itself in the gill epithelium of snook. The proteins secreted into the extracellular environment (secretome) by the parasite were identified and functionally annotated with the use of bioinformatics tools. Possible multifunctional proteins and Venom Allergen-Like proteins, with cysteine-rich domains, were identified *in silico*, whose sequence homology suggests their participation in immune evasion strategies. An experimental infection was carried out to evaluate the expression of immune system genes in snook gills. Gill samples were sent to Azenta (USA) for RNA sequencing. Seq2Fun algorithm was used to obtain functional profiles of the transcriptomic data without reference genome. ExpressAnalyst was used to determine differential gene expression, perform statistical analyses, and visualize the results. A total of 20,106 transcripts were found and 1,430 (7%) were differentially expressed genes (DEG) between infected and control groups. We identified 860 downregulated and 570 upregulated genes. Even though the well-known pro-inflammatory cytokines remained unchanged in infected gills of *C. viridis* and transforming growth factor β (*tgfβ*) was downregulated, interleukin-17 ligands *il17d*, and *il17a/f1* genes were upregulated, indicating that Th17-like immunity is promoted by the infection with *R. viridisi*. Overexpression of plasma B cell activity markers such as immunoglobulin light chain-like genes and the v-set pre-B cell surrogate light chain 3 (*vpreb3*) was also detected in this study. The possible implications of DEGs related to calcium imbalance, hypoxia adaptation, hemostasis and immunity are discussed. Altogether, the results suggest that the infection with *R. viridisi* maintains an anti-inflammatory microenvironment in infected gills of *C. viridis* and promotes the expression of genes associated with plasma B cell proliferation.

Keywords: secretome, Monogenea, RNA-seq, immunity

1. SINOPSIS

1.1. Justificación

Los monogeneos son platelmintos con alta tasa reproductiva y especificidad hospedera, distribución global y abundancia en zonas tropicales (Klapper *et al.,* 2017). Algunos monogeneos parásitos amenazan la acuicultura, causando pérdidas económicas significativas por mortalidad, menor crecimiento y reproducción de peces en cultivo tales como la tilapia del Nilo (*Oreochromis niloticus*), la cobia (*Rachycentron canadum*), la seriola (*Seriola dumerili*) y la dorada (*Sparus aurata*) (Hoai, 2020; Amakali *et al.,* 2023; Radwan *et al.,* 2024; Riera-Ferrer *et al.,* 2024). Se ha documentado que varios monogeneos del género *Rhabdosynochus* infectan las branquias de robalos, *Centropomus* spp. (Carangiformes: Centropomidae) silvestres y cultivados (Kritsky *et al.,* 2010; Montero-Rodríguez *et al.,* 2021).

El robalo blanco del Pacífico (*Centropomus viridis*) es una especie candidata y adecuada para la acuicultura a escala comercial en jaulas marinas (Giovanni *et al.,* 2022). En particular, en el noroeste de México, el monogeneo *Rhabdosynochus viridisi* es una especie patógena que causa mortalidad en reproductores de *C. viridis* (Morales-Serna *et al.,* 2020). Los juveniles de robalo cultivados en laboratorio y a escala piloto se han visto afectados por *R. viridisi* (Morales-Serna F.N. obs. pers.). Sin embargo, la patogénesis no ha sido investigada; excepto por un estudio reciente en el que se demostró que la infección experimental de juveniles de robalo con *R. viridisi* provoca alteraciones histológicas en las branquias de los peces tales como fusión de lamela secundaria, infiltración de células mononucleares inflamatorias e hiperplasia (López-Moreno *et al.,* 2024).

En la actualidad, existe un interés creciente por comprender las interacciones moleculares entre peces de cultivo y los parásitos que los afectan con el fin de encontrar tratamientos efectivos. Por ejemplo, el desarrollo de vacunas antiparasitarias en peces requiere de una mejor comprensión de las interacciones parásito-hospedero (Shivam *et al.,* 2021). La identificación de candidatos vacunales para enfermedades parasitarias se ha visto limitada en peces (Kar *et al.,* 2022). Por eso, el estudio de posibles proteínas que podrían actuar como factores de virulencia (Riera-Ferrer *et al.,* 2024) o blancos de fármacos (Caña-Bozada *et al.,* 2022) en monogeneos, así como la

caracterización de la respuesta inmune de los peces infectados favorecerá la implementación futura de estrategias preventivas y de control de parásitos para una acuicultura sostenible y de calidad para el consumo humano.

1.2 Antecedentes

En los últimos 25 años, de acuerdo con los registros en PubMed, ha habido un aumento en la literatura científica relacionada con estudios en peces marinos (**Figura 1**), con 1288 artículos haciendo referencia a al menos un parásito de la clase Monogenea. De estos, sólo 11 estudios se centran en la expresión genética del parásito, mientras que 30 evaluaron los genes expresados en peces durante la infección con parásitos monogeneos (**Figura 2**).

La taxonomía de los monogeneos es compleja y está sujeta a continuos cambios según se integran datos morfológicos y marcadores moleculares en los estudios (Hossen *et al.*, 2022). Hasta la fecha, la clasificación taxonómica de los monogeneos, según WoRMS (https://www.marinespecies.org/) es:

Reino: Animalia
Phylum: Platyhelminthes
Subphylum: Rhabditophora
Superclase: Neodermata
Clase: Monogenea
Subclase: Monopisthocotylea
Orden: Capsalidea, Dactylogyridea, Gyrodactylidea, Monocotylidea, Montchadskyellidea
Subclase: Polyopisthocotylea
Orden: Chimaericolidea, Diclybothriidea, Mazocraeidea, Polystomatidea

Sin embargo, Brabec y colaboradores (2023), mediante análisis filogenómicos con los transcriptomas de nueve especies de monogeneos, propusieron elevar a Monopisthocotyla y Polyopisthocotyla a nivel de clase, lo que implica la desaparición de la clase Monogenea. Ambos linajes incluyen ectoparásitos. La mayoría de las especies de Monopisthocotylea presentan un

haptor simple con una sola ventosa posterior y se alimentan generalmente de tejido epidérmico y moco del hospedero, mientras que los integrantes de Polyopisthocotylea, tienen varias ventosas posteriores y son hematófagos (se alimentan de sangre).

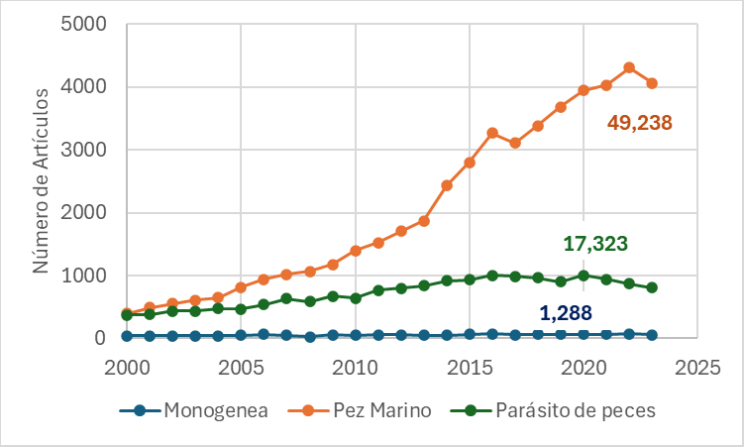

Figura 1. Artículos de investigación registrados en la base de datos PubMed desde enero de 2000 hasta el 31 de diciembre de 2023. Las palabras clave utilizadas para la recopilación de datos fueron: "Monogenea", "marine fish" y "fish monogenean parasite".

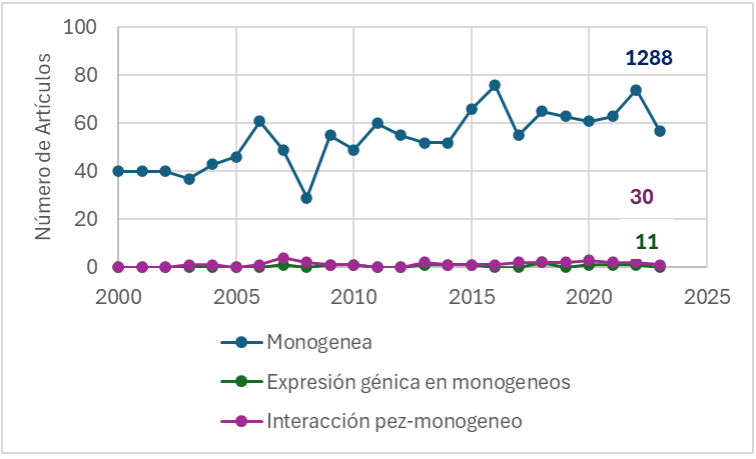

Figura 2. Artículos de investigación registrados en la base de datos PubMed desde enero de 2000 hasta enero de 2024 relacionados con la expresión genética. Las palabras clave utilizadas para la recopilación de datos fueron: "fish monogenean parasite", "gene expression in Monogenea" y "gene expression in fish infected with monogenean parasites".

Los monogeneos presentan tres secciones corporales: región cefálica, tronco y opistohaptor. El prohaptor es anterior y tiene funciones de alimentación y sensoriales, mientras que el opistohaptor

es posterior y es el principal órgano adhesivo para fijar al hospedero. Según la especie, el opistohaptor puede presentar ganchos, ventosas o pinzas (Drago y Nuñez, 2015). El alto grado de especificidad hospedera se ha asociado a adaptaciones especiales del opistohaptor y de la reproducción (Klapper *et al.,* 2017), aunque algunos monogeneos son generalistas (Lim *et al.*, 2016). Se han observado varias estrategias reproductivas en los monogeneos, tales como la oviparidad, la viviparidad y la autofecundación. La mayoría de los ovíparos depositan menos de 100 huevos/parásito/día, aunque algunas especies pueden producir más de 550 huevos/parásito/día. Por otro lado, los vivíparos, pueden desarrollarse y producir millones de crías en unas pocas semanas. La autofertilización ocurre en especies que infectan la vejiga de los anfibios y asegura el potencial reproductivo cuando un parásito se encuentra solo en un hospedero (Dinh Hoai y Hutson, 2014).

Los parásitos monogeneos son conocidos por su movilidad y capacidad de adhesión a los tejidos (Whittington y Cribb, 2001). En particular, los monopistocotileanos se mueven sobre la epidermis del cuerpo y branquias de los peces, para lo cual secretan moléculas adhesivas desde el extremo anterior. Algunos secretan estas moléculas desde el extremo posterior para complementar o reemplazar la fijación mecánica mediante ganchos y/o succión (Whittington y Cribb, 2001). Se ha empleado la microscopía óptica y electrónica para caracterizar la estructura de las células secretoras y sus productos implicados en la unión de los parásitos monogeneos *Bravohollisia rosetta* y *B. gussevi* a sus peces hospederos. Se han observado células glandulares pedunculares con dos núcleos, retículo endoplásmico granular y complejo de Golgi, las cuales producen vesículas extracelulares (VE). Estas vesículas se alteran a medida que migran desde la célula glandular hacia el reservorio haptoral y luego hacia los tejidos branquiales. El contenido de las VE se vierte hacia los reservorios mediante exocitosis. Las secreciones, introducidas por medio de los ganchos en los tejidos del hospedero, probablemente ayudan a la fijación. Las secreciones se manifiestan externamente como estructuras en forma de red y permanecen adheridas al punto de exudación, de tal forma que, a pesar de haber quitado los ganchos, los parásitos pueden permanecer anclados a los tejidos branquiales a través de estas estructuras en forma de red. Con base en esto, se postula que las secreciones en forma de red probablemente funcionen como una línea de seguridad para anclar al parásito durante el inicio de la locomoción y reducir el riesgo de desgarrar los tejidos del hospedero (Wong *et al.,* 2008).

El papel de las VE en relación con los parásitos monogeneos aún no se ha explicado

completamente, pero el análisis del transcriptoma de *Eudiplozoon nipponicum* adulto identificó varios transcritos asociados con el transporte vesicular extracelular, lo que indica que las VE podrían desempeñar un papel en las interacciones entre el hospedero y *E. nipponicum* (Vorel *et al.*, 2021). Las VE se clasifican en exosomas largos (150-600 nm) y pequeños (50-150 nm) y están delimitados por sus membranas de origen. Las VE contienen diversas moléculas, tales como proteínas, ácidos ribonucleicos (ARN) no codificantes largos y microARNs (Meldolesi, 2019), las cuales desempeñan un papel crucial en la interfaz parásito-hospedero, facilitando la infección y la supervivencia del parásito (Coakley *et al.,* 2015). Se ha postulado que la exocitosis de material sincitial (gránulos secretores, vesículas y vacuolas) constituye un mecanismo de protección de algunas especies de monogeneos poliopistocotileanos contra daños inmunológicos, iónicos y osmóticos causados por la secreción de moco branquial. En *Sparicotyle chrysophrii*, la observación de EV en la interfase tegumento-sincitio-membrana laminar podría sugerir un mecanismo protector (Riera-Ferrer *et al.,* 2024).

La combinación de diferentes técnicas microscópicas como la microscopía de barrido láser confocal, la microscopía electrónica de transmisión y la microscopía electrónica de barrido ha permitido localizar varios tipos de vesículas y cuerpos secretores/excretores, incluidos los adheridos a las membranas superficiales del tegumento en *E. nipponicum*. Se observaron glándulas unicelulares gigantes acumulándose predominantemente en la región apical y del haptor. La capa muscular se organizó con los músculos circulares externos y longitudinales internos entretejidos en forma de cesta. Se observaron también músculos perpendiculares adicionales anclados al tegumento. Se detectaron abundantes músculos dentro de las crestas tegumentarias, que presumiblemente ayudan a fijar el parásito entre las laminillas branquiales (Valigurová *et al.*, 2021). La **Figura 3** esquematiza la presencia de glándulas unicelulares que producen VE cerca del haptor, así como el proceso de endocitosis por las células hospederas.

Las moléculas secretadas por los parásitos monogeneos pueden interactuar con las respuestas inmunitarias del hospedero (Riera-Ferrer *et al.,* 2024). Ilgová y colaboradores (2021) revisaron los principales mecanismos inmunomoduladores de los monogeneos y las respuestas inmunitarias de los peces hospederos, encontrando que la mayoría de las investigaciones se centran en los mecanismos de defensa del hospedero más que las moléculas específicas del parásito involucradas en la interacción. Existen estudios que se enfocan en dilucidar los genes en el hospedero involucrados en la resistencia a los parásitos monogeneos mediante los análisis QTL (Quantitative

Trait Locus, por sus siglas en inglés). Se ha documentado que algunos QTLs se localizan en grupos de ligamiento que contienen las regiones de los receptores tipo Toll (TLR) y los complejos principales de histocompatibilidad (MHC I y MHC II), importantes en las respuestas inmunes innata y adaptativa (Ozaki *et al.*, 2013).

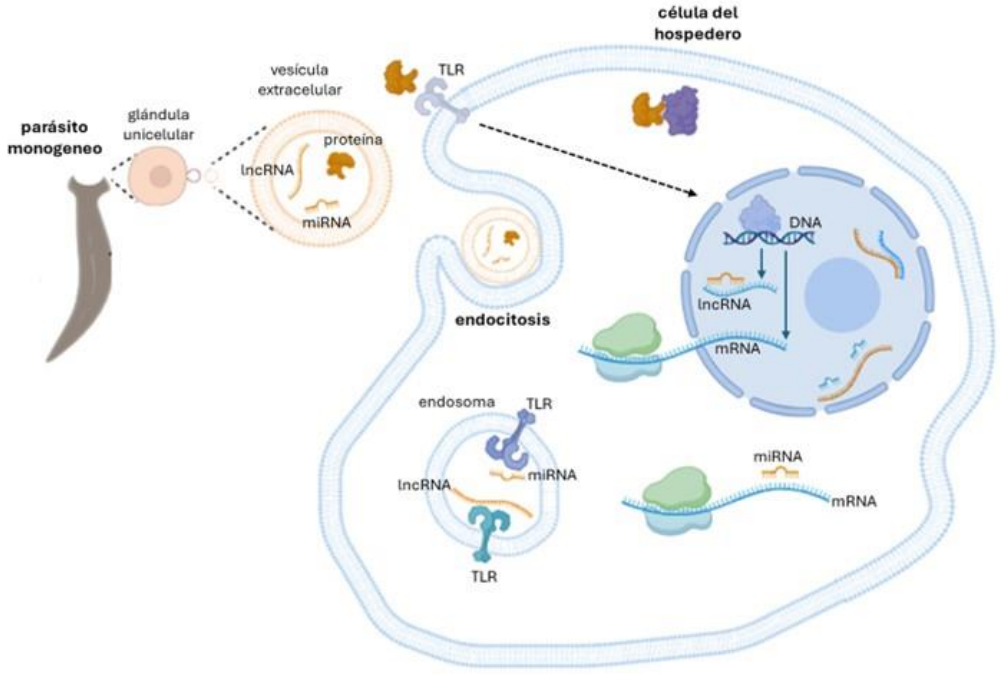

Figura 3. Interfaz del parásito monogeneo y una de las células del hospedero. lncRNA: ARN no codificante largo; miRNA: microRNA; TLR: receptor tipo Toll; mRNA: ARN mensajero; DNA: ácido desoxirribonucleico. Los parásitos monogeneos presentan glándulas unicelulares que producen vesículas extracelulares con diferentes biomoléculas en su interior. Una vez endocitosis por células del hospedero, las vesículas liberan su contenido a los endosomas o al citoplasma. Las biomoléculas del parásito como los miRNAs, lncRNAs y proteínas secretadas pueden interactuar con los TLRs u otros receptores de la célula hospedera y promover la expresión de genes. También pueden silenciar genes a través de mecanismos que involucran los lncRNA y miRNA. Elaboración propia.

Los TLR son una familia crucial de receptores que actúan en la primera línea de defensa de la inmunidad innata. Los TLR se activan a través de la unión a patrones moleculares asociados a daños (DAMPs) que son moléculas endógenas peligrosas liberadas por células y tejidos dañados, patrones moleculares asociados a patógenos (PAMPs) que incluyen las moléculas de la superficie o que liberan los patógenos extracelulares, así como a patrones moleculares asociados a

xenobióticos (XAMPs). Los TLR desempeñan un papel clave en la vinculación de la inmunidad innata y adaptativa (El-Zayat *et al.*, 2019). Los TLR son glicoproteínas transmembranales de tipo I que se componen del dominio repetido extracelular rico en leucina (LRR), el dominio de transmembrana (TM) y un dominio citoplasmático del receptor Toll/interleucina-1 (TIR). El dominio LRR reconoce componentes bacterianos y virales, incluidos lípidos, lipoproteínas, proteínas y ácidos nucleicos. Posterior a la estimulación con PAMP, el dominio TIR intracelular recluta una serie de proteínas adaptadoras (ej. TRIL: Toll-like receptor adaptor molecule 2) y activa cascadas de señalización inmune, incluidas las vías de respuesta primaria de diferenciación mieloide 88 (MyD88) dependiente e independiente de MyD88 (Nie *et al.*, 2018). Luego, se activan factores de transcripción como el factor nuclear kappa, potenciador de la cadena ligera de células B activadas (NF-κB), proteína quinasa activada por mitógenos (MAPK), proteína activadora-1 (AP-1) y miembros de la familia reguladora de interferón (IRF), lo que resulta en la producción de citocinas inflamatorias, quimiocinas y/o péptidos antimicrobianos (Shan *et al.*, 2021).

En los últimos años, la identificación de 21 tipos de TLRs (TLR1, TLR2, TLR3, TLR4, TLR5, TLR5S, TLR7, TLR8, TLR9, TLR13, TLR14, TLR18, TLR19, TLR20, TLR21, TLR22, TLR23, TLR25, TLR26, TLR27, y TLR28) en varias especies de peces ha despertado el interés en explorar el potencial de los TLR como dianas para aumentar la inmunidad y la resistencia a las enfermedades en los peces. Los TLR se clasifican según su localización celular, en TLR de la membrana celular (TLR2-1, TLR2-6, TLR4, TLR5 y TLR10) y en TLR intracelulares o sensores de ácidos nucleicos (TLR3, TLR7, TLR8 y TLR9), detectados en el retículo endoplásmico (RE), endosomas y lisosomas (Mahapatra *et al.*, 2023). Algunos peces teleósteos tienen los ortólogos de TLR de mamíferos (TLR1, TLR2, TLR3, TLR5, TLR7, TLR8 y TLR9) y otros TLR específicos de peces (TLR5, TLR14, TLR18-20 y TLR22-28 solubles) (Shan *et al.*, 2021).

Los TLR se expresan tanto en las células inmunes como en las no inmunes. En peces, se ha observado la expresión diferencial de TLR tras la infección con diversos patógenos. Por ejemplo, en la carpa común (*Cyprinus carpio*), la expresión de TLR19 aumentó significativamente en los órganos relacionados con el sistema inmunológico después de la infección experimental con la bacteria *Aeromonas hydrophila* (Shan *et al.*, 2021). En el pez cebra (*Danio rerio*), después de la inmunización parenteral con micobacterias inactivadas, se detectó un aumento significativo en la expresión de *tlr2* y *tlr4* (Ferreras-Colino *et al.*, 2024). La expresión de *tlr2*, *tlr9* y *tlr19* aumentó en branquias de *Ictalurus punctatus* luego de la infección con el parásito ciliado *Ichthyophthirius*

multifiliis (Zhao *et al.*, 2013). Por otro lado, se observó la sobreexpresión de los genes *tlr4, tlr5, tlr20* y *tlr22* en *Carassius auratus* después de la infección con el monogeneo *Dactylogyrus intermedius* (Tu *et al.*, 2016). Sin embargo, el impacto específico de los parásitos monogeneos en la expresión de TLR en branquias de peces no está bien documentado y requiere más investigación.
Los MHC clase I codifican proteínas que se expresan en la superficie de todas las células nucleadas, es decir, la mayoría de las células somáticas, y presentan péptidos de antígenos intracelulares. Los MHC clase II codifican proteínas que presentan péptidos derivados de antígenos extracelulares y se expresan en las células presentadoras de antígenos (APCs, por sus siglas en inglés) como las células dendríticas, células de Kupffer, linfocitos B y células mieloides derivadas de monocitos, así como en las células presentadoras de antígenos no profesionales: células endoteliales sinusoidales del hígado, hepatocitos, células estrelladas hepáticas, colangiocitos, fibroblastos, células endoteliales linfáticas, células endoteliales sanguíneas, mastocitos y neutrófilos (Mehrfeld *et al.*, 2018; Santambrogio *et al.*, 2019). Los MHC se encuentran sólo en vertebrados con mandíbulas. Las estructuras de los MHC clase II probablemente representan la estructura ancestral de las moléculas MHC. Yamaguchi y Dijkstra (2019) propusieron un modelo para la evolución temprana de los MHC, en el cual, la molécula MHC ancestral, se derivó de una molécula tipo inmunoglobulina con solo la cadena pesada, que se cicló entre la superficie celular y el compartimiento endosomal. Los genes MHC muestran una notable variación en el número de copias tanto inter- como intra-específicas (Bentkowski y Radwan, 2019). El polimorfismo de los MHC aparentemente evoluciona de manera adaptativa, debido a la alta tasa relativa de sustituciones no sinónimas dentro del sitio de unión a los péptidos derivados de antígenos. Se ha planteado que el polimorfismo está impulsado por la coevolución con los patógenos que se seleccionan para evadir el reconocimiento por las proteínas del MHC. Las evidencias apuntan a que los patógenos imponen una selección significativa en los MHC e impulsan los cambios en las frecuencias alélicas de los MHC en las poblaciones naturales. Sin embargo, los mecanismos de selección específicos que dan forma a la extraordinaria diversidad de genes MHC siguen siendo controversiales (Radwan *et al.*, 2020).
La función principal de los MHC clásicos es la presentación de antígenos. Aunque en la vía de procesamiento y presentación de antígenos participan diversas moléculas, los MHC de clases I y II tienen el rol principal. Estas moléculas tienen una función similar: presentan péptidos cortos en la superficie celular. Las diferencias entre ambas radican en el origen de los péptidos que presentan

y en las células receptoras que son activadas. Los MHC I presentan péptidos de fuentes endógenas o intracelulares, mientras que los MHC II presentan péptidos derivados de antígenos exógenos o extracelulares. También existe la denominada presentación cruzada, en la que las moléculas MHC I pueden presentar antígenos exógenos. Además, los antígenos endógenos pueden ser presentados por MHC II cuando se degradan mediante autofagia (Kelly y Trowsdale, 2019; Nesmiyanov, 2021). Las moléculas MHC I se ensamblan en el retículo endoplasmático (RE) y como son inestables en ausencia del ligando peptídico, requieren de la asociación de varias proteínas. La cadena pesada se estabiliza por la chaperona calnexina, previo a su asociación con la β-2 microglobulina. Luego, las proteínas calreticulina, Erp57, proteína disulfuro isomerasa y tapasina, estabilizan las moléculas ensambladas en el lumen del RE. La tapasina interactúa con el transportador asociado a la presentación de antígenos (TAP), el cual transloca los péptidos desde el citoplasma al lumen del RE. Antes de entrar el RE, los péptidos antigénicos se originan de la degradación de proteínas propias defectuosas o de origen viral, mediada por los proteasomas (el proteasoma 26S que se expresa en la mayoría de las células o el inmunoproteasoma de las células inmunes principalmente), con o sin la participación de las ubiquitinas. Las proteínas intracelulares también se pueden degradar en vías alternativas al proteasoma-TAP como la escisión por proteasas independientes o en cooperación con el proteosoma, la degradación asociada al ER y las rutas vesiculares asociadas a la autofagia y a la lisozima. Los péptidos pequeños, de hasta 18 aminoácidos, se introducen al RE mediante TAP y se escinden por las aminopeptidasas residentes del retículo endoplasmático ERAP1 y ERAP2, para optimizar la afinidad del péptido, estabilizar la estructura de la molécula de MHC I y permitir la liberación del complejo de carga de péptido. Luego, cada producto peptídico, generalmente de 8 a 10 aminoácidos, se carga en el surco de unión de la molécula MHC I con la ayuda del complejo de carga de péptido. El complejo péptido-MHC (pMHC) estable, atraviesa el complejo de Golgi y se transporta a la superficie celular donde puede ser reconocido por los receptores de las células inmunes, como el receptor de células T (TCR) de los linfocitos T CD8+ citotóxicos y el receptor similar a inmunoglobulinas (KIR) de las células asesinas naturales (NK) (D'Souza *et al.*, 2019; Perez *et al.*, 2019; Serçinoğlu y Ozbek, 2020).

El TCR contiene una región variable única que le permite discriminar entre los complejos pMHC antigénicos y los irrelevantes (incluyendo los propios), con considerable sensibilidad y especificidad (Wilcox, 2016). Una vez que el linfocito T reconoce al pMHC mediante el TCR y se forma el complejo TCR-p-MHC, se desencadenan respuestas contra la célula que presenta el

péptido antigénico. Los linfocitos T CD8, particularmente, se activan y se convierten en citotóxicos, debido a que liberan gránulos que contienen el ligando FAS, perforinas y granzimas. El ligando FAS se une a FAS y a través de los dominios proteicos de muerte activa la caspasa 8, que corta y activa a la caspasa 3 con la subsecuente inducción de apoptosis. Por otro lado, las perforinas forman canales porosos en la membrana de la célula a través de los cuales pasa la granzima, la cual entra al citoplasma y activa a la caspasa 3, induciendo así la apoptosis. De estas formas, los linfocitos T eliminan las células infectadas con microorganismos o con proteínas anómalas, mediante la activación de su propia maquinaria de autodestrucción (Golstein y Griffiths, 2018).

La función primaria de los MHC II es presentar péptidos derivados de la degradación de proteínas endocitadas a los linfocitos T CD4. Las cadenas nacientes sintetizadas en el RE, se unen a la cadena invariante li, que estabiliza el heterodímero. Luego, este complejo se transloca a través del complejo de Golgi a la vía endocítica en un compartimiento. Durante la acidificación en las vesículas endocíticas, se activan las proteasas lisosomales denominadas catepsinas S y L, las cuales digieren a li, dejando un péptido residual (CLIP) unido al MHC II. Posteriormente, el CLIP es intercambiado por el péptido antigénico, mediante las chaperonas HLA-DM y HLA-DO. Los péptidos presentados, provienen de proteínas endocitadas mediante fagocitosis, pinocitosis, endocitosis mediada por receptores, o incluso fagolisosomas de procesos de autofagia. Los complejos pMHC se presentan en la superficie a los linfocitos T CD4 para iniciar la respuesta inmune adaptativa (Jurewicz y Stern, 2019; Tumer *et al.*, 2021).

Las células T CD4 naïve se activan en los tejidos linfáticos luego de que se forma el complejo TCR-p-MHC y las APCs proveen las señales coestimuladoras en el contexto de una infección. Luego, las células T proliferan y se diferencian en subconjuntos específicos de los linfocitos T colaboradores (Th), los cuales efectúan distintas respuestas inmunes dirigidas contra el patógeno específico. Por ejemplo, los linfocitos Th1, Th2 y Th17, liberan citocinas que actúan sobre macrófagos y neutrófilos y potencian las respuestas inmunes innatas inflamatorias frente a los patógenos. Los Treg, reducen la inflamación e inducen tolerancia en células dendríticas para propiciar la reparación de tejidos. Por otro lado, los Tfh secretan interleucinas que promueven la maduración de la afinidad y cambio de clases de los anticuerpos en las células B, frente a patógenos extracelulares. Además, luego de que se erradica la infección, la mayoría de las células T efectoras se eliminan por apoptosis y persisten algunas células T CD4 de memoria que proveen una respuesta

aumentada frente a infecciones secundarias (Hilligan y Ronchese, 2020; Raphael *et al.*, 2020). La interconexión entre la respuesta innata y la adaptativa, se da gracias a la interacción de las células T CD4 activadas con las células B (Sun *et al.*, 2020).

En los peces, se considera que la función de las células T colaboradoras es similar a las de los mamíferos, por la presencia de TCRs, así como genes similares a los CD4. Las células que expresan CD4 se activan en presencia de patógenos y liberan citocinas. De las especies de peces estudiadas a la fecha, con excepción del bacalao del Atlántico (*Gadus morhua*), presentan péptidos en los MHC clase II a los linfocitos T CD4. Las células T CD4 en peces tienen diversas funciones como estimular a los macrófagos para promover su actividad microbicida, activar a las células B para la producción de anticuerpos e incrementar la inmunidad celular. Estas células también reclutan los neutrófilos, basófilos y eosinófilos al sitio de inflamación (Ashfaq *et al.*, 2019).

El sistema inmune innato de los peces, de manera similar a los mamíferos, es la primera línea de defensa que actúa rápidamente contra los patógenos en un corto periodo de tiempo y no provee protección duradera. Sin embargo, a diferencia de los mamíferos, la inmunidad innata en los peces es fundamental para prevenir la entrada de los patógenos. Esto es debido a la ineficiencia de la respuesta adaptativa, ya que los linfocitos de peces tienen una proliferación y maduración lenta, además del limitado repertorio de anticuerpos de baja afinidad y la poca complejidad de las estructuras de los órganos linfoides y consecuente formación de memoria (Kordon *et al.*, 2018).

Tanto los peces condrictios como los teleósteos carecen de médula ósea, el principal sitio de hematopoyesis en mamíferos, así como de los centros germinales especializados en la proliferación, diferenciación y selección de células B maduras con elevada afinidad de anticuerpos. En los peces teleósteos, la región principal de hematopoyesis es el riñón anterior (cefálico). En esta zona se encuentran los progenitores de células B y células plasmáticas, mientras que las células B maduras se localizan en el riñón posterior y en el bazo. Se ha propuesto que las células B maduras se liberan a la sangre, donde encuentran al antígeno y se diferencian en células plasmáticas, que luego migran al riñón cefálico. El bazo se considera el único órgano linfoide secundario en peces teleósteos y se ha sugerido que es el sitio de la estimulación antigénica. En los peces condrictios, el órgano de Leydig (una estructura glandular asociada al esófago) y el órgano epigonal (adherido a las gónadas) son los sitios principales de hematopoyesis y producción de células B. Los teleósteos y los condrictios desarrollan memoria inmunológica y varios estudios indican la presencia de anticuerpos neutralizantes inducidos frente a virus, bacterias y parásitos. Sin embargo, el tiempo

de respuesta de las IgM de teleósteos es más lento que en mamíferos (Smith *et al.,* 2019).

Las cadenas alfa de los MHC I se reconocen como elementos cruciales en la inmunidad adaptativa de vertebrados; por lo cual, los genes MHC Ia se han caracterizado en varios peces, aunque pocos estudios evidencian sus funciones en teleósteos. Los patrones de expresión del MHC Ia del lenguado japonés *Paralichthys olivaceus*, en condiciones fisiológicas normales y en retos experimentales con la bacteria intracelular *Edwardsiella tarda*, la bacteria extracelular *Vibrio anguillarum* y el virus infeccioso de necrosis del bazo y riñón (ISKNV, por sus siglas en inglés) mostraron que este gen se sobreexpresa durante la infección y que los niveles dependen de la naturaleza del patógeno, el tipo de tejido y el tiempo de infección. Se ha sugerido que el riñón cefálico, el bazo y las branquias juegan un rol importante en la respuesta inmune adaptativa mediante la vía del MHC I en *P. olivaceus*. La expresión máxima del MHC I inducida por el ISKNV fue superior a la inducida por las bacterias. Al parecer, el MHC I está involucrado en la presentación cruzada de los antígenos exógenos de *V. anguillarum* (Wang *et al.,* 2019). Por otro lado, el reto de la carpa (*Cyprinus carpio*) con el herpesvirus Koi, conllevó a una subexpresión de los ARNm de MHC Ia, quizás como mecanismo de evasión inmune del virus (Reichert *et al.,* 2016). Los niveles de expresión de MHC II y la carga parasitaria no variaron significativamente durante la infección primaria o re-infección del pez *Carassius auratus* con el parásito monogeneo *Gyrodactylus kobayashii*. Sin embargo, los peces moderadamente infectados mostraron una expresión elevada de los genes interleucina 1 beta (IL1β) y factor de necrosis tumoral alfa (TNFα), lo cual indica que la inmunidad innata es indispensable para controlar la infección parasitaria (Zhou *et al.,* 2018). Otro estudio indicó que los genes del MHC II se sobreexpresan en tejidos inmunes, piel y mucosa de las branquias de *Epinephelus coioides* infectado con el parásito protozoario *Cryptocaryon irritans*, lo que indica que la presentación de antígenos pudiera ocurrir intensamente en la piel y en las branquias infectadas, es decir, la respuesta inmune específica se da en los tejidos mucosales (Deng *et al.,* 2020).

Se han reportado alteraciones branquiales graves, como congestión sanguínea, hemorragia sanguínea, fusión de laminillas secundarias, elevación epitelial, erosión branquial y secreciones mucosas, en tilapia *Oreochromis niloticus* infectada con ectoparásitos (Suliman *et al.,* 2021). Se ha detectado la hipersecreción de moco neutro y el aumento de la diferenciación de las células caliciformes que conducen a una hiperplasia en las laminillas branquiales de *Sparus aurata* infectado con el monogeneo *Sparicotyle chrysophrii* (Riera-Ferrer *et al.,* 2024). Los tejidos de

branquias de *Sparus aurata* en contacto con *S. chrysophrii* mostraron regulación de menos genes (700) que las porciones de branquias que no estuvieron en contacto con el parásito (1235), muy probablemente debido a un efecto silenciador local del parásito. En ese mismo estudio se observó que los procesos de apoptosis, inflamación y proliferación celular predominaron en las branquias (Piazzon *et al.,* 2019). Recientemente, se ha observado la infiltración de células inflamatorias mononucleares e hiperplasia en el tejido branquial de *C. viridis* después de una infección experimental primaria con *R. viridisi* (López-Moreno *et al.,* 2024).

El número de estudios que evalúan la expresión de genes de monogeneos o peces infectados con monogeneos es limitado. De ellos, la mayoría se enfoca en los monogeneos hematófagos poliopistocotilideos. Por otro lado, las técnicas de secuenciación RNA-seq y cromatografía acoplada a espectrometría de masas posibilitan el estudio de transcriptomas y secretomas de los monogeneos para elucidar los genes y proteínas responsables de la invasión e inmunomodulación, u otros factores de virulencia (Vorel *et al.,* 2021). Varios marcadores puntuales evidencian que los monogeneos secretan proteínas que interfieren con la respuesta inmune del hospedero (Ilgová *et al.,* 2021). Teniendo en cuenta que el éxito de los ectoparásitos depende de los mecanismos de evasión del sistema inmune del hospedero para permanecer el mayor tiempo posible adheridos y que las proteínas tanto del hospedero como del parásito juegan un papel importante en dichos mecanismos, proponemos profundizar en las interacciones moleculares entre el parásito *R. viridisi* y su hospedero *C. viridis*, como sistema de equilibrio entre la acción parasitaria y la respuesta inmunológica.

1.5 Hipótesis

El ectoparásito *Rhabdosynochus viridisi* expresa proteínas asociadas a la evasión del sistema inmune del robalo (*Centropomus viridis*) y altera los niveles de expresión de genes pro-inflamatorios en las branquias.

1.3 Objetivo General

Dilucidar las principales estrategias de penetración, fijación y modulación de la respuesta inmune que emplea el parásito monogeneo *Rhabdosynochus viridisi* para establecerse en el epitelio branquial de su hospedero, el robalo *Centropomus viridis*

1.4 Objetivos Específicos

1. Identificar *in silico* las proteínas secretoras del parásito *R. viridisi* asociadas a la adhesión, proteólisis, inducción de apoptosis y potencialmente inmunomoduladoras
2. Evaluar los perfiles de expresión de genes pro- y anti-inflamatorios del robalo inducidos por la infección con *R. viridisi*
3. Caracterizar los Complejos Principales de Histocompatibilidad (MHC) y los receptores tipo Toll (TLR) del robalo *Centropomus viridis*

1.6 Sección Integradora

La presente tesis está integrada por cinco capítulos que incluyen: la sinopsis de la tesis (Capítulo 1), un artículo original publicado (Capítulo 2), un artículo de investigación original sometido a revisión para su publicación (Capítulo 3), el capítulo de resultados y discusión general (Capítulo 4); y finalmente las Conclusiones y Recomendaciones, la Bibliografía citada y los Anexos (secciones 5, 6, 7 y 8, respectivamente). Los artículos se presentan en el idioma que fueron publicados o enviados. En los siguientes párrafos se describen brevemente las publicaciones que forman parte de los capítulos 2 y 3 de esta tesis.

El primer artículo se titula “Predicted secretome of the monogenean parasite *Rhabdosynochus viridisi*: hypothetical molecular mechanisms for host-parasite interactions” y cubrió el primer

objetivo de la tesis. En esta publicación se documentan 1,655 proteínas secretoras (PS) identificadas *in silico* a partir del transcriptoma de adultos de *R. viridisi* aislados de robalos infestados en el Laboratorio de Parasitología del CIAD, Mazatlán. Se alinearon las secuencias con BlastX frente a la base de datos UniprotKB para la anotación funcional. Además, los dominios de las proteínas sin anotación se examinaron con Pfam y se analizaron las posibles rutas metabólicas en las que participan. Se encontraron proteínas posiblemente involucradas en la penetración (5 proteasas), la fijación (14 proto-cadherinas y 2 integrinas) y la regulación del estrés oxidativo (1 superóxido dismutasa, 3 oxidoreductasas, 1 peroxidasa y 2 oxidasas). Se identificaron seis supuestas proteínas multifuncionales y proteínas tipo Venom. ECPred predijo 223 enzimas (13.5%) y 1315 proteínas no enzimáticas (79.5%) del secretoma de *R. viridisi*. Encontramos predominancia de inhibidores de metalopeptidasas, así como una sobrerrepresentación de hidrolasas. Se predijo un total de 1045 (63%) proteínas antigénicas con un umbral de 0.5. Estos resultados sugieren que las proteínas secretoras de *R. viridisi* están involucradas en estrategias de evasión inmune y algunas pueden contribuir a la inmunogenicidad.

El segundo artículo titulado "Transcriptomic analysis of immune-related genes in Pacific white snook (*Centropomus viridis*) gills infected with the monogenean parasite *Rhabdosynochus viridisi*" y abarca el segundo y el tercer objetivo de la tesis. Como parte de este trabajo se realizó un ensayo de infección experimental con el objetivo de identificar los genes expresados diferencialmente (GEDs) en branquias de juveniles infectados con respecto al grupo control (no infectado). Veinticuatro días después de que se clocaron huevos de *R. viridisi* en los tanques del grupo infectado, se recolectaron los arcos branquiales más externos de los peces para la posterior secuenciación de ARN. El algoritmo Seq2Fun y la plataforma ExpressAnalyst se usaron para la obtención de los perfiles funcionales de los datos transcriptómicos, el análisis de expresión diferencial de genes, los análisis estadísticos y la visualización de los resultados. Se encontraron un total de 20,106 transcritos, de los cuales 1,430 (7%) fueron GEDs. De estos, 860 (60%) genes estuvieron sub-regulados y 570 (40%) sobre-regulados. Trece vías canónicas de la base de datos de la Enciclopedia de Genes y Genomas de Kioto (KEGG, por sus siglas en inglés) resultaron sobrerrepresentadas (FDR < 0.05), aun cuando la mayoría de los GEDs estuvieron sub-regulados, lo que sugiere la inactivación de las vías.

Las funciones de la mayoría de los GEDs con mayor cambio encontrados en este estudio no se comprenden bien en los peces. Aunque las principales citocinas proinflamatorias permanecieron

sin cambios en las branquias infectadas de *C. viridis* y el factor de crecimiento transformante β (*tgfβ*) se reguló negativamente, los ligandos de interleucina-17 *il17d* e *il17a/f1*, así como el receptor de quimiocina 2 con motivo CXC (*cxcr2*) presentaron regulación positiva, lo que indica que la infección con *R. viridisi* promueve la inmunidad similar a Th17. En este estudio también se detectó la sobreexpresión de marcadores de actividad de células B plasmáticas, como genes similares a cadenas ligeras de inmunoglobulina y la cadena ligera sustituta 3 de células pre-B v-set (*vpreb3*). Se detectó la expresión constitutiva de varios TLR, sin embargo, solo *tlr1* se encontró sobre-expresado en las branquias infectadas. Se concluye que las proteínas secretoras de *R. viridisi* y los daños ocasionados por el parásito alteran la expresión de genes relacionados con el desequilibrio del calcio, la adaptación a la hipoxia, la hemostasia y la proliferación de células B plasmáticas en branquias de *C. viridis*, pero mantienen un estado de inactivación de las respuestas pro-inflamatorias.

2. PREDICTED SECRETOME OF THE MONOGENEAN PARASITE *Rhabdosynochus viridisi*: HYPOTHETICAL MOLECULAR MECHANISMS FOR HOST-PARASITE INTERACTIONS

Marian Mirabent-Casals 1, Víctor Hugo Caña-Bozada 1, Francisco Neptalí Morales-Serna 2 and Alejandra García-Gasca 1

1 Centro de Investigación en Alimentación y Desarrollo, Avenida Sábalo Cerritos s/n, Mazatlán 82112, Sinaloa, Mexico

2 Instituto de Ciencias del Mar y Limnología, Universidad Nacional Autónoma de México, Av. Joel Montes Camarena s/n, Mazatlán 82040, Sinaloa, Mexico

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Predicted Secretome of the Monogenean Parasite *Rhabdosynochus viridisi*: Hypothetical Molecular Mechanisms for Host-Parasite Interactions

Marian Mirabent-Casals [1], Víctor Hugo Caña-Bozada [1], Francisco Neptalí Morales-Serna [2] and Alejandra García-Gasca [1,*]

[1] Centro de Investigación en Alimentación y Desarrollo, Avenida Sábalo Cerritos s/n, Mazatlán 82112, Sinaloa, Mexico
[2] Instituto de Ciencias del Mar y Limnología, Universidad Nacional Autónoma de México, Av. Joel Montes Camarena s/n, Mazatlán 82040, Sinaloa, Mexico
* Correspondence: alegar@ciad.mx

Abstract: Helminth parasites secrete several types of biomolecules to ensure their entry and survival in their hosts. The proteins secreted to the extracellular environment participate in the pathogenesis and anthelmintic immune responses. The aim of this work was to identify and functionally annotate the excretory/secretory (ES) proteins of the monogenean ectoparasite *Rhabdosynochus viridisi* through bioinformatic approaches. A total of 1655 putative ES proteins were identified, 513 (31%) were annotated in the UniProtKB/Swiss-Prot database, and 269 (16%) were mapped to 212 known protein domains and 710 GO terms. We identified six putative multifunctional proteins. A total of 556 ES proteins were mapped to 179 KEGG pathways and 136 KO. ECPred predicted 223 enzymes (13.5%) and 1315 non-enzyme proteins (79.5%) from the secretome of *R. viridisi*. A total of 1045 (63%) proteins were predicted as antigen with a threshold 0.5. We also identified six venom allergen-like proteins. Our results suggest that ES proteins from *R. viridisi* are involved in immune evasion strategies and some may contribute to immunogenicity.

Keywords: Platyhelminthes; genomics; peptidase; bioinformatics; secretome; marine fish

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1. Introduction

Monogeneans are ectoparasitic flatworms commonly found on the gills of marine and freshwater fish. Monogenean infections can cause excess mucosal secretion, epithelial damage, hemorrhage, osmotic problems, and gill atrophy leading to respiratory system failure [1]. In addition, lesions caused by monogenean parasites can facilitate secondary infections by bacteria, which can lead to the death of the fish host [2]. Some monogenean species belonging to the Diplectanidae family have been responsible for diseases and mortality in finfish aquaculture [3–6].

Several diplectanids of the genus *Rhabdosynochus* have been reported infecting the gill lamellae of wild and cultured snooks, *Centropomus* spp. (Perciformes: Centropomidae) [7,8]. Particularly, in northwestern Mexico, the diplectanid *Rhabdosynochus viridisi* has been reported causing mortality in a broodstock of Pacific white snook, *Centropomus viridis* [9]. This fish species is a well-suited candidate for marine cage aquaculture [10]. Additionally, snooks reared in laboratory and in a pilot commercial-scale farm have been affected by *R. viridisi* (Morales-Serna F.N. pers. obs.). Therefore, fish–parasite interactions should be better understood to support future research focused on the development of treatments to prevent or control monogenean infections in snooks.

Parasites secrete several biomolecules to ensure their entry and survival in the host. In particular, the proteins secreted to the extracellular environment, also known as secretome or excretory/secretory (ES) proteins, participate in the pathogenesis and immune

responses [11]. Although the secreted proteins could participate in the homeostasis of the parasite or its defense against other pathogens (e.g., part of the integument against bacteria), a critical role in the interaction with the host has been proposed. Some ES proteins induce apoptosis [12] or cause proteolysis in order to remodel tissues for invasion [13], and others act as immunomodulators to evade the host's immune system for parasite establishment and adaptation [14,15]. Immune evasion is a strategy used by pathogenic organisms to maximize their probability of transmission to a fresh host. It may involve (1) hiding from the immune system (e.g., antigen mimicry or masking, diversity/polymorphism, variation of parasite antigens); (2) interfering with the function of the immune system (e.g., blocking pattern recognition receptors (PRRs) and downstream signaling, inhibition of humoral factors); or (3) destroying elements of the immune system (e.g., induction of apoptosis of immune cells) [16–18].

The identification and analysis of ES proteins in parasitic flatworms have mainly been carried out in species of cestodes and trematodes that mostly infect mammals [19–22], whereas monogeneans have received less attention [23,24]. To further our understanding of monogeneans and their interaction with their fish hosts, we need better knowledge about their secreted proteins. Experimental studies focused on the extraction of these proteins in monogeneans may be challenging due to their small size (in the range of micrometers) and the difficulty of obtaining sufficient individuals to purify the required amounts of proteins. In this case, analysis in silico may be useful to predict ES proteins and to guide future experiments [25]. Thus, based on a recently released transcriptome of *R. viridisi* [26], the aim of the present work was to characterize in silico the ES proteins. We analyzed the possible roles of secretory antigens in immunity against this monogenean, with emphasis on recent developments regarding their immunomodulatory action and potential involvement in evasion of the host immune defense.

2. Results

2.1. In Silico Identification and Functional Annotation of ES Proteins

A total of 1655 non-redundant ES proteins from *R. viridisi* were predicted (Supplementary Materials). Only 513 proteins (31%) were annotated in the UniProtKB/Swiss-Prot database (Supplementary Materials) and 182 (11%) showed sequence similarity to proteins reported in WormBase Parasite (Supplementary Materials). Eighteen proteins (including three C-type lectins and five peptidases) showing high similarity to fish (Teleostei) and helminth (Lophotrochozoa) proteins were included in the secretome (Supplementary Table S1).

Of the 1655 predicted ES proteins, 269 (16%) were mapped to 212 known protein domains. The most represented protein domains were immunoglobulin, leucine-rich repeat domain (involved in protein–protein and protein–ligand interactions), and zinc finger (involved in protein–nucleic acid interactions), which are also present in non-ES proteins (Supplementary Materials). Lectin C-type domain (carbohydrate-binding), CAP domain, and serine and cysteine peptidase domains, related to immune-regulatory proteins, were also found. We identified six putative multifunctional proteins (Supplementary Table S2). Two of them, thioredoxin and thrombospondin 1, are included in MultitaskProtDB.

We mapped 269 (16%) ES proteins to 710 GO terms (229 Biological Process, 239 Cellular Component, and 242 Molecular Function categories). The most enriched GO terms (Figure 1) in *R. viridisi* were: cell (GO:0005623), cell part (GO:0044464), organelle (GO:0043226), membrane (GO:0016020), and membrane part (GO:0044425) at the Cellular Component category; anatomical structure development (GO:0048856) at the Molecular Function category, and cellular process (GO:0009987), cellular metabolic process (GO:0008152), and nitrogen compound metabolic process (GO:0006807) at Biological Process category. Similarly, we mapped 2419 (6.3%) non-ES proteins to 6324 GO terms (2057 Biological Process, 2133 Cellular Component, and 2134 Molecular Function categories). As shown in Figure 1, the GO terms with higher representation of ES proteins with respect to non-ES proteins were endomembrane system, cell periphery, plasma membrane, plasma

membrane part, membrane, membrane part, intrinsic component of membrane, extracellular region part, extracellular region, anatomical structure development, anatomical structure morphogenesis, and multicellular organism development (20% of proteins or more). More ES proteins than non-ES proteins were assigned to the GO terms cell surface, extracellular organelle, extracellular space, cell junction, regulation of developmental process, immune effector process, interspecies interaction between organisms, biological adhesion, and cell adhesion.

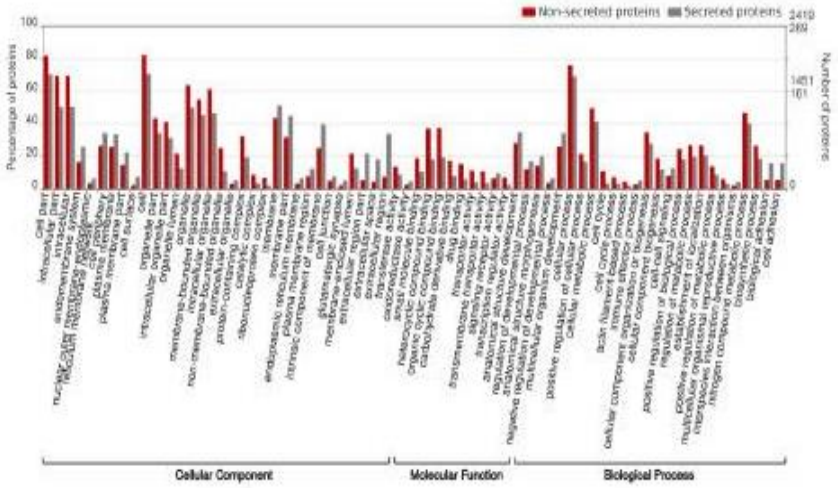

Figure 1. Gene ontology enrichment analysis of secreted and non-secreted proteins as compared to the transcriptome from *R. viridisi*. Only significantly (Pearson Chi-Square test p-value < 0.05) overrepresented GO terms are shown in this figure.

In total, 556 ES proteins were mapped to 179 KEGG pathways and 136 KO (Supplementary Materials). Several ES proteins were associated with human papillomavirus infection, proteoglycans and other pathways in cancer, and Alzheimer disease. These pathways were also present in non-ES proteins. Cathepsins B, C, and D were reported in the KEGG apoptosis pathway [PATH: ko04210]. KEGG BRITE objects related to membrane interactions (Membrane trafficking [BR:ko04131], Transporters [BR:ko02000]), were overrepresented in both ES and non-ES proteins. Thirty-four ES proteins were annotated as cell adhesion molecules (Supplementary Table S3).

ECPred predicted 223 enzymes (13.5%), 1315 non-enzyme proteins (79.5%), and 117 proteins without any prediction (7%) from the secretome of *R. viridisi* (Supplementary Materials). The putative enzymes were classified according to six classes of the Enzyme Commission (EC). Most of the predicted enzymes were represented by hydrolases (53%), followed by transferases (30%) and oxidoreductases (10%) (Figure 2A). Only 41% of the hydrolases were classified into EC subclasses (Figure 2B): 23% esterases (EC 3.1), 15% peptidases (EC 3.4), and 3% glycosylases (EC 3.2). Of the 1655 ES proteins, 12 were identified as Carbohydrate-active enzymes (CAZymes). They were included into nine different families (Supplementary Table S4) of the three major classes: five glycoside hydrolases, five glycosyltransferases, and two polysaccharide lyases. MEROPS analysis of the ES proteins resulted in the identification of 27 peptidases and 14 peptidase inhibitors (Supplementary Materials). Cysteine and serine peptidases were the most represented. The peptidase sub-families found in the secretome are shown in Figure 3. The I93 subfamily of peptidase inhibitors was the most abundant. ECPred predicted four glycosylases and 18 peptidases; however, dbCAN2 predicted five glycosylases and the alignment against the MEROPS database resulted in 27 peptidases in the secretome of *R. viridisi*.

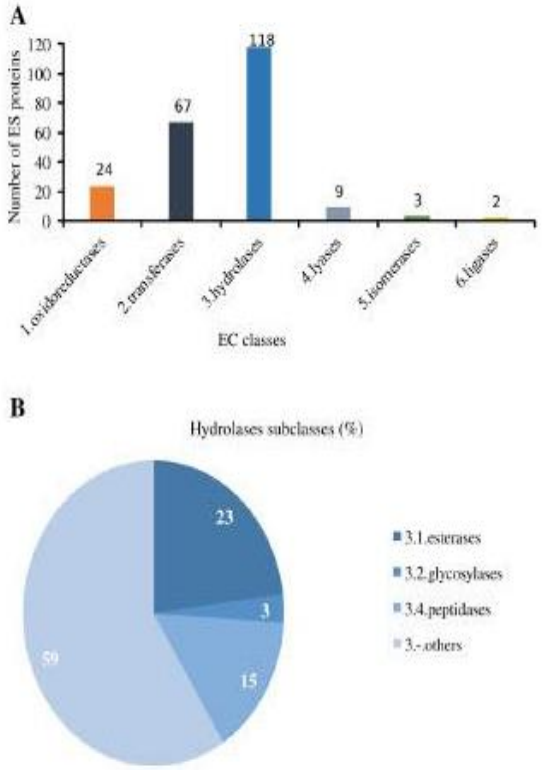

Figure 2. ES proteins of *R. viridisi* with enzymatic function predicted by ECPred: (**A**) Enzyme Commission (EC) classes, and (**B**) subclasses of hydrolases.

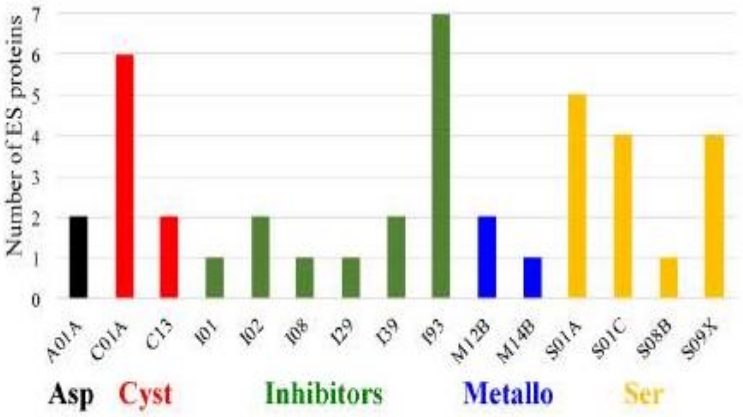

Figure 3. Peptidase families (Asp: aspartic peptidases, Cyst: cysteine peptidases, Inhibitors, Metallo: metallopeptidases, Ser: serine peptidases), and subfamily identifiers in predicted ES proteins.

2.2. *Antigenicity Prediction of ES Proteins*

A total of 1045 (63%) ES proteins were predicted as antigens with a threshold of 0.5. Of these, 43 had a score higher than 0.9 (Supplementary Table S5). Of those, only six were functionally annotated (Collagen alpha-2(I) chain, GTPase NRas, Collagen alpha-2(IV) chain, Beta-1,4-galactosyltransferase 2, Inactive histone-lysine N-methyltransferase 2E, and Transcription factor 12). The proteins with unknown function were submitted to Phyre 2 and the Protein Data Bank (PDB) template name with higher confidence (>90%) and

percentage of identity (>25%) was selected. Table 1 shows the six most antigenic proteins and VAL proteins.

Table 1. The ES proteins of *R. viridisi* with potential immunomodulatory functions.

Putative Immunomodulators	Protein ID	Annotation or PDB Template Name	Vaxijen Score
More antigenic proteins	DN725_c0_g1_i1.p1	Collagen alpha-2(I) chain	1.3983
	DN18884_c0_g6_i2.p1	Unknown: nigellin-1.1	1.3867
	DN2184_c0_g1_i6.p6	Unknown: fkbp-type peptidyl-prolyl cis-trans isomerase slyd	1.3846
	DN146924_c0_g3_i1.p1	Unknown: SH3-like barrel	1.2806
	DN9007_c0_g1_i4.p2	Unknown: protein phosphatase 1 regulatory subunit 3a	1.2422
	DN8473_c0_g5_i3.p1	Unknown: ribosomal protein L14e	1.2028
Venom allergen-like (VAL) proteins	DN106_c0_g1_i4.p14	GLIPR1-like protein 1	0.6280
	DN1638_c0_g1_i10.p1	Pathogenesis-related protein	0.5865
	DN1553_c0_g1_i1.p1	Peptidase inhibitor 16	0.6481
	DN2123_c1_g1_i5.p1	Unknown: venom allergen-like protein 4	0.4954
	DN2793_c0_g1_i1.p7	Pre-mRNA-splicing factor ISY1 homolog	0.4541
	DN537_c0_g1_i4.p3	Scoloptoxin SSD976	0.4269

3. Discussion

This is the first study predicting secreted proteins of a monogenean member of the Diplectanidae family. Previously, Caña-Bozada et al. [23] identified the ES proteins in four monogenean species, *Eudiplozoon nipponicum*, *Gyrodactylus salaris*, *Neobenedenia melleni*, and *Protopolystoma xenopodis*. Similarly, our present work suggests that various predicted ES proteins from *R. viridisi* are potentially involved in adhesion, penetration, incorporation of nutrients, and immunomodulation of host cells (Figure 4), which are functions with important roles in pathogenesis [14]. GO annotations of ES proteins from *R. viridisi* agree in most represented Molecular Functions and Biological Process categories with those previously reported for other helminths [25]. However, the annotation of membrane and membrane part in the Cellular Component category is only similar to other Platyhelminthes, including monogeneans [23]. For example, the secretion of cadherins, protocadherins, integrins, and collagen alpha chains (Supplementary Table S3) could be related to cell–cell adhesion after the physical insertion of haptors in the epithelial tissue, important to anchor and transient attachment for locomotion, achieved in monogeneans through cooperation between adhesive secretion [27] and the haptor [28].

The overrepresentation of hydrolases in the secretome of parasitic helminths has been reported elsewhere [25] and could be associated with proteolysis. Cytosolic cathepsins B and D could induce apoptosis in T cells [29]. These processes, proteolysis and apoptosis, contribute to the penetration of the parasite and destruction of host cells and immune receptors [17]. Putative cathepsins, essential for survival and presenting low homology to fish proteins, have been previously considered as potential drug targets due to their overrepresentation in ES proteins of other monogenean species [23]. We found the presence of cathepsins B, C, D, and L in both ES and non-ES proteins from *R. viridisi*. It has been reported that cathepsins L are important for nematodes and can degrade haemoglobin, serum albumin, immunoglobulins, fibronectin, collagen I, and laminin under acidic conditions, and its enzymatic activity is host-specific [30]. Also, cathepsins L have been characterized in the monogeneans *N. melleni* [31] and *E. nipponicum* [32]. *Eudiplozoon nipponicum* abundantly expresses cathepsins B, D, L1, and L3, which play a critical role in haemoglobin processing and immunomodulation [24]. *Rhabdosynochus viridisi*, like other mucus-feeding

monogeneans, could use elastase-like serine peptidases for extracellular digestion [32] instead of cathepsins, so it remains to verify the possible role of these peptidases in the secretome.

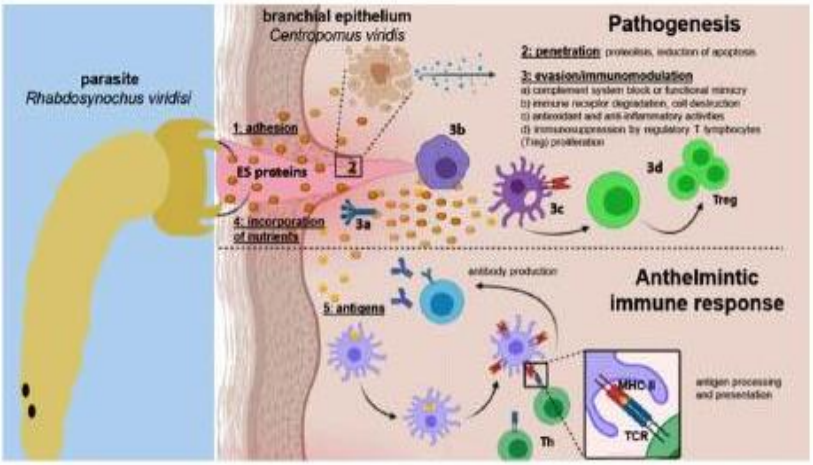

Figure 4. Excretory/secretory (ES) proteins from *Rhabdosynochus viridisi* could be involved in (1) adhesion, (2) penetration and destruction of branchial epithelium, (3) immune evasion strategies (in monocytes 3b, dendritic cells 3c, and T lymphocytes 3d), (4) nutrition, and (5) immunogenicity (the antigens are taken by Antigen Presenting Cells and then presented to T helper lymphocytes, which activate B cells for antibody production and secretion). Created in BioRender.com.

A predominance of inhibitors of the subfamily I93 was observed in the secretome of *R. viridisi*. This subfamily contains inhibitors of metallopeptidases that have been proposed to participate in extracellular matrix turnover, tissue remodeling, and other cellular processes in parasitic helminths [33]. In addition, the serpins (serine peptidase inhibitors I01, I02, I08) and cystatin (cysteine peptidase inhibitor I29) secreted by *R. viridisi*, could block complement activation, prevent inflammation, and induce immunosuppression by subverting Th1 mechanisms and drawing the immune system towards a Th2/Treg response, as has been reported for *E. nipponicum* [34,35]. Inhibitors of serine and cysteine peptidases, belonging to family I29, are highly transcribed and secreted by adult parasites of *E. nipponicum*, although they are not present in the secretome in large quantities [24].

The multifunctional proteins thioredoxin and thrombospondin 1 (TSP-1) found in the *R. viridisi* secretome could be related to pathogen virulence activity [36]. Thioredoxins of nematodes are ES proteins with antioxidant, anti-inflammatory, and anti-apoptotic activities. They also modify monocytes and epithelial cells, binding and inducing the time-dependent release of cytokines [37]. Thioredoxin has been also reported in the secretome of the monogenan *E. nipponicum* [24]. TSP-1 interacts with other extracellular matrix proteins to regulate cellular behavior. Moreover, it is secreted as a compensatory mechanism for controlling inflammation and protecting tissues from excessive damage [38]. TSP-1 has not been reported in monogeneans or other platyhelminths, but it is known that thrombospondin 2 (TSP-2) in the trematodes *Clonorchis sinensis* and *Fasciola hepatica* is a virulence and immunomodulation-related transcript that causes transforming growth factor beta stimulation [39]. In humans, TSP-1 and TSP-2 can interact with various ligands, such as structural components of the extracellular matrix, cytokines, cellular receptors, growth factors, proteases, and other stromal cell proteins [40]. This is the first report of thrombospondin 1 in a monogenean parasite.

The 18 predicted ES proteins from *R. viridisi* with high similarity to fish and helminths proteins possibly have mimicry function. For instance, Hebert et al. [41] observed that mimicry candidate proteins from the behavior-altering cestode *Schistocephalus solidus* had specific sequence similarity with proteins of the host (the threespine stickleback, *Gasteros-*

teus aculeatus). It has been suggested that a pathogen can mimic and substitute host proteins in order to hijack the host cellular processes [42]. Therefore, we would expect that potential mimicry proteins secreted by *R. viridisi* could be involved in immune-regulatory functions.

The most represented protein domains in the *R. viridisi* secretome were immunoglobulin (Ig), zinc finger, and leucine-rich repeats. Ig-like domain functions in *Caenorhabditis elegans* include cell–cell recognition, cell–surface receptors, muscle structure, and the immune system [43]. Zinc finger proteins participate in numerous physiological processes, such as cell proliferation, differentiation, and apoptosis, thereby maintaining tissue homeostasis. They are also implicated in transcriptional regulation, ubiquitin-mediated protein degradation, signal transduction, actin targeting, DNA repair, cell migration, and numerous other processes [44]. Particularly, zinc finger proteins are required for chromosome-specific pairing and synapsis during meiosis in *C. elegans* [45] and for survival of adult worms of *Schistosoma japonicum* [46]. The potential functions of zinc finger proteins in immune system regulation, both at the transcriptional and post-transcriptional levels, have been proposed recently [47]. Likewise, leucine-rich repeat domains are present in several immune receptors of animals and also participate in protein–protein interactions [48].

The most represented KEGG pathway, human papillomavirus (HPV) infection, could be associated with immune evasion. The HPV oncoproteins downregulate the expression of proinflammatory cytokines and chemokines, and upregulate the expression of immunosuppressive genes in host cells. They also degrade host proteins [49]; for example, we found the E3 ubiquitin-protein ligase UBR4 in the secretome, which seems to be involved in removing pro-apoptotic and pro-inflammatory molecules via the ubiquitin–proteasome system [50]. Several KEGG BRITE objects in ES proteins were related to membrane proteins suggesting a role in host–pathogen interactions.

Monogenean-specific antigens [51,52] and fish antibodies have been detected in several infections [53,54]. However, these studies are limited due to the difficulty of obtaining anti-Fc antibodies specific for different fish species, and high-quality protein extracts of small parasites, which are difficult to maintain in culture. The detection and identification of parasite antigens and humoral responses is important for vaccine development, and bioinformatic predictions have become essential. The present study suggests that most (63%) of the ES from *R. viridisi* are potential antigens and, therefore, could be processed and presented to T cell receptors, as well as recognized by host antibodies. According to the bioinformatic prediction of Gomez et al. [55], ES proteins of the platyhelminth *Taenia solium* are enriched in antigenic regions as compared to non-ES proteins. Antigens promoting antibody response could alleviate infection throughout antibody-dependent cellular cytotoxicity (ADCC) mechanisms [56]. For example, some structures of *Schistosoma* spp. and *Fasciola* spp., covered by antibodies, are destroyed by toxic proteins and reactive species released by cells with Fc receptors [57].

The most probable antigen predicted by Vaxijen was annotated as collagen alpha-2(I) chain. The collagen alpha-2(IV) chain also presented a score higher than 0.9. Recently, it was reported that human collagen alpha-2 type I stimulates collagen synthesis, wound healing, and elastin production in normal human dermal fibroblasts [58]. Type IV collagen, the most abundant constituent of the basement membrane, is highly conserved among vertebrates and invertebrates, regulating cell adhesion and migration [59]. Other most probable antigens are apparently unique to the species *R. viridisi*, since they did not have functional annotation by sequence homology. Species-specific antigens are common to members of a single species that improve the efficiency of immunological diagnosis and immunoprophylaxis of helminthic diseases [60]. Further research to identify antigens from the ES proteins in the tegument of monogeneans could shed light on the development of better strategies to prevent or control fish diseases. These antigens, such as membrane or tegumental proteins, have been proposed as vaccine candidates due to the probability of interaction with the host's immune system [61–64].

VAL proteins were also identified. These proteins are generally secreted in parasitic stages [65] and there is an increasing interest in characterizing their immunological

properties to design anthelmintic vaccines [66–68]. They are ubiquitous ES products that paralyze plants and animals, and have been previously named as sperm-coating protein/Tpx/antigen 5/pathogenesis-related-1/Sc (SCP/TAPS), cysteine-rich secretory proteins/antigen 5/pathogenesis-related 1 (CAP), or activation-associated secreted proteins (ASPs); the CAP domain seems to be conserved in evolution, allowing the binding of small hydrophobic ligands, but little is known regarding endogenous ligands [65]. There are no experimental studies of VAL proteins from monogenean species, and only 41 VAL transcripts from *N. melleni* have been reported [69]. Of the six VAL proteins of *R. viridisi*, only the peptidase inhibitor 16 (PI16) showed similarity to unnamed protein products from *P. xenopodis*, which is another monogenean species. PI16 suppresses the chemokine chemerin activation [70], which impairs endothelial cell inflammation [71]. GLIPR1-like protein 1 has been reported in ES from other parasites, suggesting a role in cell adhesion [72,73]. Pathogenesis-related proteins form a protective barrier against invasive pathogens, at least in plants [74]. Pre-mRNA-splicing factor ISY1 homolog is a component of the spliceosome C complex required for the selective processing of microRNAs (miRNAs) during embryonic stem cell differentiation. It is also involved in pre-mRNA splicing in the nucleus [75]. Scoloptoxin SSD976 is a voltage-gated calcium channel inhibitor [76] that could impair Ca^{2+} signaling, T cell activation, and proliferation in fish [77]. Venom allergen-like protein 4 (VAL-4) has been reported as secreted by other helminths. VAL-4 presents lipid-binding properties and can sequester small hydrophobic ligands; this could be a mechanism to modulate immune responses in the host [65]. It remains to be determined if there is functional homology of these VALs in the context of monogenean–fish interaction, at least to verify their role in the parasite life cycle and modulation of the host.

The present study provides an in silico characterization of the secretome of *R. viridisi*; nevertheless, it has some limitations. The identified ES proteins of *R. viridisi* depend on the quality of the annotated transcriptome, and it is possible that proteins presenting high homology to fish belong to the host. Moreover, there is a lack of data from monogenean proteins, and annotation based only on sequence homology is not totally reliable. It is necessary to verify the presence of the secreted proteins through a proteomic approach, and to identify key virulence factors by functional studies, which are limited due to the size and difficulty of handling *R. viridisi* specimens. Antigenicity prediction is based on a human host, whose immune system is different from fish; the latter is composed of low-affinity antibodies, while antigen presentation and ADCC remain unverified.

4. Materials and Methods

Protein sequences were retrieved from an available *R. viridisi* transcriptome [26]. The specimens used for that transcriptome were all adults isolated from the gills of their fish host *C. viridis*, reared in laboratory conditions [26]. The predicted ES proteins were identified following the bioinformatic workflow of Gahoi et al. [25], which filters sequences based on signal peptide, discarding sequences with transmembrane regions, subcellular localization of mitochondrial proteins, endoplasmic reticulum retention signal, and GPI-anchor proteins.

To rule out contaminant sequences, the ES proteins were aligned using BLASTP against the NCBI non-redundant protein database (E-value < 1×10^{-5}). The sequences that were best hits with Protostomia (taxid: 33,317) were retrieved, and the remaining sequences were considered contaminants. Proteins with similar E-value among Teleostei, Lophotrochozoa, and Platyhelminthes bases were included. ES proteins were annotated based on Uniprot-SwissProt and WormBase Parasite [78] databases, using BLASTP (E-value < 1×10^{-4}). ES proteins without any homologue in the NCBI non-redundant protein database were considered as the specific secretome of *R. viridisi*.

The domains were identified with HMMSCAN v3.1.b2 [79] using the Pfam database as reference [80]. Possible multifunctional proteins (with two or more domains associated with different biochemical functions) were identified using the domain annotation and the Multitask ProtDB-II protein database [81]. The Gene Ontology (GO) terms were retrieved

with the PANNZER2 server [82] and were subsequently plotted and analyzed with the WEGO 2.0 server [83] by Pearson Chi-Square test, using the entire proteome as the reference group. A significant enrichment was considered when the p-value < 0.05. The GO terms were updated with QuickGO [84]. KEGG Orthology (KO) IDs and KEGG pathways were retrieved from the Trinotate annotation [26].

Enzymes were classified with the ECPred v1.1 software [85], using the option "weighted", according to the Enzyme Commission (EC) nomenclature based on the type of catalyzed reaction: oxidoreductases (EC 1), transferases (EC 2), hydrolases (EC 3), lyases (EC 4), isomerases (EC 5), and ligases (EC 6). CAZymes were identified with the dbCAN2 server [86]. Peptidases and peptidase inhibitors were identified by aligning ES proteins against the MEROPS database (merops_scan.lib) [87], using BLASTP (E-value < 1×10^{-4}). Antigenic proteins were predicted with VaxiJen 2.0 server [88]. Those proteins with score higher than 0.9 were subjected to a fold recognition analysis with Phyre2 [89] for functional information. Venom allergen-like (VAL) proteins, which have cysteine-rich secretory proteins, antigen 5, and pathogenesis-related 1 (CAP) domain, were also analyzed with VaxiJen.

5. Conclusions

The present work represents hypothetical molecular mechanisms of host–parasite interactions of the monogenean *R. viridisi* with its host. We identified potential proteins secreted by this parasite and discussed their possible contribution to the pathogenesis and anthelmintic responses. Using bioinformatic analysis, we found that most of the ES from *R. viridisi* are potential antigens. This is the second study reporting the presence of VAL transcripts in a monogenean parasite. Further research is necessary to confirm the presence and function of these ES proteins in vivo.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/parasitologia3010004/s1; Supplementary File S1: Predicted ES proteins of *Rhabdosynochus viridisi*; Supplementary File S2: Annotation of ES proteins of *R. viridisi* based on Uniprot-SwissProt database; Supplementary File S3: Annotation of ES proteins of *R. viridisi* based on WormBase Parasite database; Supplementary File S4: Annotation of ES and non-ES proteins of *R. viridisi* based on Pfam domains; Supplementary File S5: Annotation of ES and non-ES proteins of *R. viridisi* based on KEGG pathways; Supplementary File S6: Annotation of ES proteins of *R. viridisi* based on ECPred; Supplementary File S7: Annotation of ES proteins of *R. viridisi* based on MEROPS database. Supplementary Tables file, containing Table S1: Proteins with similarity between fish and parasites; Table S2: Possible multifunctional proteins found in predicted ES proteins of *R. viridisi*; Table S3: Adhesion proteins identified in *R. viridisi*; Table S4: Families of carbohydrate-active enzymes (CAZymes) in predicted ES proteins of *R. viridisi*; Table S5: Most antigenic ES proteins from *R. viridisi*.

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3. TRANSCRIPTOMIC ANALYSIS OF IMMUNE-RELATED GENES IN PACIFIC WHITE SNOOK (*Centropomus viridis*) GILLS INFECTED WITH THE MONOGENEAN PARASITE *Rhabdosynochus viridisi*

Marian Mirabent-Casals 1, Víctor Hugo Caña-Bozada 1, Francisco Neptalí Morales-Serna 2, Juan Manuel Martínez-Brown 1, Rosa María Medina-Guerrero 1, Rubi Hernández Cornejo 1 y Alejandra García-Gasca 1

1 Centro de Investigación en Alimentación y Desarrollo, Avenida Sábalo Cerritos s/n, Mazatlán 82112, Sinaloa, México

2 Instituto de Ciencias del Mar y Limnología, Universidad Nacional Autónoma de México, Av. Joel Montes Camarena s/n, Mazatlán 82040, Sinaloa, México

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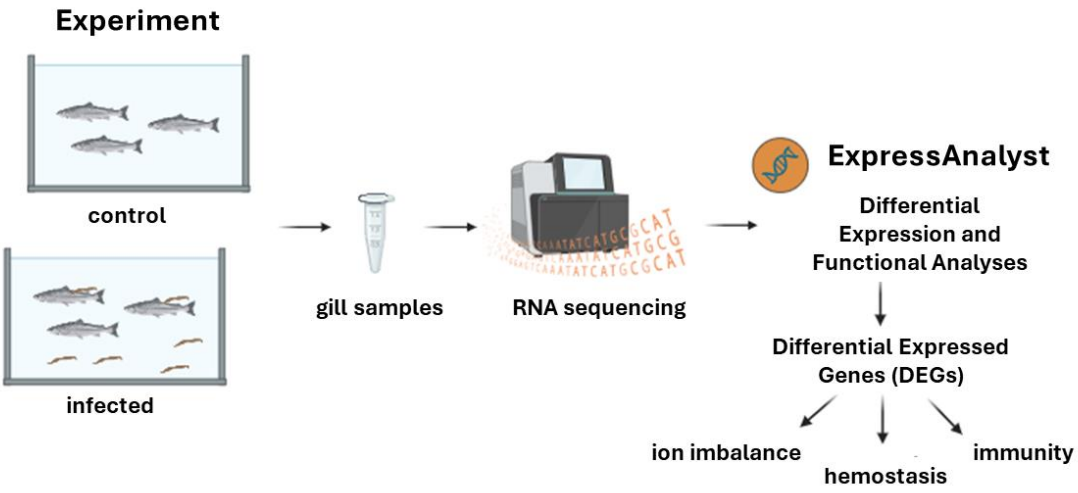
Experiment
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ExpressAnalyst
Differential Expression and Functional Analyses
Differential Expressed Genes (DEGs)
ion imbalance
hemostasis
immunity

Transcriptomic analysis of immune-related genes in Pacific white snook (*Centropomus viridis*) gills infected with the monogenean parasite *Rhabdosynochus viridisi*

Marian Mirabent-Casals [1], Víctor Hugo Caña-Bozada [2], Francisco Neptali Morales-Serna [3], Juan Manuel Martínez-Brown [4], Rosa María Medina-Guerrero [2], Rubi Hernández Cornejo [1] and Alejandra García-Gasca [1,*]

[1] Molecular Biology and Tissue Culture Laboratory, Centro de Investigación en Alimentación y Desarrollo, Avenida Sábalo Cerritos s/n, Mazatlán 82112, Sinaloa, Mexico; mmirabent220@estudiantes.ciad.mx ; rhernandez@ciad.mx ; alegar@ciad.mx

[2] Laboratory of Parasitology, Centro de Investigación en Alimentación y Desarrollo, Avenida Sábalo Cerritos s/n, Mazatlán 82112, Sinaloa, Mexico; victorcana1991@hotmail.com; rmedina@ciad.mx

[3] Instituto de Ciencias del Mar y Limnología, Universidad Nacional Autónoma de México, Av. Joel Montes Camarena s/n, Mazatlán 82040, Sinaloa, Mexico; neptali@ola.icmyl.unam.mx

[4] Laboratory of Reproduction and Marine Fish Hatchery, Centro de Investigación en Alimentación y Desarrollo, Avenida Sábalo Cerritos s/n, Mazatlán 82112, Sinaloa, Mexico; juan.martinez@ciad.mx

*alegar@ciad.mx Author to whom correspondence should be addressed.

Abstract

The parasite *Rhabdosynochus viridisi* (Platyhelminthes: Monopisthocotyla) infects the Pacific white snook *Centropomus viridis* gills and can cause adverse effects in aquaculture facilities. The immune responses of Pacific white snook to monogenean infections are not well understood. Thus, this study aimed to identify differentially expressed genes (DEGs) in the gills of Pacific white snook juveniles experimentally infected with *R. viridisi*, emphasizing in immune-related genes and pathways activated or suppressed during the infection. RNA sequencing was performed on the gills of uninfected (control) and infected fish. Seq2Fun was selected without a reference transcriptome. ExpressAnalyst was used for differential expression and functional analyses. A total of 20,106 transcripts were found and 1430 (7 %) were DEGs between infected and control groups. We identified 860 (60%) down-regulated and 570 (40%) upregulated genes. Thirteen canonical pathways from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database were overrepresented (FDR < 0.05), and most of the DEGs were downregulated, suggesting inactivation of these pathways. The functions of most of the DEGs with higher fold change found in this study are not well understood in fish. Even though the well-known pro-inflammatory cytokines remained unchanged in infected gills of *C. viridis*, and transforming growth factor β (*tgfβ*) was downregulated, interleukin-17 ligands *il17d*, and *il17a/f1*, as well as CXC motif chemokine receptor 2 (*cxcr2*) genes were upregulated, indicating that the infection with *R. viridisi* promotes Th17-like immunity. Overexpression of plasma B cell activity markers such as immunoglobulin light chain-like genes and the v-set pre-B cell surrogate light chain 3 (*vpreb3*) was also detected in this study. The possible implications of DEGs related to calcium imbalance, hypoxia adaptation, hemostasis, and immunity are discussed. These results will support future studies aimed at improving the prevention and treatment of monogenean infections in finfish aquaculture.

Keywords: *Rhabdosynochus viridisi*, aquaculture, transcriptomics, immunity, *Centropomus viridis*

Highlights

- *Rhabdosynochus viridisi* alters gene expression patterns in the gills of Pacific white snook
- *R. viridisi* infection inactivates several pathways involved in cell survival and function in parasitized gills
- Differentially expressed genes were associated with calcium imbalance, deviations from normoxia and hemostatic conditions
- Plama B cell proliferation and Th17-like immunity genes were overexpressed in infected gills

1. Introduction

Freshwater and marine fishes of tropical areas are commonly parasitized by monogeneans (phylum Platyhelminthes). These parasites typically have high reproductive rates, high host specificity, and a direct (single-host) life cycle. Some monogenean species represent a threat to the finfish aquaculture industry, as they can lead to reduced growth and reproduction rates, decreased fitness, increased mortality, and substantial economic losses [1, 2]. For instance, the Nile tilapia (*Oreochromis niloticus*) infected with *Dactylogyrus* spp, showed severe gill tissue damage, leading to significant changes in hemato-biochemical, immune, antioxidant, genotoxic, and pathological indices [2]. *Neobenedenia melleni* caused a 40% mortality rate in farmed cobia (*Rachycentron canadum*), resulting in an estimated production loss of 284 million tons, whereas *Benedenia seriolae* and *Zeuxapta seriolae*, occasioned massive mortalities (39 million tons) for greater amberjack (*Seriola dumerili*) [3]. *Sparicotyle chrysophrii* brought significant economic losses in the aquaculture of gilthead seabream (*Sparus aurata*) [4]. Likewise, *Rhabdosynochus viridisi* is considered a threat to the aquaculture of Pacific white snook (*Centropomus viridis*) [5].

Monogeneans primarily attach to the mucus-coated external structures of fish, such as gills, fins, or skin. Once attached to the tissue, they feed on the blood or mucus and epithelial cells of the host. During direct contact, these parasites expose their tegument and secrete extracellular vesicles, releasing various biomolecules that could interact with the humoral and cellular responses of the host's immune system [4]. In a previous study, we predicted that excretory-secretory proteins released by *R. viridisi* include protease inhibitors such as serpins, cystatin, as well as the multifunctional proteins thioredoxin and thrombospondin 1, which may participate in preventing inflammation through the modulation of the complement system, T lymphocytes, and monocytes [6].

Gene expression studies can reveal how fish respond to monogenean infections and how parasites use their molecular arsenal to survive and reproduce. Gene expression and regulation have not been extensively studied in monogeneans due to technical challenges for sample collection, DNA/RNA extraction, limited genomic resources, or species-specific differences [7]. However, there is an increasing number of studies that use quantitative Polymerase Chain Reaction, or RNA sequencing to explore fish-monogenean interactions [8, 9, 10, 11, 12]. Identifying differentially expressed genes (DEGs), specific immune-related genes, and pathways activated or suppressed during infection provide insights into potential

targets for immune modulation [13, 14]. To better understand the immune response of fish infected with monogeneans, several studies have measured the expression of particular genes, such as the cytokines interleukins 1 beta (*il1β*), 8 and 10 (*il8* and *il10*), tumoral necrosis factor alpha (*tnfα*), and immunoglobulins chains genes [5, 8]. Transcriptomic analysis may provide a comprehensive view of the molecular mechanisms of fish to combat parasite infections; however, this approach has been poorly applied in cases involving monogeneans [9]. Thus, based on high-throughput sequencing, the present study aimed to identify DEGs in the gills of the Pacific white snook infected with *R. viridisi*, emphasizing on immune-related genes and pathways activated or suppressed during the infection.

2. Materials and Methods

2.1 Experimental design and tissue sampling

Adults and eggs of *R. viridisi* were obtained as described by Caña-Bozada et al., [15]. Fifteen-centimeter-long cotton threads with eggs of *R. viridisi* were introduced into a 400 L reservoir tank with 35 Pacific white snook juveniles to spread the infection. After 26 days, 40 threads were placed in the reservoir tank and were kept there for 72 hours to collect the eggs. After that time, ten threads were randomly selected and examined under a stereomicroscope to confirm the presence of new eggs and quantify them. Adult parasites collected from the gills of three fish were also examined under a stereomicroscope. *R. viridisi* was identified by observing the sclerotized structures of adult specimens [16].

Healthy fish juveniles from the same cohort were produced in the Pilot Marine Fish Hatchery at CIAD-Mazatlán and collocated in two 400 L tanks (about 50 fish per tank) at the Parasitology Laboratory of CIAD-Mazatlán. Although apparently uninfected, these fish received a formalin bath (200 µL/L for 40 minutes) to eliminate any potential parasite infection. After that, fish were transferred to two 400 L tanks in a seawater flow-through system with aeration to be acclimatized for two weeks at 25 ± 1°C, 34.7 ± 0.3 ups, and 5.2 ± 0.08 mg/L of dissolved oxygen. They were fed a commercial diet (Skretting pellet of 4 mm) twice daily until apparent satiety. After acclimatization, the fish were placed into six 400 L tanks. The experiment consisted of two groups: uninfected (control, C) and infected (treated, T), each with three replicates.

The successful propagation of *R. viridisi* in the reservoir tank allowed the collection of threads with a large number of eggs (> 300) to proceed with the experimental infection. Ten

threads with approximately 300 eggs of *R. viridisi* each were introduced in each tank of the infected group. After 24 days, the infection was confirmed by detecting new monogenean eggs and adults in the gills of fish from the infected group. Therefore, we proceeded with tissue sampling. A total of 30 fish (5 fish per tank) were collected and anesthetized by four minutes of immersion in a clove oil solution in seawater (75 µL/L). Once anesthetized, the fish were killed following the recommendations of the Guidelines for the Euthanasia of Animals [17]. Subsequently, all gill arches from both gill chambers were extracted.

For RNA sequencing analysis, a portion of the outermost gill arch was collected from each control and infected fish. Four pools of gill portions were formed for each group (C and T). Each tank had a representative pool (C1–C3 and T1–T3) consisting of gill portions from four fish, only the fourth pool (C4 and T4) included gill portions from three fish. Each pool was deposited in a 1.5 mL microtube with RNAlater ® and preserved at -20 °C for further analysis. The remaining gill arches were collected in 70% alcohol for subsequent parasite quantification. All fish euthanized were measured and weighed. At this time, the fish measured 19 ± 1 cm in total length and weighed 56 ± 12 g.

The Fulton (FCF) and Bagenal and Tesch condition factors (BTCF) were determined for control and infected fish using the following formulas:

$$FCF = \frac{100 * W}{L^3} \quad (1) \qquad BTCF = \frac{100 * W}{L^{\beta}} \quad (2)$$

W: weight; L: length; β: slope of the linear regression of the length-weight relationship: logW = α + β*logL. A T-test was performed to detect differences between infected and control condition factors. Pearson correlations between the condition factors and the number of parasites per fish were determined.

2.2 *RNA sequencing, Bioinformatics and Statistics*

RNA extraction, library preparation and sequencing with Illumina MiSeq platform (2 x 150 bp paired-end configuration) were conducted in Azenta Life Science (South Plainfield, USA). ExpressAnalyst [18, 19] was used for the Differential Expression Analysis (DEA) of the gill samples of *C. viridis*. For this, the raw reads downloaded from Azenta server were uploaded to the ExpressAnalyst Docker. The algorithm Seq2Fun was selected without reference transcriptome to perform quality control and map the reads to the "fishes" database (downloaded on April 24, 2024) from EcoOmics DataBase (EODB available https://www.ecoomicsdb.ca/). The "fishes" database contained protein sequences from 62

fish species. Seq2Fun algorithm performed translated searches of the RNAseq reads against this specific database, eliminating the possibility of annotation of orthologs from the parasite or other organisms. The Seq2Fun output files were a count table (**Supplementary File S1**), and an ortholog annotation table (**Supplementary File S2**).

The count table was uploaded to the web server ExpressAnalyst (https://www.expressanalyst.ca/ExpressAnalyst/uploads/TableUploadView.xhtml) with the following specifications: Specify organism = Generic/Species independent; Analysis Type = Differential Expression; Data Type = Counts (bulk RNA-seq); ID Type = Seq2Fun Ortholog ID; Metadata included. The annotation libraries in ExpressAnalyst are updated yearly, based on the latest ID versions available from NCBI (Entrez, RefSeq), Ensembl, and Uniprot. This pipeline was selected because perfoms similar to *de novo* transcriptome assembly in less time [19]. Unannotated reads, reads with a count lower than four or with variance percentile rank lower than 15 (those with stable expression values across conditions), were filtered out and the remaining reads were normalized using the Relative Log Expression Normalization method. Simple Metadata, Limma Statistical Method without robust trend adjustment, and Specific Comparison between infected versus control groups were selected for DEA. Limma was selected since it uses moderated t-statistic (two-sided) and performs better than edgeR and DEseq2. DEGs were considered when the fold change (FC) threshold was two or more ($\log2|FC| \geq 1$) and the False Discovery Rate (FDR) cut-off was 0.05 ($FDR \leq 0.05$). Overrepresentation analysis (ORA) and Gene Set Enrichment Analysis (GSEA) were used to identify significant KEGG pathways, Biological Processes and Molecular Functions from the Gene Ontology (GO) database.

3. Results

All fish of the control group remained free of parasites during the experiment. On the other hand, all fish in the treated group were infected, with a median of 69 monogeneans per fish. The mean weight, length and parasite count per sampled fish are shown in **Supplementary File S3**. Infected fish presented $\beta = 2.84$, whereas uninfected fish had $\beta = 2.64$. There were no significant differences in the Fulton's condition factor between the control and infected fish. However, the Bagenal and Tesch condition factor was significantly higher in the control group (**Table 1**). No correlation between any condition factor and the number of parasites per fish was observed.

Table 1. T-test comparison of condition factors (mean ± standard deviation) between control and infected groups and Pearson correlation coefficients between condition factors and the number of parasites per fish.

	T-test			Pearson Correlation	
Condition Factor	**Control**	**Infected**	**p-value**	**ρ**	R^2
Fulton (β = 3)	0.72 ± 0.06	0.72 ± 0.05	0.99	-0.16	0.025
Bagenal and Tesch	2.09 ± 0.14	1.16 ± 0.08	< 0.001	-0.1	0.011

Raw sequencing reads of each experiment sample were deposited in the NCBI Sequence Read Archive (SRA) database and can be accessed through Bioproject PRJNA1116595. The accession numbers SAMN41537589, SAMN41537590, SAMN41537591, and SAMN41537592 correspond to the uninfected gills, whereas SAMN41537593, SAMN41537594, SAMN41537595, and SAMN41537596 are from infected gills. The Bioproject will be available after publication. The quality control parameters for the Seq2Fun analysis of each sample are reported in **Table 2**. In this work, the clean read rates (CRR) indicated that more than 96 % of reads were retained after filtering out the low-quality reads. CRR values should be more than 90 % for each sample [19]. The mapping read rates (MRR) were lower than 50 % in all the samples, which is consistent with the fact that Seq2Fun mapped reads without a reference transcriptome and not all genes are expressed in gills. The mapping core ortholog rates (MCOR) were above 95 % in all samples. This indicated that there was an adequate match between the sequences from *C. viridis* and the other fish included in the EODB. The sequences of core orthologs are present in more than 90 % of species in the database [19].

Table 2. RNA-seq read processing results for Seq2Fun. CRR: Clean Reads Rate; MRR: Mapping Reads Rate; MCOR: Mapping Core Ortholog Rate; C1, C2, C3, and C4 samples correspond to the control group; T1, T2, T3, and T4 correspond to the infected group.

Sample	Raw Reads	Clean Reads	CRR (%)	Mapped Reads	MRR (%)	MCOR (%)
C1	22, 630, 273	21, 863, 715	96.61	7, 767, 520	35.53	96.21
C2	24, 509, 222	23, 773, 691	97.0	10, 921, 700	45.94	96.29
C3	27, 967, 329	27, 174, 821	97.17	12, 830, 550	47.21	96.52
C4	26, 800, 969	26, 068, 450	97.27	12, 772, 282	49.0	97.28
T1	27, 745, 837	26, 975, 407	97.22	12, 287, 330	45.55	96.14
T2	25, 255, 272	24, 558, 468	97.24	11, 269, 853	45.89	95.27
T3	25, 295, 266	24, 542, 967	97.03	10, 551, 147	42.99	94.94
T4	23, 923, 886	23, 232, 485	97.11	11, 277, 940	48.54	95.31

The Principal Component Analysis (PCA) plot of the normalized raw counts shows differential gene expression between the infected and control fish. The PCA indicated that the infection explained 42.1% of the total variation (**Figure 1**).

A total of 20,106 transcripts were identified in the RNA-seq reads of *C. viridis*. After normalization, transcripts with low counts and low variance were filtered out, and 15,503 were retained for further statistical and functional analysis (**Supplementary File S4**). Among them, 1430 DEGs were detected. The volcano plot (**Figure 2**) shows the statistical significance versus the magnitude of change in the expression of the transcripts. Even though the number of downregulated genes was higher (860) than the upregulated genes (570), there were more significant transcripts with higher expression levels (log2FC > 4) than the ones with lower expression (log2FC < -4). **Table 3** highlights the top 20 (ten upregulated and ten downregulated) DEGs.

Thirteen pathways were considered overrepresented with FDR < 0.05 (**Supplementary File S5**). The top ten significantly overrepresented KEGG pathways had a high distribution of DEGs around log2 FC of -2, suggesting a suppression or inactivation (**Figure 3**). However, except for the Arrhythmogenic right ventricular cardiomyopathy pathway (KEGG: hsa05412), the others presented at least one upregulated gene. The significant KEGG pathways analyzed by GSEA are presented in **Supplementary File S6**.

Table 3. Top 20 differentially expressed genes (+ upregulated, - downregulated)

EntrezID	Protein Product symbol	Protein Name	Log2FC	*FDR*
s2f_0015643001	SI:DKEY-30J10.5	uncharacterized protein	+8.0	0.0298
s2f_0024425001	ROA2	heterogeneous nuclear ribonucleoprotein a2	+7.5	0.0076
s2f_0000081022	GLIPR2	golgi-associated plant pathogenesis-related protein 1-like	+6.6	0.0107
s2f_0028015001		trypsin-like	+6.5	0.0045
s2f_0001863005	SI:DKEY-85K7.10	endonuclease domain-containing 1 protein-like	+6.4	0.0009
s2f_0011066001		cap-gly domain-containing linker protein 4-like	+6.2	0.0059
s2f_0011731001	TMEM44	transmembrane protein 44 isoform x1	+5.9	0.0077
s2f_0002098001	myadml2	myeloid-associated differentiation marker-like protein 2	+5.6	0.0049
s2f_0000302005	S100A1	protein s100-a1-like	+5.6	7.72E-05
s2f_0000441010	PDIA2	protein disulfide-isomerase a2	+5.5	4.88E-06
s2f_0001359008	SI:DKEY-202L22.6	interferon-induced very large gtpase 1-like	-5.5	0.0006
s2f_0010615001	ZACN	zinc-activated ligand-gated ion channel	-4.3	0.0045
s2f_0000583002	TGM5L	protein-glutamine gamma-glutamyltransferase 5-like	-4.2	0.0049
s2f_0000583008	TGM4	protein-glutamine gamma-glutamyltransferase 4	-4.0	0.0035
s2f_0004881006	HRH4	histamine h3 receptor-like	-4.0	0.0035
s2f_0016519001	SI:DKEY-19B23.8	low density lipoprotein receptor adapter protein 1-like	-3.7	0.0091
s2f_0029211001		(genbank) unnamed protein product	-3.6	0.029
s2f_0002382003	SPRY4	protein sprouty homolog 4	-3.6	0.0043
s2f_0000159024	CYP26A1	cytochrome p450 26a1	-3.4	0.0186
s2f_0005533005	FAM163A	protein fam163a-like	-3.2	0.0263

Figures 4 and **5** represent the top 20 Biological Processes and top 20 Molecular Functions from the Gene Ontology database (GO:BP and GO:MF), respectively. The entire list of genes (not just DEGs) was compared with each gene set through GSEA ranked by t-statistics and FDR < 0.05. Biological processes such as DNA replication (GO:0006260) and chromosome segregation (GO:0007059) had several up-regulated genes, indicating cell proliferation, whereas cell adhesion (GO:0007155) presented most of the down-regulated DEGs. Calcium, sodium and potassium ion transport (GO:0006816, GO:0006814, GO:0006813), angiogenesis (GO:0001525), blood coagulation (GO:0007596) and hemostasis (GO:0007599) were down-regulated (Supplementary File S7). Significant up-regulated molecular functions included endopeptidase (GO:0004175), isomerase (GO:0016853), nuclease (GO:0004518) and ligase (GO:0016874) activity (**Supplementary File S8**).

A customized heatmap was plotted with the significant DEGs of the cytokine-cytokine receptor interaction pathway to analyze whether the parasite *R. viridisi* regulates the expression of pro- and anti-inflammatory cytokines or their respective receptors (**Figure 6**). We can visually assess whether a DEG has a relatively uniform difference across all samples in each group or if the difference was driven by a more heterogeneous response. Erythropoietin receptor (*epor*), interferon lambda receptor 1 (*ifnlr1*), tumor necrosis factor receptor superfamily, member a (*tnfrsfa*), and member 19 (*tnfrsf19*), atypical chemokine receptor 3b (*ackr3*), chemokine (C-C motif) receptors 9a and 9b (*ccr9a* and *ccr9b*), interleukin 20 receptor beta (*il20rβ*) genes were down-regulated in infected gills (mainly from T2 and T4), while interleukin 13 receptor, alpha 2 (*il13rα2*), interleukin 9 receptor (*il9r*), chemokine (C-X-C motif) receptor 2 (*cxcr2*), and chemokine (C-C motif) receptor 11.1 (*ccr11.1*) were clearly up-regulated in T1. Nerve growth factor beta (*ngfβ*), transforming growth factor β (*tgfβ*), growth hormone receptor (*ghr*), interleukin 11a (*il11a*), erythropoietin (*epo*), and growth differentiation factors 5, 6, and 10a (*gdf5*, *gdf6* and *gdf10a*, respectively) were down-regulated. Thrombopoietin (*thpo*), chemokine (C-C motif) ligand 33, duplicate 2 (*ccl33.2*) interleukin-17 ligands genes *il17d*, *il17a/f1*, and *il17a/f2* were upregulated in infected gills.

We also found that immunoglobulin lambda-like-1 (*igll1*), 5 immunoglobulin lambda-1 light chain-like (s2f_0009908003, s2f_0009908004, s2f_0009908005, s2f_0009908006, s2f_0001205009), immunoglobulin light iota constant 1, s1 (*igic1s1*), immunoglobulin iota chain-like (s2f_0007396003), secreted immunoglobulin domain 1 (*sid1*), and v-set pre-B cell

surrogate light chain 3 (*vpreb3*) genes were upregulated in infected gills. Toll-like receptor 1 (*tlr1*) was the only upregulated gene (log2FC = +1.7, FDR = 0.01) among the TLRs in *C. viridis*.

4. Discussion

A hypothesis of interest is whether the monogenean parasites promote the upregulation of anti-inflammatory cytokines and downregulation of pro-inflammatory cytokines, causing overall immunosuppression in infected fish. Ilgová et al. [8] reviewed and synthesized the main immunomodulatory mechanisms of monogeneans, mainly polyophistocotyleans, and the immune responses of host fish, showing differences in cytokine expression depending on the fish-monogenean model. In the present study, the impact of a monophistocotylean monogenean, *R. viridisi*, on the immune response of a marine fish, *C. viridis*, was analyzed. Inflammatory response (GO:0006954) was overrepresented among the significant up-regulated biological processes (Supplementary File S7), however, the expression patterns of the main pro-inflammatory cytokines such as *il1β*, *il6*, *il8*, *il10*, and *tnfα*, had no change in the gills of *C. viridis* infected with *R. viridisi*, which is consistent with a previous work [5].

We found that erythropoietin *epo*, its receptor *epor*, and transforming growth factor β (*tgfβ*) were downregulated in infected gills of *C. viridis*. Another study demonstrated that hypoxic conditions can induce *epo* expression in the gills of *Gymnocypris dobula* [20]. In mammals, EPOR gene product highly expressed on the monocyte/macrophage membranes, exerts cytoprotective effects on non-erythroid cells, directly inhibits naïve and memory conventional T cell proliferation by signaling through the homodimer EPOR, induces TGFβ synthesis by monocytes, and converts naïve CD4+ T cells into functional iTreg by stimulating local production of functionally active TGFβ by antigen-presenting cells [21]. The thrombopoietin (*thpo*) and thrombopoietin receptor (*mpl*) were upregulated. The THPO-MPL axis promotes platelet and megakaryocyte development in mammals, and has potential thrombocyte formation in fish as a hemostatic mechanism after a period of stress, leading to a rapid decrease in clotting time and promoting inflammation [22]. CXCR2 is the receptor for the chemokine CXC motif chemokine ligand 8 (CXCL8)/interleukin-8 (IL-8), and their interaction triggers the activation of the ERK MAPk signaling [23].

Th17 cell differentiation (map04659) pathway was overrepresented in the GSEA (**Supplementary File 6**). It has been reported after nodavirus infection in European sea bass (*Dicentrarchus labrax*) the overexpression of *il17d* in the brain, the target tissue of the viral

replication [24]. IL17D induces the expression of *il8* from endothelial cells to attract neutrophils, basophils, and T-cells, and mediates the early local recruitment of innate immune cells, promoting inflammation [25]. Gene *il8* presented no significant fold change in our study. Genes involved in IL-17/Th17 response were particularly enriched among DEGs in guppies (*Poecilia reticulata*) infected with the monogenean *Gyrodactylus bullatarudis*, indicating a crucial role in immune cell differentiation and mucosal integrity preservation [26]. Suppression of local inflammation has also been associated with IL-17 during viral infections [24, 27]. Overall, it seems that Th17-like immunity is promoted by the infection with *R. viridisi* with a *tgfβ* independent mechanism.

Adaptive immune response (GO:0002250) was significantly overrepresented in the GSEA of GO (**Supplementary File S7**). In our work, we detected the overexpression of *vprepb3* and Ig light chain-like genes of isotype lambda, which had been identified before in fish [28]. Pre-B and mature B cells secrete VprepB3 [29]. Fish B lymphocytes express immunoglobulins (Igs) on their surface and secrete antigen-specific antibodies in response to immune challenges such as microbial infections [28]. The role of Ig in ectoparasite infections in fish is not well understood. Teleost Igs elicit a mammalian-like mucosal immune response in gill-associated lymphoid tissue [30]. Mucosal immune system in teleost fish is characterized by secretory Igs (such as IgT and IgM highly expressed in gill mucus), which, once secreted by B plasma cells, exert their function against pathogens in a relatively antigen-rich (commensal microbial communities) and tolerogenic environment [31]. **Figure 7** summarizes the main responses found in infected gills after the infection with *R. viridisi*.

In our study, it is evident that the parasite *R. viridisi* induced a shift in the gene expression patterns in the gills of Pacific white snook. Similarly, Piazzon et al. [32] showed that the gills of gilthead sea bream (*Sparus aurata*) parasitized with the monogenean *Sparicotyle chrysophrii* overexpressed genes related to the immune response such as cytokines, chemokines, immunoglobulins, complement factors, and that processes such as apoptosis, inflammation and cell proliferation dominated gills. These authors also reported that non-parasitized gill portions of infected *S. aurata* exhibited a higher number of DEGs than parasitized gill portions, indicating less response in the area of the parasite attachment. In our work, the fact that more genes were down-regulated than up-regulated, suggests that *R. viridisi* is able to induce inactivation of several pathways in parasitized gills of *C. viridis*. Primary immunodeficiency (map05340) pathways could be overrepresented in infected gills

of *C. viridis* due to the disruption of the cellular immunity, which can increase the fish susceptibility to secondary infections.

Severe gill alterations such as blood congestion, hemorrhage, fusion of secondary lamella, epithelial lifting, gill erosion, and mucus secretions have been reported on tilapia *O. niloticus* infected with ectoparasites [33]. Infiltration of mononuclear inflammatory cells and hyperplasia were observed in the gill tissue of *C. viridis* after a primary infection with *R. viridisi* [5]. In this study, the infection did not alter the hypoxia-inducible factors genes (*hif*). However, hypoxia-inducible factor 1-alpha inhibitor (*hif1an)* was upregulated in infected gills (log2FC = +1.2; FDR = 0.016), suggesting a possible mechanism of modulation, which simulates normoxia even in hypoxic conditions. This result is contrary to the obtained by Piazzon et al. [32], where the authors found down-regulation of the *hif1an* in *S. aurata* infected with *S. chrysophrii*, attributing it a positive effect to tolerate the parasite-induced hypoxia. The HIF signaling network system is related to the PI3K-Akt pathway, and both play a crucial role in hypoxia adaptation in fish [34, 35].

The osmo-respiratory mechanisms involving ionic and osmotic gradients from seawater to blood, are poorly understood in marine fish [36]. The gill of marine fish is composed of several cell types, including pavement cells, which cover most of the respiratory surface and are interspersed with mucus cells (goblet cells). The chloride cells are also known as mitochondria-rich cells or ionocytes and may be involved in the active transepithelial ionic movements in fish, and the metabolism associated with the high oxygen demand of gill tissue [37, 38]. Freshwater species directly obtain most of their calcium from the environment through the gills, while marine fish uptake calcium mainly through the intestine [39]. Hypoxia led to a more rapid *in vivo* accumulation of calcium in tissues mediated by a significant accumulation at the gill of the Pacific hagfish *Eptatretus stoutii*, showing a role for calcium in prolonged hypoxia tolerance [40].

Calcium participates in muscle fibers contraction and the nervous system's excitability; thus, an imbalance could alter fish mobility. **Figure 3** shows that the top ten significant overrepresented pathways are mainly inactivated due to the high numbers of downregulated genes. The hypertrophic cardiomyopathy (HCM) pathway in fish, similar to other vertebrates, is associated with the regulation of heart muscle growth and function, resulting in impaired cardiac function [41, 42]. The molecular mechanisms of HCM are complex and can involve altered calcium cycling and sarcomeric calcium sensitivity,

increased fibrosis, disturbed biomechanical stress sensing, and impaired cardiac energy homeostasis [43]. The calcium signaling pathway was also inactivated in our study (**Figure 3**). **Figure 4** is consistent and shows several GO related to ion transport with a tendency to inactivate. Protein s100-a1-like (S100A1) is a calcium-binding protein implicated in cardiac and neurological functions [44, 45]. In this study, *s100a1* showed elevated expression in infected compared to uninfected gills, suggesting a role in calcium homeostasis.

The functions of the heterogeneous nuclear ribonucleoprotein a2 (*roa2*), transmembrane protein 44 (*tmem44*), protein disulfide-isomerase a2 (*pdia2*), zinc-activated ligand-gated ion channel (*zacn*), protein-glutamine gamma-glutamyltransferase 5-like (*tgm5l*), protein-glutamine gamma-glutamyltransferase 4 (*tgm4*), histamine h3 receptor-like (*hrh4*), protein sprouty homolog 4 (*spry4*), cytochrome p450 26a1 (*cyp26a1*), and protein fam163a-like (*fam163a*) in fish are not well studied. These genes presented a higher fold change in *C. viridis* infected with *R. viridisi* (**Table 3**). In mammals, ROA2 acts as a nuclear "reader" by binding N6-methyladenosine (m6A)-containing pri-miRNAs, and m6A-containing pre-mRNAs, and regulates mRNA splicing [46]. It also activates the innate immune response, and translocates to the cytoplasm, where it activates the TANK-binding kinase 1 (TBK1) - interferon regulatory factor 3 (IRF3) pathway, leading to interferon-alpha/beta production [47]. The function of TMEM44 in mammals remains unknown. PDIA2 in humans acts as a chaperone by folding the nascent proteins in the endoplasmic reticulum and inhibiting aggregation of misfolded proteins due to its ability to form and rearrange disulfide bonds [48]. ZACN is a zinc-activated ligand-gated ion channel, a member of the Cys-loop receptor superfamily with physiological functions in mammals [49].

Transglutaminases catalyze the protein cross-linking and the conjugation of polyamines to specific proteins, which are important for several functions. It has been reported that medaka (*Oryzias latipes*) mutants deficient in the enzyme transglutaminase 2 (TG2) had movement retardation [50]. In vertebrates, TG2 activates the master transcriptional regulator of stress-responsive genes, heat shock transcription factor (HSF) 1, and TG2 ablation leads to the downregulation of the Wnt/β-catenin pathway [51]. We found that *tgm4* and *tgm5l* (coding for transglutaminases) (**Table 3**) and several genes of the Wnt pathway were significantly downregulated in infected snooks (**Figure 4**), while *hsf2* and *hsf4* remained unchanged (**Supplementary File S4**).

HRH4 is a histamine receptor localized in myeloid cell lines of mammals, such as mast cells, basophils, and eosinophils. HRH4 participates in inflammation and immune responses by inducing mast cell degranulation and the expression of various inflammatory cytokines and chemokines [52]. SPRY4 suppresses the insulin receptor, the epidermal growth factor-receptor transduced mitogen-activated protein kinase (EGF-MAPK) signaling pathway, and the extracellular-signal-regulated kinase (ERK) activation in mammals [53, 54]. CYP26A1 is a cytochrome p450 involved in the metabolism of all-trans retinoic acid that plays a role in the oxidative metabolism of xenobiotics [55]. It has been reported that *fam163a* overexpression facilitated ERK phosphorylation and promoted the expression of *cyclin D1* increasing the cellular proliferation of lung squamous cell carcinomas cells [56]. Even though *fam163a* is downregulated in infected snooks, several genes are participating in significant pathways related to cellular division and proliferation (**Figure 4**). It is notable that the golgi-associated plant pathogenesis-related protein 1-like (*glipr2*) gene, which may be involved in positive regulation of the ERK cascade and the epithelial cell migration, was upregulated in this study, and it was downregulated in the gills of the marine fish *Sillago sihama* after one hour of hypoxia [57].

Finally, the significant differences in the Bagenal and Tesch condition factor (BTCF) between control and infected fish indicates that *R. viridisi* can negatively affect the health status of Pacific white snook. Similarly, farmed tilapia *O. niloticus* exhibited low BTCF values (≤ 0.25) associated with the high infection levels of the monogeneans *Cichlidogyrus* sp. and *Gyrodactylus* sp., and the protozoans *Trichodina* sp. and *Vorticella* sp. [58]. Based on these results, the use of BTCF, which eliminates the effects of allometry from the estimation of fish condition, is recommended to evaluate the impact of ectoparasites on fish health.

5. Conclusion

The infection with *R. viridisi* maintains a tolerogenic microenvironment, without eliciting pro-inflammatory cytokines expression in infected gills of white snook, and promotes the overexpression of genes associated with the proliferation of thrombocytes and plasma B cells. Further analyses are required to elucidate the functions of the most differentially regulated genes in fish, and to corroborate if the adaptive immune responses are activated against the parasite or other associated microbial pathogens.

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Supplementary Materials: Supplementary File S1: Count table; Supplementary File S2: Ortholog annotation; Supplementary File S3: Intensity and Condition Factors data; Supplementary File S4: Statistical Analysis of Transcripts; Supplementary File S5: ORA of KEGG pathways results. Supplementary File S6: GSEA of KEGG pathways; Supplementary File S7: GSEA of GO:BP; Supplementary File S8: GSEA of GO: MF. Available at https://data.mendeley.com/datasets/r77r5c99sc/1

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Figure captions

Figure 1. Principal Component Analysis of the samples (raw counts after normalization), shows that the monogenean parasitic infection explained 42.1% of the total variation.

Figure 2. Volcano plot representing 1430 differentially expressed genes of the infected group compared to the control. Red: 570 upregulated genes; Blue: 860 downregulated genes (FDR < 0.05, log2|FC| ≥ 1).

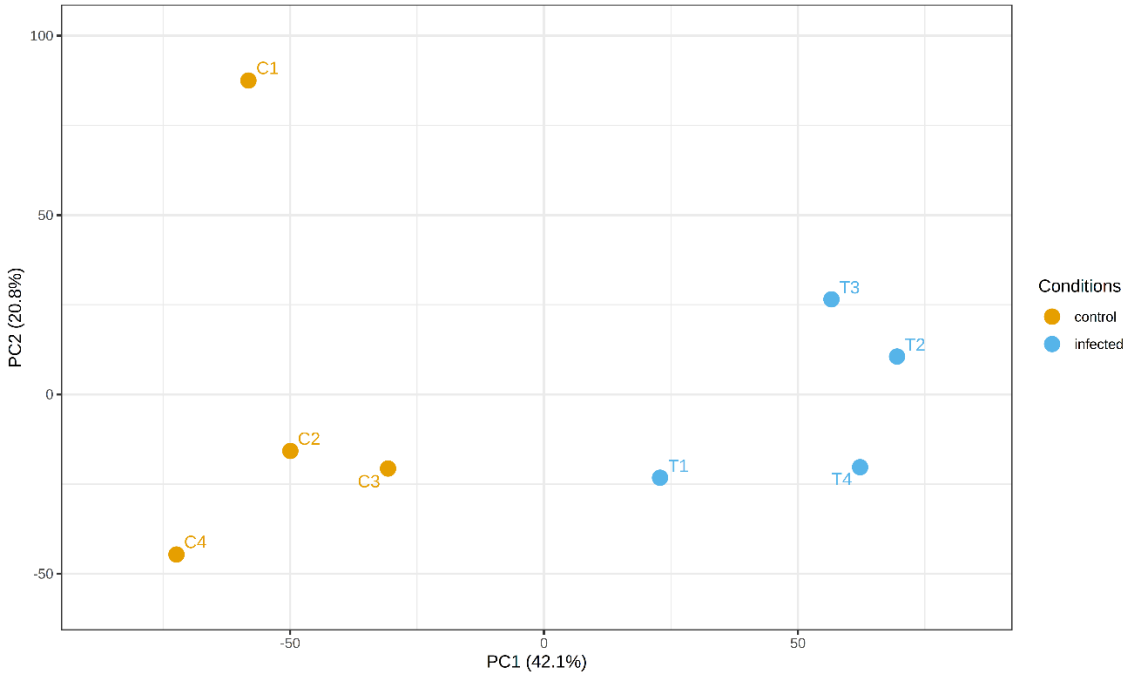
Figure 3. Ridgeline represents the top 10 KEGG pathways. ORA enrichment analysis compared the list of DEGs with gene sets corresponding to KEGG pathways. The raw p-value cutoff was 0.05, and the data was sorted by p-value. Red circles represent upregulated DEGs and blue circles represent the downregulated DEGs of each pathway.

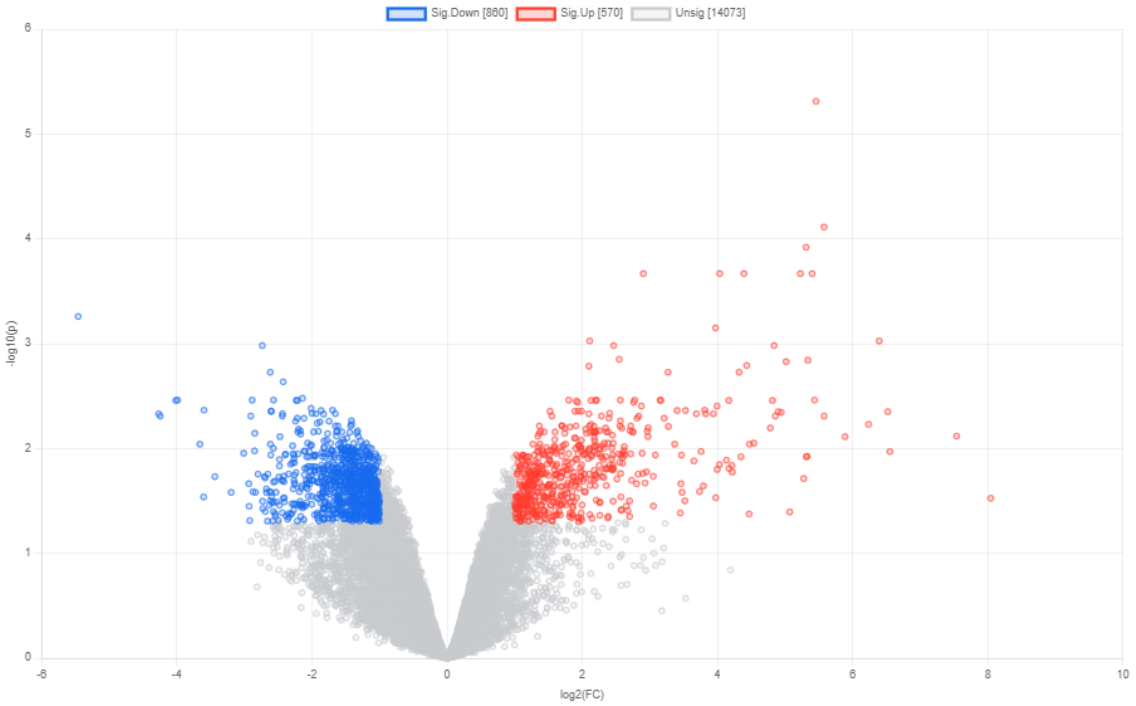
Figure 4. Ridgeline represents the top 20 Biological Processes from the Gene Ontology database (GO:BP). GSEA analysis was performed with the Rank T-statistic (p-value < 0.05). The processes were sorted by the p-values. Red circles represent genes with positive fold change and blue circles represent genes with negative fold change.

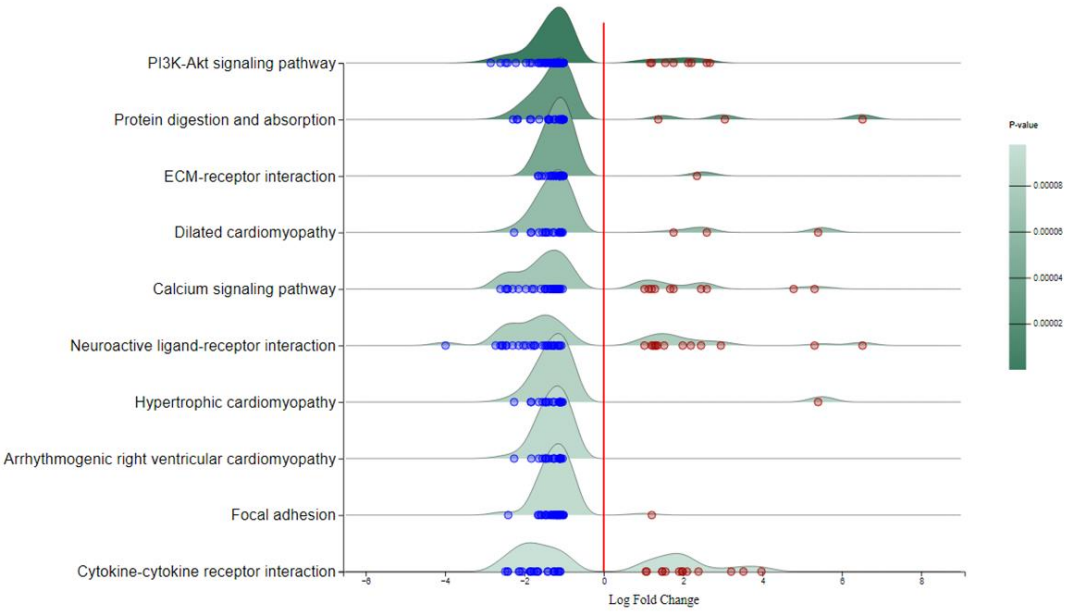
Figure 5. Ridgeline represents the top 20 Molecular Functions from the Gene Ontology database (GO:MF). GSEA analysis was performed with the Rank T-statistic (p-value < 0.05). The functions were sorted by the p-values. Red circles represent genes with positive fold change, and blue circles represent genes with negative fold change.

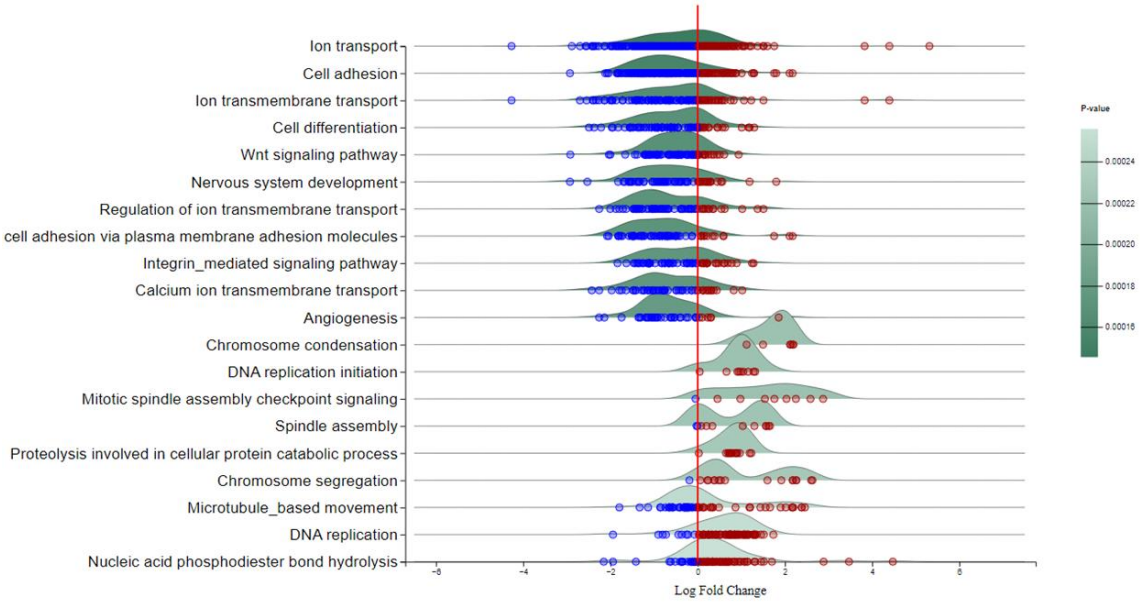
Figure 6. Heatmap of the cytokine-cytokine receptor interaction-related DEGs. Ward's method was used for clustering samples. Gene expression data are scaled to z-scores. Each row has a mean of 0 and a standard deviation of 1.

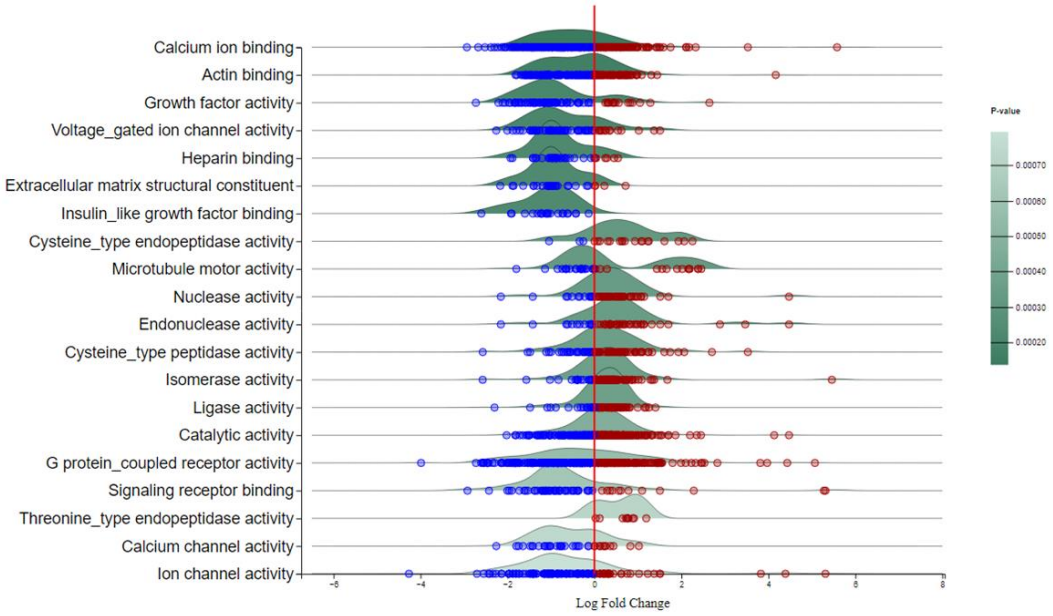
Figure 7. Main responses triggered by the parasite *R. viridisi* in infected gills of *C. viridis* according to this study: calcium imbalance, tissue repair, innate immunity suppression, hemostasis and adaptative immunity activation. Red color means upregulated genes. Blue color represents downregulated genes.

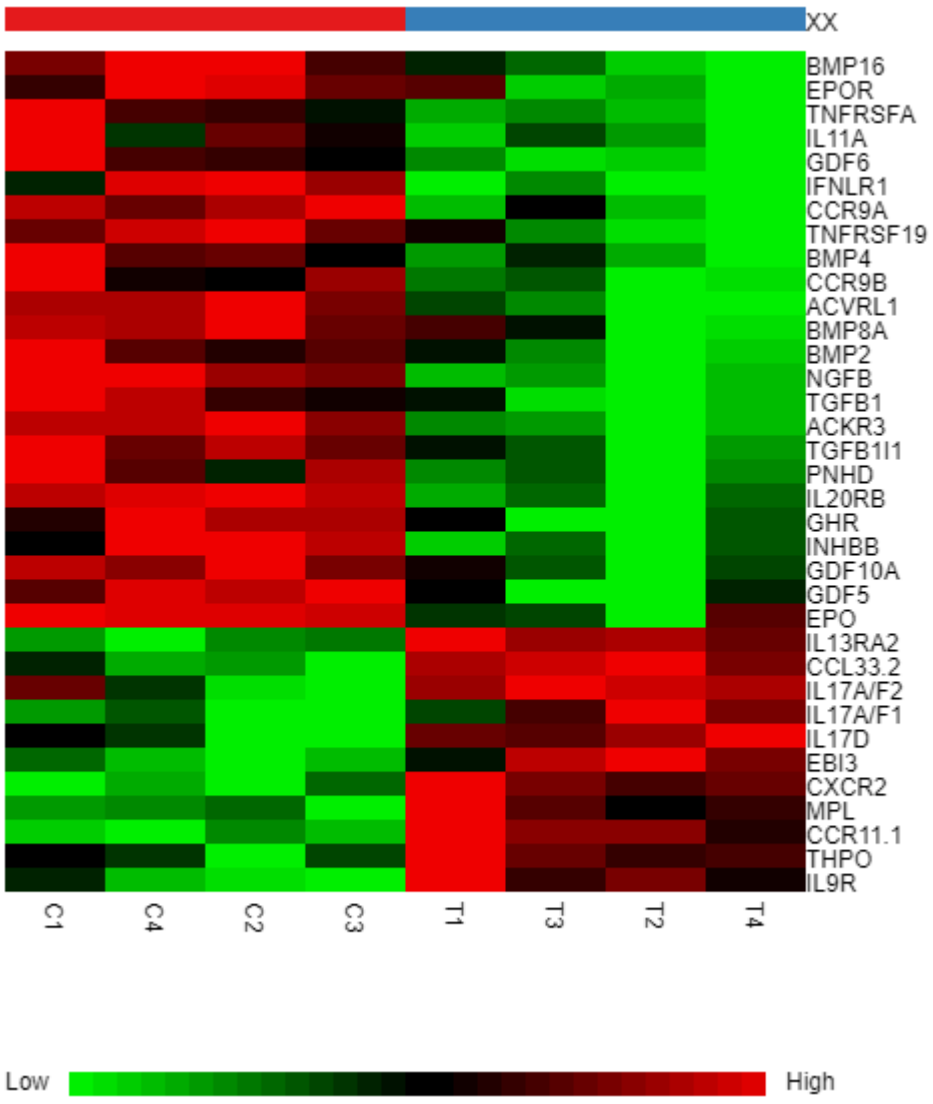

100
50
0
-50
PC2 (20.8%)
C1
C2
C3
C4
T1
T2
T3
T4
-50
0
50
PC1 (42.1%)
Conditions
control
infected

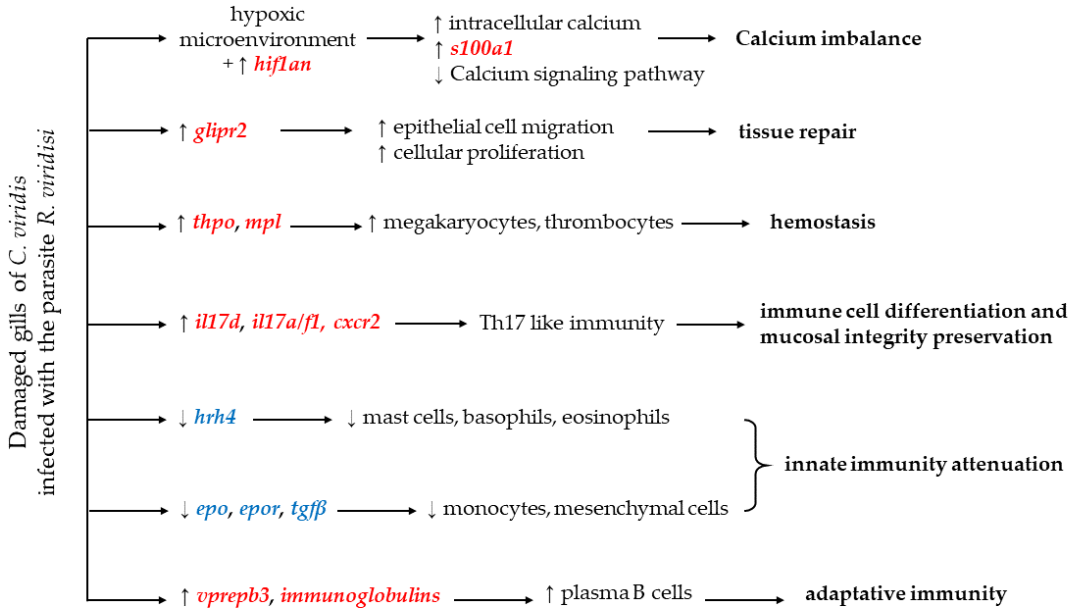

Sig.Down [860]
Sig.Up [570]
Unsig [14073]
-log10(p)
log2(FC)
6
5
4
3
2
1
0
-6
-4
-2
0
2
4
6
8
10


PI3K-Akt signaling pathway
Protein digestion and absorption
ECM-receptor interaction
Dilated cardiomyopathy
Calcium signaling pathway
Neuroactive ligand-receptor interaction
Hypertrophic cardiomyopathy
Arrhythmogenic right ventricular cardiomyopathy
Focal adhesion
Cytokine-cytokine receptor interaction
−6
−4
−2
0
2
4
6
8
Log Fold Change
P-value
0.00008
0.00006
0.00004
0.00002


Ion transport
Cell adhesion
Ion transmembrane transport
Cell differentiation
Wnt signaling pathway
Nervous system development
Regulation of ion transmembrane transport
cell adhesion via plasma membrane adhesion molecules
Integrin_mediated signaling pathway
Calcium ion transmembrane transport
Angiogenesis
Chromosome condensation
DNA replication initiation
Mitotic spindle assembly checkpoint signaling
Spindle assembly
Proteolysis involved in cellular protein catabolic process
Chromosome segregation
Microtubule_based movement
DNA replication
Nucleic acid phosphodiester bond hydrolysis
−6
−4
−2
0
2
4
6
Log Fold Change
P-value
0.00024
0.00022
0.00020
0.00018
0.00016


Calcium ion binding
Actin binding
Growth factor activity
Voltage_gated ion channel activity
Heparin binding
Extracellular matrix structural constituent
Insulin_like growth factor binding
Cysteine_type endopeptidase activity
Microtubule motor activity
Nuclease activity
Endonuclease activity
Cysteine_type peptidase activity
Isomerase activity
Ligase activity
Catalytic activity
G protein_coupled receptor activity
Signaling receptor binding
Threonine_type endopeptidase activity
Calcium channel activity
Ion channel activity
-6
-4
-2
0
2
4
6
8
Log Fold Change
P-value
0.00070
0.00060
0.00050
0.00040
0.00030
0.00020


XX
BMP16
EPOR
TNFRSFA
IL11A
GDF6
IFNLR1
CCR9A
TNFRSF19
BMP4
CCR9B
ACVRL1
BMP8A
BMP2
NGFB
TGFB1
ACKR3
TGFB1I1
PNHD
IL20RB
GHR
INHBB
GDF10A
GDF5
EPO
IL13RA2
CCL33.2
IL17A/F2
IL17A/F1
IL17D
EBI3
CXCR2
MPL
CCR11.1
THPO
IL9R
C1
C4
C2
C3
T1
T3
T2
T4
Low
High


Damaged gills of C. viridis
infected with the parasite R. viridisi
hypoxic microenvironment + ↑ hif1an
↑ intracellular calcium
↑ s100a1
↓ Calcium signaling pathway
Calcium imbalance
↑ glipr2
↑ epithelial cell migration
↑ cellular proliferation
tissue repair
↑ thpo, mpl
↑ megakaryocytes, thrombocytes
hemostasis
↑ il17d, il17a/f1, cxcr2
Th17 like immunity
immune cell differentiation and mucosal integrity preservation
↓ hrh4
↓ mast cells, basophils, eosinophils
↓ epo, epor, tgfβ
↓ monocytes, mesenchymal cells
innate immunity attenuation
↑ vprepb3, immunoglobulins
↑ plasma B cells
adaptative immunity

4. RESULTADOS Y DISCUSIÓN GENERAL

Existe un creciente interés por desarrollar la acuicultura marina para consumo humano y esto conlleva a identificar los patógenos que afectan la especie de cultivo en particular y a buscar métodos de control efectivos (Buchmann, 2022). El robalo *Centropomus viridis* es un pez marino con grandes potenciales para la acuicultura en jaulas. Los reproductores y juveniles de *C. viridis* han sido afectados por eventos de infección con el monogeneo *Rhabdosynochus viridisi*. La administración de compuestos químicos como el praziquantel o antibióticos a los peces infectados con monogeneos no ha sido del todo eficaz contra los huevos del parásito y tiene otras limitaciones tales como déficit de ingestión de los compuestos por la baja palatabilidad de los alimentos, contaminación del medio circundante, dispersión de los compuestos químicos y desarrollo de poblaciones resistentes a los fármacos en uso (Arya y Singh, 2017). Por otro lado, la obtención y evaluación de inmunoestimulantes y vacunas, así como la aplicación de métodos de control genético contra monogeneos, se han visto limitados por la escasa información sobre las interacciones moleculares entre parásito y hospedero (Buchmann y Lindenstrøm, 2002), así como los mecanismos de evasión del sistema inmune y los antígenos específicos del parásito que desencadenan la respuesta inmune protectora de memoria en los peces (Shivam *et al.,* 2021).

En este trabajo, se identificaron 1655 posibles proteínas secretoras (PS) de *R. viridisi* a partir del transcriptoma de especímenes adultos. El análisis bioinformático funcional de estas PS indicó su probable participación en la adhesión, penetración, incorporación de nutrientes e inmunomodulación de las células del hospedero. Los resultados fueron similares a las predicciones realizadas para otras especies de monogeneos como *Eudiplozoon nipponicum*, *Gyrodactylus salaris* y *Neobenedenia melleni* (Caña-Bozada *et al.,* 2021). El presente estudio encontró una sobrerrepresentación de las hidrolasas en el secretoma de *R. viridisi*, lo cual es consistente con lo observado en otros helmintos parásitos (Gahoi *et al.,* 2019). En particular, se encontraron las catepsinas citosólicas B, C, D y L, las cuales podrían participar en procesos de proteólisis e inducción de apoptosis. Ambos procesos contribuyen a la penetración del parásito, a la destrucción de receptores proteicos y a regulación de las poblaciones celulares del sistema inmunológico del pez (Zakeri *et al.,* 2018). También se identificaron posibles peptidasas tipo serina, las cuales *R. viridisi* podría utilizar para llevar a cabo la digestión extracelular del moco y tejido epitelial del pez

(Jedličková *et al.*, 2018). El secretoma de *R. viridisi* incluyó varios inhibidores de la subfamilia I93 conocidos como inhibidores de metalopeptidasas. Se ha propuesto que estos inhibidores están involucrados en el recambio de la matriz extracelular y la remodelación de tejidos en helmintos parásitos (Cantacessi *et al.*, 2013). Además, las posibles serpinas y cistatina secretadas por *R. viridisi* (inhibidores de serina y cisteína peptidasas respectivamente) podrían bloquear la activación del complemento, prevenir la inflamación por modulación de monocitos e inducir inmunosupresión en *C. viridis*, de manera similar a lo informado para *E. nipponicum* (Roudnický *et al.*, 2018; Ilgová *et al.*, 2020). Las proteínas multifuncionales tiorredoxina y trombospondina 1 encontradas en el secretoma de *R. viridisi* podrían contribuir también a la patogenicidad a través del control de la inflamación (Ditgen *et al.*, 2016; Haçarız *et al.*, 2015).

Se identificaron 18 PS de *R. viridisi* con elevada similitud de secuencia de proteínas de peces (**Anexo 1**), sugiriendo su función de mimetismo. Entre ellas estuvieron catepsinas, receptores de manosa, proteínas con dominios tipo lectina y otras con potencial actividad enzimática. Estas proteínas no se pueden adjudicar con certeza al pez o al parásito, a menos que se identifiquen las secuencias en los genomas de ambas especies. No obstante, se sabe que los parásitos pueden liberar proteínas similares a las de su hospedero como mecanismo de evasión o con fines inmunorreguladores (Hebert *et al.*, 2015; Mei y Zhang, 2020). Es de destacar que la proteína *Glioma pathogenesis-related protein 1* (GLIPR1) fue una de las PS tipo VAL (*Venom allergen-like*, por sus siglas en inglés) de *R. viridisi* con potencial antigénico. A su vez, *glipr2* fue uno de los genes más sobre-regulados en las branquias de *C. viridis* infectadas con *R. viridisi*. GLIPR1 se ha reportado en el secretoma de otros parásitos, indicando un rol en la adhesión celular (Sotillo *et al.*, 2019), mientras que *glipr2* se ha detectado en branquias de otro pez marino (*Sillago sihama*) sometido a 1 hora de hipoxia (Saetan *et al.*, 2020). Al menos en mamíferos, GLIPR2 funge como regulador positivo de la migración de células epiteliales y regulador negativo de la autofagia (Zhao *et al.*, 2020). La expresión de *glipr2* se encontró enriquecida en monocitos/macrófagos, células NK y linfocitos T en proliferación, sugiriendo un rol potencial en el reclutamiento de células inmunes y alteraciones en el microambiente (Lin *et al.*, 2024).

En el presente trabajo, la respuesta inflamatoria (GO:0006954) se mostró sobrerrepresentada en las branquias de *C. viridis* infectado con *R. viridisi*; sin embargo, los patrones de expresión de las principales citocinas proinflamatorias, como *il1β*, *il6*, *il8*, *il10* y *tnfα*, no tuvieron cambio, lo cual concuerda con un trabajo previo (López-Moreno *et al.*, 2024). En el secretoma de *R. viridisi*, la

ruta KEGG más representada fue la infección por el virus del papiloma humano (VPH), la cual podría estar asociada con la evasión inmune. Las oncoproteínas del VPH regulan negativamente la expresión de citocinas y quimiocinas proinflamatorias y regulan positivamente la expresión de genes inmunosupresores en las células hospederas (Scarth *et al.*, 2021). Esto pudiera explicar uno de los mecanismos de inmunomodulación. Por otro lado, los procesos biológicos tales como replicación del ADN (GO:0006260) y segregación cromosómica (GO:0007059) presentaron varios GED sobre-regulados, indicando proliferación celular. Los marcadores de monocitos, mastocitos, basófilos y eosinófilos estuvieron sub-regulados, y la vía de diferenciación de células Th17 (map04659) sobrerrepresentada. Se detectó también en este estudio la sobre-expresión de ligandos de la interleucina 17 (IL-17). Por lo anterior, se puede inferir una proliferación de células epiteliales y linfocitos Th17. En otro estudio, con juveniles de *Poecilia reticulata* infectados con el monogeneo *Gyrodactylus bullatarudis*, los genes implicados en la respuesta IL-17/Th17 estuvieron particularmente enriquecidos, lo que indica un papel crucial en la reparación tisular, diferenciación de las células inmunes y la preservación de la integridad de la mucosa (Konczal *et al.*, 2020).

Se detectó la expresión constitutiva de los *tlr2*, *tlr3*, *tlr5*, *tlr7*, *tlr8*, *tlr9*, *tlr13*, *tlr18*, *tlr21* y *tlr22* en las branquias, sin embargo, solo *tlr1* se encontró sobreexpresado en las branquias infectadas. Se ha reportado sobreexpresión de *tlr1* en riñón del pez gato amarillo híbrido (*Pelteobagrus fulvidraco* × *P. vachelli*) en respuesta a la infección con la bacteria *Aeromonas hydrophila*, indicando un rol en la respuesta inflamatoria (Guo *et al.*, 2023). Los PAMPs para los TLR1, TLR7, TLR8, TLR13-R20 y TLR23–27 en peces aún no se han caracterizado (Sahoo, 2020). Los TLR pueden activar las células presentadoras de antígenos, las células secretoras de anticuerpos y también participan en el inicio de la presentación de antígenos a las células T, por lo cual son receptores clave en la activación de las respuestas inmunitaria innata y adaptativa específica (Mahapatra *et al.*, 2023). Por otro lado, se identificaron también los genes *clip*, *ciita*, *mhc1ula*, y *mhc1laa*, los cuales no presentaron cambios en las branquias infectadas. CIITA es necesario y suficiente para inducir la expresión de *mhc ii* (León Machado y Steimle, 2021). Sin embargo, no se detectó ningún gen de *mhc ii* en las branquias de *C. viridis*. En los peces teleósteos, la familia de genes *mhc* clase I se encuentra expandida y localizada en distintos cromosomas, mientras que los *mhc* clase II se han perdido en algunas especies (Johnstone y Chaves-Pozo, 2022). Además, el número de copias funcionales de genes *mhc ii β* puede variar entre especies (desde una copia en salmónidos hasta más de 10 copias en los cíclidos), así como entre poblaciones e individuos de la misma población

(Gerdol *et al.,* 2019). En una búsqueda preliminar de los genes del *mhc* clase I y II de *C. viridis* (**Anexo 2**), a partir de un transcriptoma obtenido previamente (Llera-Herrera, 2019), se encontraron secuencias de los genes *mhc i* y *mhc ii*. El transcriptoma incluyó órganos con funciones inmunes tales como riñón cefálico, bazo e hígado. Esto sugiere que, al momento de la toma de las muestras en este trabajo, no había suficientes células presentadoras de antígenos en las branquias.

La respuesta inmune adaptativa (GO:0002250) estuvo significativamente sobrerrepresentada en las branquias de *C. viridis* infectadas con *R. viridisi* (**Anexo 3**). En este trabajo se observó una sobreexpresión de *vprepb3* y genes similares a las cadenas ligeras de inmunoglobulinas (Ig) del isotipo lambda identificadas anteriormente en peces (Fillatreau *et al.,* 2013), lo que indica una posible abundancia y activación de células B plasmáticas (Rodig *et al.,* 2010). Los linfocitos B de peces secretan anticuerpos específicos (como las IgT e IgM altamente expresadas en el moco) en respuesta a desafíos inmunes como infecciones microbianas (Fillatreau *et al.,* 2013; Yu *et al.,* 2020). Sin embargo, no se comprende bien el papel de las Ig en las infecciones por ectoparásitos. Algunos antígenos específicos de monogeneos (Monni y Cognetti-Varriale, 2001; Hatanaka *et al.,* 2005) y los anticuerpos de peces (Leeksma et al., 2002; Sasaki et al., 2003) se han identificado durante procesos de infección. Sin embargo, estos estudios son limitados debido a la dificultad de obtener anticuerpos anti-Fc específicos para diferentes especies de peces y extractos proteicos de alta calidad a partir de monogeneos, ya que estos por lo general son pequeños y difíciles de mantener en cultivo. En este trabajo se realizó la predicción bioinformática de antigenicidad con el software Vaxijen y la mayoría (63%) de las PS de *R. viridisi* resultaron ser antígenos potenciales. El reconocimiento de dichos antígenos por parte de los anticuerpos de *C. viridis*, podría promover la citotoxicidad celular dependiente de anticuerpos como mecanismo para eliminar los parásitos. No obstante, no se detectó sobreexpresión de las cadenas pesadas de las Ig en este estudio y queda por demostrar si los anticuerpos expresados en *C. viridis* están dirigidos a los antígenos de los parásitos monogeneos o antígenos microbianos. Se comprobó recientemente que la infección de *Sparus aurata* con el monogeneo *Sparicotyle chrysophrii* modula la microbiota de las branquias y favorece las infecciones microbianas secundarias (Toxqui-Rodríguez *et al.,* 2024). La vía de inmunodeficiencia primaria (mapa05340) estuvo sobrerrepresentada en las branquias infectadas de *C. viridis*. Las alteraciones de la inmunidad celular y humoral pueden aumentar la susceptibilidad de los peces a las infecciones secundarias.

En el secretoma de *R. viridisi* se identificó la escoloptoxina SSD976, la cual es un inhibidor de los

canales de calcio dependiente de voltaje (Liu *et al.,* 2012) que podría afectar la señalización de Ca2+ en peces (Wei *et al.,* 2020). La vía de señalización del calcio también se inactivó en este estudio y el gen *s100a1* que codifica una proteína de unión a calcio mostró una expresión elevada en branquias infectadas en comparación con las no infectadas, lo que sugiere un papel en la homeostasis del calcio. El calcio participa en la contracción de las fibras musculares y en la excitabilidad del sistema nervioso; por lo tanto, un desequilibrio podría alterar la movilidad de los peces y la homeostasis (Frey *et al.,* 2011). El transporte de iones de calcio, sodio y potasio (GO:0006816, GO:0006814, GO:0006813), así como los procesos biológicos de angiogénesis (GO:0001525), coagulación sanguínea (GO:0007596) y hemostasia (GO:0007599) se regularon a la baja en branquias infectadas, sugiriendo un desbalance iónico y circulatorio.

En este estudio, no se encontraron diferencias significativas en el factor de condición de Fulton (FCF), por lo que se determinó el factor de condición de Bagenal y Tesch (FCBT), el cual elimina los efectos de la alometría en la estimación de la condición de los peces (**Anexo 4**). Los FCBT de juveniles de *C. viridis* infectados con *R. viridisi* fueron significativamente menores a los de los peces no infectados, lo que indica un peor estado de salud en los peces infectados. Sin embargo, no se encontró ninguna correlación entre los factores de condición y la densidad de parásitos (**Anexo 5**).

5. CONCLUSIONES GENERALES

La presente investigación presenta los posibles mecanismos moleculares de la interacción entre el monogeneo *R. viridisi* y su hospedero *C. viridis*. Durante la infección, los parásitos adultos podrían secretar diferentes proteínas antigénicas, enzimas y otras proteínas multifuncionales asociadas a la patogénesis. La infección con *R. viridisi* es capaz de inducir la inactivación de varias vías metabólicas en branquias parasitadas y alterar procesos biológicos tales como el transporte iónico y la hemostasia. En las branquias infectadas se mantiene un microambiente no inflamatorio, ya que no se modifican los niveles de expresión de las citocinas proinflamatorias, los receptores tipo Toll (excepto *tlr1*) ni los genes del complejo principal de histocompatibilidad. Además, se promueve la sobreexpresión de genes asociados con la proliferación de células B plasmáticas y linfocitos Th17, que pudieran participar en respuestas antihelmínticas, antimicrobianas o en la reparación tisular.

6. RECOMENDACIONES

El presente estudio proporciona una caracterización *in silico* del secretoma de *R. viridisi* así como el análisis de la expresión diferencial y funcional de los genes detectados en branquias infectadas con respecto a las no infectadas. No obstante, presenta limitaciones que generan nuevas preguntas para futuras investigaciones. Como no se disponen de los genomas del parásito monogeneo ni del robalo *C. viridis* y en la toma de muestras del parásito puede haber restos de branquias del pez, no es posible atribuir con certeza los transcritos de las proteínas secretoras a *R. viridisi*. La identificación de los factores clave de virulencia y de antígenos se ha limitado en parte debido al tamaño pequeño de los especímenes de *R. viridisi* y a la dificultad para manipularlos. Los estudios que faciliten el cultivo de estos organismos, la obtención de datos proteómicos y funcionales posibilitarán confirmar la presencia y actividad de las proteínas secretoras *in vivo*. Además, la identificación de miRNAs y lncRNAs secretados por *R. viridisi* y los análisis de co-expresión con genes del robalo, ampliarán la comprensión del rol de estas biomoléculas en la modulación de la respuesta celular y humoral. Por otro lado, se requieren más análisis para dilucidar las funciones de los genes regulados de manera más diferencial en las branquias de *C. viridis* y para corroborar si las respuestas inmunes adaptativas se activan contra el parásito u otros patógenos microbianos asociados a infecciones secundarias. La presentación de antígenos a los receptores de los linfocitos T y la citotoxicidad dependiente de anticuerpo no se ha comprobado en peces. Estos estudios contribuirán al desarrollo de vacunas antiparasitarias o terapias inmuno-moduladoras para los peces de interés comercial.

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8. ANEXOS

8.1. Proteínas con Similitud de Secuencia entre Peces y Helmintos (Correspondiente al capítulo 2)

Supplementary Table S1. Proteins with similarity between fish and parasites. E-values were obtained from the alignment of the sequences of *R. viridisi* against sequences from different taxa using the NCBI database.

Protein ID	Teleostei E-value	Lophotrochozoa E-value	Platyhelminthes E-value
DN106_c0_g1_i4.p14	2e-21 peptidase inhibitor	3e-23 protein BOX15	1e-23 protein BOX15
DN5221_c0_g1_i2.p1	3e-15 C-type lectin	5e-14 lectin/receptor	4e-07 protein BOX15
DN12056_c0_g1_i1.p1	4e-07 protocadherin	3e-08 protocadherin	1e-08 protocadherin
DN5544_c0_g1_i11.p6	3e-09 C-type lectin	8e-07 C-type lectin	3e-07 C-type lectin
DN4578_c0_g2_i10.p1	3e-83 cathepsin	2e-87 cathepsin	2e-87 cathepsin
DN51_c2_g1_i1.p1	6e-29 disintegrin and metalloproteinase domain	4e-25 disintegrin and metalloproteinase domain	2e-24 BOX15
DN59_c0_g2_i1.p4	1e-40 diphosphomevalonate decarboxylase	7e-48 diphosphomevalonate decarboxylase	1e-40 diphosphomevalonate decarboxylase
DN700_c0_g1_i10.p1	2e-09 macrophage mannose receptor	2e-09 macrophage mannose receptor	8e-06 C-type lectin
DN70202_c0_g1_i1.p1	2e-104 cathepsin	2e-104 cathepsin	7e-105 BOX15/cathepsin
DN7233_c0_g1_i15.p5	5e-12 nuclear pore complex protein	2e-10 BOX15	8e-11 BOX15
DN75_c0_g1_i1.p2	5e-04 serine protease	2e-06 chymotrypsinogen	0.018 BOX15
DN890_c0_g1_i12.p4	6e-07 stromal interaction molecule	7e-08 stromal interaction molecule	7e-08 BOX15
DN1020_c0_g1_i6.p4	3e-31 C-type lectin	4e-41 C-type lectin	5e-39 C-type lectin
DN1169_c1_g1_i8.p1	3e-09 serine protease	8e-10 chymotrypsin-like elastase	4e-08 peptidase/serine protease
DN1300_c0_g1_i5.p2	3e-22 secretory phospholipase A2	4e-26 phospholipase A2	2e-21 BOX15/ phospholipase A2
DN1349_c0_g1_i9.p4	1e-20 endoplasmic reticulum oxidoreductin 1 (ERO1)-like protein	1e-22 ERO1-like protein	8e-19 ERO1-like protein
DN13993_c0_g1_i3.p1	8e-11 C-type mannose receptor	6e-10 galactose-specific lectin	7e-09 lectin
DN5361_c0_g1_i3.p1	4e-37 frizzled-8-like	2e-40 frizzled-8	8e-41 frizzled-8

8.2. Alineamiento Básico (BLAST) para la Identificación de Transcritos de MHC en el Transcriptoma del Robalo *C. viridis* Obtenido Previamente (Llera-Herrera, 2019) (Correspondiente al capítulo 4)

Protein	**NCBI Accession**	**Specie**	**Gene ID**	**E-value**
MHC I	ADX01349.1	*Lutjanus sanguineus*	DN36239_c0_g1	**2.67 e-156**
	ABH08806.1	*Lutjanus campechanus*		5.38 e-49
MHC II a	AAD54154.1	*Lates calcarifer*	DN34820_c0_g1	2.30 e-32
	ABG77303.1	*Lutjanus campechanus*	DN35990_c0_g1	1.78 e-53
	AFI57500.1	*Lutjanus argentimaculatus*	DN35990_c0_g1	**6.66 e-88**
MHC II b	ABG79768.1	*Lutjanus campechanus*	DN34183_c10_g1	2.07 e-19
	AFI57501.1	*Lutjanus argentimaculatus*	DN34132_c2_g2	**1.13 e-99**

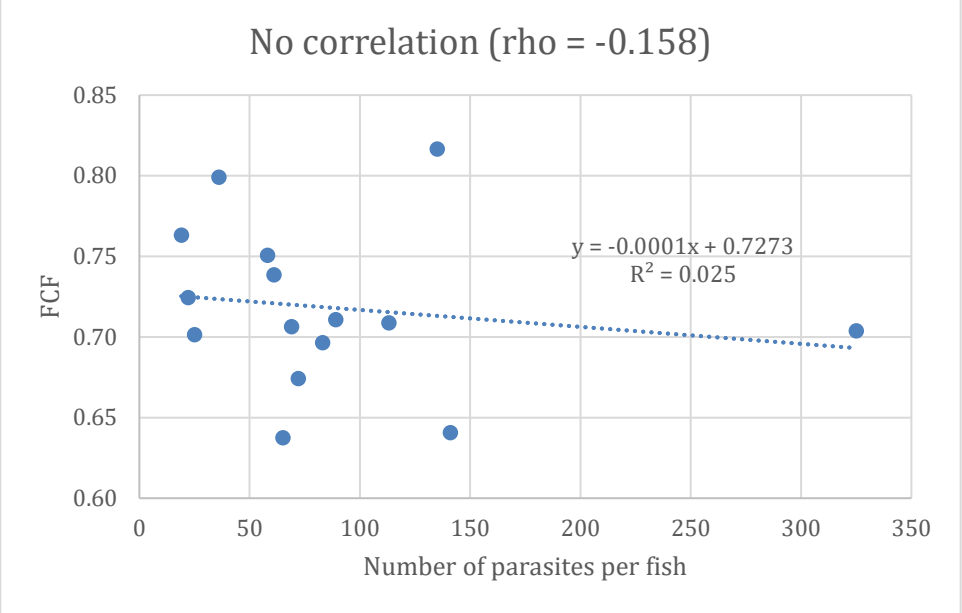
8.3. Análisis de Enriquecimiento (GSEA) de los Términos de Ontología de Genes Correspondientes a Procesos Biológicos (Correspondiente al capítulo 3)

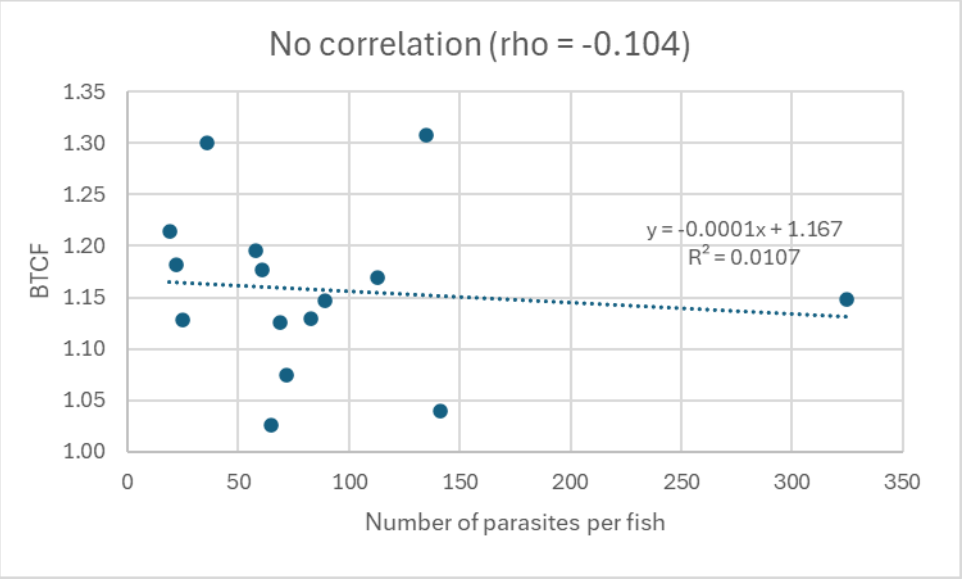
Name	Total	Hits	EnrichmentScore	Pval	Padj
Ion transport	1404	303	-0.4379	0.000117	0.008903
Cell adhesion	694	201	-0.5595	0.000123	0.008903
Ion transmembrane transport	697	113	-0.529	0.000134	0.008903
Cell differentiation	378	78	-0.5783	0.000139	0.008903
Wnt signaling pathway	149	75	-0.5729	0.00014	0.008903
Homophilic cell adhesion via plasma membrane adhesion molecules	295	67	-0.594	0.000142	0.008903
Regulation of ion transmembrane transport	174	68	-0.615	0.000142	0.008903
Nervous system development	161	63	-0.575	0.000143	0.008903
Calcium ion transmembrane transport	155	48	-0.6229	0.000146	0.008903
Calcium ion transport	125	48	-0.5707	0.000146	0.008903
Angiogenesis	56	37	-0.6169	0.000149	0.008903
Hemostasis	30	14	-0.7464	0.000162	0.008903
Chromosome condensation	50	6	0.9495	0.000244	0.009825
Kinetochore assembly	43	6	0.9566	0.000244	0.009825
Mitotic spindle assembly checkpoint signaling	39	9	0.9017	0.000253	0.009825
Proteolysis involved in cellular protein catabolic process	87	16	0.8746	0.000262	0.009825
Chromosome segregation	150	17	0.8247	0.000264	0.009825
Double_strand break repair via homologous recombination	126	18	0.7582	0.000268	0.009825
DNA replication	605	71	0.6522	0.000341	0.01153
Nucleic acid phosphodiester bond hydrolysis	496	83	0.5063	0.00036	0.01153
Cell division	514	111	0.6733	0.000397	0.01153
Cellular response to DNA damage stimulus	515	124	0.5096	0.00041	0.01153
Cell cycle	594	135	0.6268	0.000433	0.01153
Negative regulation of endopeptidase activity	491	52	-0.518	0.000435	0.01153
DNA repair	684	142	0.4849	0.000443	0.01153
Sodium ion transport	144	34	-0.6106	0.000454	0.01153
Extracellular matrix organization	122	17	-0.7319	0.000483	0.01179
Translation	939	211	0.3602	0.000576	0.01357
Microtubule_based movement	319	61	0.5154	0.000653	0.01487
DNA replication initiation	63	9	0.8736	0.000758	0.01587
Spindle assembly	67	11	0.8733	0.000768	0.01587
Blood coagulation	56	23	-0.6491	0.00077	0.01587
Potassium ion transmembrane transport	317	74	-0.4897	0.000843	0.01687
Inflammatory response	131	39	0.5347	0.00122	0.02301
Sodium ion transmembrane transport	124	28	-0.6234	0.00122	0.02301
Regulation of signal transduction	76	22	-0.6288	0.001693	0.02958
Negative regulation of peptidase activity	247	19	-0.6515	0.001726	0.02958
Integrin_mediated signaling pathway	143	53	-0.4949	0.001748	0.02958
Adaptive immune response	53	13	0.7263	0.001803	0.02975
Potassium ion transport	258	68	-0.4783	0.001849	0.02977
Circadian rhythm	32	7	-0.8253	0.002179	0.03424
Cell communication	112	39	-0.5363	0.002659	0.04082
Negative regulation of angiogenesis	18	10	-0.767	0.003139	0.0467
Peptide cross_linking	26	7	-0.8198	0.003184	0.0467
Mitotic chromosome condensation	35	5	0.9063	0.003399	0.04877

8.4. Morfometría de los Peces Muestreados, Conteo de Parásitos y Factores de Condición de Fulton y de Bagenal and Tesch (Correspondiente al capítulo 3)

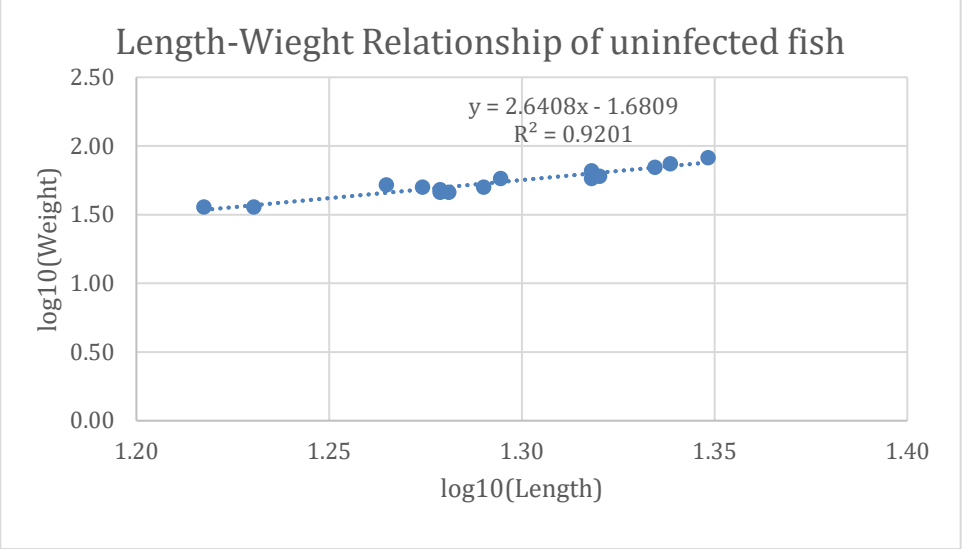
Length (cm)	log10(length)	Weight (g)	log10(weight)	Parasite Count	Fulton's CF	Bagenal and Tesch's CF
20.9	1.32	60	1.78	0	0.66	1.96
19.5	1.29	50	1.70	0	0.67	1.96
20.8	1.32	66	1.82	0	0.73	2.19
21.8	1.34	74	1.87	0	0.71	2.17
22.3	1.35	82	1.91	0	0.74	2.26
19.7	1.29	58	1.76	0	0.76	2.22
16.5	1.22	36	1.56	0	0.80	2.20
21.6	1.33	70	1.85	0	0.69	2.10
18.8	1.27	50	1.70	0	0.75	2.16
19	1.28	48	1.68	0	0.70	2.02
17	1.23	36	1.56	0	0.73	2.03
20.8	1.32	58	1.76	0	0.64	1.92
19	1.28	46	1.66	0	0.67	1.94
19.1	1.28	46	1.66	0	0.66	1.91
18.4	1.26	52	1.72	0	0.83	2.38
19	1.28	56	1.75	135	0.82	1.31
18.4	1.26	44	1.64	69	0.71	1.13
21.3	1.33	68	1.83	325	0.70	1.15
19.6	1.29	48	1.68	65	0.64	1.03
18.3	1.26	46	1.66	58	0.75	1.20
22.8	1.36	84	1.92	113	0.71	1.17
20.6	1.31	56	1.75	141	0.64	1.04
19.9	1.30	56	1.75	89	0.71	1.15
18.4	1.26	46	1.66	61	0.74	1.18
18.2	1.26	46	1.66	19	0.76	1.21
20.5	1.31	60	1.78	83	0.70	1.13
19.5	1.29	52	1.72	25	0.70	1.13
21.3	1.33	70	1.85	22	0.72	1.18
21	1.32	74	1.87	36	0.80	1.30
18.4	1.26	42	1.62	72	0.67	1.07

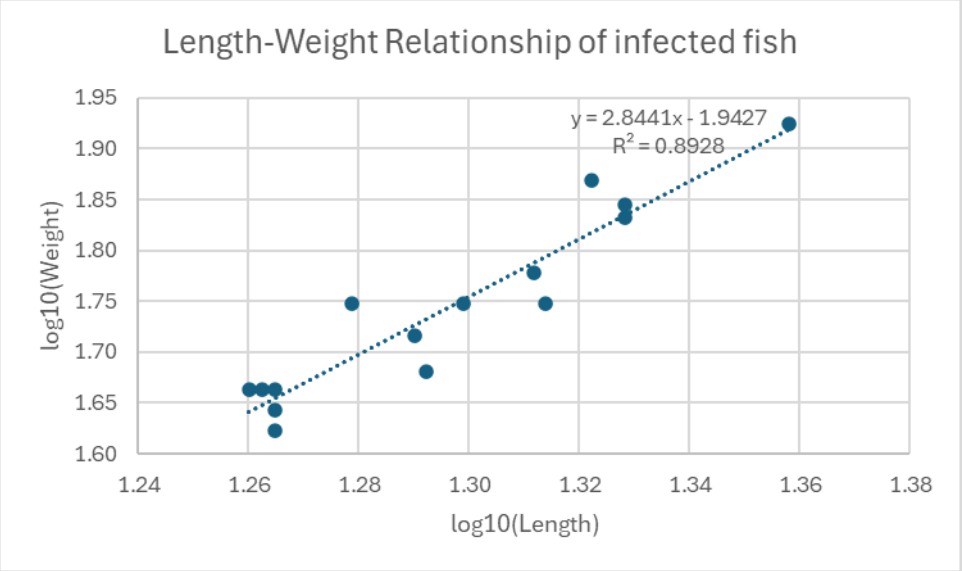
8.5. Análisis de Correlación entre los Factores de Condición de Fulton y Bagenal y Tesch y el Número de Parásitos por Pez (Correspondientes al capítulo 3)





8.6. Relación entre el Peso y la Longitud Total de los Peces Muestreados, Infectados y Control (Correspondientes al capítulo 3)





8.7. Proteínas Multifuncionales Potencialmente Secretadas por *R. viridisi* (Correspondiente al capítulo 2)

Supplementary Table S2. Possible multifunctional proteins found in predicted ES proteins of *R. viridisi*.

Protein ID	Pfam ID	Description	Reported in MultitaskProtDB
DN116_c0_g1_i15.p1	PF02225.23	Protease A domain	
	PF01532.21	Glycosyl hydrolase family 47	
DN1289_c0_g1_i2.p1	PF04777.14	Erv1 / Alr family (mitochondrial sulfhydryl oxidase)	Thioredoxin (*Escherichia coli*)
	PF00085.21	Thioredoxin (reduction of other proteins by cysteine thiol-disulfide exchange)	
DN2691_c0_g1_i1.p1	PF06468.14	Spondin_N (involved in patterning axonal growth trajectory)	Thrombospondin 1 (*Homo sapiens*)
	PF00090.20	Thrombospondin type@2 (Adhesive glycoprotein that mediates cell-to-cell and cell-to-matrix interactions. Binds heparin.)	
	PF00014.24	Kunitz inhibitor domain (protease inhibitor)	
DN2999_c0_g1_i1.p1	PF01549.25	ShK domain-like (Stichodactyla toxin that blocks potassium channels)	
	PF00094.26	von Willebrand factor type D domain (growth regulator/binding domain)	
DN30683_c0_g2_i14.p2	PF14543.7	Xylanase inhibitor N-terminal	
	PF00026.24	Eukaryotic aspartyl protease	
DN466_c0_g1_i12.p1	PF01186.18	Lysyl oxidase	
	PF00530.19	Scavenger receptor cysteine-rich domain (endocytic receptors that mediate non-opsonic phagocytosis in response to foreign ligands)	

8.8. Proteínas de Adhesión Indentificadas en el Secretoma de *R. viridisi* (Correspondiente al capítulo 2)

Supplementary Table S3. Adhesion proteins identified in *R. viridisi*.

Protein ID	Uniprot ID	Annotation
DN100674_c1_g2_i1.p1	Q5RJH3	Cadherin-12
*DN919_c0_g1_i11.p6	Q9Y5E4	Protocadherin beta-5
DN76720_c0_g1_i2.p5	Q96JQ0	Protocadherin-16
*DN2904_c1_g1_i10.p1	Q96JQ0	Protocadherin-16
*DN3831_c0_g1_i12.p2	Q9Y5G1	Protocadherin gamma-B3
*DN76720_c0_g1_i1.p1	Q9Y5F6	Protocadherin gamma-C5
*DN76720_c0_g1_i1.p2	P58365	Cadherin-23
*DN46_c0_g1_i1.p1	Q91Y11	Protocadherin alpha-9
*DN1162_c0_g1_i11.p1	Q08174	Protocadherin-1
*DN3858_c0_g2_i1.p1	Q08174	Protocadherin-1
*DN3291_c0_g2_i1.p1	Q2PZL6	Protocadherin Fat 4
*DN12056_c0_g1_i1.p1	Q9Y5G0	Protocadherin gamma-B5
*DN1190_c0_g1_i3.p1	Q9Y5G8	Protocadherin gamma-A5
DN522_c1_g1_i6.p1	Q6WYY1	Protocadherin-11 X-linked
DN7461_c0_g3_i2.p1	Q5DRF1	Protocadherin alpha-13
DN1457_c0_g1_i1.p1	Q6KEQ9	Protocadherin-11 X-linked
DN3273_c0_g2_i1.p1	Q5DRC1	Protocadherin gamma-A10
DN25713_c0_g1_i5.p4	Q9H251	Cadherin-23
DN134_c0_g1_i1.p2	A5Z1X6	Integrin beta-1
*DN1069_c0_g1_i11.p1	P05107	Integrin beta-2
*DN2351_c0_g1_i1.p1	Q06274	Integrin alpha-5
DN20_c0_g1_i2.p1	P33730	E-selectin
**DN2061_c0_g1_i12.p2	Q18823	Laminin-like protein lam-2
DN3022_c0_g1_i1.p1	Q29058	Galectin-4
*DN7800_c0_g1_i1.p1	Q24292	Protein dachsous/ protocadherin-16/23
* DN602_c1_g1_i1.p1	Q9VW71	Fat-like cadherin-related tumor suppressor homolog/ protocadherin Fat
* DN191_c0_g1_i4.p2	O89026	Roundabout homolog 1
* DN6333_c0_g4_i2.p1	G5EBF1	Protein sax-3
* DN721_c0_g1_i1.p1	Q9HCK4	Roundabout homolog 2
**DN2063_c0_g1_i13.p1	Q9UQP3	Tenascin-N
**DN557_c0_g1_i3.p1	P08120	Collagen alpha-1(IV) chain
**DN725_c0_g1_i1.p1	P02466	Collagen alpha-2(I) chain
**DN725_c0_g1_i2.p1	P02466	Collagen alpha-2(I) chain
**DN1296_c0_g1_i5.p1	P02466	Collagen alpha-2(I) chain

*KEGG BRITE, object "cell adhesion molecules" [ko04515]; **KEGG "focal adhesion" [PATH:ko04510]

8.9. Familias de Enzimas cuyos Sustratos son Carbohidratos, Predichas en el Secretoma de *R. viridisi* (Correspondiente al capítulo 2)

Supplementary Table S4. Families of carbohydrate-active enzymes (CAZymes) in predicted ES proteins of *R. viridisi*.

Protein ID	HMMER E-value	Coverage	HMM Profile
DN116_c0_g1_i15.p1	3.2e-138	0.99	GH47: Glycoside Hydrolase Family 47 (Activity: α-mannosidase (EC 3.2.1.113))
DN1423_c0_g1_i10.p2	7.3e-61	0.63	GH5_27: Glycoside Hydrolase Family 5 / Subf 27 (Activity: β-glucosidase (EC 3.2.1.21); β-mannosidase (EC 3.2.1.25))
DN1977_c0_g1_i14.p1	1.1e-154	0.99	GH31: Glycoside Hydrolase Family 31 (Activity: α-glucosidase (EC 3.2.1.20); α-galactosidase (EC 3.2.1.22); α-mannosidase (EC 3.2.1.24))
DN2542_c2_g1.i1.p1	1.6e-89	0.97	GH20: Glycoside Hydrolase Family 20 (Activity: β-hexosaminidase (EC 3.2.1.52); β-1,6-N-acetylglucosaminidase (EC 3.2.1.-))
DN3756_c0_g1_i1.p1	5.8e-39	0.98	GT31: Glycosyl Transferase Family 31 (Activity: chondroitin β-1,3-glucuronyltransferase (EC 2.4.1.226); chondroitin β-1,4-N-acetylgalactosaminyltransferase (EC 2.4.1.-))
DN502_c0_g1_i1.p5	6.8e-38	0.98	GT31
DN6559_c0_g1_i2.p1	4.8e-21	0.73	GT4: GlycosylTransferase Family 4 (Activity: α-glucosyltransferase (EC 2.4.1.52))
DN716_c0_g2_i12.p1	2.5e-129	0.99	GH47
DN834_c0_g1_i18.p2	1.6e-47	0.62	GT27: GlycosylTransferase Family 27 (Activity: polypeptide α-N-acetylgalactosaminyltransferase (EC 2.4.1.41))
DN151_c2_g1_i1.p2	DIAMOND		GT0
DN3825_c0_g1_i5.p1	Hotpep		PL0
DN725_c0_g1_i2.p1	Hotpep		PL0

GH: Glycoside Hydrolase, GT: Glycosyl Transferase, PL: Polysaccharide Lyase

8.10. Proteínas más Antigénicas de *R. viridisi* Predichas por Vaxijen (Correspondiente al capítulo 2)

Supplementary Table S5. Most antigenic ES proteins from *R. viridisi* (VaxiJen score > 0.9).

Protein ID	Annotation	Score
DN725_c0_g1_i1.p1	Collagen alpha-2(I) chain	1.3983
DN18884_c0_g6_i2.p1	Unknown nigellin-1.1	1.3867
DN2184_c0_g1_i6.p6	Unknown fkbp-type peptidyl-prolyl cis-trans isomerase slyd	1.3846
DN725_c0_g1_i2.p1	Collagen alpha-2(I) chain	1.3310
DN146924_c0_g3_i1.p1	Unknown SH3-like barrel	1.2806
DN9007_c0_g1_i4.p2	Unknown protein phosphatase 1 regulatory subunit 3a	1.2422
DN8473_c0_g5_i3.p1	Unknown Ribosomal protein L14e	1.2028
DN1296_c0_g1_i5.p1	GTPase NRas	1.1934
DN557_c0_g1_i3.p1	Collagen alpha-2(IV) chain (Nematoda)	1.1569
DN93160_c0_g2_i1.p1	Unknown beta-Grasp (ubiquitin-like)	1.1100
DN102334_c1_g2_i1.p1	Unknown PA1123-like	1.1017
DN28732_c0_g3_i1.p1	Unknown Flavodoxin-like	1.0883
DN19332_c0_g1_i7.p1	Unknown patellamide protein	1.0872
DN49560_c0_g1_i1.p1	Beta-1,4-galactosyltransferase 2	1.0820
DN8509_c0_g1_i3.p1	unknown	1.0786
DN33919_c0_g3_i1.p1	unknown	1.0510
DN98968_c0_g3_i1.p1	unknown	1.0417
DN1329_c1_g1_i12.p5	Inactive histone-lysine N-methyltransferase 2E	1.0151
DN30584_c0_g1_i1.p1	unknown	1.0057
DN11034_c2_g2_i1.p1	unknown	1.0029
DN12500_c1_g2_i1.p1	unknown	1.0007
DN46661_c0_g1_i1.p1	unknown	0.9913
DN18130_c1_g1_i1.p1	unknown	0.9897
DN90688_c0_g3_i1.p2	unknown	0.9897
DN75293_c0_g1_i1.p1	unknown	0.9874
DN69511_c0_g1_i1.p1	unknown	0.9873
DN17841_c0_g1_i1.p2	unknown	0.9814
DN111655_c0_g4_i1.p1	unknown	0.9658
DN180090_c0_g1_i1.p1	unknown	0.9604
DN121379_c0_g3_i1.p1	unknown	0.9571
DN1001_c0_g1_i1.p4	Transcription factor 12	0.9432
DN15568_c3_g2_i1.p1	unknown	0.9359

DN18483_c0_g4_i3.p1	unknown	0.9352
DN16250_c1_g5_i1.p1	unknown	0.9179
DN140269_c1_g1_i2.p1	unknown	0.9158
DN1236_c1_g1_i10.p3	unknown	0.9147
DN15610_c0_g2_i1.p1	unknown	0.9105
DN5197_c0_g2_i3.p3	unknown	0.9075
DN29218_c0_g1_i1.p1	unknown	0.9035
DN51918_c0_g1_i1.p1	unknown	0.9025
DN7673_c0_g3_i1.p1	unknown	0.9025
DN98683_c0_g1_i1.p1	unknown	0.9017
DN1882_c0_g1_i10.p1	unknown	0.9006
DN43568_c0_g3_i1.p1	unknown	0.9000

8.11. Análisis de Sobrerrepresentación (ORA) de las Rutas KEGG (Correspondiente al capítulo 3)

KEGG pathway	Total	Expected	Hits	P.Value	FDR
PI3K-Akt signaling pathway	326	30.1	60	1.47E-07	6.11E-05
Protein digestion and absorption	107	9.87	27	9.67E-07	0.000201
ECM-receptor interaction	93	8.58	24	2.49E-06	0.000345
Calcium signaling pathway	218	20.1	40	1.84E-05	0.0019
Neuroactive ligand-receptor interaction	245	22.6	43	2.80E-05	0.00213
Dilated cardiomyopathy	100	9.22	23	3.08E-05	0.00213
Arrhythmogenic right ventricular cardiomyopathy	84	7.75	20	5.91E-05	0.00345
Focal adhesion	207	19.1	37	6.65E-05	0.00345
Cytokine-cytokine receptor interaction	203	18.7	36	9.80E-05	0.00452
Hypertrophic cardiomyopathy	95	8.76	21	0.000123	0.00512
Amoebiasis	88	8.12	19	0.000351	0.0132
Taste transduction	48	4.43	12	0.00109	0.0377
Small cell lung cancer	97	8.95	19	0.00124	0.0395
Pathways in cancer	512	47.2	67	0.00211	0.0618
Hematopoietic cell lineage	80	7.38	16	0.00233	0.0618
Human papillomavirus infection	326	30.1	46	0.00238	0.0618
Basal cell carcinoma	68	6.27	14	0.00323	0.0788
Cell cycle	125	11.5	21	0.00501	0.109
Salivary secretion	57	5.26	12	0.00511	0.109
Progesterone-mediated oocyte maturation	79	7.29	15	0.00525	0.109
Complement and coagulation cascades	72	6.64	14	0.00553	0.109
Serotonergic synapse	85	7.84	15	0.0104	0.197
Regulation of lipolysis in adipocytes	48	4.43	10	0.011	0.198
cGMP-PKG signaling pathway	143	13.2	22	0.0117	0.202
cAMP signaling pathway	187	17.2	27	0.0129	0.214
Hippo signaling pathway	164	15.1	24	0.0157	0.25
Staphylococcus aureus infection	69	6.36	12	0.023	0.345
Styrene degradation	3	0.277	2	0.0239	0.345
Cholinergic synapse	94	8.67	15	0.0248	0.345
Viral myocarditis	70	6.46	12	0.0255	0.345
Renin-angiotensin system	13	1.2	4	0.0262	0.345
Renin secretion	55	5.07	10	0.0273	0.345
Intestinal immune network for IgA production	40	3.69	8	0.0274	0.345
Oocyte meiosis	97	8.95	15	0.0319	0.39
Primary immunodeficiency	35	3.23	7	0.038	0.45
TGF-beta signaling pathway	100	9.22	15	0.0405	0.466
JAK-STAT signaling pathway	127	11.7	18	0.0436	0.473
Vascular smooth muscle contraction	101	9.32	15	0.0436	0.473
Axon guidance	190	17.5	25	0.0444	0.473
Melanoma	60	5.53	10	0.0465	0.483

8.12. Análisis de Enriquecimiento (GSEA) de las Rutas Metabólicas KEGG (Correspondiente al capítulo 3)

Name	Total	Hits	EnrichmentScore	Pval	Padj
ECM-receptor interaction	374	90	-0.6507	1.90E-12	7.11E-10
Focal adhesion	717	200	-0.5018	5.83E-10	1.09E-07
PI3K-Akt signaling pathway	1291	310	-0.4445	3.76E-09	4.68E-07
Hypertrophic cardiomyopathy	289	84	-0.5819	1.14E-08	1.06E-06
Antigen processing and presentation	337	64	0.6371	1.92E-08	1.44E-06
Axon guidance	595	177	-0.4824	1.06E-07	6.63E-06
MAPK signaling pathway	1023	269	-0.4333	1.45E-07	7.72E-06
Proteasome	162	42	0.6909	1.96E-07	9.18E-06
DNA replication	214	37	0.7095	2.31E-07	9.59E-06
Breast cancer	403	134	-0.4982	3.32E-07	1.24E-05
Influenza A	845	144	0.4596	4.27E-07	1.45E-05
Primary immunodeficiency	92	34	0.7063	8.37E-07	2.61E-05
Wnt signaling pathway	637	154	-0.4709	1.74E-06	5.00E-05
Cell cycle - yeast	456	70	0.5581	2.16E-06	5.38E-05
Proteoglycans in cancer	794	203	-0.4375	3.08E-06	7.21E-05
Lysosome	528	135	0.4415	3.76E-06	8.26E-05
Epstein-Barr virus infection	800	195	0.3885	4.42E-06	8.84E-05
Arrhythmogenic right ventricular cardiomyopathy	181	75	-0.5484	4.49E-06	8.84E-05
Dilated cardiomyopathy	306	90	-0.5176	4.92E-06	9.19E-05
Hippo signaling pathway	452	154	-0.4591	5.99E-06	0.000107
Cell cycle	518	120	0.4504	7.68E-06	0.000131
Adrenergic signaling in cardiomyocytes	619	120	-0.4836	1.26E-05	0.000204
Cushing syndrome	453	135	-0.4609	1.69E-05	0.000263
Calcium signaling pathway	1237	207	-0.4237	1.80E-05	0.000269
Autoimmune thyroid disease	193	45	0.5949	2.22E-05	0.000319
Circadian entrainment	378	77	-0.53	2.50E-05	0.000347
Complement and coagulation cascades	266	65	-0.5429	2.79E-05	0.000367
Protein processing in endoplasmic reticulum	821	160	0.4058	2.84E-05	0.000367
Tuberculosis	899	166	0.3911	3.00E-05	0.000374
Oxidative phosphorylation	537	123	0.4344	3.39E-05	0.000409
cAMP signaling pathway	972	180	-0.4285	3.92E-05	0.000459
Allograft rejection	160	42	0.609	4.90E-05	0.000556
TGF-beta signaling pathway	368	94	-0.4917	5.44E-05	0.000598
Base excision repair	138	34	0.6319	6.02E-05	0.000643
Parkinson disease	1232	256	0.3408	8.09E-05	0.00084
Basal cell carcinoma	162	64	-0.5331	8.48E-05	0.000857
Homologous recombination	246	38	0.6022	0.000115	0.001132
GnRH secretion	169	54	-0.5488	0.000152	0.001461
Phagosome	615	155	0.3782	0.000161	0.001506
Cortisol synthesis and secretion	185	50	-0.5614	0.000173	0.001575
Graft-versus-host disease	154	34	0.6026	0.000211	0.001874
Hepatocellular carcinoma	478	151	-0.4186	0.000224	0.001951
Spliceosome	546	127	0.4005	0.000241	0.002045
Asthma	80	20	0.7113	0.000247	0.002054
Ras signaling pathway	968	197	-0.4013	0.000255	0.002076
Nucleotide excision repair	232	42	0.5699	0.00027	0.002107
Biosynthesis of nucleotide sugars	193	37	0.5861	0.00027	0.002107
Parathyroid hormone synthesis, secretion and action	336	94	-0.475	0.000294	0.002245
Chemical carcinogenesis - receptor activation	701	152	-0.4147	0.000377	0.002821
Fanconi anemia pathway	244	51	0.5272	0.000428	0.003136

Name	Total	Hits	EnrichmentScore	Pval	Padj
Melanogenesis	423	87	-0.4698	0.000483	0.003471
Th17 cell differentiation	332	102	0.4044	0.000513	0.003539
Relaxin signaling pathway	540	111	-0.4376	0.000516	0.003539
Gastric cancer	464	140	-0.4176	0.00052	0.003539
Meiosis - yeast	369	59	0.4737	0.00072	0.004764
Human papillomavirus infection	1221	313	-0.358	0.000726	0.004764
Amino sugar and nucleotide sugar metabolism	384	47	0.5187	0.000772	0.004976
Nicotine addiction	143	21	-0.6667	0.000995	0.006305
Necroptosis	647	125	0.3733	0.001053	0.006486
Ribosome	728	130	0.3566	0.001058	0.006486
Apelin signaling pathway	469	102	-0.4371	0.00109	0.006575
Protein digestion and absorption	780	102	-0.4361	0.00114	0.006766
Hedgehog signaling pathway	270	52	-0.5217	0.001353	0.007909
Rap1 signaling pathway	868	197	-0.377	0.001399	0.008052
Fc gamma R-mediated phagocytosis	332	94	0.4115	0.001472	0.008343
Aldosterone synthesis and secretion	437	76	-0.4683	0.001856	0.01036
cGMP-PKG signaling pathway	706	137	-0.4045	0.001905	0.01048
Insulin resistance	371	100	-0.4256	0.00204	0.01106
Oocyte meiosis	695	90	0.3991	0.002527	0.0135
Prion disease	1150	250	0.2993	0.002663	0.01391
Mismatch repair	157	22	0.6317	0.002678	0.01391
Natural killer cell mediated cytotoxicity	362	75	0.4073	0.002851	0.01461
Rheumatoid arthritis	316	81	0.413	0.003019	0.01526
Aminoacyl-tRNA biosynthesis	131	41	0.5107	0.003192	0.01592
Huntington disease	1098	288	0.2845	0.003554	0.01749
Inflammatory bowel disease	170	52	0.4568	0.003735	0.01814
Signaling pathways regulating pluripotency of stem cells	399	131	-0.4015	0.003887	0.01864
Prostate cancer	341	90	-0.4296	0.004095	0.01939
NF-kappa B signaling pathway	424	114	0.3575	0.004249	0.01987
Fluid shear stress and atherosclerosis	643	125	-0.3945	0.004708	0.02174
Pyrimidine metabolism	219	52	0.4456	0.005415	0.0247
Steroid biosynthesis	77	17	0.6407	0.006205	0.02785
Staphylococcus aureus infection	279	65	0.4035	0.006255	0.02785
Circadian rhythm	182	30	-0.5626	0.006481	0.02851
Vascular smooth muscle contraction	559	96	-0.4166	0.006606	0.02855
Salivary secretion	433	55	-0.4694	0.006642	0.02855
Cocaine addiction	196	38	-0.5188	0.007191	0.03056
AGE-RAGE signaling pathway in diabetic complications	502	97	-0.4056	0.007413	0.03115
Oxytocin signaling pathway	659	124	-0.3776	0.00792	0.03259
Regulation of actin cytoskeleton	818	186	-0.3568	0.007943	0.03259
Morphine addiction	255	66	-0.4555	0.008017	0.03259
C-type lectin receptor signaling pathway	513	102	0.3417	0.00898	0.03611
Systemic lupus erythematosus	1015	75	0.379	0.009164	0.03646
Glucagon signaling pathway	468	80	-0.4192	0.009576	0.0377
Cardiac muscle contraction	287	76	-0.4214	0.009797	0.03792
N-Glycan biosynthesis	142	46	0.4431	0.009836	0.03792
B cell receptor signaling pathway	275	71	0.3731	0.009995	0.03814
Insulin secretion	295	62	-0.4596	0.01028	0.03883
Melanoma	190	59	-0.452	0.01053	0.03914
Intestinal immune network for IgA production	145	40	0.4746	0.01059	0.03914
Bile secretion	249	43	-0.4955	0.01067	0.03914
Non-homologous end-joining	49	13	0.6764	0.01095	0.03975
Malaria	205	45	-0.4736	0.0112	0.04001
Terpenoid backbone biosynthesis	134	24	0.556	0.01123	0.04001
Amyotrophic lateral sclerosis	1513	341	0.265	0.01141	0.04026
Fc epsilon RI signaling pathway	178	53	0.424	0.01174	0.04102
IL-17 signaling pathway	360	84	0.3691	0.01296	0.04464
Cytosolic DNA-sensing pathway	182	44	0.4389	0.01301	0.04464
Gastric acid secretion	445	51	-0.4622	0.01425	0.04842
Dopaminergic synapse	539	101	-0.3993	0.01437	0.04842

8.13. Análisis de Enriquecimiento (GSEA) de los Términos de Ontología de Genes de las Funciones Moleculares (Correspondiente al capítulo 3)

Name	Total	Hits	ichmentSc	Pval	Padj
Actin binding	619	197	-0.4379	0.000123	0.008395
Ion channel activity	534	115	-0.5135	0.000133	0.008395
Growth factor activity	260	80	-0.6415	0.00014	0.008395
Voltage_gated ion channel activity	173	69	-0.6063	0.000144	0.008395
Heparin binding	84	37	-0.6928	0.000152	0.008395
Extracellular matrix structural constituent	145	28	-0.7329	0.000156	0.008395
Insulin_like growth factor binding	46	20	-0.7341	0.000159	0.008395
Voltage_gated calcium channel activity	41	17	-0.7477	0.000164	0.008395
Cysteine_type endopeptidase activity	132	21	0.7204	0.000268	0.01036
Microtubule motor activity	124	35	0.652	0.000286	0.01036
Endonuclease activity	259	51	0.5614	0.000303	0.01036
Peptidase inhibitor activity	301	22	-0.6656	0.000315	0.01036
Serine_type endopeptidase activity	924	90	0.4413	0.000366	0.01056
Cysteine_type peptidase activity	513	94	0.4613	0.000367	0.01056
Structural constituent of ribosome	751	135	0.3954	0.000439	0.0117
Calcium channel activity	93	33	-0.6101	0.000458	0.0117
Transmembrane signaling receptor activity	435	103	-0.4549	0.000677	0.01633
Threonine_type endopeptidase activity	25	10	0.8548	0.000731	0.01633
Ligase activity	503	99	0.4177	0.000745	0.01633
Extracellular ligand_gated ion channel activity	172	24	-0.6352	0.000946	0.01978
Protein dimerization activity	1191	129	-0.4217	0.001051	0.02102
Isomerase activity	454	93	0.4176	0.001099	0.02106
Sodium channel activity	80	13	-0.7555	0.001173	0.02159
Nuclease activity	281	57	0.4829	0.001562	0.02764
Heme binding	1249	68	-0.4766	0.001861	0.0317
DNA_binding transcription factor activity, RNA polymerase II_specific	1738	163	-0.3968	0.002041	0.03353
G protein activity	25	20	-0.6552	0.00257	0.04077
Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	809	47	-0.5107	0.002673	0.04099
Signaling receptor binding	176	60	-0.4819	0.002768	0.04107