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TESIS DOCTORAL

PATOGENIA DE LA ENTEROMIXOSIS DEL RODABALLO: INTERACCIÓN PARÁSITO-HOSPEDADOR Y RESPUESTA INMUNITARIA

Fdo. Paolo Ronza

Departamento de Ciencias Clínicas Veterinarias

Programa de Doctorado "Investigación Básica y Aplicada en Ciencias Veterinarias"

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Presentada por D. Paolo Ronza

Alumno del Programa de Doctorado "Investigación Básica y Aplicada en Ciencias Veterinarias"

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RESUMEN

El filo Myxozoa alberga numerosas especies parasitarias que ocasionan importantes pérdidas económicas en acuicultura. La enteromixosis del rodaballo, causada por *Enteromyxum scophthalmi*, es una de las más devastadoras enfermedades provocadas por mixosporidios, siendo un factor limitante para la producción de esta especie. La enfermedad cursa como un síndrome caquectizante, asociado a enteritis descamativa y depleción leucocitaria, cuya gravedad se incrementa a lo largo de la infección. Actualmente no existen tratamientos efectivos frente a la enteromixosis y hay diferentes aspectos de la patogenia que todavía se desconocen. El objetivo de esta Tesis Doctoral ha sido profundizar en el conocimiento de la relación parásito-hospedador, mediante un abordaje multidisciplinario, combinando estudios histopatológicos y análisis moleculares.

Se ha investigado la implicación en la patogenia de la enfermedad de una de las principales citoquinas pro-inflamatorias, el factor de necrosis tumoral alfa (TNFα). Para este fin, se optimizó una técnica inmunohistoquímica, mediante la cual se describió por primera vez la inmunolocalización del TNFα en diferentes tejidos de rodaballo. Se combinó el uso de esta técnica con el análisis de la expresión génica del TNFα en rodaballos sanos y parasitados en diferentes fases de la infección. Los resultados demostraron la participación de esta citoquina en la respuesta frente a *E. scophthalmi*, y su posible involucración en el desarrollo de las lesiones.

Por otra parte, se analizaron los cambios a nivel transcriptómico inducidos en el hospedador en distintas etapas de la enfermedad, cuyo estudio no había sido abordado previamente. Se ha empleado la actual herramienta de elección para este tipo de estudios, RNA-seq, relacionando los datos obtenidos con el cuadro morfopatológico y el curso de la enfermedad. Esos trabajos han permitido avanzar en el conocimiento de la interacción parásito-hospedador, aportando información novedosa sobre la respuesta inmunitaria del rodaballo y su papel en la enfermedad, así como sobre los mecanismos de infección y estrategias de evasión de *E. scophthalmi*. Asimismo, se han esclarecido los mecanismos moleculares responsables de los cambios morfológicos y la fisiopatología de la enfermedad avanzada.

En conjunto, los resultados obtenidos en esta Tesis Doctoral proporcionan una valiosa base hacia el diseño de métodos de prevención y control de la enteromixosis.

PALABRAS CLAVE

Enteromixosis, rodaballo, patogenia, histopatología, expresión génica

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RESUMO

O filo Myxozoa alberga numerosas especies parasitarias que ocasionan importantes perdas económicas na acuicultura. A enteromixose do rodaballo, causada por *Enteromyxum scophthalmi*, é unha das máis devastadoras enfermidades provocadas por mixosporidios, sendo un factor limitante para a produción desta especie. A enfermidade cursa como un síndrome caquectizante, asociado a enterite descamativa e depleción leucocitaria, cuxa gravidade aumenta ao longo da infección. Actualmente non existen tratamentos efectivos fronte á enteromixose e hai diferentes aspectos da patoxenia que aínda se descoñecen. O obxectivo desta Tese Doutoral foi afondar no coñecemento da relación parasito-hospedador, mediante unha abordaxe multidisciplinaria, combinando estudos histopatolóxicos e análises moleculares.

Explorouse a implicación na enfermidade dunha das principais citoquinas pro-inflamatorias, o factor de necrose tumoral alfa (TNFα). Para este fin, optimizouse unha técnica inmunohistoquímica, por medio da cal se describiu por primeira vez a inmunolocalización do TNFα en varios tecidos de rodaballo. Este método combinouse ca análise de expresión xénica do TNFα en rodaballos sans e parasitados en distintas fases da infección. Os resultados demostraron a participación desta citoquina na resposta fronte a *E. scophthalmi* e revelaron a súa posible implicación no desenvolvemento das lesións.

Investigáronse, tamén dun xeito novidoso, os cambios a nivel transcriptómico inducidos no hospedador en distintas etapas da enfermidade. Empregouse a actual ferramenta de elección para este tipo de análises, RNA-seq, relacionando os datos obtidos có cadro morfopatolóxico e o curso da enfermidade. Estes traballos permitiron progresar no coñecemento da interacción parásito-hospedador, aportando novas informacións sobre a resposta inmunitaria do rodaballo e o seu papel na enfermidade, así como sobre os mecanismos de infección e estratexias de evasión de *E. scophthalmi*. Asemade, esclarecéronse os mecanismos moleculares responsables dos cambios morfolóxicos e da fisiopatoloxía da enfermidade avanzada.

En conxunto, os resultados obtidos nesta Tese Doutoral proporcionan unha valiosa base cara o deseño de métodos de prevención e control da enteromixose.

PALABRAS CHAVE

Enteromixose, rodaballo, patoxenia, histopatoloxía, expresión xénica

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ABSTRACT

The phylum Myxozoa includes numerous species that parasitize fish, causing important economic losses in aquaculture. Turbot enteromyxosis, caused by *Enteromyxum scophthalmi*, is one of the most devastating diseases caused by myxozoan, being a limiting factor for turbot production. The disease develops as a cachectic syndrome, associated to catarrhal enteritis and leukocytic depletion, whose severity increases along the infection course. To date, no effective treatment exists against enteromyxosis and there are different unknown aspects concerning its pathogenesis. The aim of this PhD Thesis was to deepen the knowledge of host-parasite interaction, through a multidisciplinary approach, combining histopathological studies and molecular analyses.

The implication in the pathogenesis of the disease of a major pro-inflammatory cytokine, the tumor necrosis factor alpha (TNFα), was investigated. To this end, an immunohistochemical technique was set up, allowing, for the first time, the description of TNFα immunolocalization in different turbot tissues. This method was combined with TNFα gene expression analysis in control and diseased turbot at different stages of the infection. The results obtained demonstrated the participation of this cytokine in the immune response to *E. scophthalmi*, postulating its involvement in the development of the lesions.

On the other hand, the transcriptomic changes induced in the host at different stages of the disease were analyzed. This aspect was neither previously addressed. The current technology of choice for this kind of study, RNA-seq, was employed, and the resulting data analyzed in relation to the morphopathological picture and the disease course. These works allowed advancing in the knowledge of host-parasite interaction, providing novel information on turbot immune response and its role in the disease, as well as on the infection mechanisms and evasion strategies of *E. scophthalmi*. Also, the molecular basis of the morphological changes and physiopathology of the advanced disease have been elucidated.

Altogether, the results obtained in this PhD Thesis provide a valuable basis toward the design of preventive and therapeutic measures for enteromixosis.

KEYWORDS

Enteromyxosis, turbot, pathogenesis, histopathology, gene expression

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LISTADO DE PUBLICACIONES DERIVADAS DE LA TESIS

El desarrollo de la presente Tesis Doctoral ha conllevado la publicación de los siguientes artículos científicos:

-Robledo, D.*, **Ronza, P.***, Harrison, P.W., Losada, A.P., Bermúdez, R., Redondo, M.J., Pardo, B.G., Sitjà-Bobadilla, A., Quiroga, M.I., Martínez, P. "RNA-seq analysis reveals significant transcriptome changes in turbot (*Scophthalmus maximus*) suffering severe enteromyxosis". BMC Genomics 2014, 15:1149 (*primera autoría compartida). Impact factor: 3,99 (2014).

-Ronza, P., Losada, A.P., Villamarín, A., Bermúdez, R., Quiroga, M.I. "Immunolocalization of tumor necrosis factor alpha in turbot (*Scophthalmus maximus*, L.) tissues". Fish and Shellfish Immunology 2015, 45: 470-476. Impact factor: 2,67 (2014).

-Ronza, P., Bermúdez, R., Losada, A.P., Sitjà-Bobadilla, A., Pardo, B.G., Quiroga, M.I. "Immunohistochemical detection and gene expression of TNFα in turbot (*Scophthalmus maximus*) enteromyxosis". Fish and Shellfish Immunology 2015, 47: 368-376. Impact factor: 2,67 (2014).

-Ronza, P.*, Robledo, D.*, Bermúdez, R., Losada, A.P., Pardo, B.G., Sitjà-Bobadilla, A., Quiroga, M.I., Martínez, P. "RNA-seq analysis of incipient enteromyxosis in turbot (*Scophthalmus maximus*): new insights into parasite invasion and immune evasion strategies". Sometida al International Journal for Parasitology (*primera autoría compartida). Impact factor: 3,87 (2014).

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LISTA DE ABREVIATURAS

ADN: ácido desoxirribonucleico

ARN: ácido ribonucleico

ADNc: ADN complementario

Célula NK: célula asesina natural, del inglés *natural killer*

CD2: cúmulo de diferenciación 2, del inglés *cluster of differentiation 2*

CD209: cúmulo de diferenciación 209, del inglés *cluster of differentiation 209*

KEGG: Enciclopedia de Genes y Genomas de Kioto**,** del inglés *Kyoto Encyclopedia of Genes and Genomes*

IBDs: enfermedades inflamatorias intestinales, del inglés *inflammatory bowel diseases*

IgM: inmunoglobulina M

IHQ: inmunohistoquímica

iNOS: sintasa inducible de óxido nítrico, del inglés *inducible nitric oxide synthase*

ISG: genes estimulados por interferón, del inglés *interferon-stimulated genes*

Linfocitos Th17: linfocitos T colaboradores 17, del inglés *T helper 17*

NGS: secuenciación de nueva generación, del inglés *next-generation sequencing*

PCR: reacción en cadena de la polimerasa, del inglés *polymerase chain reaction*

PE: post-exposición

PKD: enfermedad proliferativa del riñón, del inglés *proliferative kidney disease*

RIG: gen inducible por ácido retinoico, del inglés *retinoic-acid inducible gene*

RNA-seq: secuenciación de ARN, del inglés *ribonucleic acid (RNA) sequencing*

Q-PCR: PCR cuantitativa, del inglés *quantitative polymerase chain reaction*

SOCS1: supresor de la señalización de citoquinas 1, del inglés *suppressor of cytokine signalling 1*

SNPs: polimorfismos de nucleótido simple, del inglés *single nucleotide polymorphisms*

ST: estadio de desarrollo, del inglés *stage*

TNFα: factor de necrosis tumoral alfa, del inglés *tumor necrosis factor alpha*

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Introducción

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INTRODUCCIÓN: LA ENTEROMIXOSIS DEL RODABALLO

1. Definición

La enteromixosis es una enfermedad parasitaria cuyos agentes causales son organismos del género *Enteromyxum*, perteneciente al subfilo Myxozoa, reino Animalia. En el rodaballo (*Scophthalmus maximus*, L.) la enfermedad es provocada por la especie *Enteromyxum scophthalmi* (Palenzuela, Redondo y Álvarez-Pellitero, 2002).

2. Etiología

2.1. Los mixozoos

Los mixozoos (subfilo Myxozoa) son un grupo heterogéneo de endoparásitos microscópicos, que se caracterizan por la formación de esporas multicelulares. Son principalmente conocidos por ser parásitos de peces tanto de agua dulce como marina, provocando importantes pérdidas económicas en acuicultura (Yokoyama y col. 2012; Feng y col. 2014; Gómez y col. 2014). La clasificación de estos organismos continúa siendo un tema actual de debate y estudio en la biología evolutiva. Inicialmente incluidos dentro de los protozoos, fueron posteriormente reconocidos como miembro del reino Animalia (Metazoa) en base a sus características morfológicas, como la multicelularidad y la presencia de uniones celulares especializadas, y a los análisis filogenéticos del ADN ribosómico 18S (Kent y col. 2001). Actualmente, los resultados de distintas investigaciones parecen coincidir en ubicarlos como subfilo dentro del filo Cnidaria (Morris 2012; Nesnidal y col. 2013; Feng y col. 2014). Se conocen más de 2.300 especies en el grupo Myxozoa, todas ellas parásitas de vertebrados e invertebrados. Entre los vertebrados, los peces son los hospedadores más conocidos, aunque también se han descrito en anfibios, reptiles, aves y mamíferos. La morfología simple y el tamaño extremadamente reducido que presentan los mixozoos en comparación con otros cnidarios se consideran indicativos de su evolución hacia el parasitismo (Lom y Dyková 2006; Morris 2012; Yokoyama y col. 2012). Las características principales de estos organismos son las esporas multicelulares que presentan valvas protectoras, las células infectivas de morfología ameboide o esporoplasmas, y la presencia de una o varias cápsulas polares, estructuras similares a los nematocistos de los Cnidaria (Figura 1). Las cápsulas polares contienen un filamento polar (Figura 1), que se extruye cuando el parásito entra en contacto con el hospedador (Figura 2), para anclarse a los tejidos e infectarlos permitiendo la penetración de los esporoplasmas (Kent y col. 2001; Lom y Dyková 2006; Morris 2012; Yokoyama y col. 2012).

Los mixozoos se dividen en las clases Malacosporea y Myxosporea. En diferentes especies de ambas clases se ha demostrado la existencia de un ciclo de vida bifásico con alternancia entre un hospedador invertebrado y vertebrado (Figura 2), y se considera que éste es el ciclo natural de todos los mixozoos (Yokoyama y col. 2012). Se conoce

un número muy reducido de malacosporidios, incluyendo un importante parásito de salmónidos, *Tetracapsuloides brysalmonae*, agente causal de la enfermedad proliferativa del riñón (PKD) (Canning y col. 2002; Kumar y col. 2013), mientras que la inmensa mayoría de los mixozoos descritos hasta la actualidad pertenecen a los Myxosporea (Lom y Dyková 2006).

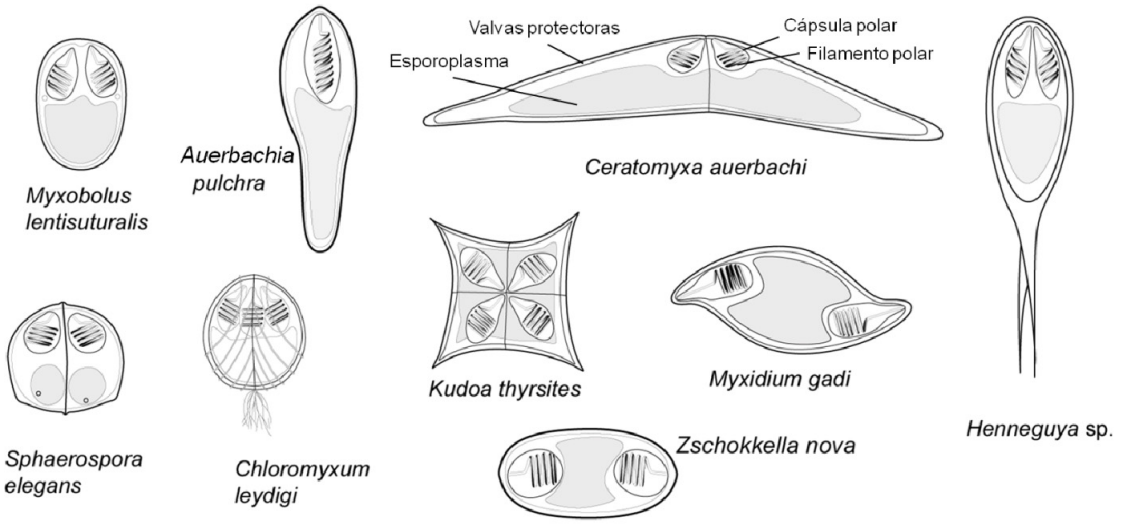


Figura 1. Mixosporas de diferentes especies de mixozoos. © Dr. Ivan Fiala.

Los mixosporidios presentan una estructura vegetativa típica, el plasmodio esporogónico, con una característica organización de "célula dentro de célula", por la que una célula madre primaria alberga en su interior una o más células secundarias originadas por endodiogenia y éstas, a su vez, pueden albergar células terciarias (Lom y Dyková 2006; Morris 2012). Las mixosporas producidas en estos plasmodios dentro del hospedador vertebrado (pez) son infectivas para el hospedador invertebrado, típicamente anélidos oligoquetos en agua dulce y poliquetos en agua salada (Yokoyama y col. 2012). En el anélido, ocurre la fase sexual del ciclo vital, con producción de gametos que se fusionan en zigotos, hasta la producción de actinosporas, que representan la fase infectiva para los peces (Kent y col. 2001; Lom y Dyková 2006) (Figura 2).

La mayoría de las especies de mixozoos descritas parecen convivir con el hospedador sin ser causa de enfermedad, sin embargo, para muchas de ellas su potencial patogénico es prácticamente desconocido al afectar peces de escaso valor económico. Por otro lado, se conocen numerosos mixozoos capaces de provocar enfermedades graves y que son causas de importantes pérdidas económicas en acuicultura de peces marinos, de agua dulce y diádromos (Yokoyama y col. 2012; Gómez y col 2014).

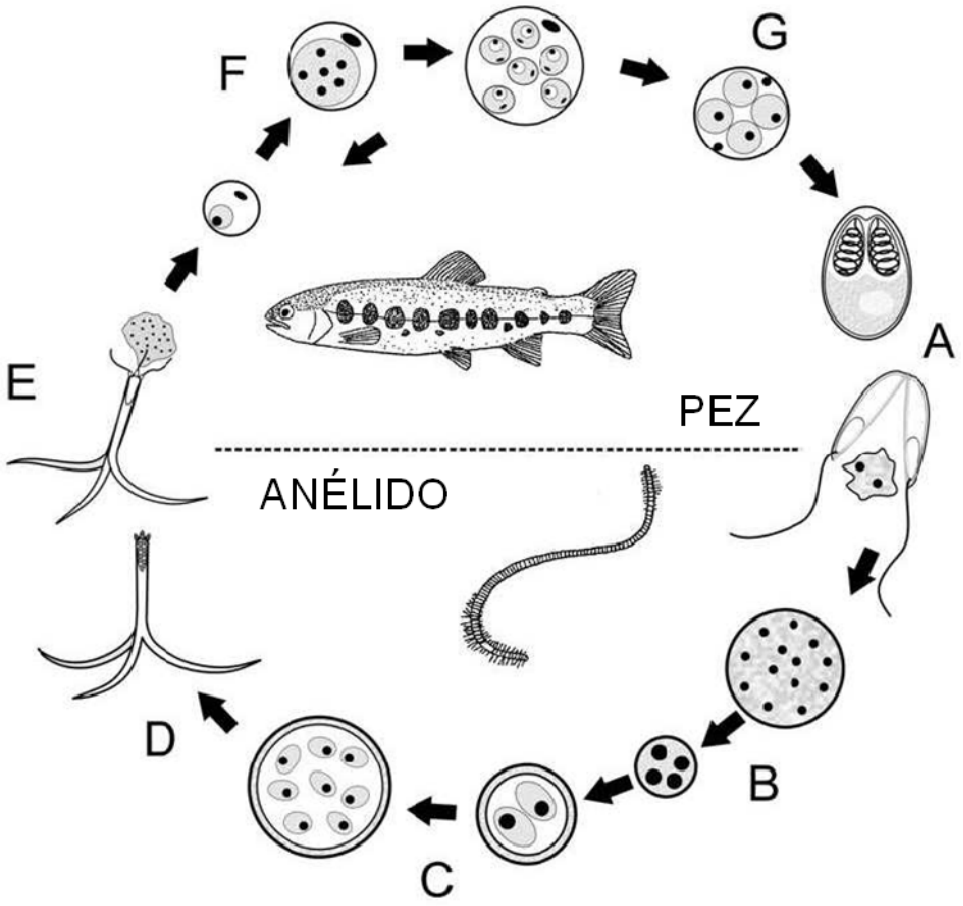


Figura 2. Diagrama del ciclo vital de los mixozoos. A) Extrusión de los filamentos polares de la mixospora para anclarse a los tejidos del hospedador invertebrado, apertura de las valvas y penetración del esporoplasma. B) Gametogonia. C) Esporogonia de la fase de actinospora. D) Actinospora madura. E) Extrusión de los filamentos polares de la actinospora al contactar con el hospedador vertebrado e infección mediante la penetración de los esporoplasmas. F) Fase proliferativa con organización "célula dentro de célula". G) Esporogonia de la fase de mixospora. Modificado de Yokoyama y col. (2012).

Según la localización en el pez, las especies parasitarias se denominan celozoicas (en el interior de cavidades corporales o de órganos) o histozoicas (en el parénquima de los tejidos), pudiendo además localizarse de forma intercelular o intracelular (Lom y Dyková 2006). Entre los mixosporidios celozoicos con más repercusión económica destacan distintas especies de los géneros *Ceratomyxa* y *Sphaerospora*, que pueden causar lesiones en la vejiga natatoria, la vesícula biliar y el aparato reproductor (Dyková y Lom 1988; Sitjà-Bobadilla y Álvarez-Pellitero 1993; Palenzuela y col. 1997; Rigos y col. 1997; Gunter y Adlard 2010). Una importante excepción dentro del género *Ceratomyxa* está constituida por la especie *C. shasta*, uno de los parásitos mejor estudiados en salmónidos, que es histozoico y provoca graves daños intestinales (Bjork y Bartholomew 2010; Bruno y col. 2013). Otras especies histozoicas relevantes pertenecen principalmente a los géneros *Henneguya*, *Kudoa*, *Myxobolus* y *Enteromyxum*. En particular, varias especies de *Henneguya* se han identificado en el músculo cardiaco en peces marinos (Yokoyama y col. 2003, 2005b; Dyková y col. 2011), mientras que en peces de agua dulce se han descrito principalmente provocando lesiones en las branquias (Naldoni y col. 2009; Lovy y col. 2011; Adriano y col. 2012). La

localización en los músculos esqueléticos es característica para numerosas especies de *Kudoa*, produciendo miolicuefacción post-mortem con la consiguiente imposibilidad de venta de los ejemplares afectados (Moran y col. 1999; Yokoyama y col. 2004; Yokoyama e Itoh 2005; Levsen y col. 2008; Matsukane y col. 2011). Del género *Myxobolus*, la especie más importante es *Myxobolus cerebralis*, el primer mixozoo del que se conoció el ciclo vital (Wolf y Markwin 1984) y agente causal de la enfermedad del torneo en salmónidos. El parásito afecta al tejido cartilaginoso provocando deformidades esqueléticas (Sarker y col. 2015). Finalmente, los mixosporidios del género *Enteromyxum* se localizan en el tracto gastrointestinal (Sitjà-Bobadilla y Palenzuela 2012). La especie *Enteromyxum fugu* es, en realidad, un parásito celozoico, ya que se localiza sobre el epitelio de revestimiento intestinal, sin penetrarlo (Tun y col. 2002; Yanagida y col. 2006), a diferencia de las otras dos especies del género, *E. leei* y *E. scophthalmi*.

2.2. El género *Enteromyxum*

El género *Enteromyxum* fue establecido por Palenzuela, Redondo y Álvarez-Pellitero en 2002, a raíz del estudio del agente causal de una enfermedad emaciativa que afectaba a los rodaballos en cría intensiva. Los análisis filogenéticos del ARN ribosómico permitieron la clasificación taxonómica de este parásito en un nuevo género y la incorporación en el mismo de la especie previamente conocida como *Myxidium leei*, también parásito del tracto gastrointestinal de peces marinos (Palenzuela y col. 2002). Sucesivamente, se demostró la pertenencia al género *Enteromyxum* del mixozoo entérico *Myxidium fugu* que, al igual que *M. leei*, se había clasificado como especie del genero *Myxidium* únicamente en base a las características morfológicas (Palenzuela y col. 2002; Yanagida y col. 2004). Hasta ahora *E. scophthalmi*, *E. leei* y *E. fugu* siguen siendo las únicas tres especies conocidas de este género (Sitjà-Bobadilla y Palenzuela 2012), perteneciente a la familia Myxidiidae, suborden Variisporina (Figura 3A) (Lom y Dyková 2006).

Las mixosporas se desarrollan en esporoblastos dispóricos y presentan forma semilunar, largas cápsulas polares que se abren en los extremos de la espora y un esporoplasma binucleado (Palenzuela y col. 2002; Yanagida y col. 2004) (Figura 3B). Para las tres especies de este género se desconoce la existencia de un hospedador invertebrado en el ciclo vital, mientras que se ha demostrado la existencia de trasmisión directa entre peces de los estadios vegetativos o trofozoitos (Diamant 1997; Redondo y col. 2002; Yasuda y col. 2002).

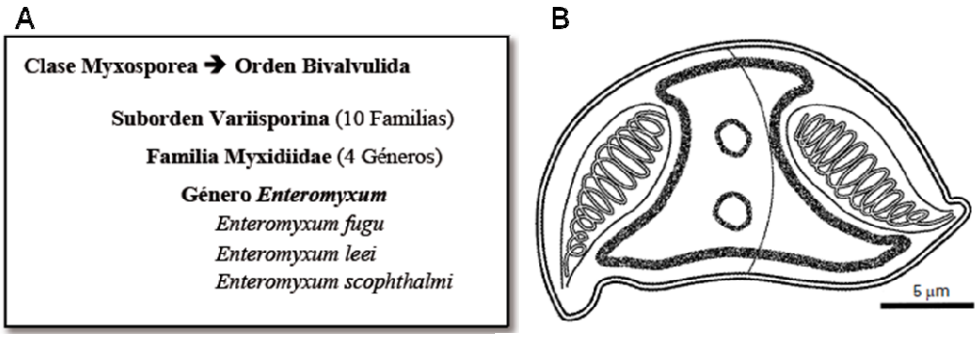


Figura 3. Clasificación del género *Enteromyxum* (A) (Lom y Dyková 2006) y esquema de la mixospora de *E. scophthalmi* (B) (Palenzuela y col. 2002).

E. fugu es un parásito celozoico del pez globo (*Takifugu rubripes*, Temminck and Schlegel), pero no provoca signos clínicos evidentes (Tun y col. 2002; Yanagida y col. 2006). Opuestamente, *E. leei* y *E. scophthalmi* son parásitos histozoicos, que se localizan en el epitelio de revestimiento del tracto gastrointestinal, y tienen un importante impacto en la acuicultura marina. Están principalmente asociados al desarrollo de un síndrome caquectizante en los peces infectados, con enteritis y alteración de la arquitectura intestinal, provocando pérdidas económicas derivadas de la mortalidad y/o empeoramiento de los indicadores de productividad (Sitjà-Bobadilla y Palenzuela 2012). *E. leei* se describió inicialmente como agente patógeno de la dorada (*Sparus aurata*, L.) (Diamant 1992) y otros espáridos (Le Breton y Marques 1995), pero su rango de hospedadores es muy amplio (más de 40 especies marinas e incluso de forma experimental en peces de agua dulce), así como su distribución geográfica, y frecuentemente se describen infecciones en nuevas especies (Padrós y col. 2001; Diamant y col. 2006; Rigos y Katharios 2010; Katharios y col. 2011; Sitjà-Bobadilla y Palenzuela 2012; Katharios y col. 2014). Se considera uno de los patógenos más devastadores en maricultura de aguas templadas (Sitjà-Bobadilla y Palenzuela 2012). La virulencia de *E. leei* varía según la especie afectada, pudiendo presentarse la enfermedad con un cuadro clínico grave asociado a elevada mortalidad, como es el caso del sargo picudo (*Diplodus puntazzo*, Cetti) y el pez globo (Yanagida y col. 2006; Álvarez-Pellitero y col. 2008a), o desarrollarse de forma crónica con adelgazamiento lento y progresivo y reducidas tasas de mortalidad, como en dorada (Fleurance y col. 2008; Sitjà-Bobadilla y col. 2008).

E. scophthalmi se ha descrito, hasta el momento, únicamente en rodaballos procedentes de piscifactorías, donde presenta una elevada virulencia (Sitjà-Bobadilla y Palenzuela 2012). A nivel experimental se observó que el lenguado senegalés (*Solea senegalensis*, Kaup) puede adquirir la infección, aunque esta especie no resultó ser muy susceptible, no manifestando signos clínicos ni desarrollo de enfermedad caquectizante (Palenzuela y col. 2007).

2.3. *Enteromyxum scophthalmi*

La presencia de un mixosporidio entérico en rodaballo se detectó por primera vez en los años 90 en Galicia, asociado a brotes epizoóticos con elevada mortalidad en granjas. La enfermedad se asemejaba a la descrita en otras especies afectadas por el parásito conocido entonces como *Myxidium leei*, aunque se encontraron diferencias en la morfología de las mixosporas (Branson y col. 1999). Los estudios morfológicos, ultraestructurales y filogenéticos realizados por Palenzuela y col. (2002) permitieron clarificar la sistemática de ambos organismos, con la creación del género *Enteromyxum*, y la denominación de *E. scophthalmi* para el agente causal del síndrome caquectizante en rodaballo y la reclasificación de *M. leei* como *E. leei*.

Los estadios vegetativos o trofozoítos de *E. scophthalmi* se desarrollan en el epitelio del tracto gastrointestinal del rodaballo hasta la formación de plasmodios esporogónicos. Sin embargo, se desconoce el hospedador invertebrado que resultaría infectado por las mixosporas producidas. En cambio, está demostrada la transmisión directa de los

trofozoítos entre peces, cuyos principales estadios infectivos son el 2 y 3 (Redondo y col. 2002, 2004). Redondo y col. (2004) han establecido una clasificación de los estadios de desarrollo (ST) de *E. scophthalmi* del 1 al 5, habiendo estadios vegetativos (ST1, ST2 y ST3) y fases esporogónicas (ST4 y ST5) (Figura 4). La organización estructural es la típicamente conocida como "célula dentro de célula", por lo que sólo el ST1 es una única célula con uno o varios núcleos y a partir del ST2 las estructuras parasitarias son multicelulares. El ST2 consiste en una célula madre principal que alberga una célula secundaria, y en el ST3 la célula principal suele albergar más células secundarias, en las que a su vez pueden desarrollarse células terciarias. A nivel ultraestructural se puede apreciar en esas células abundancia de ribosomas, retículo endoplasmático rugoso y mitocondrias, así como de reservas de nutrientes en gránulos de β-glicógeno y gotas lipídicas, reflejando la elevada actividad y requerimientos de esos estadios proliferativos. El ST4 ya es un plasmodio con esporas en formación, resultando en dos esporas originadas asincrónicamente. Las células esporogénicas se diferencian para dar lugar a células especializadas para la formación de las cápsulas polares (caspulogénicas), las valvas protectoras (valvogénicas) y los esporoplasmas (esporoplásmicas). A nivel ultraestructural es posible reconocer en esta fase las uniones celulares especializadas, que en *E. scophthalmi* consisten en hemidesmosomas que unen las células valvogénicas y capsulogénicas. El ST5 se corresponde con las esporas maduras o formadas casi por completo, a veces todavía contenidas por el plasmodio. Las mixosporas maduras presentan unas dimensiones aproximadas de 25 x 15 x 15 µm y esporoplasma binucleado (Figuras 3 y 4) (Palenzuela y col. 2002, Redondo y col. 2003a, 2004).

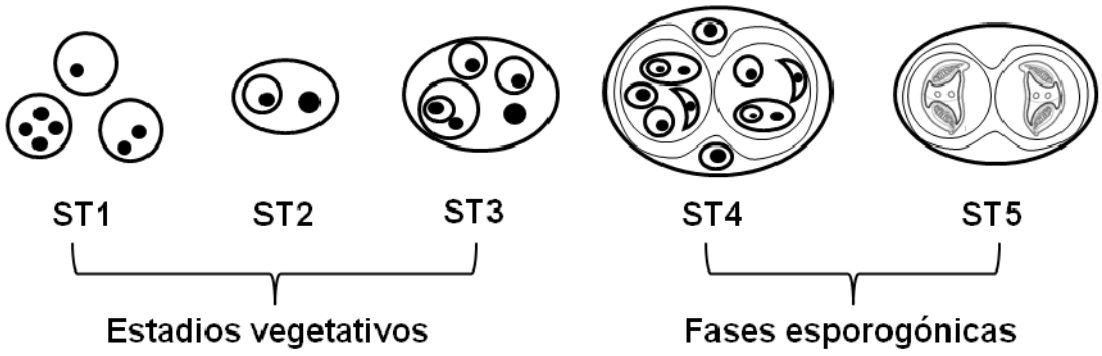


Figura 4. Estadios de desarrollo de *E. scophthalmi*. Modificado de Redondo y col. (2004).

Se han llevado a cabo varios intentos para mantener un cultivo *in vitro* de *E. scophthalmi*, en distintos medios e incluso utilizando una línea celular de rodaballo, pero no se ha conseguido ni la proliferación ni el mantenimiento de los estadios de desarrollo (Redondo y col. 2003b). Su supervivencia en agua de mar es de aproximadamente 24 horas, aunque se considera que este tiempo podría extenderse cuando el mixosporidio es expulsado con las heces de los animales enfermos, ya que los remanentes del epitelio intestinal y el mucus servirían para protegerle del choque osmótico (Redondo y col. 2002, 2003b, 2004).

3. Epidemiología

La mayor parte de la producción de rodaballo de acuicultura en la Unión Europea está concentrada en España (70,5% en 2014), y más concretamente en Galicia (99% de la producción española en 2014) (APROMAR 2015). Eso explica que los escasos datos epidemiológicos sobre infecciones naturales de los que se dispone procedan de estudios realizados en piscifactorías de esta Comunidad Autónoma. En los últimos años, otros países han introducido la acuicultura de esta especie, en particular China, que contribuyó a más del 85% de la producción mundial de rodaballo en 2013 (APROMAR 2015), pero la presencia de *E. scophthalmi* no ha sido descrita fuera de la Unión Europea hasta la actualidad.

La enfermedad afecta a rodaballos de peso superior a 50 gramos, con un pico de prevalencia en la franja entre 201 y 300 gramos, y tasas de morbilidad y mortalidad que llegan hasta el 100% en los tanques afectados (Branson y col. 1999; Redondo y col. 2004; Quiroga y col. 2006). La temperatura juega un papel importante en la infección, de modo que a temperaturas del agua más elevadas (generalmente superiores a 14 ºC) se corresponde una progresión más rápida de la enfermedad y un aumento de la mortalidad, tanto en infecciones naturales como experimentales (Branson y col. 1999; Redondo y col. 2002, 2004; Quiroga y col. 2006). Aunque no se ha podido determinar el ciclo natural del parásito, se considera que el origen de un brote epizoótico es una actinospora liberada por un hospedador intermediario invertebrado, en analogía a lo observado en otras especies de mixozoos (Branson y col. 1999; Redondo y col. 2004; Quiroga y col. 2006). En este sentido, se ha correlacionado el suministro de agua con la infección, estimándose que los peces que reciben agua previamente filtrada tienen 14 veces menos riesgo de ser infectados (Quiroga y col. 2006). Por otro lado, desde las primeras apariciones de la enfermedad se observó que las elevadas tasas de morbilidad y mortalidad de los peces podrían estar asociadas a la trasmisión horizontal directa de *E. scophthalmi* (Branson y col. 1999), sin necesidad de completar el ciclo con la fase de actinospora, lo que fue confirmado por Redondo y col. (2002) de modo experimental. Los peces infectados se consideran la principal fuente de contagio dentro de las granjas, siendo la densidad de cultivo un factor de riesgo que aumenta la disponibilidad de formas parasitarias infectivas (Redondo y col. 2002; Quiroga y col. 2006).

La trasmisión experimental de la enteromixosis se ha llevado a cabo con éxito mediante cohabitación de rodaballos sanos e infectados, a través de efluentes de tanques parasitados a sanos, y por vía oral mediante administración a rodaballos sanos de raspados intestinales procedentes de peces infectados (Redondo y col. 2002, 2004). En esos desafíos, las tasas de prevalencia y mortalidad resultaron a menudo cercanas al 100%, reflejando la elevada susceptibilidad del rodaballo al parásito (Redondo y col. 2002, 2004; Bermúdez y col. 2006a; Sitjà-Bobadilla y col. 2006; Losada y col. 2014a). Una característica observada en infecciones naturales y experimentales es la escasa formación de esporas maduras de *E. scophthalmi*, lo que sugiere, junto con la elevada virulencia del mixozoo, que el rodaballo pueda ser un hospedador accidental (Redondo y col. 2002, 2004; Bermúdez y col. 2006a).

La ingestión de estadios infectivos procedentes de las heces de ejemplares parasitados (ruta fecal-oral) se considera la principal vía de entrada, mediante la cual *E. scophthalmi* podría alcanzar directamente el tracto gastrointestinal, aparato diana de la infección (Redondo y col. 2004). Igualmente, no se puede descartar que existan otras vías de entrada, como la branquial o la cutánea (Redondo y col. 2002), descritas para otras especies de mixosporidios (Yokoyama y col. 2012; Gómez y col. 2014). Asimismo, muchos mixozoos presentan diseminación por vía hemática e incluso proliferación en el sistema circulatorio (Gómez y col. 2014), lo que no se descarta para *E. scophthalmi*, dada la observación de formas parasitarias en frotis de sangre (Redondo y col. 2004; Quiroga y col. 2006; Sitjà-Bobadilla y col. 2006).

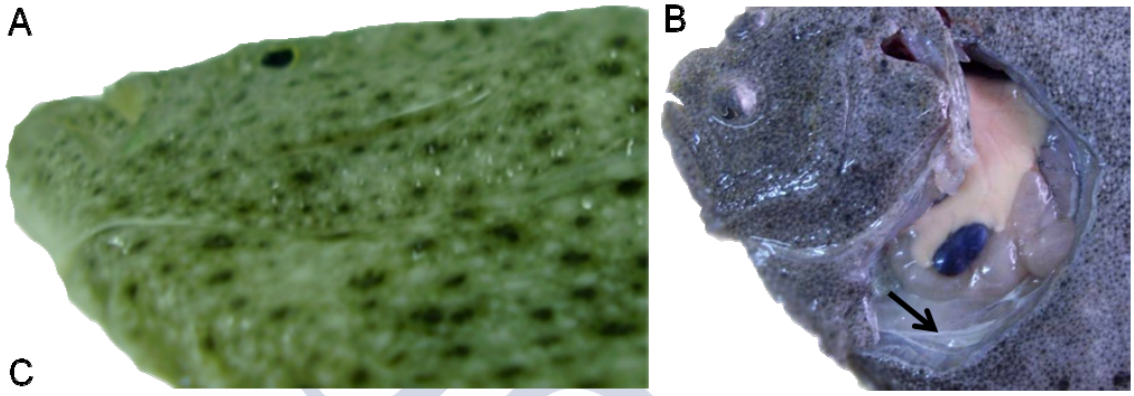
La enteromixosis se caracteriza por un largo periodo de prepatencia, pudiendo transcurrir de semanas a meses desde la exposición al mixosporidio hasta su detección histológica en el tubo digestivo y la aparición de los primeros signos clínicos (Redondo y col. 2004; Quiroga y col. 2006). La infección experimental mediante inoculación por vía oral de estadios infectivos es la que más rápido permite la observación del parásito en cortes histológicos de intestino. Las primeras detecciones se han descrito en algunos casos alrededor de los 7-8 días post-exposición (PE) (Redondo y col. 2004; Losada y col. 2014a). En cambio, las primeras formas parasitarias suelen observarse alrededor de los 20 días PE cuando la infección es transmitida por efluente o cohabitación, que son las vías que mejor reproducen las condiciones naturales (Redondo y col. 2002, 2004; Bermúdez y col. 2006a; Sitjà-Bobadilla y col. 2006; Losada y col. 2012). Aunque en líneas generales el rodaballo presenta una elevada susceptibilidad a la enfermedad, existen evidencias de diversos grados de resistencia. Se ha constatado que hay diferencias según el origen de los ejemplares (Quiroga y col. 2006), del mismo modo que algunos rodaballos sobreviven a brotes epizoóticos y adquieren anticuerpos protectores frente a nuevas infecciones (Sitjà-Bobadilla y col. 2004, 2007).

4. Cuadro clínico y morfopatológico

La enteromixosis se manifiesta clínicamente como un síndrome caquectizante, caracterizado por anorexia, pérdida de peso y letargia (Branson y col. 1999; Sitjà-Bobadilla y col. 2006; Bermúdez y col. 2010; Sitjà-Bobadilla y Palenzuela 2012). La enfermedad presenta un curso crónico y el adelgazamiento progresivo de los rodaballos se refleja macroscópicamente en la enoftalmia y la prominencia de los huesos del cráneo debido a la atrofia de músculos circundantes (Figura 5A) (Branson y col. 1999; García 1999; Bermúdez y col. 2010; Losada y col. 2014a). Por ello se denominó inicialmente como "síndrome de la cabeza hundida". Otros signos externos descritos son disnea y dilatación abdominal (Branson y col. 1999). En fases avanzadas de la enfermedad, a la apertura de la cavidad celómica (Figura 5B) es común observar ascitis y dilatación del tubo digestivo, cuya serosa puede presentar congestión o incluso focos de hemorragia. En el interior del tracto gastrointestinal, además, suele observarse presencia de líquido seromucoso (Branson y col. 1999; García 1999; Bermúdez y col. 2010; Losada y col.

2014a). Los restantes órganos no suelen mostrar lesiones significativas, aunque pueden presentar palidez (Branson y col. 1999) y de forma esporádica se ha descrito esplenomegalia (García 1999; Bermúdez y col. 2010).

En el trabajo de Bermúdez y col. (2010) se realizó una descripción histopatológica detallada de las distintas fases de desarrollo de la enteromixosis utilizando tejidos de rodaballos con infecciones naturales y experimentales, estableciendo una escala para la clasificación de los peces según la severidad de las lesiones y la carga parasitaria (Figura 5C).



C

Grado lesional	Vellosidades parasitadas: Signos externos	%	Intensidad	Estadios *E. scophthalmi*	Descamación	Células inflamatorias
Leve	-/+	<30	+	1-2	-	-/+
Moderado	++	30-80	++	2-3	+/++	+/++
Severo	+++	>80	+++	2-5	+++	+/+++

Figura 5. Hallazgos macroscópicos en rodaballos infectados por *E. scophthalmi* (A, B) y esquema de la clasificación de la infección en base al grado lesional (C). Se aprecia la apariencia de "cabeza hundida" debida a la atrofia muscular (A) y, a la apertura de la cavidad celómica (B), se observa líquido seroso (flecha), dilatación del tubo digestivo, esplenomegalia y palidez del hígado. A) Imagen del grupo investigador. B, C) Imagen y esquema modificados del artículo de Bermúdez y col. (2010).

Microscópicamente, las lesiones más características se encuentran en el tracto gastrointestinal (Figura 6), donde los trofozoítos de *E. scophthalmi* proliferan en el epitelio de revestimiento. La enfermedad está caracterizada por un cuadro de gastroenteritis catarral cuya severidad aumenta a lo largo de la infección, causando en muchos casos la muerte de los ejemplares afectados. La infección comienza típicamente en la región de los ciegos pilóricos e intestino anterior, y se disemina de forma ascendente y descendente hasta la colonización de todo el tubo digestivo, desde el esófago hasta el intestino posterior (Redondo y col. 2002, 2004; Bermúdez y col. 2010; Losada y col. 2012). En la infección leve (Figura 6a), la mayoría de las vellosidades intestinales no presentan alteraciones ni presencia de formas parasitarias visibles. En las áreas parasitadas es común observar estadios tempranos de desarrollo de *E. scophthalmi* localizados en la base del epitelio de revestimiento, ocasionalmente asociados a leves infiltrados inflamatorios mononucleares en la misma base del epitelio y en la lámina propia-submucosa (Bermúdez y col. 2010). Los primeros estadios vegetativos del parásito tienen un tamaño reducido y a menudo se presentan como estructuras basófilas de morfología esférica, difíciles de distinguir de células apoptóticas, complicando la detección

histológica de la enfermedad incipiente (Redondo y col. 2004; Bermúdez y col. 2010; Losada y col. 2014a). En esta fase, también se aprecia una mayor densidad de células mucosas y células rodlet (Bermúdez y col. 2010).

En la infección moderada (Figura 6b), hay un importante aumento de la carga parasitaria y se observan formas parasitarias en distintas fases de desarrollo a lo largo de todo el tracto gastrointestinal, aunque más numerosas en ciegos pilóricos e intestino anterior. Es posible apreciar la presencia de trofozoítos también en el epitelio glandular del estómago. La reacción inflamatoria es evidente en algunas áreas, aunque no siempre relacionada con la presencia de un número elevado de parásitos. Este infiltrado está constituido principalmente por células mononucleares, mayoritariamente linfocitos en la base del epitelio, mientras que en la lámina propia-submucosa se aprecia una población mixta de macrófagos, linfocitos y granulocitos (García 1999; Sitjà-Bobadilla y col. 2006; Bermúdez y col. 2010) y ocasionalmente agregados de melanomacrófagos (Bermúdez y col. 2010; Losada y col. 2014b). En esta fase de la enfermedad también se puede reconocer una alteración de la arquitectura del epitelio de revestimiento en algunas vellosidades intestinales y/o áreas del estómago, que muestran un característico perfil ondulado o festoneado (Bermúdez y col. 2010; Losada y col. 2014a).

Esta lesión se extiende a la práctica totalidad del tubo digestivo en la enteromixosis avanzada (Figura 6c, d, e), que se caracteriza fundamentalmente por los cambios en el epitelio de revestimiento. Éste se encuentra a menudo separado de la lámina basal, observándose un grado más o menos marcado de descamación de la mucosa al lumen intestinal, hasta dejar las vellosidades desprovistas de epitelio en los casos más graves (Branson y col. 1999; García 1999; Redondo y col. 2004; Bermúdez y col. 2010; Losada y col. 2014a). El epitelio sufre alteraciones histológicas y ultraestructurales muy marcadas; numerosos enterocitos muestran morfología apoptótica y/o necrótica, con vacuolización citoplasmática, núcleo fragmentado y/o condensación de la cromatina. Asimismo, se puede apreciar pérdida de la polarización celular y del borde en cepillo, disrupción de las conexiones célula-célula y depósito de material amorfo electrolúcido en la zona de pérdida de adhesión con la lámina basal (Bermúdez y col. 2010). Las células que se desprenden en su mayoría sufren alteraciones típicas de apoptosis, y es común detectar formas parasitarias asociadas a grupos de enterocitos en el lumen intestinal (Redondo y col. 2004; Bermúdez y col. 2010; Losada y col. 2014a). De forma esporádica se ha descrito la presencia de procesos de reepitelización de la mucosa intestinal, caracterizadas por un epitelio de tipo escamoso o cúbico bajo (García 1999; Bermúdez y col. 2010). La carga parasitaria en esta fase es elevada a lo largo del tracto gastrointestinal y hay numerosos estadios esporogónicos, aunque la cantidad de esporas maduras observable es muy baja con respecto al número de estadios de desarrollo (Branson y col. 1999; Redondo y col. 2002, 2004; Bermúdez y col. 2010). Otras lesiones que contribuyen al cuadro de enteritis son el edema de la lámina propia-submucosa con dilatación de los vasos sanguíneos y linfáticos, así como un grado variable, en ocasiones muy intenso, de infiltración inflamatoria en la que se pueden apreciar células apoptóticas (Sitjà-Bobadilla y col. 2006; Bermúdez y col. 2010; Losada y col. 2014a).

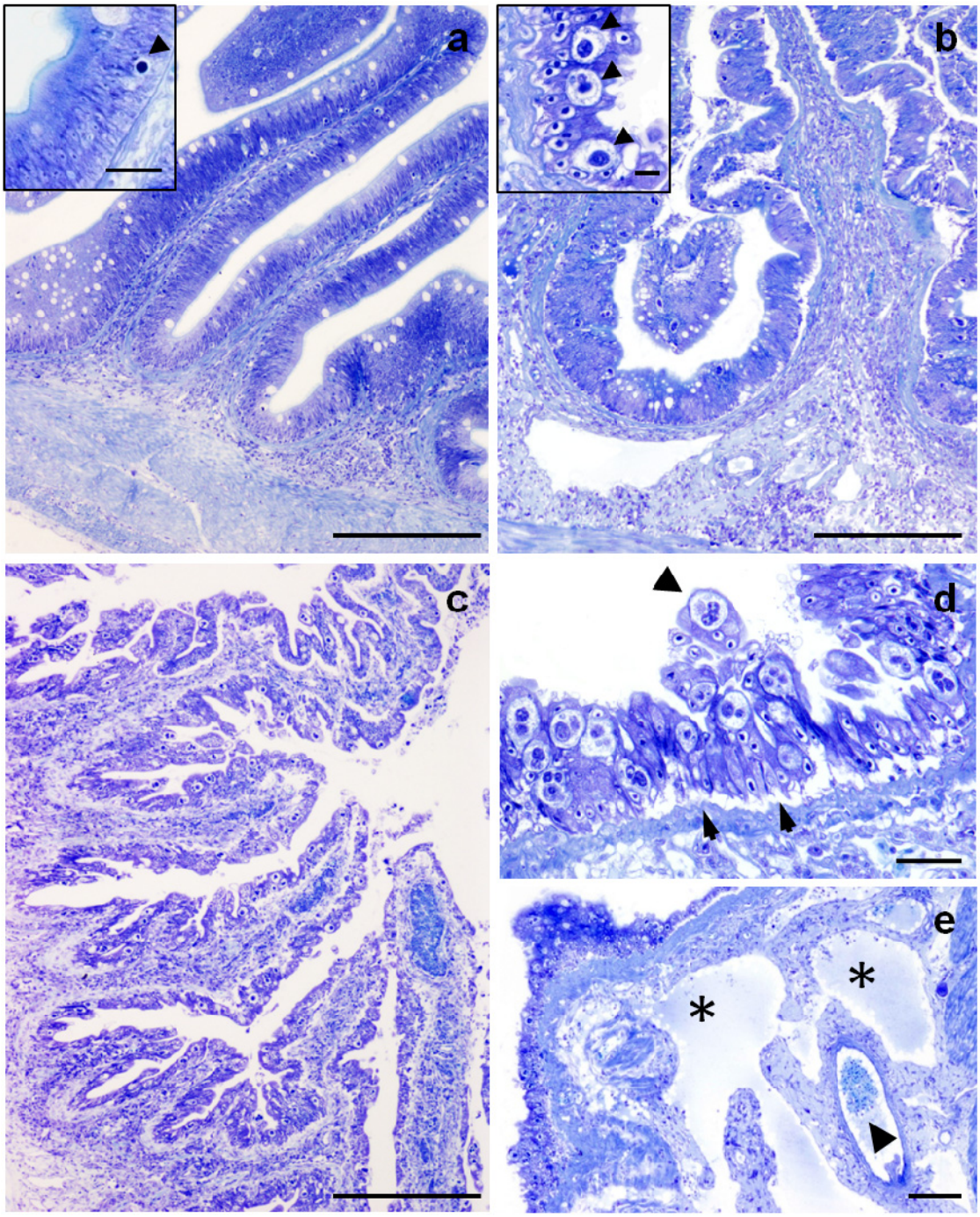


Figura 6. Secciones histológicas del intestino de rodaballos infectados por *E. scophthalmi*. a) Infección leve: las alteraciones histológicas son mínimas, constituidas por una leve infiltración inflamatoria en lámina propia-submucosa y en la base del epitelio. Pueden apreciarse estructuras basófilas redondeadas (inserto), compatibles con ST1 de *E. scophthalmi*. b) Infección moderada: se observa una evidente reacción inflamatoria, acompañada por edema de la lámina propia-submucosa. El epitelio de revestimiento muestra un perfil ondulado y pueden identificarse numerosas estructuras parasitarias (inserto, puntas de flecha) entre los enterocitos. c, d, e) Infección severa: se observa la descamación del epitelio intestinal al lumen y la presencia de infiltrados inflamatorios de grado moderado-severo (c). El epitelio de revestimiento a menudo se encuentra separado de la lámina basal (d, flechas) y pueden apreciarse estructuras parasitarias desprendiéndose del epitelio junto con los enterocitos (d, punta de flecha). En la lámina propia-submucosa es posible encontrar dilatación de los vasos sanguíneos (e, punta de flecha) y linfáticos (e, asteriscos). Imágenes del grupo investigador (a, c) y del artículo de Bermúdez y col. (2010) (b, d, e).

En los restantes órganos, la lesión más característica de la enteromixosis es la depleción leucocitaria del tejido linfohematopoyético del riñón y del bazo, que se desarrolla en las fases avanzadas de la enfermedad (Bermúdez y col. 2006a, 2010; Sitjà-Bobadilla y col. 2006; Losada y col. 2014a) (Figura 7). En los órganos linfohematopoyéticos de los peces infectados también se han descrito cambios en la densidad y la morfología de los centros de melanomacrófagos y aumento de células apoptóticas (Bermúdez y col. 2006a, 2010; Sitjà-Bobadilla y col. 2006; Ronza y col. 2013a). La presencia de los estadios iniciales de desarrollo de *E. scophthalmi* ha sido reportada en otras localizaciones diferentes al tubo digestivo, como piel y branquias (posibles vías de entrada), vasos sanguíneos (posible vía de diseminación) y órganos linfohematopoyéticos, a veces fagocitados por los macrófagos (Redondo y col. 2002, 2004; Sitjà-Bobadilla y col. 2006; Bermúdez y col. 2010; Estensoro y col. 2014). Otras localizaciones descritas esporádicamente son los conductos biliares, el páncreas y el tejido muscular, generalmente en fases avanzadas de la enfermedad, cuando la carga parasitaria es muy elevada (García 1999; Redondo y col. 2004; Bermúdez y col. 2010). De todos modos, la localización extraintestinal de las formas parasitarias no suele asociarse a alteraciones histológicas de los tejidos interesados.

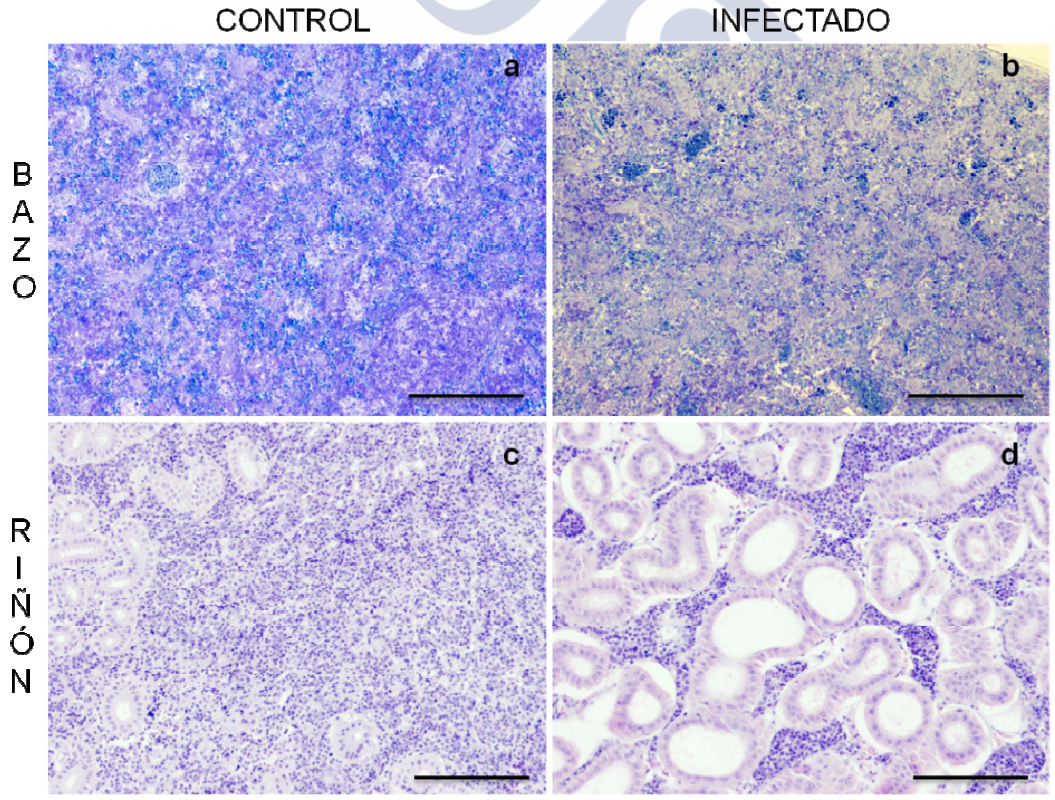


Figura 7. Secciones histológicas del bazo (a, b) y del riñón (c, d), mostrando la depleción del tejido linfohematopoyético que sufren ambos órganos en los rodaballos con enteromixosis severa (b, d). Imágenes del grupo investigador.

5. Diagnóstico, prevención y control

El diagnóstico de la enteromixosis del rodaballo se basa en la detección del agente causal, *E. scophthalmi*, ya que los signos clínicos de la enfermedad son inespecíficos. En condiciones de campo es posible observar las esporas del parásito o los trofozoítos en preparaciones en fresco, aunque en el primer caso el diagnóstico es difícil por la escasez de esporas producidas y en el segundo se requiere una amplia experiencia para llevarlo a cabo con éxito (Sitjà-Bobadilla y Palenzuela 2012). Mediante tinción rutinaria con hematoxilina-eosina se aprecian los estadios de desarrollo de *E. scophthalmi* en el epitelio digestivo y se puede evaluar el daño tisular asociado. Algunas tinciones especiales (azul de toluidina, Giemsa y/o ácido peryódico de Schiff) también pueden ser útiles para facilitar la visualización del mixosporidio (Bermúdez y col. 2010; Sitjà-Bobadilla y Palenzuela 2012). Por otro lado, el diagnóstico histopatológico es complicado en etapas muy tempranas de la infección, cuando la carga parasitaria es muy baja, pudiendo ocurrir que los cortes histológicos no abarquen los pliegues intestinales afectados, o los estadios parasitarios pasen desapercibidos o se confundan los ST1 con enterocitos apoptóticos (Redondo y col. 2004; Bermúdez y col. 2006a; Losada y col. 2014a). Existe una técnica no letal para el diagnóstico molecular de *E. scophthalmi* mediante la reacción en cadena de la polimerasa (PCR) a partir de muestras obtenidas del recto con una torunda (Quiroga y col. 2006; Sitjà-Bobadilla y col. 2007; Sitjà-Bobadilla y Palenzuela 2012). Asimismo, recientemente se han descrito dos técnicas de PCR cuantitativa a tiempo real (Piazzon y col. 2012; Alonso y col. 2015), y una técnica inmunohistoquímica con anticuerpo policlonal anti-*E. scophthalmi* (Figura 8) (Estensoro y col. 2014; Losada y col. 2014a), también dirigidas al diagnóstico y al monitoreo epidemiológico de la enteromixosis en las granjas de rodaballo.

Actualmente no existen tratamientos ni vacunas frente a la enteromixosis. Se han testado principalmente fármacos coccidiostáticos, como el toltrazuril, que indujo vacuolización en los estadios de desarrollo de *E. scophthalmi*, aunque no afectó a las esporas y no mejoró el desarrollo clínico de la enfermedad (Bermúdez y col. 2006b). Otras pruebas terapéuticas se hicieron con las combinaciones salinomicina-amprolio, robenidina-sulfamidas y narasina-nicarbazina, que en algún caso mejoraron la tasa de supervivencia de los rodaballos. Sin embargo, las tasas de prevalencia alcanzaron igualmente el 100% en todos los grupos y ninguna combinación resultó efectiva para frenar el progreso de la infección (Palenzuela y col. 2009).

El manejo en las granjas de rodaballos es la principal medida de prevención y control de la enfermedad. En particular, debería primar el tratamiento de las aguas, tanto de entrada como de salida, mediante filtración y desinfección con rayos ultravioleta u ozono, y la vigilancia epidemiológica de la enfermedad, con el sacrificio de los lotes infectados y desinfección de las instalaciones (Quiroga y col. 2006; Sitjà-Bobadilla y Palenzuela 2012).

6. Interacción parásito-hospedador

El estudio de la interacción entre *E. scophthalmi* y los tejidos de rodaballo se ha centrado a nivel del tracto gastrointestinal, diana de la infección, donde el parásito invade y coloniza el epitelio de revestimiento. De forma general, las formas parasitarias se desarrollan extracelularmente, aunque esporádicamente se han descrito estadios intracelulares, principalmente en las primeras fases de desarrollo (ST1 y ST2) (Palenzuela y col. 2002; Redondo y col. 2003a, 2004), pudiendo ser una localización preferencial en esa etapa del ciclo de vida. Mediante microscopía óptica y electrónica, se ha observado que los trofozoítos generalmente se ubican intercelularmente, relacionándose con los enterocitos circundantes mediante proyecciones citoplasmáticas intrincadas, en contacto directo con las membranas celulares del hospedador, formando estructuras similares a uniones en hendidura o *gap junction*. Estas estructuras se consideran funcionales a la fijación, comunicación y nutrición de los trofozoitos (Redondo y col. 2003a; Bermúdez y col. 2010).

La obtención de explantes de intestino de rodaballo ha permitido observar *in vitro* la invasión del epitelio por *E. scophthalmi*, que posee la capacidad de penetrar la mucosa intestinal tanto por la superficie luminal como a través de la lámina basal (Redondo y col. 2004). Esta misma metodología se utilizó para demostrar que las interacciones carbohidratos-lectinas, que juegan un papel relevante en muchas infecciones parasitarias en peces (Álvarez-Pellitero 2008b), también están involucradas en los mecanismos de adhesión y penetración de este mixosporidio (Redondo y Álvarez-Pellitero 2010a). Se estudió mediante lectinohistoquímica el patrón de los residuos de carbohidratos presente en la superficie de *E. scophthalmi* y en la zona de interacción parásito-hospedador, encontrándose mayor abundancia de estructuras glucosa-manosa, N-acetil-glucosamina, N-acetil-galactosamina y α-D-galactosa (Redondo y col. 2008). Posteriormente se demostró que la incubación de explantes intestinales con lectinas capaces de unirse a esos residuos tenía un efecto inhibitorio sobre la adhesión y penetración del parásito, sugiriendo el papel de las interacciones carbohidratos-lectinas como mecanismos de reconocimiento y diana de posibles tratamientos (Redondo y Álvarez-Pellitero 2010b). De todas formas, todavía faltan por conocer muchos aspectos de los mecanismos de infección, y, en particular, los eventos que caracterizan las primeras fases de la enteromixosis están lejos de ser esclarecidos (Sitjà-Bobadilla y Palenzuela 2012).

Se considera que, tanto en rodaballo como en otras especies parasitadas por mixosporidios del género *Enteromyxum*, la presencia de las formas parasitarias en el epitelio de revestimiento provoca una alteración de la barrera intestinal acompañada de disrupción de las uniones celulares entre enterocitos, lo que conllevaría un desequilibrio en la osmorregulación y en la absorción de nutrientes. Estas lesiones, conjuntamente con la anorexia, serían la base de la pérdida de peso y de la caquexia que muestran los peces parasitados (Sitjà-Bobadilla y Palenzuela 2012). Estos mecanismos explicarían de forma general el desarrollo de un síndrome de emaciación, común a las distintas especies parasitadas por esos mixosporidios. Sin embargo, dada la distinta

susceptibilidad observada, es evidente que los mecanismos patogenéticos varían entre especies y se necesita profundizar en cada relación parásito-hospedador. En rodaballo, son características las alteraciones cada vez más severas del epitelio de revestimiento con la progresión de la enfermedad: contorno festoneado, aumento de células con morfología apoptótica y descamación de la mucosa intestinal (Bermúdez y col. 2010). En peces con enfermedad avanzada, se observaron evidencias de pérdida de las uniones célula-célula mediante microscopía electrónica de trasmisión (Bermúdez y col. 2010), y mediante inmunohistoquímica se detectó alteración del marcaje de distintas proteínas de las uniones celulares (Ronza y col. 2013b). El incremento de la tasa de muerte celular programada se investigó mediante inmunomarcaje frente a la caspasa-3 activa (Figura 8a, b), una proteína efectora del proceso de apoptosis, demostrándose la presencia de numerosas células inmunorreactivas en el epitelio de revestimiento y entre las descamadas en el lumen en los ejemplares parasitados. A menudo, las células apoptóticas observadas se encontraban en las inmediaciones o asociadas a las estructuras parasitarias (Losada y col. 2014a) (Figura 8a). Bermúdez y col. (2010) sugirieron que la muerte celular a este nivel podría estar inducida por la pérdida de anclaje de las células a la lámina basal, un fenómeno denominado anoikis. El incremento de la apoptosis en el epitelio podría ser beneficioso para el hospedador para disminuir la carga parasitaria, pero otra hipótesis es que la descamación al lumen intestinal de los trofozoítos asociados a remanentes epiteliales podría aumentar su resistencia en el medio marino y facilitar su trasmisión a otros peces (Redondo y col. 2003a; Bermúdez y col. 2010).

Con pocas excepciones, durante la enteromixosis del rodaballo se observa un empeoramiento progresivo de los signos clínicos y de las lesiones, que conducen a la muerte de los ejemplares parasitados. La presencia del parásito y sus productos pueden contribuir al desarrollo de estas lesiones de forma directa o indirectamente debido a la propia respuesta del hospedador. La imposibilidad del cultivo *in vitro* dificulta enormemente la caracterización de *E. scophthalmi*, pero entre sus posibles mecanismos de virulencia se ha sugerido la actividad de proteasas putativas (Sitjà-Bobadilla y col. 2006; Redondo y col. 2010). Estas enzimas están involucradas en otras interacciones mixosporidio-hospedador (Gómez y col. 2014), y también se ha postulado su implicación en la patogenia de la enteromixosis de la dorada (Sitjà-Bobadilla y col. 2008; Davey y col. 2011; Estensoro y col. 2013).

Por otro lado, en rodaballo se produce una importante reacción inflamatoria a nivel local, lo que ha resultado ser la causa principal del desarrollo de las lesiones en muchas enfermedades asociadas a cuadros de enteritis descamativa (Peterson y Artis 2014; Williams y col. 2014; Kamekura y col. 2015). En los peces parasitados se ha evidenciado un aumento muy notable de inmunomarcaje de la sintasa inducible de óxido nítrico (iNOS) a nivel intestinal, implicando no solo a las células inmunitarias sino también al epitelio de revestimiento y a las células mucosas (Figura 8c). El óxido nítrico es un importante mediador en la respuesta inmunitaria de los peces, sin embargo,

como han sugerido los autores del estudio, en este caso su acción podría ser deletérea para el propio organismo y contribuir a la patogenia de la enfermedad (Losada y col. 2012).

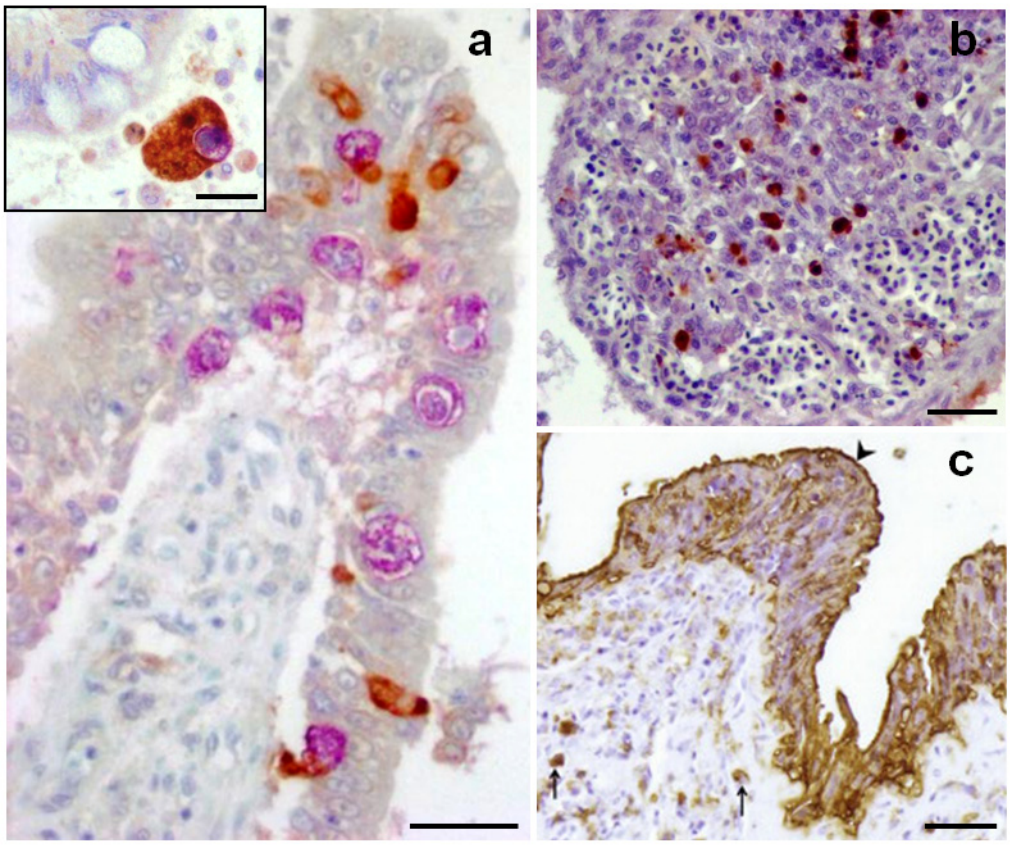


Figura 8. Estudios inmunohistoquímicos de rodaballos parasitados. a) Técnica de inmunohistoquímica doble frente a *E. scophthalmi* (en morado) y caspasa-3 activa (en marrón). Se aprecian distintas células apoptóticas en el epitelio, inmunorreactivas para la caspasa-3 activa, asociadas a la presencia de numerosas estructuras parasitarias. Inserto: Estadio de desarrollo de *E. scophthalmi* en el lumen intestinal, rodeado de varios enterocitos apoptóticos. b) Inmunomarcaje frente a caspasa-3 activa de varias células (en marrón) pertenecientes al infiltrado inflamatorio presente en la lámina propia-submucosa intestinal. c) Técnica inmunohistoquímica para la sintasa inducible de óxido nítrico (iNOS). Además del inmunomarcaje de diferentes células inflamatorias (flechas), se aprecia una intensa positividad en el epitelio de revestimiento (punta de flecha). a, b) Imágenes del artículo de Losada y col. (2014a). c) Imagen del artículo de Losada y col. (2012).

Los esfuerzos para profundizar en la respuesta de los rodaballos a la parasitación intestinal han incluido la investigación del sistema neuroendocrino, por su papel central en la función digestiva y el comportamiento alimentario, además de su interacción con el sistema inmunitario para la coordinación de la respuesta de defensa (Palmer y Greenwood-Van Meerveld 2001). A través de la puesta a punto de técnicas inmunohistoquímicas, se han estudiado los cambios de expresión de diferentes neuromoduladores en el tracto gastrointestinal de rodaballos con infección moderada. Se observaron diferencias en el número de células inmunorreactivas entre peces sanos y enfermos respecto a varios neuropéptidos (Tabla 1), que sugieren la participación de estas moléculas en la inducción o respuesta a la anorexia y en la respuesta inmunitaria (Bermúdez y col. 2007; Losada y col. 2014b). A este propósito, ambos estudios destacan

la posibilidad de una respuesta exacerbada a nivel local, una característica también observada en doradas infectadas por *E. leei*. Sin embargo, en esta especie se ha demostrado la activación de mecanismos antiinflamatorios que podrían actuar en prevenir o reducir el daño tisular (Davey y col. 2011; Pérez-Cordón y col. 2014).

Tabla 1. Lista de los neuropéptidos cuya expresión inmunohistoquímica ha sido estudiada en rodaballos sanos e infectados por *E. scophthalmi*. Datos de los artículos de Bermúdez y col. (2007) y Losada y col. (2014b).

Péptido	Funciones	Células positivas en enteromixosis
Serotonina	Regulador del apetito Estimulante de la motilidad intestinal Vasoconstrictor	Incremento*
Colecistoquinina	Regulador de la motilidad intestinal Regulador del vaciado gástrico Inhibidor del apetito Estimulante de la secreción pancreática	Incremento**
Leu-encefalina	Inhibidor del vaciado gástrico Inhibidor de la motilidad y secreción intestinal Modulación de la respuesta inflamatoria	Incremento*
Met-encefalina	Inhibidor del vaciado gástrico Inhibidor de la motilidad y secreción intestinal Modulación de la respuesta inflamatoria	Incremento
Sustancia P	Estimulante de la motilidad intestinal Vasodilatador Estimulante del sistema inmunitario	Incremento
Somatostatina	Inhibidor de la producción de ácidos gástricos Inhibidor de la secreción pancreática Inhibidor de la respuesta inmunitaria	Incremento**
Bombesina	Estimulante de la producción de ácidos gástricos Estimulante de la motilidad intestinal Inhibidor del vaciado gástrico y del apetito	Disminución*
Glucagón	Hiperglucemiante y lipolítico Inhibidor del apetito	Disminución*
Péptido relacionado con el gen de la calcitonina	Inhibidor de la motilidad intestinal Vasodilatador Inhibidor de la respuesta inmunitaria	Disminución
Péptido intestinal vasoactivo	Inhibidor de la motilidad intestinal Vasodilatador Inhibidor de la respuesta inmunitaria	Disminución*

* = resultado significativo ($p < 0.05$); ** = resultado altamente significativo ($p < 0.01$).

Asimismo, la enteromixosis induce en el rodaballo una respuesta a nivel sistémico, con activación de la respuesta inmunitaria innata y adaptativa. A través de estudios serológicos se observó la activación, en mayor o menor medida, de varios mecanismos propios de la inmunidad innata en respuesta a la infección, como el estallido respiratorio, la lisozima y el sistema del complemento (Sitjà-Bobadilla y col. 2006). También se apreció un incremento significativo de células positivas al marcaje de la iNOS a lo largo de la enfermedad en el bazo y el riñón, los principales órganos hematopoyéticos (Losada y col. 2012). Por otro lado, los análisis hematológicos mostraron un progresivo aumento de la serie granulocítica, mientras que los linfocitos sufrían una paulatina disminución durante el curso de la enfermedad (Sitjà-Bobadilla y col. 2006).

El papel de la respuesta adaptativa se abordó en un estudio inmunohistoquímico utilizando un anticuerpo específico frente a la IgM de rodaballo, observándose un pico inicial en el número de células positivas en bazo a los 20 días PE, y posteriormente en riñón a los 40 días PE. Sin embargo, en los puntos de muestreo sucesivos, el número de células positivas disminuía en los dos órganos, en coincidencia con el desarrollo de la depleción leucocitaria. En cambio, a nivel intestinal, las células inmunorreactivas mostraron un progresivo incremento, posiblemente debido a la migración de estas células desde los órganos linfoides, alcanzando los máximos valores a los 76 días PE (Bermúdez y col. 2006a). También se ha observado que el rodaballo es capaz de producir anticuerpos específicos frente a *E. scophthalmi*, que mostraron un efecto protector y confirieron resistencia a la reinfección en algunos ejemplares (Sitjà-Bobadilla y col. 2004, 2007). Sin embargo, las investigaciones apuntan a que en la mayoría de los casos la respuesta humoral es tardía y no resulta efectiva para frenar el desarrollo de la enfermedad (Bermúdez y col. 2006a; Sitjà-Bobadilla y col. 2006), e incluso los rodaballos mueren sin que se detecte producción de anticuerpos específicos (Sitjà-Bobadilla y col. 2006).

La ineficacia de la respuesta inmunitaria del rodaballo para contrarrestar la progresión de la infección se ha relacionado con un posible fallo en la conexión entre la respuesta innata y la adaptativa. A esta situación podría contribuir el desarrollo de un estado de inmunodepresión, reflejado por la depleción leucocitaria observada en los órganos hematopoyéticos (Bermúdez y col. 2006a; Sitjà-Bobadilla y col. 2006). La patogenia de esta lesión no está esclarecida, aunque la apoptosis se ha sugerido entre las posibles causas. Se ha descrito un incremento de células apoptóticas durante la infección, tanto en bazo como en riñón. Asimismo, se ha postulado que la apoptosis sufrida por las células inflamatorias a nivel intestinal (Figura 8b) podría asociarse a la acentuada migración leucocitaria desde los órganos linfohematopoyéticos hacia el tracto digestivo, contribuyendo a provocar la depleción (Bermúdez y col. 2006a, 2010; Sitjà-Bobadilla y col. 2006; Losada y col. 2014a).

7. Futuras direcciones

En líneas generales, hay diferentes aspectos comunes a las enfermedades causadas por mixozoos que necesitan ser estudiados en mayor profundidad. La imposibilidad del cultivo *in vitro* y las incógnitas sobre el ciclo de vida son cuestiones comunes a diferentes especies (Gómez y col. 2014, Sitjà-Bobadilla y col. 2015), que parecen de resolución compleja. Por otra parte, la búsqueda de herramientas de prevención y tratamiento frente a los mixozoos pasa por un mayor conocimiento de la interacción parásito-hospedador. Se necesita un análisis más exhaustivo de las rutas de entrada, mecanismos de reconocimiento y de respuesta inmunitaria activados por el hospedador, así como de las estrategias usadas por los parásitos para evadirlos (Gómez y col. 2014; Sitjà-Bobadilla y col. 2015). En la enteromixosis, todavía hay escasos conocimientos sobre estos eventos característicos de las primeras fases de la infección, que resultan en

la invasión y colonización del epitelio del tracto gastrointestinal (Sitjà-Bobadilla y Palenzuela 2012). Asimismo, se considera primordial la identificación de las rutas y moléculas clave que determinan la susceptibilidad y resistencia a las mixosporidiosis en las distintas especies, así como el grado de intensidad de la enfermedad resultante (Sitjà-Bobadilla y col. 2015). A este propósito, existen diferentes hipótesis a clarificar sobre el papel de la respuesta inmunitaria en la enteromixosis del rodaballo tanto en el desarrollo de las lesiones (Bermúdez y col. 2007; Losada y col. 2012, 2014a, 2014b), como acerca de los posibles factores de resistencia (Sitjà-Bobadilla y col. 2004, 2007).

Una de las moléculas clave en la respuesta inmunitaria, de la cual no se ha estudiado su implicación en la enteromixosis del rodaballo, es el factor de necrosis tumoral alfa (TNFα). Este constituye una de la principales citoquinas que actúan en la respuesta innata, en la base de la inducción y modulación de la cascada inflamatoria (Parameswaran y Patial 2010). En numerosas investigaciones se ha observado que su papel en la respuesta inmunitaria parece estar conservado en los teleósteos (García-Castillo y col. 2004; Goetz y col. 2004; Grayfer y col. 2008; Roca y col. 2008; Lam y col. 2011). La enteromixosis en rodaballo se caracteriza por un cuadro de enteritis catarral, asociado a un importante aumento de la expresión de la iNOS y del número de células apoptóticas (Bermúdez y col. 2010; Losada y col. 2012, 2014a). La capacidad del TNFα para promover la producción de óxido nítrico ha sido probada en rodaballo (Ordás y col. 2007), y su papel como mediador principal para la inducción de apoptosis está ampliamente descrito en mamíferos (Hehlgans y Pfeffer 2005; Parameswaran y Patial 2010). Además, en distintas investigaciones se ha hipotetizado que el desarrollo de las lesiones intestinales podría estar relacionado con una respuesta local desproporcionada (Bermúdez y col. 2007; Losada y col. 2012, 2014a, 2014b). El TNFα juega un papel importante en distintas patologías de mamíferos caracterizadas por una respuesta inflamatoria exacerbada (Bradley 2008; Waters y col. 2013). En particular, ha sido directamente relacionado con la descamación del epitelio de revestimiento y la disfunción de la barrera intestinal en las enfermedades inflamatorias intestinales (IBDs) (Watson y Hughes 2012; Leppkes y col. 2014). Asimismo, se considera entre los mayores responsables del desarrollo de caquexia (Morley y col. 2006; Argilés y col. 2011), un cuadro también típico de la enteromixosis en fase avanzada.

Muchas de las investigaciones recientes sobre la enfermedad han utilizado con éxito las técnicas inmunohistoquímicas (IHQ) para caracterizar la expresión de moléculas de interés en tejidos sanos y enfermos, permitiendo avances importantes en el conocimiento de patogenia de la enfermedad (Bermúdez y col. 2006a, 2007; Losada y col. 2012, 2014a, 2014b). La IHQ es una de las técnicas más difundida en histopatología, tanto para fines diagnósticos como de investigación, de gran utilidad por la capacidad de visualización de un producto de interés en el contexto tisular (Ramos-Vara 2008). Por otra parte, los estudios genómicos en peces han permitido poner de manifiesto la existencia de muchos de los genes relacionados con la respuesta inmunitaria en mamíferos, sugiriendo una mayor similitud de la esperada en la respuesta

de defensa, y ayudando a esclarecer la evolución de varios mecanismos biológicos (Zhu y col. 2013; Buchmann 2014). Sin embargo, para una caracterización exhaustiva del sistema inmunitario y su funcionamiento, se precisa un mayor número de estudios morfopatológicos, cuya escasez es debida precisamente a la falta de marcadores específicos (Koppang y col. 2007; Mulero y col. 2008; Randelli y col. 2008). En ese sentido, es deseable que se siga impulsando la puesta a punto de técnicas inmunohistoquímicas y anticuerpos relacionados con el sistema inmunitario de teleósteos, que permitan elucidar los mecanismos de la respuesta inmunitaria.

Por otra parte, en los últimos años hemos sido testigos del notable desarrollo de las denominadas ciencias "ómicas". Se considera que estamos asistiendo al paso de la era "genómica" a la era post-genómica, que se caracteriza por tres elementos principales (Martínez-Gómez y col. 2012). Éstos son el conocimiento del genoma completo de cada vez más especies y el consiguiente desplazamiento del centro de gravedad de los estudios del ADN al ARN, es decir, del genoma al transcriptoma. Finalmente, el elemento principal que caracteriza la era post-genómica es el desarrollo de tecnologías de secuenciación de alto rendimiento llamadas de nueva o siguiente generación (NGS), que han permitido una secuenciación más eficiente, rápida y barata de los ácidos nucleicos.

Actualmente la herramienta de elección para los estudios transcriptómicos a gran escala es RNA-seq (secuenciación de ARN). Esta herramienta se basa en la aplicación de las tecnologías NGS para secuenciación masiva del ADN complementario (ADNc) obtenido del ARN extraído de tejidos de interés. El paso sucesivo es el ensamblaje de los fragmentos de corta secuencia resultantes (*reads*) para la identificación de los genes correspondientes, facilitado en el caso de disponer de un genoma de referencia. Posteriormente, mediante la cuantificación de los transcriptos presentes es posible estimar los niveles de expresión génica (Figura 9) (Wang y col. 2009; Nookaew y col. 2012).

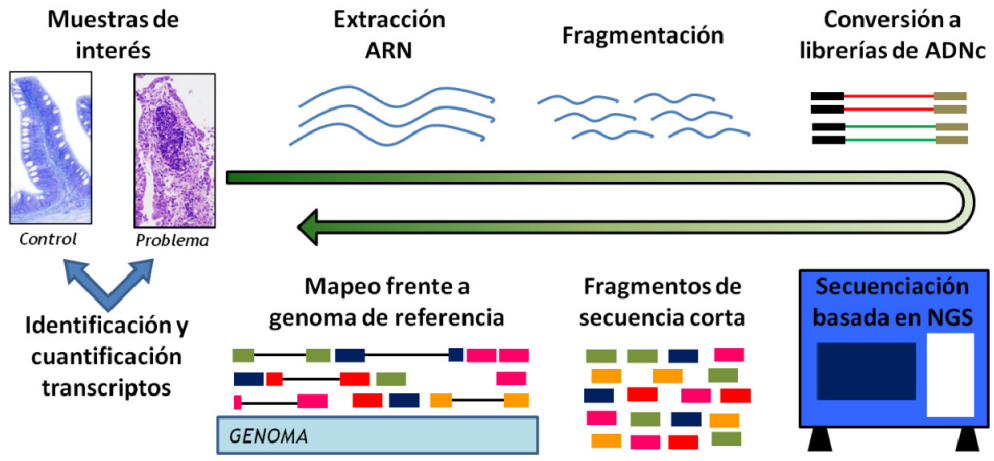


Figura 9. Representación esquemática del protocolo de trabajo de un análisis de expresión génica mediante RNA-seq cuando se dispone de un genoma de referencia.

Esta tecnología presenta numerosas ventajas respecto a las utilizadas anteriormente, como los *microarrays*, proporcionando una cobertura completa de los transcriptos con una elevada sensibilidad, especificidad y reproducibilidad (Wang y col. 2009). Asimismo, permite la detección e identificación de genes desconocidos, variantes de *splicing* y polimorfismos de nucleótido simple (SNPs) (Marioni y col. 2008; Morozova y col. 2009; Nookaew y col. 2012). La aplicación de RNA-seq está resultando de gran utilidad en medicina humana para la investigación de la patogenia y la búsqueda de biomarcadores de enfermedades complejas (Sutherland y col. 2011; Berman y col. 2012; Sinicropi y col. 2012; Costa y col. 2013; Zhang y col. 2014), así como para la caracterización de los agentes patógenos (Westermann y col. 2012). En patología de animales acuáticos, también se ha reconocido el potencial de esta herramienta (Qian y col. 2014; Sitjà-Bobadilla y col. 2015) y se está implementando con éxito para el estudio de la interacción patógeno-hospedador (Li y col. 2012; Peatman y col. 2013; Valenzuela-Miranda y col. 2015; Hasanuzzaman y col. 2016).

En el caso del rodaballo, la disponibilidad del genoma, que ha sido publicado recientemente (Figueras y col. 2016), es una gran ventaja para el óptimo aprovechamiento de la tecnología RNA-seq. Hasta la fecha, se desconocen por completo los efectos inducidos por la enteromixosis en el rodaballo a nivel de expresión génica. La integración de los datos obtenidos por transcriptómica con las observaciones morfopatológicas es esencial para profundizar en la interacción parásito-hospedador y esclarecer la patogenia de la enfermedad.

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Objetivos

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OBJETIVOS

Esta Tesis Doctoral se enmarca en las actividades del Proyecto AGL2009-13282-C02-02 "Control de las enteromixosis del rodaballo y espáridos. Nuevas aproximaciones basadas en la caracterización genética, antigénica y estructural de los parásitos y de la interacción parásito-hospedador. ENTEROMYXCONTROL" del Ministerio de Ciencia e Innovación (desde 2011 Ministerio de Economía y Competitividad). El objetivo general de esta línea de investigación es profundizar en diferentes aspectos relacionados con la interacción patógeno-hospedador, para elucidar los mecanismos patogénicos de la enteromixosis e identificar posibles dianas terapéuticas.

En esta Tesis Doctoral se han centrado los esfuerzos en estudiar la posible implicación del TNFα en la enteromixosis y en el análisis de los cambios en los tejidos del rodaballo a nivel transcriptómico en relación al cuadro morfopatológico observado en distintas fases de la enfermedad.

Por lo tanto los objetivos específicos de esta Tesis Doctoral son:

-Optimizar una técnica inmunohistoquímica para la detección del TNFα en tejidos de rodaballo.

-Analizar la inmunolocalización del TNFα en diferentes tejidos de ejemplares sanos.

-Evaluar el papel del TNFα en la patogenia de la enteromixosis mediante su estudio inmunohistoquímico y análisis de expresión génica en rodaballos sanos y enfermos.

-Determinar los cambios a nivel transcriptómico inducidos por *E. scophthalmi* en el rodaballo en distintas etapas de la enfermedad mediante la aplicación de la tecnología RNA-seq.

-Analizar los resultados obtenidos con RNA-seq en relación con el cuadro morfopatológico de la enteromixosis.

-Caracterizar los principales mecanismos patogenéticos y genes candidatos implicados en la enfermedad mediante un abordaje multidisciplinario.

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Publicaciones

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ARTÍCULO I: IMMUNOLOCALIZATION OF TUMOR NECROSIS FACTOR ALPHA IN TURBOT (*SCOPHTHALMUS MAXIMUS*, L.) TISSUES

Ronza, P.[1]; Losada, A.P.[1]; Villamarín, A.[2]; Bermúdez, R.[3]; Quiroga, M.I.[1]

[1]Departamento de Ciencias Clínicas Veterinarias, Facultad de Veterinaria, Universidade de Santiago de Compostela, 27002 Lugo.

[2]Departamento de Bioquímica y Biología Molecular, Facultad de Veterinaria, Universidade de Santiago de Compostela, 27002 Lugo.

[3]Departamento de Anatomía y Producción Animal, Facultad de Veterinaria, Universidade de Santiago de Compostela, 27002 Lugo.

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Full length article

Immunolocalization of tumor necrosis factor alpha in turbot (*Scophthalmus maximus*, L.) tissues

Paolo Ronza [a], Ana Paula Losada [a], Antonio Villamarín [b], Roberto Bermúdez [c], María Isabel Quiroga [a, *]

[a] Departamento de Ciencias Clínicas Veterinarias, Facultad de Veterinaria, Universidad de Santiago de Compostela, 27002, Lugo, Spain
[b] Departamento de Bioquímica y Biología Molecular, Facultad de Veterinaria, Universidad de Santiago de Compostela, 27002, Lugo, Spain
[c] Departamento de Anatomía y Producción Animal, Facultad de Veterinaria, Universidad de Santiago de Compostela, 27002, Lugo, Spain

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Abstract

Tumor necrosis factor alpha (TNFα) is a cytokine involved in a broad spectrum of cellular and organismal responses. Its main function, as a potent pro-inflammatory mediator, has been demonstrated in numerous teleost species and there are many reports on the modulation of TNFα gene expression under pathological conditions. Nevertheless, there is still scarce knowledge about the tissue distribution and type of cells that express this cytokine in fish species, which would help to further investigate its biological activities. These studies are hampered by the lack of molecular markers for teleost that hinder the development of morphological techniques, like immunohistochemistry. The aim of this work was to develop an immunohistochemical technique for the detection of TNFα in paraffin-embedded organs from healthy turbot (*Scophthalmus maximus*), an economically-important marine fish species. A commercial anti-human TNFα antibody, whose specificity was confirmed by western blot analysis, was used. Immunoreactive cells were observed in higher numbers in the lymphohaematopoietic organs, kidney, spleen and thymus, although TNFα-positive cells were also present in the digestive tract, liver, heart, gills and skin. Similarly to non-fish species, monocytes/macrophages appeared to be the main producers of this cytokine; nevertheless, the presence of immunoreactive rodlet cells in different tissues was also reported. The nature and distribution of the labelled cells appeared to be related with a strategic localization for defence response to antigenic challenge. The relative abundance of TNFα-positive cells in the lymphohaematopoietic organs also suggests that this cytokine may have a broader role in the normal physiology of those organs. The immunohistochemical technique allowed the *in situ* characterization of TNFα expression, representing a valid tool to investigate the immune response of turbot.

Keywords: Tumor necrosis factor alpha; immunohistochemistry; turbot; immune system; cytokine; monocyte/macrophage; rodlet cell.

1. Introduction

Tumor necrosis factor alpha (TNFα) is a pleiotropic cytokine acting in the regulation of a wide range of biological activities, including immune response, maintenance of homeostasis of the immune system, apoptosis, cell proliferation and differentiation (Goetz *et al.* 2004; Hehlgans and Pfeffer 2005; Parameswaran and Patial 2010). It plays a pivotal role in orchestrating the inflammatory reaction, and is considered as key for the induction and modulation of clinical signs and lesions in different mammal diseases (Bradley 2008; Parameswaran and Patial 2010; Waters *et al.* 2013). A number of recent studies have demonstrated that many of the molecules acting in the regulation of inflammatory processes in mammals (pattern recognition receptors, chemokines and cytokines) are present in fish, where they showed similar functions in innate immunity (Koppang *et al.* 2007; Lieschke and Trede 2009; Zhu *et al.* 2013; Buchmann 2014). TNFα gene has been cloned and identified from a range fish species (Hirono *et al.* 2000; Laing *et al.* 2001; García-Castillo *et al.* 2002; Saeij *et al.* 2003; Zou *et al.* 2003;

Nascimento *et al.* 2007; Grayfer *et al.* 2008; Kim *et al.* 2009; Lam *et al.* 2011), including turbot (*Scophthalmus maximus*, L.) (Ordás *et al.* 2007), a marine flatfish of growing economical importance in world aquaculture. There are many reports demonstrating analogous functions of teleost TNFα with its mammalian counterparts (García-Castillo *et al.* 2004; Goetz *et al.* 2004; Grayfer *et al.* 2008; Roca *et al.* 2008; Lam *et al.* 2011) and modulation of its expression in has been detected by RT-PCR in response to bacterial (Schwenteit *et al.* 2013), viral (Montes *et al.* 2010) and parasitic (Pennacchi *et al.* 2014) diseases, as well as in toxicological (Ma *et al.* 2014) and vaccine trials (Raida and Buchmann 2008; Fredriksen *et al.* 2011). Nevertheless, morphological studies of fish immune system are scarce due to the lack of specific markers and, alongside the impressive advances in genomics of bony fish, the development of morphopathological techniques is necessary to further gain insights in fish immunology and pathology (Koppang *et al.* 2007; Mulero *et al.* 2008; Randelli *et al.* 2008). Immunohistochemistry presents the advantage of showing the expression of target molecules in the context of tissue morphology, allowing the correlation with the histological findings and the characterization of the immunoreactive cells. As reported for other fish species, mammalian and turbot TNFα molecules shares conserved features, but differently from other teleosts (Zou *et al.* 2002; Savan and Sakai 2004; Kadowaki *et al.* 2009; Lam *et al.* 2011), turbot does not seem to present several isoforms of this cytokine (Ordás *et al.* 2007). Few immunochemical assays have been performed for TNFα detection in fish, and these reports only focused on its expression in damaged areas and none gave a comprehensive picture of tissue immunolocalization of TNFα (Komatsu *et al.* 2009; Yousaf *et al.* 2012; Fleming *et al.* 2010; McCarthy *et al.* 2013). In one of these reports (Fleming *et al.* 2010), a monoclonal antibody anti-human TNFα was used, which seems to cross-react with zebrafish (*Danio rerio*, Hamilton). The aim of this study was to set up an immunohistochemical technique to detect TNFα in paraffin-embedded tissues from turbot, analyzing the distribution of this cytokine in organs of healthy specimens. For this purpose, a commercial polyclonal antibody anti-human TNFα was employed and its specificity tested by western blot analysis.

2. Materials and methods

2.1. Fish and sampling procedures

For this study, 10 juvenile turbot (240 g mean weight) were obtained from a fish farm in north-western Spain. Fish were euthanized by overexposure to tricaine methane sulfonate (MS222, Sigma-Aldrich, Denmark) and necropsied. For histological examination and immunohistochemistry, tissues samples from kidney, spleen, thymus, digestive tract, liver, heart, gills, brain and skin were fixed in Bouin's fluid at 4 ºC during 18 hours and paraffin-embedded. Histological analysis was performed on H&E and toluidine blue-stained sections. For western blot analysis, tissue samples (1 g) were collected from the aforementioned organs, except the gills and thymus, and kept at -20ºC until processed. Moreover, western blot analysis was performed on three pooled sample of kidney and spleen, kept at -20 ºC in RNAlater, from turbot experimentally

infected with *Aeromonas salmonicida* (AS) at 7 days-post-infection, gently donated by the research group Acuigen from the University of Santiago de Compostela (Spain) (Millán *et al.* 2011).

2.2. Immunohistochemistry

Thin sections (3 µm thick) were obtained from all the sampled organs. The sections were placed on slides treated with silane to improve section adherence and dried overnight at 37 ºC. After deparaffination with xylene (two 5-min washes) and hydration through a graded ethanol series, slides were incubated with a peroxidase-blocking solution (Dako, Glostrup, Denmark) for 30 minutes to quench endogenous peroxidase activity. Antigen retrieval was performed by heating under pressure with sodium citrate buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0).The optimal working dilution for primary antibody (rabbit polyclonal antibody to TNFα, ab6671, Abcam, Cambridge, UK) was determined to be 1:600 with an incubation time of 2 h at room temperature. Then, slides were incubated during 30 min with horseradish peroxidase (HRP)-labelled polymer conjugated to rabbit secondary antibody and peroxidase reaction was developed using a diaminobenzidine-positive chromogen (EnVision+ System-HRP kit, K 4011, Dako). All incubations were performed in a humid chamber at room temperature, and the sections were washed three times for 5 min in 0.1 M phosphate buffered saline containing 0.05% Tween-20 between all subsequent steps. For microscopic observation, sections were counterstained with haematoxylin, dehydrated and coverslipped with DePeX mounting medium. Tissue sections of swine were used as positive controls. In sections included as negative controls, primary antibody was replaced with PBS or antibody diluent. As well, the technique was performed on kidney and spleen from two marine teleosts, which present a high amino acid sequence homology for TNFα with turbot: European sea bass (*Dicentrarchus labrax,* L.) (77% identity) and gilthead sea bream (*Sparus aurata*, L.) (75% identity).

2.3. Western blotting

Western blot analysis was performed to assess the specificity of the anti-TNFα antibody on protein extracts from anterior and trunk kidney, spleen, liver, heart and brain. To add further evidence, the technique was also carried out comparing on the same membrane kidney and spleen from AS-infected and healthy turbot. The immunogen employed to produce this antibody (recombinant full length TNFα protein, ab140754, Abcam) was used as positive control. Tissues were homogenized in ice-cold homogenization buffer (20 mM Tris-HCl, 150 mM NaCl, 1mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 10 mM MNaF, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml pepstatin A, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1% triton X-100, pH7.4). Homogenates were centrifuged at 17,000 rpm for 30 minutes at 4 ºC, and supernatants filtered through gauze and frozen in aliquots at -20º C until required. Protein concentrations were determined by Bradford method according to manufacturer's instruction (Bio-Rad, Hercules, CA, USA). Samples of protein extracts and control protein (recombinant full length TNF-α protein) were mixed with 1/3 vol of 4X SDS sample buffer (250 mM Tris-HCl, 8% SDS,

40% glycerol, 20% β-mercaptoethanol, pH 6.8) and denatured by heating at 95 ºC for 5 minutes. Approximately 50 µg of total protein per tissue and 300 ng of control protein were resolved by SDS-PAGE using 10% polyacrylamide slabs-gel and proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore, Bedford, MA, USA) by applying a 300 mA current for 2 h and 30 min at 4 ºC. After blocking for 6 h at room temperature with 5% non-fat dry milk in 20 mM TTBS (Tris–HCl buffer, pH 7.5, 0.15 M NaCl, 0.1% Tween 20), membranes were incubated overnight at 4º C with the anti-TNF-α primary antibody diluted 1:2000 with TTBS. The blots were then incubated for 1 h at room temperature with anti-rabbit secondary antibody conjugated to horseradish peroxidase (Sigma–Aldrich, St Louis, MO, USA) diluted 1:50,000 with TTBS and, finally, developed with the chemiluminiscent HRP substrate (Millipore) and exposed to X-ray film (Curix RP2 Plus; Agfa-Gevaert, Mortsel, Belgium) for a few seconds. Membranes were washed 5 times for 10 min with TTBS between subsequent steps. Negative control was performed by substituting primary antibody with TTBS.

Additionally, for all tested samples a SDS-PAGE electrophoresis and gel staining with Coomassie Brillant Blue R (Sigma-Aldrich) was carried out. In this case, 3 µg of control protein were loaded.

3. Results

3.1. Histological analysis

The histological evaluation of the sampled tissues did not show any significant pathological alteration or presence of pathogenic agents.

3.2. Immunohistochemistry

The expected negative or positive reactions were observed in the negative or positive controls, respectively. TNFα expression was detected in all studied turbot organs except the brain, although the major lymphoid organs presented the higher number of immunoreactive cells (Figure 1a, c, e), especially kidney and spleen. The typical TNFα-positive ($TNF\alpha^+$) cell, showing specific cytoplasmic and membrane staining, was rounded, mononuclear with rounded or slightly indented nucleus, between 7 and 11 microns in size, with an irregular cytoplasm. These cells were consistent with macrophage and melanomacrophages (Figure 1b, d, f). In blood vessels of different organs immunoreactive rounded cells were often observed, compatible with circulating monocytes (Figure 2b, c, d). Moreover, another cell type that usually showed a positive staining for TNFα were rodlet cells, mainly located in the epithelia of the digestive tract, renal tubules and hepatic bile ducts (Figure 2f, g), but also sporadically seen in thymus parenchyma.

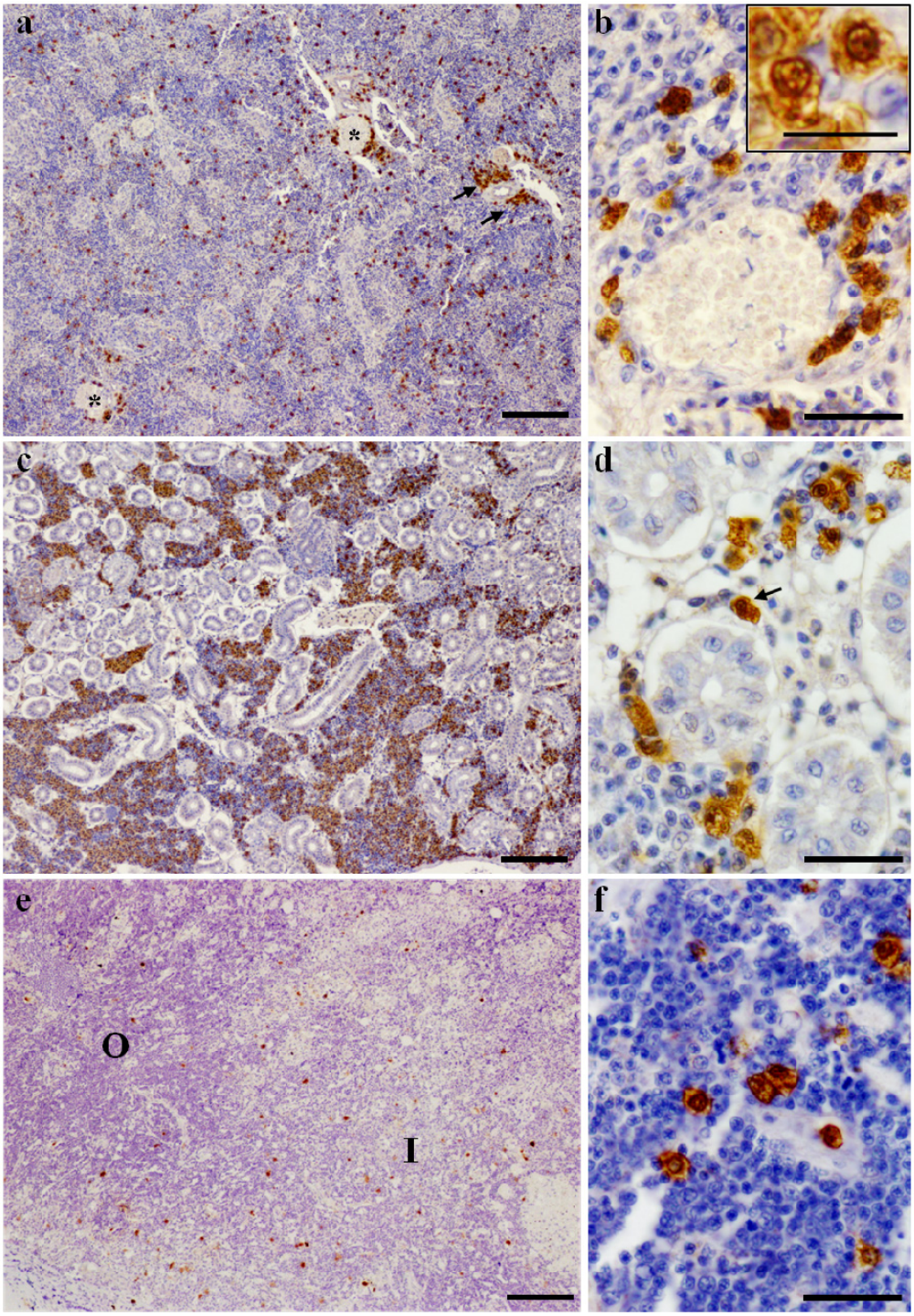

Figure 1. Photomicrographs of turbot spleen (a, b), kidney (c, d) and thymus (e, f) showing reactivity against the commercial polyclonal TNFα antibody. a) Scattered immunopositive cells in the splenic parenchyma, which tend to cluster around the melanomacrophage centres (asterisk) and blood vessels (arrows) (bar = 100 μm). b) TNFα+ cells surrounding a melanomacrophage centre in the spleen (bar = 20 μm). Inset: Higher magnification of labelled macrophage-like cells (bar = 10 μm) c) Immunoreactive cells in the lymphohaematopoietic interstitial tissue of the kidney (bar = 100 μm). d) Higher magnification of renal tissue showing TNFα+ cells between the tubules. Note the association of some cells with the peritubular capillaries, resembling peritubular macrophages (arrow) (bar = 20 μm). e) Immunolabelled cells randomly distributed in the outer (O) and inner (I) zone of the thymus parenchyma (bar = 100 μm). f) Higher magnification of TNFα+ cells in the thymus. Notice their shape and size, consistent with macrophages, in comparison with the thymocytes (bar = 20 μm).

Regarding the distribution of TNFα$^+$ cells in turbot tissue, in spleen immunoreactive cells were scattered throughout the parenchyma but tended to form clusters around the blood vessels and melanomacrophage centres (Figure 1a,b). In kidney, a high number of cells in the lymphohaematopoietic interstitial tissue were positive; some of them were closely associated with the peritubular capillaries, consistent with peritubular macrophages (Figure 1c,d). In the thymus, TNFα$^+$ cells compatible with macrophage were detected in both outer and inner part of the organ, following a random distribution (Figure 1e,f). In the digestive tract, labelled macrophage-like cells appeared in the lamina propria (Figure 2a) of the different regions, but were rarely observed in high numbers, being especially scarce in oesophagus and stomach. Also, in most specimens, it was possible to observe some TNFα$^+$ rodlet cells in the lining epithelium (Figure 2f) from oesophagus to hindgut, while immunoreactivity of enteroendocrine-like cells was occasionally noticed. In liver, scarce immunoreactive cells resembling intraparenchymal macrophages were noticed in most of studied samples, associated with hepatic sinusoids (Figure 2b). In sections from heart, in addition to several TNFα$^+$ monocyte-like cells in heart cavities, some endocardial cells from atrium and, to a less extent, ventricle showed positivity to anti TNFα antibody (Figure 2c). As well, positivity of endothelial cells in capillary from different organs was noticed. In gills, most of TNFα$^+$ cells resembled monocytes within the central venous sinus of primary lamellae and the capillary network of secondary lamellae, although in some sections, few labelled cells consistent with resident macrophages were also observed within the stratified epithelium of primary lamellae (Figure 2d). Finally, a scarce number of rounded immunoreactive cells were observed within the non-keratinizing stratified squamous epithelium of the skin (Figure 2e). Non-specific background staining of connective tissue was occasionally noticed in different tissues.

In spleen and kidney from *Dicentrarchus labrax* and *Sparus aurata*, similar results to turbot tissues were obtained: labelled cells were mainly consistent with macrophage- and melanomacrophage-like and monocyte-like cells in the parenchyma and blood vessels of both organs. Also, some immunoreactive rodlet cells were observed in the epithelium of renal tubules.

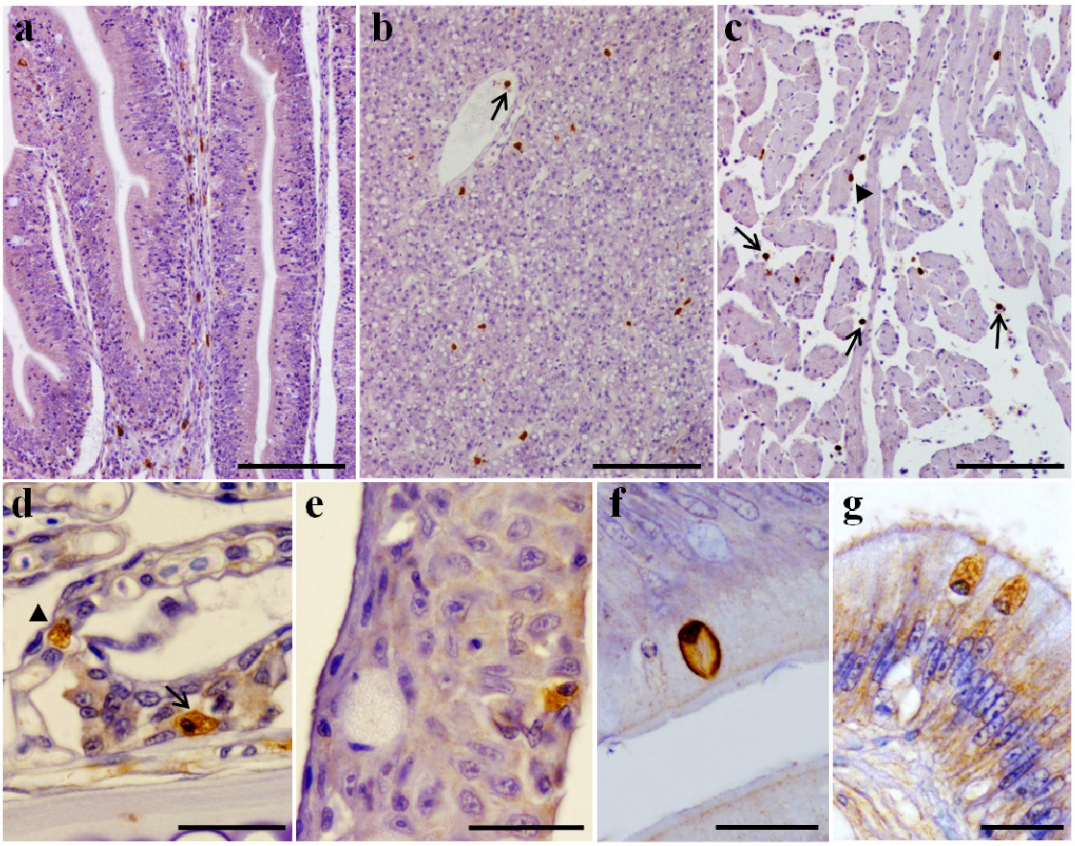

Figure 2. Photomicrographs of turbot intestine (a, f), liver (b, g), heart (c), gills (d) and skin (e) showing reactivity against the TNFα antibody. a) Immunopositive cells in the intestinal lamina propria-submucosa (bar = 100 µm). b) Macrophage-like TNFα+ cells observed in the hepatic parenchyma. Note also the presence of a labelled monocyte-like cell in a blood vessel (arrow) (bar = 100 µm). c) Cardiac ventricle containing several immunoreactive monocyte-like cells (arrow). In the heart, some endocardial cells also showed positivity to TNFα (arrowhead) (bar = 100 µm). d) TNFα+ monocyte-like cell in the capillary network of a secondary lamella (arrowhead) and macrophage-like cell within the epithelium of a primary lamella (arrow) (bar = 20 µm). e) Stratified squamous epithelium of the skin with a macrophage-like immunoreactive cell near the basement membrane (bar = 20 µm). f) Strong positivity to TNFα in a rodlet cell located in the apical part of the intestinal epithelium (bar = 20 µm). g) Immunolabelled rodlet cells within the epithelium of a bile duct (bar = 20 µm).

3.3. Western blotting

Western blot analysis revealed TNFα expression only in kidney and spleen samples from healthy turbot (Figure 3a). In both anterior and trunk kidney the signal intensity was very strong, giving a thick band, while in spleen the band was more thin. The main bands showed a molecular weight of approximately 27 kDa, reaching about 30 kDa in the upper part of kidney's bands. The band for the control protein was about 17 kDa, as expected by product's specification (Figure 3). No specific signal was observed in the remaining organs or in negative control.

When tested in kidney and spleen samples from AS-infected fish, the assay showed an increased signal of the 27 kDa band in spleen, while in kidney this was comparable with the sample from healthy fish (Figure 3b).

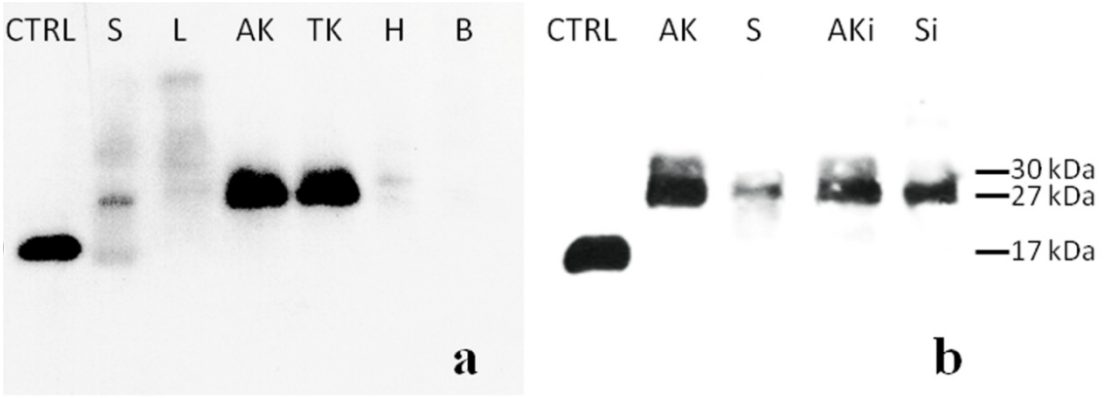

Figure 3. Western blot analysis of TNFα expression using the commercial polyclonal TNFα antibody. a) Reactivity to TNFα ha was tested in protein extracts from different organs of healthy turbot and b) from head kidney and spleen of healthy and *A. salmonicida* (AS)-infected turbot. The same amount of protein extract (50 µg) from each organ was loaded. In both assays the immunogen used to produce the antibody, with a declared molecular weight of 17 kDa, was included as positive control. S = spleen; L = liver; AK= anterior kidney; TK = trunk kidney; H = heart; B =brain; AKi = anterior kidney from AS-infected turbot; Si = spleen from AS-infected turbot.

4. Discussion

In this study, we report the development of an immunohistochemical technique for the detection of turbot TNFα and the distribution of labelled cells in organs from healthy specimens. The specificity of the commercial polyclonal antibody in recognizing this molecule in turbot has been tested by western blot analysis. Turbot TNFα was cloned and molecularly characterized by Ordás *et al.* (2007), who found a predicted molecular weight of 28 kDa for the encoded peptide. This is consistent with our results showing the presence of labelled bands in kidney and spleen with a 27 kDa approximate molecular weight. Only these two organs gave a positive result with western blot analysis, likely due to the small amount of this cytokine present in healthy organs (Pedrera *et al.* 2007; Bradley 2008), especially those which do not have a primary lymphoid function. When TNFα expression was tested with protein extracts from spleen and kidney of AS-infected turbot, an increased intensity of TNFα band was detected in spleen. Increased expression of TNFα during bacterial infection is well demonstrated, and has been reported in the spleen of teleosts challenged with AS (Schwenteit *et al.* 2013; Zanuzzo *et al.* 2015). Also, the immunohistochemical technique enabled the detection of $TNF\alpha^+$ cells in kidney and spleen from *D. labrax* and *S. aurata*. The high homology of the TNFα amino acid sequence between the three species would favor the occurrence of biological cross-reactivity (Scheerlinck 1999), and recombinant turbot TNFα was previously found able to promote leukocytes recruitment when injected in seabream *S. aurata* (Ordás *et al.* 2007).

TNFα is known to be mainly produced by cells of the monocytic/macrophages linage (Goetz *et al.* 2004; Parameswaran and Patial 2010) and the results we obtained would confirm this also in turbot. The typical $TNF\alpha^+$ cell was consistent with monocytes/macrophage and was found, with the exception of the brain, in all studied organs.

Western blot analysis in healthy turbot showed a strong expression of the cytokine in kidney, in accordance with the immunohistochemical findings showing a large portion of labelled

cells in the interstitial lymphohaematopoietic areas of this organ. Myeloid cells from head kidney have been described to be the mainly producers of this cytokine in fish (Goetz *et al.* 2004). Conversely, Ordás *et al.* (2007) did not observe constitutive expression of TNFα in turbot head kidney by RT-PCR, while this was detected in many other fish species (Laing *et al.* 2001; García-Castillo *et al.* 2002; Zou *et al.* 2003; Savan and Sakai 2004; Nascimento *et al.* 2007; Grayfer *et al.* 2008; Lam *et al.* 2011; Schwenteit *et al.* 2013), and also in turbot by our research group (unpublished data). TNFα constitutive expression in the main lymphohaematopoietic organ of teleosts would be an expected result, comparing with that observed for mammals' bone marrow (Jiang *et al.* 1994; Cluitmans *et al.* 1995). As well, the spleen, which as kidney presents vital immunological and haematopoietic functions in teleosts (Rauta *et al.* 2012), was the second organ for number of TNFα$^+$ cells, and TNFα expression was also detected by western blot. Another main lymphoid organ, the thymus, presented a discrete number of labelled cells, predominantly macrophage-like. Constitutive expression of TNFα in this organ has been documented in mammals (Deman *et al.* 1992; Giroir *et al.* 1992; Wolf and Cohen 1992; Grech *et al.* 2000) and in several teleosts (Zou *et al.* 2003; Hino *et al.* 2006; Nascimento *et al.* 2007; Lam *et al.* 2011). Also, Hino *et al.* (2006) reported that similarly to mammalian species, this cytokine is implicated in T cell development in the thymus of rainbow trout (*Oncorhynchus mykiss,* Walbaum). Mammalian TNFα is involved in cell differentiation and has been related with both lympho- and haematopoiesis (Witsell and Schook 1992; Zhang *et al.* 1995; Dybedal *et al.* 2001), as well as in the maintenance, compartmentalization and leukocyte movement in lymphohaematopoietic organs (Sedgwick *et al.* 2000; Tumanov *et al.* 2010). The widespread expression of TNFα we observed in lymphoid organs from healthy turbot, especially in the kidney, suggests its constitutive production, possibly to play analogous roles to the mammalian counterpart.

On the other hand, the existence of a preformed intracellular pool of TNFα has been demonstrated in rainbow trout macrophages. This pool would be ready to be released after an immunological challenge, being not necessarily related with gene expression (Roher *et al.* 2011). The major role played by this cytokine as an early mediator of innate immunity, regulating the production and release of other mediators and promoting cell activation and leukocyte migration, has been demonstrated in different teleost species (Zou *et al.* 2002; García-Castillo *et al.* 2004; Ordás *et al.* 2007; Grayfer *et al.* 2008; Kadowaki *et al.* 2009; Kim *et al.* 2009). In this work, labelled cells were observed in typical organs and localization for antigen processing and presentation in fish, like surrounding melanomacrophages centres and blood vessels in the spleen and in renal interstitial tissue and portal macrophages (Agius and Roberts 2003; Rauta *et al.* 2012; Failde *et al.* 2013; Iliev *et al.* 2013; Coscelli *et al.* 2014). Moreover, TNFα$^+$ cells consistent with phagocytes of the reticulo-endothelial system were observed in liver and heart, both performing a scavenging role in fish (Dalmo *et al.* 1997). Immunopositive cells were also found in organs in direct contact with the external environment, like skin, gills and the gastrointestinal tract, which are also known to present resident cellular defence against the continuous antigenic stimuli (Press and Evensen 1999). As well, monocytes-like immunostained cells were found in the heart cavities and blood vessels of the different organs. However, the number of TNFα$^+$ cells in not primary lymphoid

organs was low, as reported in other studies using healthy animals (Salguero *et al.* 2001; Pedrera *et al.* 2007), probably representing a portion of cells with pre-stored TNFα ready to be released or being activated by some general stimulus.

A similar reasoning can be applied to the immunostaining of rodlet cells, mainly observed within the lining epithelium of the intestine. These granule-containing secretory cells, which still present many unknown aspects, are generally associated with epithelial tissues (Reite 2005), although their presence has been documented also in the thymus parenchyma of some teleost species, like zebrafish (Siderits and Bielek 2009) and turbot (Vigliano *et al.* 2011). Several authors have demonstrated the involvement of these cells in the immune response (Manera and Dezfuli 2004; Reite 2005; Reite and Evensen 2006; Mazon *et al.* 2007; Schultz *et al.* 2014), particularly in parasitic infections (Palenzuela *et al.* 1999; Reite and Evensen 2006; Dezfuli *et al.* 2007; Sitjà-Bobadilla *et al.* 2008; Bermúdez *et al.* 2010). Reite (2005) has suggested that these cells join mast cells/eosinophilic granule cells (MCs/EGCs) in case of parasite infection fighting at epithelial level. In turbot, MCs/EGCs are really hard to be seen even in case of parasitization (authors' personal observations), and in a study aimed to label stored histamine in this kind of cells, not only immunohistochemical results were negative, but the authors did not recognize any MCs/EGCs in turbot gills and intestine by HE and Giemsa staining (Mulero *et al.* 2007). While it has been extensively demonstrated that mammalian MCs presents stored TNFα (Gordon and Galli 1990; Frangogiannis *et al.* 1998; Bischoff *et al.* 1999; Gibbs *et al.* 2001), there are no data on its expression in MCs/EGCs or rodlet cells of teleost. As well, there is a paucity of data about immunohistochemical markers labelling rodlet cells; to our knowledge, only reactivity to S100 in turbot (Vigliano *et al.* 2009) and piscidin in tilapia (*Oreochromis niloticus*, L.) (Silphaduang *et al.* 2006) have been documented. The strategic location at epithelial surfaces supports a role in host defense for rodlet cells and the immunohistochemical findings reported here suggest that a portion of this cell population expresses TNFα also in physiological conditions, especially at intestinal level.

5. Conclusions

The immunohistochemical technique with a commercial polyclonal antibody recognizing TNFα in turbot tissues showed a good specificity. The findings of this work suggest that, similarly to other species, cells of monocyte/macrophage lineage are the main producers of this cytokine in turbot. The positivity of rodlet cells adds further evidence supporting their role in the defence response. The distribution of immunoreactive cells points towards a physiological expression of this cytokine in strategic locations for antigenic challenge in the different tissues. On the other hand, its predominant expression in lymphohaematopoietic organs suggests that TNFα may play a role, as seen in mammals, in lymphohaematopoiesis, leukocyte movement or in the organ maintenance and spatial organization. The developed immunohistochemical technique is proposed as a valuable tool for investigating the role of TNFα in turbot immune system under physiological and pathological conditions.

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References

Agius, C., Roberts, R.J., 2003. Melano-macrophage centres and their role in fish pathology. J Fish Dis 26, 499-509.

Bermúdez, R., Losada, A.P., Vázquez, S., Redondo, M.J., Álvarez-Pellitero, P., Quiroga, M.I., 2010. Light and electron microscopic studies on turbot *Psetta maxima* infected with *Enteromyxum scophthalmi*: histopathology of turbot enteromyxosis. Dis Aquat Organ 89, 209-221.

Bischoff, S.C., Lorentz, A., Schwengberg, S., Weier, G., Raab, R., Manns, M.P., 1999. Mast cells are an important cellular source of tumour necrosis factor alpha in human intestinal tissue. Gut 44, 643-652.

Bradley, J.R., 2008. TNF-mediated inflammatory disease. J Pathol 214, 149-160.

Buchmann, K., 2014. Evolution of innate immunity: clues from invertebrates via fish to mammals. Front Immunol 5, 459.

Cluitmans, F.H., Esendam, B.H., Landegent, J.E., Willemze, R., Falkenburg, J.H., 1995. Constitutive in vivo cytokine and hematopoietic growth factor gene expression in the bone marrow and peripheral blood of healthy individuals. Blood 85, 2038-2044.

Coscelli, G.A., Bermúdez, R., Losada, A.P., Faílde, L.D., Santos, Y., Quiroga, M.I., 2014. Acute *Aeromonas salmonicida* infection in turbot (*Scophthalmus maximus* L.). Histopathological and immunohistochemical studies. Aquaculture 430, 79-85.

Dalmo, R.A., Ingebrigtsen, K., Bøgwald, J., 1997. Non-specific defence mechanisms in fish, with particular reference to the reticuloendothelial system (RES). J Fish Dis 20, 241-273.

Deman, J., Martin, M.T., Delvenne, P., Humblet, C., Boniver, J., Defresne, M.P., 1992. Analysis by in situ hybridization of cells expressing mRNA for tumor-necrosis factor in the developing thymus of mice. Dev Immunol 2, 103-109.

Dezfuli, B.S., Giari, L., Shinn, A.P., 2007. The role of rodlet cells in the inflammatory response in *Phoxinus phoxinus* brains infected with *Diplostomum*. Fish Shellfish Immunol 23, 300-304.

Dybedal, I., Bryder, D., Fossum, A., Rusten, L.S., Jacobsen, S.E., 2001. Tumor necrosis factor (TNF)-mediated activation of the p55 TNF receptor negatively regulates maintenance of cycling reconstituting human hematopoietic stem cells. Blood 98, 1782-1791.

Failde, L.D., Losada, A.P., Bermúdez, R., Santos, Y., Quiroga, M.I., 2013. *Tenacibaculum maritimum* infection: Pathology and immunohistochemistry in experimentally challenged turbot (*Psetta maxima* L.). Microb Pathog.

Fleming, A., Jankowski, J., Goldsmith, P., 2010. In vivo analysis of gut function and disease changes in a zebrafish larvae model of inflammatory bowel disease: a feasibility study. Inflamm Bowel Dis 16, 1162-1172.

Frangogiannis, N.G., Lindsey, M.L., Michael, L.H., Youker, K.A., Bressler, R.B., Mendoza, L.H., Spengler, R.N., Smith, C.W., Entman, M.L., 1998. Resident cardiac mast cells degranulate and

release preformed TNF-α, initiating the cytokine cascade in experimental canine myocardial ischemia/reperfusion. Circulation 98, 699-710.

Fredriksen, B.N., Saevareid, K., McAuley, L., Lane, M.E., Bogwald, J., Dalmo, R.A., 2011. Early immune responses in Atlantic salmon (*Salmo salar* L.) after immunization with PLGA nanoparticles loaded with a model antigen and beta-glucan. Vaccine 29, 8338-8349.

García-Castillo, J., Pelegrín, P., Mulero, V., Meseguer, J., 2002. Molecular cloning and expression analysis of tumor necrosis factor alpha from a marine fish reveal its constitutive expression and ubiquitous nature. Immunogenetics 54, 200-207.

García-Castillo, J., Chaves-Pozo, E., Olivares, P., Pelegrín, P., Meseguer, J., Mulero, V., 2004. The tumor necrosis factor alpha of the bony fish seabream exhibits the in vivo proinflammatory and proliferative activities of its mammalian counterparts, yet it functions in a species-specific manner. Cell Mol Life Sci 61, 1331-1340.

Gibbs, B.F., Wierecky, J., Welker, P., Henz, B.M., Wolff, H.H., Grabbe, J., 2001. Human skin mast cells rapidly release preformed and newly generated TNF-α and IL-8 following stimulation with anti-IgE and other secretagogues. Exp Dermatol 10, 312-320.

Giroir, B.P., Brown, T., Beutler, B., 1992. Constitutive synthesis of tumor necrosis factor in the thymus. Proc Natl Acad Sci USA 89, 4864-4868.

Goetz, F.W., Planas, J.V., MacKenzie, S., 2004. Tumor necrosis factors. Dev Comp Immunol 28, 487-497.

Gordon, J.R., Galli, S.J., 1990. Mast cells as a source of both preformed and immunologically inducible TNF-alpha/cachectin. Nature 346, 274-276.

Grayfer, L., Walsh, J.G., Belosevic, M., 2008. Characterization and functional analysis of goldfish (*Carassius auratus* L.) tumor necrosis factor-alpha. Dev Comp Immunol 32, 532-543.

Grech, A.P., Riminton, D.S., Gabor, M.J., Hardy, C.L., Sedgwick, J.D., Godfrey, D.I., 2000. Increased thymic B cells but maintenance of thymic structure, T cell differentiation and negative selection in lymphotoxin-alpha and TNF gene-targeted mice. Dev Immunol 8, 61-74.

Hehlgans, T., Pfeffer, K., 2005. The intriguing biology of the tumour necrosis factor/tumour necrosis factor receptor superfamily: players, rules and the games. Immunology 115, 1-20.

Hino, K., Nakamura, O., Yoshiura, Y., Suetake, H., Suzuki, Y., Watanabe, T., 2006. TNF induces the growth of thymocytes in rainbow trout. Dev Comp Immunol 30, 639-647.

Hirono, I., Nam, B.H., Kurobe, T., Aoki, T., 2000. Molecular cloning, characterization, and expression of TNF cDNA and gene from Japanese flounder *Paralychthys olivaceus*. J Immunol 165, 4423-4427.

Iliev, D.B., Thim, H., Lagos, L., Olsen, R., Jorgensen, J.B., 2013. Homing of Antigen-Presenting Cells in Head Kidney and Spleen - Salmon Head Kidney Hosts Diverse APC Types. Front Immunol 4, 137.

Jiang, S., Levine, J.D., Fu, Y., Deng, B., London, R., Groopman, J.E., Avraham, H., 1994. Cytokine production by primary bone marrow megakaryocytes. Blood 84, 4151-4156.

Kadowaki, T., Harada, H., Sawada, Y., Kohchi, C., Soma, G., Takahashi, Y., Inagawa, H., 2009. Two types of tumor necrosis factor-alpha in bluefin tuna (*Thunnus orientalis*) genes: Molecular cloning and expression profile in response to several immunological stimulants. Fish Shellfish Immunol 27, 585-594.

Kim, M.S., Hwang, Y.J., Yoon, K.J., Zenke, K., Nam, Y.K., Kim, S.K., Kim, K.H., 2009. Molecular cloning of rock bream (*Oplegnathus fasciatus*) tumor necrosis factor-alpha and its effect on the respiratory burst activity of phagocytes. Fish Shellfish Immunol 27, 618-624.

Komatsu, K., Tsutsui, S., Hino, K., Araki, K., Yoshiura, Y., Yamamoto, A., Nakamura, O., Watanabe, T., 2009. Expression profiles of cytokines released in intestinal epithelial cells of the rainbow trout, *Oncorhynchus mykiss*, in response to bacterial infection. Dev Comp Immunol 33, 499-506.

Koppang, E.O., Fischer, U., Satoh, M., Jirillo, E., 2007. Inflammation in fish as seen from a morphological point of view with special reference to the vascular compartment. Curr Pharm Des 13, 3649-3655.

Laing, K.J., Wang, T., Zou, J., Holland, J., Hong, S., Bols, N., Hirono, I., Aoki, T., Secombes, C.J., 2001. Cloning and expression analysis of rainbow trout *Oncorhynchus mykiss* tumour necrosis factor-alpha. Eur J Biochem 268, 1315-1322.

Lam, F.W., Wu, S.Y., Lin, S.J., Lin, C.C., Chen, Y.M., Wang, H.C., Chen, T.Y., Lin, H.T., Lin, J.H., 2011. The expression of two novel orange-spotted grouper (*Epinephelus coioides*) TNF genes in peripheral blood leukocytes, various organs, and fish larvae. Fish Shellfish Immunol 30, 618-629.

Lieschke, G.J., Trede, N.S., 2009. Fish immunology. Curr Biol 19, R678-R682.

Ma, J., Li, X., Li, Y., Li, Y., Niu, D., 2014. Toxic Effects of Paraquat on Cytokine Expression in Common Carp, *Cyprinus carpio* L. J Biochem Mol Toxicol 28, 501-509.

Manera, M., Dezfuli, B.S., 2004. Rodlet cells in teleosts: a new insight into their nature and functions. J Fish Biol 65, 597-619.

Mazon, A.F., Huising, M.O., Taverne-Thiele, A.J., Bastiaans, J., Verburg-van Kemenade, B.M., 2007. The first appearance of Rodlet cells in carp (*Cyprinus carpio* L.) ontogeny and their possible roles during stress and parasite infection. Fish Shellfish Immunol 22, 27-37.

McCarthy, U., Casadei, E., Wang, T., Secombes, C.J., 2013. Red mark syndrome in rainbow trout *Oncorhynchus mykiss*: investigation of immune responses in lesions using histology, immunohistochemistry and analysis of immune gene expression. Fish Shellfish Immunol 34, 1119-1130.

Millán, A., Gómez-Tato, A., Pardo, B.G., Fernández, C., Bouza, C., Vera, M., Alvarez-Dios, J.A., Cabaleiro, S., Lamas, J., Lemos, M.L., Martínez, P., 2011. Gene expression profiles of the spleen, liver, and head kidney in turbot (*Scophthalmus maximus*) along the infection process with *Aeromonas salmonicida* using an immune-enriched oligo-microarray. Mar Biotechnol (NY) 13, 1099-1114.

Montes, A., Figueras, A., Novoa, B., 2010. Nodavirus encephalopathy in turbot (*Scophthalmus maximus*): inflammation, nitric oxide production and effect of anti-inflammatory compounds. Fish Shellfish Immunol 28, 281-288.

Mulero, I., Sepulcre, M.P., Meseguer, J., García-Ayala, A., Mulero, V., 2007. Histamine is stored in mast cells of most evolutionarily advanced fish and regulates the fish inflammatory response. Proc Natl Acad Sci USA 104, 19434-19439.

Mulero, I., Pilar Sepulcre, M., Roca, F.J., Meseguer, J., García-Ayala, A., Mulero, V., 2008. Characterization of macrophages from the bony fish gilthead seabream using an antibody against the macrophage colony-stimulating factor receptor. Dev Comp Immunol 32, 1151-1159.

Nascimento, D.S., Pereira, P.J.B., Reis, M.I.R., do Vale, A., Zou, J., Silva, M.T., Secombes, C.J., dos Santos, N.M.S., 2007. Molecular cloning and expression analysis of sea bass (*Dicentrarchus labrax* L.) tumor necrosis factor-α (TNF-α). Fish Shellfish Immunol 23, 701-710.

Ordás, M.C., Costa, M.M., Roca, F.J., López-Castejón, G., Mulero, V., Meseguer, J., Figueras, A., Novoa, B., 2007. Turbot TNFalpha gene: molecular characterization and biological activity of the recombinant protein. Mol Immunol 44, 389-400.

Palenzuela, O., Álvarez-Pellitero, P., Sitjà-Bobadilla, A., 1999. Glomerular disease associated with *Polysporoplasma sparis* (Myxozoa) infections in cultured gilthead sea bream, *Sparus aurata* L. (Pisces: Teleostei). Parasitology 118, 245-256.

Parameswaran, N., Patial, S., 2010. Tumor necrosis factor-alpha signaling in macrophages. Crit Rev Eukaryot Gene Expr 20, 87-103.

Pedrera, M., Sánchez-Cordón, P.J., Romero-Trevejo, J.L., Raya, A.I., Núnez, A., Gómez-Villamandos, J.C., 2007. Cytokine expression in paraffin wax-embedded tissues from conventional calves. J Comp Pathol 136, 273-278.

Pennacchi, Y., Leef, M.J., Crosbie, P.B., Nowak, B.F., Bridle, A.R., 2014. Evidence of immune and inflammatory processes in the gills of AGD-affected Atlantic salmon, *Salmo salar* L. Fish Shellfish Immunol 36, 563-570.

Press, C.M., Evensen, Ø., 1999. The morphology of the immune system in teleost fishes. Fish Shellfish Immunol 9, 309-318.

Raida, M.K., Buchmann, K., 2008. Bath vaccination of rainbow trout (*Oncorhynchus mykiss* Walbaum) against *Yersinia ruckeri*: effects of temperature on protection and gene expression. Vaccine 26, 1050-1062.

Randelli, E., Buonocore, F., Scapigliati, G., 2008. Cell markers and determinants in fish immunology. Fish Shellfish Immunol 25, 326-340.

Rauta, P.R., Nayak, B., Das, S., 2012. Immune system and immune responses in fish and their role in comparative immunity study: a model for higher organisms. Immunol Lett 148, 23-33.

Reite, O.B., 2005. The rodlet cells of teleostean fish: their potential role in host defence in relation to the role of mast cells/eosinophilic granule cells. Fish Shellfish Immunol 19, 253-267.

Reite, O.B., Evensen, O., 2006. Inflammatory cells of teleostean fish: a review focusing on mast cells/eosinophilic granule cells and rodlet cells. Fish Shellfish Immunol 20, 192-208.

Roca, F.J., Mulero, I., López-Munoz, A., Sepulcre, M.P., Renshaw, S.A., Meseguer, J., Mulero, V., 2008. Evolution of the inflammatory response in vertebrates: fish TNF-alpha is a powerful activator of endothelial cells but hardly activates phagocytes. J Immunol 181, 5071-5081.

Roher, N., Callol, A., Planas, J.V., Goetz, F.W., MacKenzie, S.A., 2011. Endotoxin recognition in fish results in inflammatory cytokine secretion not gene expression. Innate Immun 17, 16-28.

Saeij, J.P., Stet, R.J., de Vries, B.J., van Muiswinkel, W.B., Wiegertjes, G.F., 2003. Molecular and functional characterization of carp TNF: a link between TNF polymorphism and trypanotolerance? Dev Comp Immunol 27, 29-41.

Salguero, F.J., Mekonnen, T., Ruiz-Villamor, E., Sanchez-Cordon, P.J., Gomez-Villamandos, J.C., 2001. Detection of monokines in paraffin-embedded tissues of pigs using polyclonal antibodies. Vet Res 32, 601-609.

Savan, R., Sakai, M., 2004. Presence of multiple isoforms of TNF alpha in carp (*Cyprinus carpio* L.): genomic and expression analysis. Fish Shellfish Immunol 17, 87-94.

Scheerlinck, J.-P.Y., 1999. Functional and structural comparison of cytokines in different species. Vet Immunol Immunopathol 72, 39-44.

Schultz, A.G., Jones, P.L., Toop, T., 2014. Rodlet cells in Murray cod, *Maccullochella peelii peelii* (Mitchell), affected with chronic ulcerative dermatopathy. J Fish Dis 37, 219-228.

Schwenteit, J.M., Breithaupt, A., Teifke, J.P., Koppang, E.O., Bornscheuer, U.T., Fischer, U., Gudmundsdottir, B.K., 2013. Innate and adaptive immune responses of Arctic charr (*Salvelinus alpinus*, L.) during infection with *Aeromonas salmonicida* subsp. *achromogenes* and the effect of the AsaP1 toxin. Fish Shellfish Immunol 35, 866-873.

Sedgwick, J.D., Riminton, D.S., Cyster, J.G., Korner, H., 2000. Tumor necrosis factor: a master-regulator of leukocyte movement. Immunol Today 21, 110-113.

Siderits, D., Bielek, E., 2009. Rodlet cells in the thymus of the zebrafish *Danio rerio* (Hamilton, 1822). Fish Shellfish Immunol 27, 539-548.

Silphaduang, U., Colorni, A., Noga, E.J., 2006. Evidence for widespread distribution of piscidin antimicrobial peptides in teleost fish. Dis Aquat Organ 72, 241-252.

Sitjà-Bobadilla, A., Calduch-Giner, J., Saera-Vila, A., Palenzuela, O., Álvarez-Pellitero, P., Pérez-Sánchez, J., 2008. Chronic exposure to the parasite *Enteromyxum leei* (Myxozoa: Myxosporea) modulates the immune response and the expression of growth, redox and immune relevant genes in gilthead sea bream, *Sparus aurata* L. Fish Shellfish Immunol 24, 610-619.

Tumanov, A.V., Grivennikov, S.I., Kruglov, A.A., Shebzukhov, Y.V., Koroleva, E.P., Piao, Y., Cui, C.Y., Kuprash, D.V., Nedospasov, S.A., 2010. Cellular source and molecular form of TNF specify its distinct functions in organization of secondary lymphoid organs. Blood 116, 3456-3464.

Vigliano, F.A., Bermúdez, R., Nieto, J.M., Quiroga, M.I., 2009. Development of rodlet cells in the gut of turbot (*Psetta maxima* L.): Relationship between their morphology and S100 protein immunoreactivity. Fish Shellfish Immunol 26, 146-153.

Vigliano, F.A., Losada, A.P., Castello, M., Bermúdez, R., Quiroga, M.I., 2011. Morphological and immunohistochemical characterisation of the thymus in juvenile turbot (*Psetta maxima*, L.). Cell Tissue Res 346, 407-416.

Waters, J.P., Pober, J.S., Bradley, J.R., 2013. Tumour necrosis factor in infectious disease. J Pathol 230, 132-147.

Witsell, A.L., Schook, L.B., 1992. Tumor necrosis factor alpha is an autocrine growth regulator during macrophage differentiation. Proc Natl Acad Sci U S A 89, 4754-4758.

Wolf, S.S., Cohen, A., 1992. Expression of cytokines and their receptors by human thymocytes and thymic stromal cells. Immunology 77, 362-368.

Yousaf, M.N., Koppang, E.O., Skjodt, K., Kollner, B., Hordvik, I., Zou, J., Secombes, C., Powell, M.D., 2012. Cardiac pathological changes of Atlantic salmon (*Salmo salar* L.) affected with heart and skeletal muscle inflammation (HSMI). Fish Shellfish Immunol 33, 305-315.

Zanuzzo, F.S., Urbinati, E.C., Rise, M.L., Hall, J.R., Nash, G.W., Gamperl, A.K., 2015. *Aeromonas salmonicid*a induced immune gene expression in Aloe vera fed steelhead trout, *Oncorhynchus mykiss* (Walbaum). Aquaculture 435, 1-9.

Zhang, Y., Harada, A., Bluethmann, H., Wang, J.B., Nakao, S., Mukaida, N., Matsushima, K., 1995. Tumor necrosis factor (TNF) is a physiologic regulator of hematopoietic progenitor cells: increase of early hematopoietic progenitor cells in TNF receptor p55-deficient mice in vivo and potent inhibition of progenitor cell proliferation by TNF alpha in vitro. Blood 86, 2930-2937.

Zhu, L.Y., Nie, L., Zhu, G., Xiang, L.X., Shao, J.Z., 2013. Advances in research of fish immune-relevant genes: a comparative overview of innate and adaptive immunity in teleosts. Dev Comp Immunol 39, 39-62.

Zou, J., Wang, T., Hirono, I., Aoki, T., Inagawa, H., Honda, T., Soma, G.I., Ototake, M., Nakanishi, T., Ellis, A.E., Secombes, C.J., 2002. Differential expression of two tumor necrosis factor genes in rainbow trout, *Oncorhynchus mykiss*. Dev Comp Immunol 26, 161-172.

Zou, J., Secombes, C.J., Long, S., Miller, N., Clem, L.W., Chinchar, V.G., 2003. Molecular identification and expression analysis of tumor necrosis factor in channel catfish (*Ictalurus punctatus*).Dev Comp Immunol 27, 845-858.

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ARTÍCULO II: IMMUNOHISTOCHEMICAL DETECTION AND GENE EXPRESSION OF TNFα IN TURBOT (*SCOPHTHALMUS MAXIMUS*) ENTEROMYXOSIS

Ronza, P.[1]; Bermúdez, R.[2]; Losada, A.P.[1]; Sitjà-Bobadilla, A.[3]; G. Pardo, B.[4]; Quiroga, M.I.[1]

[1]Departamento de Ciencias Clínicas Veterinarias, Facultad de Veterinaria, Universidade de Santiago de Compostela, 27002 Lugo.

[2]Departamento de Anatomía y Producción Animal, Facultad de Veterinaria, Universidade de Santiago de Compostela, 27002 Lugo.

[3]Instituto de Acuicultura Torre de la Sal (IATS-CSIC), 12595 Ribera de Cabanes, Castellón.

[4]Departamento de Genética, Facultad de Veterinaria, Universidade de Santiago de Compostela, 27002 Lugo.

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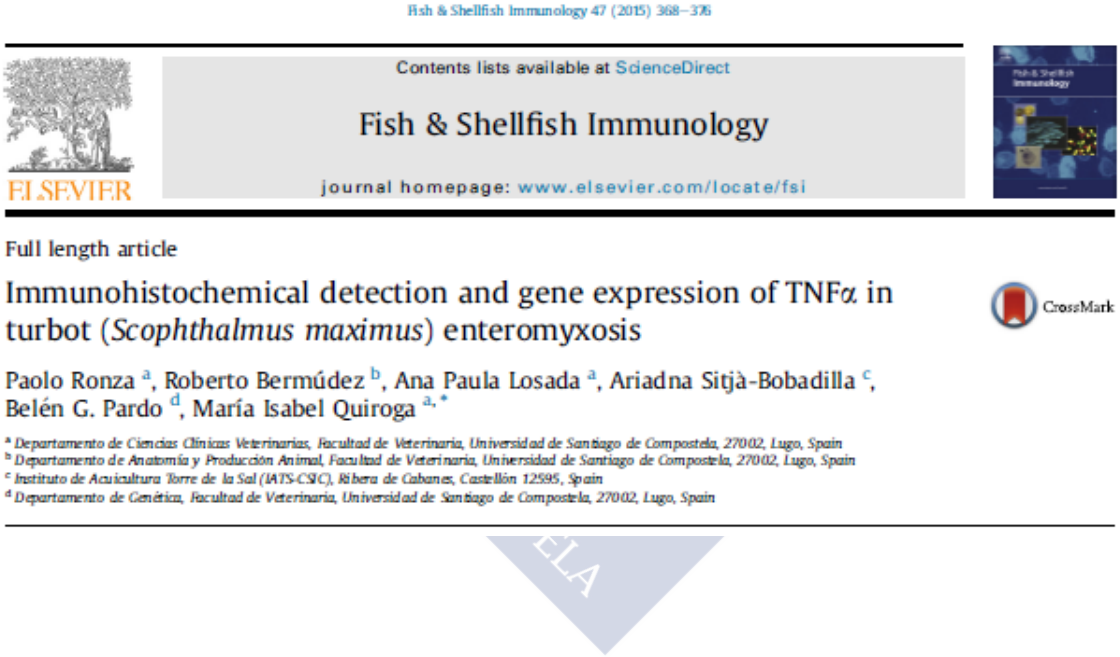

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Full length article

Immunohistochemical detection and gene expression of TNFα in turbot (*Scophthalmus maximus*) enteromyxosis

Paolo Ronza [a], Roberto Bermúdez [b], Ana Paula Losada [a], Ariadna Sitjà-Bobadilla [c], Belén G. Pardo [d], María Isabel Quiroga [a, *]

[a] Departamento de Ciencias Clínicas Veterinarias, Facultad de Veterinaria, Universidad de Santiago de Compostela, 27002, Lugo, Spain
[b] Departamento de Anatomía y Producción Animal, Facultad de Veterinaria, Universidad de Santiago de Compostela, 27002, Lugo, Spain
[c] Instituto de Acuicultura Torre de la Sal (IATS-CSIC), Ribera de Cabanes, Castellón 12595, Spain
[d] Departamento de Genética, Facultad de Veterinaria, Universidad de Santiago de Compostela, 27002, Lugo, Spain

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Abstract

Enteromyxum scophthalmi (Myxozoa) constitutes one of the most devastating pathogens for turbot (*Scophthalmus maximus*, L.) aquaculture. This parasite causes a severe intestinal parasitosis that leads to a cachectic syndrome with high morbidity and mortality rates for which no therapeutic options are available. Presence of inflammatory infiltrates, increased apoptotic rates and epithelial detaching have been described at intestinal level, as well as leukocyte depletion in lymphohaematopoietic organs. Previous investigations on enteromyxosis in turbot showed the high susceptibility of this species to the parasite and reported the existence of a dysregulated immune response against the parasite. The pleiotropic cytokine tumour necrosis factor alpha (TNFα) plays a major role in immune response and is involved in a wide range of biological activities. In teleost, the gene expression of this cytokine has been found regulated under several pathological conditions. Teleost TNFα shows some analogous functions with its mammalian counterparts, but the extent of its activities is still poorly understood. Cytokines are generally considered as a double-edge sword and TNFα has been implicated in the pathogenesis of different inflammatory diseases as well as in wasting syndromes described in mammals. The aim of this work was to analyse the expression of TNFα during enteromyxosis with molecular (Q-PCR) and morphological (immunohistochemistry) tools. Kidney, spleen and pyloric caeca from turbot with moderate and severe infections were analysed and compared to healthy naïve fish. TNFα expression was increased in both spleen and kidney in the earlier stages of the disease, whereas in severely infected fish, the expression decreased, especially in kidney. At the intestinal level, an increase in the number of TNFα-positive cells was noticed, which was proportional to the infiltration of inflammatory cells. The results demonstrate the involvement of TNFα in the immune response to *E. scophthalmi* in turbot, which could be related to the development of the clinic signs and lesions.

Keywords: Tumour necrosis factor alpha; cytokine; turbot; enteromyxosis; inflammatory response; Q-PCR; immunohistochemistry

1. Introduction

Enteromyxosis caused by the myxozoan parasite *Enteromyxum scophthalmi* poses a serious threat for turbot (*Scophthalmus maximus*, L.) aquaculture. Parasitic forms invade the digestive tract, being the infection first detected in pyloric caeca or anterior intestine and subsequently spreading along the entire gut (Redondo *et al.* 2004; Bermúdez *et al.* 2010). In contrast to other myxozoan species, *Enteromyxum* spp. can be directly transmitted from fish to fish, and up to now, no effective therapeutic options are available to control this parasitosis (Redondo *et al.* 2002; Sitjà-Bobadilla and Palenzuela, 2012). The disease leads to a cachectic syndrome characterized by weight loss, anorexia and amyotrophy (Sitjà-Bobadilla and Palenzuela, 2012). Turbot presents elevated rate of morbidity and mortality and the disease can affect up to 100% of fish in a farming unit (Branson *et al.* 1999; Quiroga *et al.* 2006). Compared to gilthead sea bream (*Sparus aurata*, L.) infected by *E. leei*, turbot shows a higher susceptibility to enteromyxosis associated to more severe lesions (Sitjà-Bobadilla and Palenzuela, 2012). Microscopically, the main lesion is catarrhal enteritis of increasing severity throughout the

disease, characterized by severe inflammatory infiltrates and detachment of the lining epithelium, along with high parasite burden in late stages of the disease. In these stages, leukocyte depletion in the lymphohaematopoietic organs is also a common finding (Sitjà-Bobadilla *et al.* 2006; Bermúdez *et al.* 2010; Losada *et al.* 2014a). Previous studies on the immune response and host-parasite interaction have provided evidences of a dysfunctional immune response against the parasite. Turbot appears unable to mount an effective systemic adaptive response (Bermúdez *et al.* 2006; Sitjà-Bobadilla *et al.* 2006, 2007; Robledo *et al.* 2014b), while locally the immune response seems to be exacerbated, contributing to the development of lesions (Losada *et al.* 2012, 2014a; Robledo *et al.* 2014b). Tumour necrosis factor alpha (TNFα) is a cytokine that acts in a broad range of signalling events within cells, being involved in cell activation, proliferation death and survival (Waters *et al.*, 2013). It plays a pivotal role in the organization and functions of the immune system, mainly as a major pro-inflammatory cytokine, acting at early stages of the inflammatory reaction and orchestrating the subsequent cascade of events (Bradley 2008; Waters *et al.* 2013). This cytokine has been described as a double-edge sword, since its functions are essential for a proper immune response, but it is also clearly associated with the development of clinical signs and lesions in different human diseases (Aggarwal 2003; Hehlgans and Pfeffer 2005; Morley *et al.* 2006; Bradley 2008; Durán 2008). TNFα is clearly implicated in the pathogenesis of inflammatory bowel diseases (IBDs) (Leppkes *et al.* 2014), which share with enteromyxosis the dysregulated immune response and the intestinal lesions, as well as in wasting diseases (Morley *et al.* 2006), characterized as enteromyxosis by anorexia, weight loss and amyotrophy. For these conditions, the immunomodulatory therapies, often consisting in specific blockade on TNFα action, have raised in many cases as the most effective (Marcora *et al.* 2006; Argiles *et al.* 2011; Arijs *et al.* 2011; van Schaik *et al.* 2014). Also in a model of IBD described in zebrafish (*Danio rerio*, Hamilton), TNFα expression was found increased and immunomodulatory therapies showed positive results (Fleming *et al.* 2010). In fact, in the different fish species where TNFα has been identified, this cytokine showed similar immune-related functions to its mammalian counterpart (García-Castillo *et al.* 2004; Goetz *et al.* 2004; Hino *et al.* 2006; Grayfer *et al.* 2008; Roca *et al.* 2008; Lam *et al.* 2011). Nevertheless, the complex and widespread biological activities accomplished in mammals are still poor described in teleosts. The regulation of its expression has been reported in several piscine parasitic diseases (Mladineo and Block 2010; Heinecke and Buchmann 2013; Pennacchi *et al.* 2014), including enteromyxosis by *E. leei* in gilthead sea bream (Pérez-Cordón *et al.* 2014). The aim of this study was to investigate the involvement of TNFα in turbot enteromyxosis combining Q-PCR and immunohistochemistry to analyse its expression in target organs (pyloric caeca, kidney and spleen).

2. Materials and methods

2.1. Experimental design and histopathology

The experimental setup and sampling procedures were previously described (Robledo y col. 2014a). Briefly, recipient (R) turbot were experimentally-infected by oral route (Redondo y col. 2002) and tissue samples were collected at different time points in Bouin's fluid and RNAlater for histopathological and molecular techniques, respectively. The status of control (C, not

exposed to infection) and R fish was assessed by light microscopy on H&E and toluidine blue stained sections. R fish were classified into three groups (slight, moderately and severely infected) according to the histopathological grading described by Bermúdez *et al.* (2010). For this study, spleen, kidney and pyloric caeca from 8 C and 8 R turbot at 24 and 42 days post-inoculation (DPI) were used. In order to increase the uniformity of the samples, R turbot at 24 DPI were chosen among those graded as moderately infected and R turbot at 42 DPI among those graded as severely infected. The experiment was carried out in accordance with national (Royal Decree RD1201/2005, for the protection of animals used in scientific experiments) and institutional regulations (CSIC, IATS Review Board) and the current European Union legislation on handling experimental animals.

2.2. Immunohistochemical detection of TNFα

Paraffin sections (3 μm thick) from Bouin's fixed tissue samples were dewaxed in xylene and rehydrated through a graded ethanol series. IHC was carried out with a previously developed protocol (Ronza *et al.* 2015), using an automated stainer (Dako Autostainer, Dako, Glostrup, Denmark) after the antigen retrieval step, in order to standardize the immunostaining. Briefly, primary antibody (1:600 working dilution, rabbit polyclonal antibody to human TNFα, ab6671, Abcam, Cambridge, UK) was incubated during 2 h at room temperature. After 30 min incubation with a HRP-labelled secondary antibody, the peroxidase reaction was developed with a diaminobenzidine-positive chromogen (EnVision+ System-HRP kit, K 4011; Dako), achieving the desired signal after 1 min of incubation. The sections were washed three times for 5 min in 0.1 M phosphate buffered saline containing 0.05% Tween-20 between all subsequent steps. After counterstaining with haematoxylin, sections were unloaded by the Autostainer, dehydrated and coverslipped with DePeX mounting medium (Gurr®, BDH Prolabo, VWR International, Ltd. UK). In order to test the specificity of the immunoreaction, positive (swine tissue) and negative (replacement of the primary antibody by PBS) controls were included.

2.3. Gene expression

Tissue samples preserved in RNAlater were kept at 4ºC during 24 h and stored at -20ºC until RNA extraction. Total RNA was extracted from tissues of C and R fish using TRIZOL Reagent (Life Technologies, Carlsbad, CA, USA) according to manufacturer's recommendations. RNA was quantified using NanoDrop® ND-1000 spectrophotometer (NanoDrop® Technologies Inc.) and its quality was checked in an Agilent BioAnalizer (Agilent Technologies, USA). Good quality RNA (RIN > 7.5) was reverse transcribed (1 μg) into cDNA by random primers using AffinityScript Multiple Temperature cDNA Synthesis kit following the supplier's protocol (Agilent Technologies). The Q-PCR analysis was carried out in a MX3005P thermocycler (Stratagene) using 2 μl of cDNA per reaction and 300 nM of each primer in a final volume of 20 μl according to the Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent Technologies) manufacturer's instructions. The constitutively expressed ribosomal protein S4 (RPS4), proved to be stably expressed in turbot (Millán *et al.* 2011; Robledo *et al.* 2014b), was chosen as the house-keeping gene for sample normalisation. TNFα primers (sense: 5'-GGGTGGATGTGGAAGGTGAT-3'; antisense: 5'-GGCCTCTGTTTGGCTTGACT-3') were designed based on the mRNA sequence of turbot TNFα (GenBank accession number

FJ654645) (Ordás *et al.* 2007). Each sample was performed in triplicate for accuracy and error estimation including one reverse-transcription-negative control for each gene. Fluorescence readings at the end of each cycle were used to estimate threshold cycle values (Ct). Values were normalized to RPS4 and fold change in transcript level determined with the relative quantitative method (ΔΔCt) (Livak and Schmittgen 2001) using data from C fish as reference values. Prior to quantitative analysis, a standard curve was constructed using six serial dilutions of cDNA (from 1,000 to 0.01 ng) and the efficiency of each primer set was determined. Efficiencies of 90–110% were obtained by primer optimization. Each sample was analysed for primer–dimer, contamination, or mispriming by inspection of their dissociation curves.

2.4. Statistical analysis

The statistical analysis of gene expression was performed with SPSS Statistics 20.0 software (SPSS Inc., Chicago, Illinois, USA). Data were expressed as mean ± SEM, and significance of differences was determined by Student's *t*-test, after checking that data from C and R fish follow a normal distribution using Shapiro-Wilk test. Results were considered significant at $p < 0.05$.

3. Results

3.1. Histopathology

The 8 R turbot at 24 DPI selected for this study presented scarce parasitic forms in the lining epithelium of different regions of the gastrointestinal tract, more numerous in pyloric caeca and anterior intestine. In these regions, mild inflammatory infiltrates constituted by mononuclear cells were commonly observed in the lamina propria-submucosa and at the basis of the epithelium (Figure 1a, c). These cells were mainly consistent with lymphocytes within the lining epithelium, while the infiltrates in the lamina propria-submucosa were constituted by a heterogeneous population of macrophages and lymphocytes (Figure 1c). In those areas where the presence of *E. scophthalmi* and inflammatory cells was higher, some intestinal folds showed changes in epithelial architecture (Figure 1a) and some enterocytes presented apoptotic features. No significant histopathological changes were present in other organs.

On the other hand, the 8 R turbot at 42 DPI sampling point showed a heavy parasitic load along the entire gut lining epithelium, which presented the typical scallop shape and numerous areas of epithelial detachment (Figure 1b). Mononuclear cells, mainly consistent with lymphocytes, were seen infiltrating the epithelium and the lamina-propria submucosa appeared thickened and edematous, with severe mixed inflammatory infiltrates (Figure 1b). Leukocyte accumulation in blood vessels and dilatation of blood and lymphatic vessels were also often observed. Most enterocytes and detached cells in the intestinal lumen, showed altered morphology, consistent with apoptosis, like rounding up or shrinkage and nuclear alterations, namely hypertrophied nuclei, chromatin condensation, degradation of the nuclear envelope or nuclear fragmentation (Figure 1d). Also, numerous inflammatory cells in the lamina propria-submucosa undergoing apoptotic death were reported. The histological diagnosis was moderate to severe parasitic catarrhal enteritis. In other organs, the most striking lesion was the leukocyte depletion found in the spleen and in the kidney, being generally more evident in the latter organ (Figure 1f).

C turbot from both sampling points did not show any significant histological alteration in the sampled tissues.

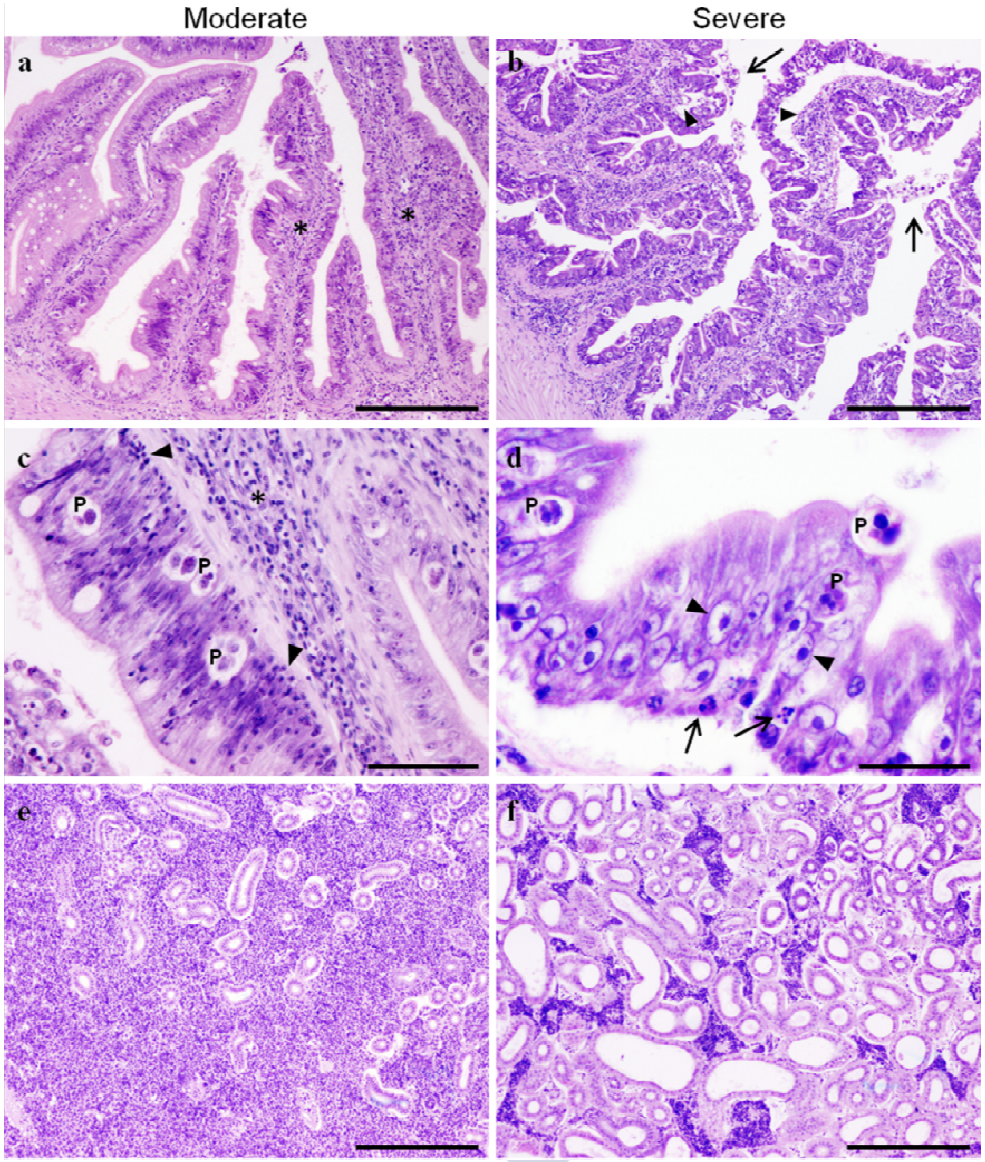

Figure 1. Histopathological findings in moderately (a, c, e) and severely (b, d, f) *Enteromyxum scophthalmi*-infected turbot. Stained with H&E. a) Section of pyloric caeca presenting features of moderate enteromyxosis, with some intestinal folds (asterisks) presenting more severe inflammatory infiltrates with associated changes in epithelial architecture (Scale bar = 200 µm). b) Pyloric caeca of a severely infected turbot, showing high parasite load and inflammatory infiltration of the lamina propria-submucosa. Note the marked alteration of the lining epithelium, which is also detached from the basal lamina in some areas (arrowheads), as well as sloughed enterocytes can be appreciated into the intestinal lumen (arrows) (Scale bar = 200 µm). c) Higher magnification of pyloric caeca showing the infiltration of the epithelium (arrowheads) and the lamina propria-submucosa (asterisk) by mononuclear inflammatory cells, associated to the presence of the parasites (P) (Scale bar = 50 µm). d) Higher magnification illustrating the presence of cells with apoptotic features in the epithelium of pyloric caeca, which harbours several parasitic forms (P). Some of the cells at the basis of the epithelium are shrunken with pyknotic, fragmented nuclei (arrows) while other present hypertrophied nuclei (arrowheads) (Scale bar = 20 µm). e) Section of the kidney of a moderately infected turbot, which do not present significant histological alterations (Scale bar = 200 µm). f) Evident depletion of the lymphohaematopoietic interstitial tissue in the kidney of a turbot with severe enteromyxosis (Scale bar = 200 µm).

3.2. Immunohistochemical detection of TNFα

Immunoreactivity against TNFα antibody was found in the three studied organs from both C and R turbot. A comparison between the different conditions is shown in Figure 2 and the relative density of TNFα-positive ($TNF\alpha^+$) cells summarized in Table 1.

Table 1. Relative density of $TNF\alpha^+$ cells in control, moderately and severely *Enteromyxum scopthalmi*-infected turbot.

FISH STATUS	SPLEEN	KIDNEY	PYLORIC CAECA	
			MØ-like	RC
Control	++	+++	+	+
Moderate	++++	+++/++++	++	++
Severe	+/++	++	+++	+

Relative density: +, low; ++, medium; +++, high; ++++, very high. MØ = macrophage, RC= rodlet cells.

In the spleen, immunoreactive macrophage-like cells were observed in all analysed sections, and generally tended to cluster around blood vessels and melanomacrophage centres (Figure 3a). Nevertheless, in moderately infected fish, a marked increase in $TNF\alpha^+$ cells was observed, which were diffusely distributed in the splenic parenchyma (Figure 2). In severely infected specimens, on the contrary, the density of $TNF\alpha^+$ cells was comparable to C fish, with some areas of the spleen showing high concentration of labelled cells, but diminished in the areas suffering from cellular depletion (Figure 2).

In kidney, numerous cells of the lymphohaematopoietic interstitial tissue were $TNF\alpha^+$ in C fish (Figure 2). Turbot with moderate infection showed a similar or slightly augmented number of immunoreactive cells, which often showed a more intense immunoreaction compared to C turbot (Figure 2). In turbot with severe infection, which showed an evident cellular depletion affecting the interstitial tissue, the number of $TNF\alpha^+$ cells was generally scarce (Figures 2 and 3b). In all the studied fish, rodlet cells within the epithelium of renal tubules were occasionally immunostained (Figure 3b), without a clear association with the healthy status of the specimens.

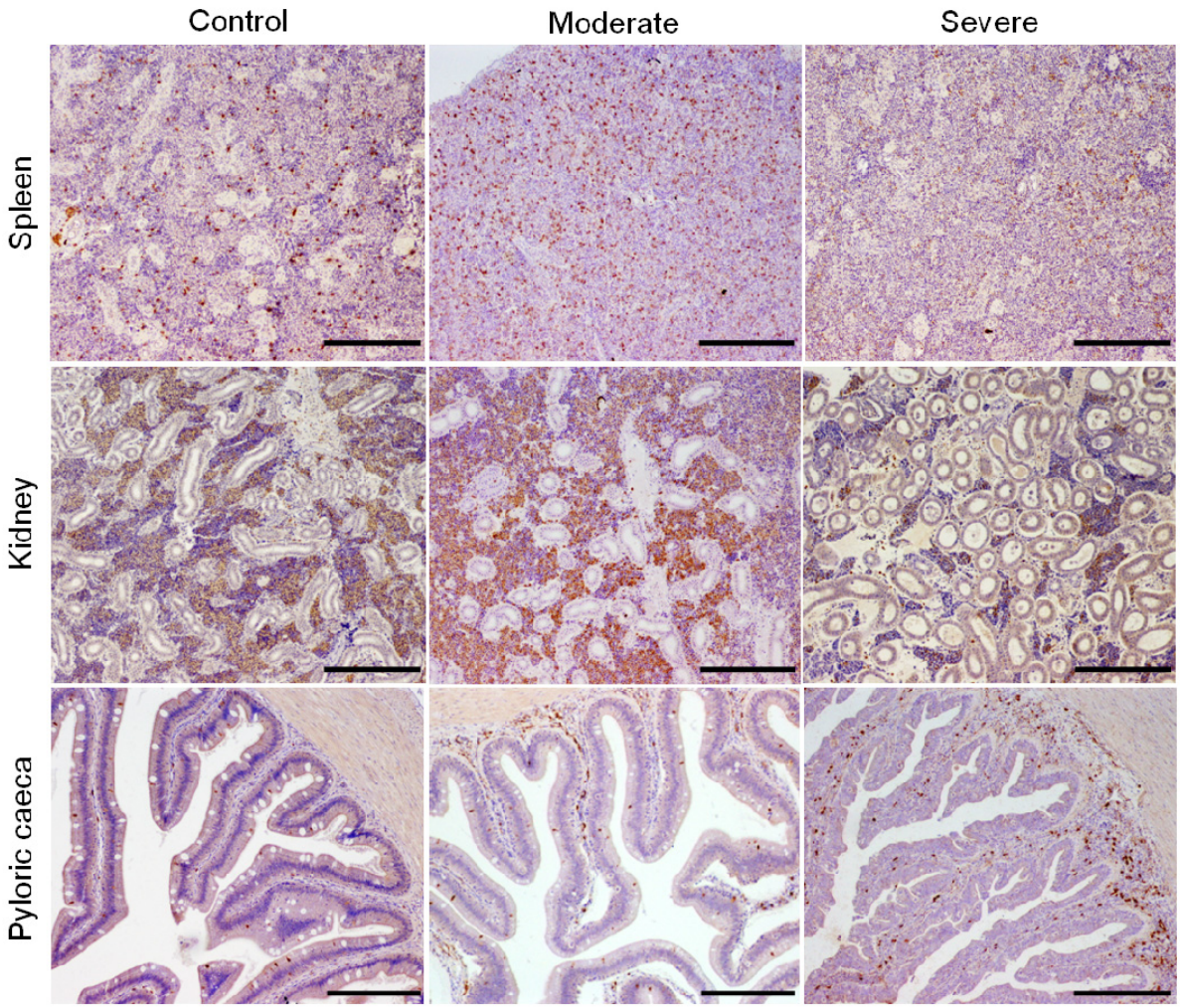

Figure 2. Comparative photomicrographs of spleen, kidney and pyloric caeca from control and parasitized turbot immunostained for TNFα (Scale bars = 200 µm).

In pyloric caeca, the main TNFα$^+$ cell types were rounded macrophage-like cells in the lamina propria-submucosa and bullet-shape rodlet cells within the lining epithelium (Figures 2 and 3c, d, e, f). In C fish, scarce numbers of immunoreactive cells were observed (Figure 1). Moderately infected turbot showed increased numbers of TNFα$^+$ macrophage-like cells in the lamina-propria submucosa (Figures 2 and 3c), that further augmented in those severely infected, in association with the inflammatory infiltrates in the lamina propria-submucosa (Figures 2 and 3e). By contrast, TNFα+ rodlet cell numbers were higher in turbot with moderate infection (Figure 3d) than in those with severe infection, where this cell type was rarely observed. In the severely infected group, the lining epithelium often suffered critical damage, and TNFα+ cells were also found between those sloughed off into the intestinal lumen (Figure 3e). Also, monocyte-like immunoreactive cells were commonly observed in blood vessels (Figure 3f).

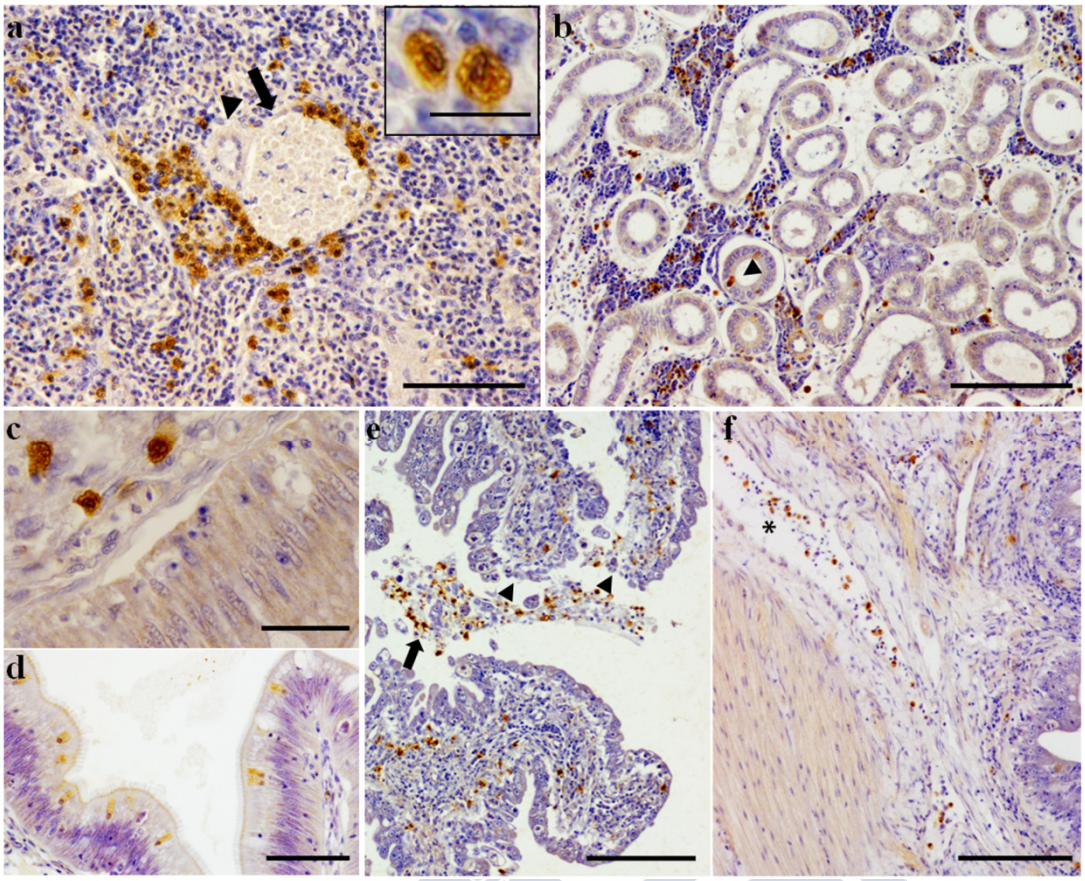

Figure 3. Immunohistochemical detection of TNFα. a) TNFα$^+$ macrophage-like cells clustering around a melanomacrophage centre (arrow) and an arteriole (arrowhead) in the spleen of a control fish (Scale bar = 50 μm). Higher magnification of labelled macrophage-like cells (Scale bar = 10 μm). b) Photomicrographs of kidney from a severely *Enteromyxum scophthalmi*-infected turbot showing a serious cell depletion in the interstitial tissue associated to dilatation of renal tubules. Note the rounded immunoreactive cells in the lymphohaematopoietic interstitial tissue and a TNFα$^+$ rodlet cell (arrowhead) in the epithelium of a tubule (Scale bar = 100 μm). c, d) Pyloric caeca of moderately infected turbot. TNFα$^+$ macrophage-like cells in the lamina propria-submucosa (c, Scale bar = 20 μm) and high concentration of TNFα$^+$ rodlet cells in the lining epithelium (d, Scale bar = 50 μm). e, f) Pyloric caeca of severely *E. scophthalmi*-infected fish (Scale bars = 100 μm). Intestinal folds showing a high parasitic burden in the damaged lining epithelium, which presents areas of epithelial detachment (e, arrowheads). TNFα$^+$ cells can be seen between the inflammatory infiltrates in the lamina propria-submucosa, and also in the intestinal lumen (e, arrow) together with sloughed enterocytes and cellular debris. Notice the presence of numerous immunoreactive monocyte-like cells in a blood vessel (f, asterisk) located in the lamina propria-submucosa.

3.3. Gene expression

TNFα expression was detected in the three organs of all the analysed fish. Figure 4 shows the TNFα expression pattern in the three organs of R and C in the two sampling points. The expression of TNFα in the kidney and spleen of moderately infected turbot was significantly higher than in C fish ($p < 0.05$), whereas no significant differences were found in severely infected turbot. In this latter group, however, it is interesting to denote that TNFα expression was decreased in the kidney, though not statistically enough ($p = 0.052$). In pyloric caeca, there was a general increasing trend in both infected groups, especially in moderately infected turbot, but the high individual variability resulted in no statistically significant differences.

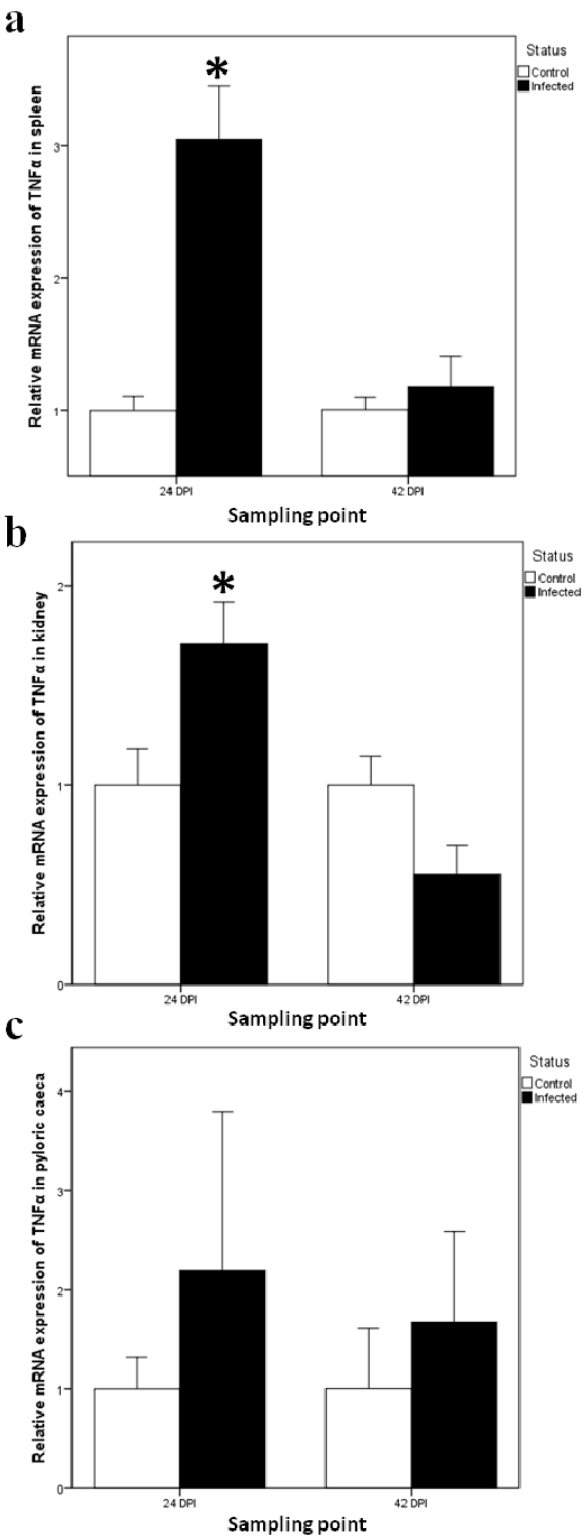

Figure 4. Bar graphs showing TNFα transcript levels in spleen (a), kidney (b) and pyloric caeca (c) from control (white bars) and *Enteromyxum scophthalmi*-infected (black bars) turbot at 24 (moderate infection) and 42 (severe infection) DPI. The transcript levels of TNFα in control fish was used as references values (values >1 or <1 indicate increase or decrease with respect to the reference). Asterisks (*) indicate statistically significant differences ($p < 0.05$) between control and infected groups from the same sampling point.

4. Discussion

Turbot enteromyxosis is characterized by catarrhal enteritis of increasing severity alongside the disease course. The histopathological findings observed in the infected fish represent the characteristic evolution of the disease, including the development of moderate to severe cellular depletion in the lymphohaematopoietic organs (Bermúdez *et al.* 2010). At intestinal level, progressive increase of the parasite load and inflammatory infiltration were observed, associated to alteration of the lining epithelium architecture, enhanced presence of apoptotic figures and epithelial detachment. In moderately infected turbot, the parasitization and lesions development were more emphasized in pyloric caeca and anterior intestine, in accordance with previous observations reporting the beginning of the infection in these regions (Redondo *et al.* 2004; Bermúdez *et al.* 2010). As well, the results confirmed the oral route as the most effective way for infecting turbot, with more homogeneous prevalence rates and lesions (Redondo *et al.* 2002, 2004) which allowed the selection of a proper number of specimens with analogous lesions for this study. Different reports point towards the involvement of a dysregulated response in the pathogenesis of turbot enteromyxosis (Bermúdez *et al.* 2006; Losada *et al.* 2012; Robledo *et al.* 2014a). The multifunctional cytokine TNFα is considered a key mediator of host response to infection (Waters *et al.* 2013), and this role should be confirmed also in teleosts, where changes in TNFα expression have been reported in bacterial, viral and parasitic diseases (Montes *et al.* 2010; Tanekhy *et al.* 2010; Schwenteit *et al.* 2013; Pennacchi *et al.* 2014; Pérez-Cordón *et al.* 2014). In turbot, TNFα has been cloned by Ordás *et al.* (2007), who have also performed gene expression assays and studied some functions of the obtained recombinant protein (rTNFα). rTNFα was able to recruit and activate inflammatory cells, as well to enhance nitric oxide production by macrophages. In the current study, the main lymphohaematopoietic organs (spleen and kidney) showed an increased gene expression of TNFα in turbot with moderate infection, which was coincident with the results of the immunohistochemistry. This demonstrates the involvement of this cytokine in the development of the immune response against *E. scophthalmi* in this stage of the infection. Turbot kidney physiologically presents numerous $TNF\alpha^+$ cells in the lymphohaematopoietic interstitial tissue (Ronza *et al.* 2015), so their increase in infected specimens did not appear dramatic, but a more intense labelling was often noticed. Spleen, on the other hand, presented the highest increase in TNFα gene expression and a remarkable increase of immunoreactive cells. This fact may reflect the major role of this organ in antigen trapping and presentation in teleosts (Rauta *et al.* 2012), which is probably enhanced in this phase of the disease. Therefore, TNFα may be suggested to drive the induction of a systemic response against enteromyxosis in turbot, as seen in mammalian species (Bradley 2008; Waters *et al.* 2013), by activating and recruiting inflammatory cells to the site of infection. Immunohistochemistry showed the progressive increase in $TNF\alpha^+$ cells in pyloric caeca of infected fish, including the mobilization of labelled monocyte-like cells in blood vessels. On the other hand, TNFα gene expression in this location did not result significantly different from control in any sampling point and just an increasing trend was noticed. Although the higher individual variability might have influenced this result, the lack of a clear increase in TNFα gene expression also suggests that part of the numerous TNFα-containing cells in the intestine of infected turbot

may have been recruited from other localizations. The gene expression would occur before these cells reach the digestive tract, where they arrive containing a preformed pool of TNFα. The existence of a preformed intracellular pool of TNFα, ready to be released and not necessarily associated with gene expression, have been described in rainbow trout (*Oncorhynchus mykiss*) macrophages (Roher *et al.* 2011).

Intestinal rodlet cells were previously shown to be TNFα^{+} (Ronza *et al.* 2015) and to increase in numbers in early stages of turbot enteromyxosis (Bermúdez *et al.* 2010). In this study, an increase in the number of TNFα^{+} rodlet cells was observed in pyloric caeca of moderately infected turbot, supporting the hypothesis of their role in the defence response against the parasite. As well, the paucity of immunoreactive rodlet cells noticed in fish with severe infection is in accordance with the decrease of this cell type previously observed in highly parasitized turbot, and attributed to the damage in the epithelium that becomes unable to support these cells (Bermúdez *et al.* 2010).

In mammals, TNFα is involved in the establishment of the inflammatory reaction and in the physiologic and pathologic adaptation of the cells to inflammation at intestinal level (Leppkes *et al.* 2014). Particularly, this cytokine plays a critical role in the pathophysiology of IBDs, being involved in intestinal epithelial shedding and barrier dysfunction (Marchiando *et al.* 2011; Watson and Hughes 2012). These conditions are characterized by a dysregulated immune response, and monoclonal antibodies against TNFα have been proved as effective tools for treatment (Arijs *et al.* 2011; van Schaik *et al.* 2014). Also, TNFα expression was found increased in a zebrafish larvae model of IBD, and these fish showed a positive response to immunomodulatory treatment (Fleming *et al.* 2010). In turbot enteromyxosis, scalloped shape of the intestinal epithelium and detachment of the lining epithelium are characteristic lesions (Bermúdez *et al.* 2010), as well as there are evidences of alterations in the expressions of the cell junctions proteins (Ronza *et al.* 2013). TNFα has been demonstrated to cause loss of intestinal epithelial barrier by acting in the modulation of tight-junctions (Leppkes *et al.* 2014), a lesion that might explain the pathophysiology of enteromyxosis in different species (Ishimatsu *et al.* 2007; Sitjà-Bobadilla and Palenzuela 2012). In gilthead sea bream, experimentally-infected by *E. leei*, TNFα expression was found increased in the intestine both at 17 and 64 DPI (Pérez-Cordón *et al.* 2014), nonetheless this species does not show severe detachment of intestinal cells (Fleurance *et al.* 2008) and the disease usually has a subclinical development and not very high mortality rates (Sitjà-Bobadilla and Palenzuela 2012). Nonetheless, the analysis of immune-relevant genes expression suggested that an anti-inflammatory phase occurs in gilthead sea bream enteromyxosis that may mitigate the deleterious effects of a prolonged intestinal inflammation (Sitjà-Bobadilla *et al.* 2008; Pérez-Cordón *et al.* 2014). This would not occur in turbot enteromyxosis where several genes involved in promoting inflammation were found still up-regulated at intestinal level in late stages of the disease (Robledo *et al.* 2014a). As well, increased expression of inducible nitric oxide synthase (iNOS) has been reported in severe infection (Losada *et al.* 2012). Modulation of iNOS expression by TNFα has been documented in mammal species (Nandi *et al.* 2010), as well as rTNFα has been shown to enhance nitric oxide production by macrophage in turbot

(Ordás *et al.* 2007). Nitric oxide may be an additional factor contributing to epithelial injury by altering cell junctions and inducing apoptosis of enterocytes (Monteiro *et al.* 2004; Chokshi *et al.* 2008; Losada *et al.* 2012).

Apoptosis has been recognized by means of histological (Bermúdez *et al.* 2010), immunohistochemical (Losada *et al.* 2014a) and molecular (Robledo *et al.* 2014a) techniques as one of the mechanism that plays a main role in the pathogenesis of turbot enteromyxosis at intestinal level. Apoptotic cells, immunoreactive to active caspase-3, are increased in the lining epithelium and between the inflammatory infiltrates of the intestine of infected fish (Losada *et al.* 2014a). In addition, the gene codifying for caspase-3 and other pro-apoptotic genes, included members of TNF family, were found up-regulated in pyloric caeca of severely parasitized fish (Robledo *et al.* 2014a). The turbot employed in the present work also showed an enhanced presence of apoptotic enterocytes associated to the presence of the parasite and the inflammatory reaction, as well as, apoptotic inflammatory cells where often observed in lamina propria-submucosa of severely infected fish. The importance of TNFα signalling in modulating programmed cell death of intestinal cells has been reported in several mammalian parasitic diseases (Panaro *et al.* 2007; Castellanos-Gonzalez *et al.* 2008; Bienvenu *et al.* 2010) and in IBDs (Gunther *et al.* 2013). Whether apoptosis may significantly affect lymphohaematopoietic organs of parasitized turbot, on the contrary, is still unclear (Bermúdez *et al.* 2010; Losada *et al.* 2014a; Robledo *et al.* 2014a). In this study, we did not appreciate a clear increase in the apoptotic rate in kidney and spleen from infected fish, altough slightly enhanced figures were reported for some specimen. In any case, the biology of TNFα signalling is complex, being involved in both cell survival and apoptosis (Aggarwal 2003; Hehlgans and Pfeffer 2005). Regarding apoptosis of leukocytes, for example, blockade of TNFα is postulated to be beneficial in IBDs by promoting T lymphocyte apoptosis (Mudter and Neurath 2007), whereas in other diseases, like swine fever, this cytokine was found responsible for leukocytic apoptosis and the consequent lymphoid depletion (Sánchez-Cordón *et al.* 2005). In turbot enteromyxosis, leukocytic death by apoptosis in the intestine may trigger the migration of these cells from lymphohaematopoietic organs, and this mechanism together with the local action of apoptosis were suggested to contribute to the cell depletion observed in kidney and spleen (Bermúdez *et al.* 2006, 2010; Sitjà-Bobadilla *et al.* 2006; Losada *et al.* 2014a). In the current study, after the initial significant increase in moderately infected fish, a subsequent decreasing trend in TNFα expression was detected in the kidney of severely infected fish by both the techniques used. According to the histological and immunohistochemical results, this decrease seems to be mostly due to the severe cell depletion suffered by this organ. In fact, the number of $TNF\alpha^+$ cells appeared proportionally not so scarce, given the severe loss of interstitial tissue. Similar findings were observed in the spleen of these animals, though less pronounced. Cellular depletion in lymphohaematopoietic organs is a main lesion of advanced enteromyxosis in turbot, and is involved in dysfunctions of the immune response, such as the decrease of IgM-positive cells (Bermúdez *et al.* 2006) or the depression of several immune-related genes (Robledo *et al.* 2014a). Interestingly, the same lesion occurs in *E. leei*-infected sharpsnout sea bream (*Diplodus puntazzo*, Cetti) (Álvarez-Pellitero *et al.* 2008), a species that, as turbot, presents a high susceptibility to this

myxozoan parasitosis, but not in diseased gilthead sea bream. In this sense, it is noteworthy to highlight that in *E. leei*-infected gilthead sea bream no significant differences in TNFα expression were found in blood or lymphohaematopoietic organs (head kidney and spleen) at any time point (17 and 64 DPI in anal infection and 113 DPI in effluent infection) (Sitjà-Bobadilla *et al.* 2008; Pérez-Cordón *et al.* 2014). In fact, all the changes found in the expression of cytokines were at the local intestinal level. The action of TNFα as positive or negative regulator of haematopoiesis is still poorly understood, even in mammals, where it appears to depend on a delicate balance of length of exposure to TNFα, progenitor cell type, stage of cell cycle and presence of other regulators (Schuettpelz and Link 2013; Waters *et al.* 2013). Nevertheless, evidence exists that chronic inflammatory cytokine signalling may lead to haematopoietic stem cell dysfunction (Schuettpelz and Link 2013).

Enteromyxoses in different species, including turbot, present a chronic course leading to a cachectic syndrome with weight loss, anorexia and muscle atrophy (Sitjà-Bobadilla and Palenzuela 2012). Inflammatory cytokines are considered the main mediators of cachexia (Morley *et al.* 2006; Argiles *et al.* 2011). TNFα in particular, which was formerly named as cachectin (Beutler *et al.* 1985), promotes different catabolic responses, inducing muscle loss, anorexia and down-regulation of the expression of anabolic hormones (Reid and Li 2001; Marcora *et al.* 2006; Morley *et al.* 2006; Cawthorn and Sethi 2008). Therefore, immunomodulatory therapies aimed to block the synthesis or action of TNFα and other inflammatory cytokines have been tested in several wasting diseases (Marcora *et al.* 2006; Argiles *et al.* 2011), including parasitosis (Truyens *et al.* 1995), with promising results. In enteromyxosis-induced cachectic syndrome, the implication of the immune response interacting with the neuroendocrine system is under debate (Bermúdez *et al.* 2007; Estensoro *et al.* 2009, 2011; Losada *et al.* 2014b; Robledo *et al.* 2014a). The results of this study suggest that a possible involvement of TNFα in the pathophysiology of cachexia deserves further attention.

5. Conclusions

In the current work the regulation of the expression of the multifunctional cytokine TNFα during the infection of turbot by *E. scophthalmi* has been demonstrated. The combined use of Q-PCR and immunohistochemistry provided more feasible results and a more comprehensive picture of TNFα dynamics during the disease. The increased expression detected in earlier stages of enteromyxosis in spleen and kidney indicates the involvement of TNFα in the development of the immune response, probably driving the recruitment of inflammatory cells in the intestine, the target organ of the parasite. In this location, the accumulation of the inflammatory infiltrates containing $TNF\alpha^+$ cells suggests a prolonged exposure to TNFα that may be involved in the development of the lesions, namely apoptosis, epithelial shedding and intestinal barrier dysfunction. In advanced stages of enteromyxosis, the decreasing trend in $TNF\alpha^+$ cell numbers in both lymphohaematopoietic organs and of gene expression in kidney reflects the observed cell depletion. Turbot enteromyxosis appears to be characterized by a dysfunctional, exacerbated immune response. In similar conditions observed in other species

TNFα plays a main role in the pathogenesis. This cytokine acts in the regulation of a wide spectrum of biological activities, which include immune response and haematopoiesis, but also feeding behaviour and metabolism. The extent of its implication in the development of the different clinical signs and lesions associated to enteromyxosis should be further addressed. This can set the basis for the implement of immunomodulatory therapies aimed to control this important parasitosis.

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References

Aggarwal, B.B., 2003. Signalling pathways of the TNF superfamily: a double-edged sword. Nat Rev Immunol 3, 745-756.

Álvarez-Pellitero, P., Palenzuela, O., Sitjà-Bobadilla, A., 2008. Histopathology and cellular response in *Enteromyxum leei* (Myxozoa) infections of *Diplodus puntazzo* (Teleostei). Parasitol Int 57, 110-120.

Argiles, J.M., Busquets, S., Lopez-Soriano, F.J., 2011. Anti-inflammatory therapies in cancer cachexia. Eur J Pharmacol 668 Suppl 1, S81-86.

Arijs, I., De Hertogh, G., Machiels, K., Van Steen, K., Lemaire, K., Schraenen, A., Van Lommel, L., Quintens, R., Van Assche, G., Vermeire, S., Schuit, F., Rutgeerts, P., 2011. Mucosal gene expression of cell adhesion molecules, chemokines, and chemokine receptors in patients with inflammatory bowel disease before and after infliximab treatment. Am J Gastroenterol 106, 748-761.

Bermúdez, R., Vigliano, F., Marcaccini, A., Sitjà-Bobadilla, A., Quiroga, M.I., Nieto, J.M., 2006. Response of Ig-positive cells to *Enteromyxum scophthalmi* (Myxozoa) experimental infection in turbot, *Scophthalmus maximus* (L.): A histopathological and immunohistochemical study. Fish Shellfish Immunol 21, 501-512.

Bermúdez, R., Vigliano, F., Quiroga, M.I., Nieto, J.M., Bosi, G., Domeneghini, C., 2007. Immunohistochemical study on the neuroendocrine system of the digestive tract of turbot, *Scophthalmus maximus* (L.), infected by *Enteromyxum scophthalmi* (Myxozoa). Fish Shellfish Immunol 22, 252-263.

Bermúdez, R., Losada, A.P., Vázquez, S., Redondo, M.J., Álvarez-Pellitero, P., Quiroga, M.I., 2010. Light and electron microscopic studies on turbot *Psetta maxima* infected with *Enteromyxum scophthalmi*: histopathology of turbot enteromyxosis. Dis Aquat Organ 89, 209-221.

Beutler, B., Greenwald, D., Hulmes, J.D., Chang, M., Pan, Y.C., Mathison, J., Ulevitch, R., Cerami, A., 1985. Identity of tumour necrosis factor and the macrophage-secreted factor cachectin. Nature 316, 552-554.

Bienvenu, A.-L., Gonzalez-Rey, E., Picot, S., 2010. Apoptosis induced by parasitic diseases. Parasit Vectors 3, 106.

Bradley, J.R., 2008. TNF-mediated inflammatory disease. J Pathol 214, 149-160.

Branson, E., Riaza, A., Álvarez-Pellitero, P., 1999. Myxosporean infection causing intestinal disease in farmed turbot, *Scophthalmus maximus* (L.), (Teleostei: Scophthalmidae). J Fish Dis 22, 395-399.

Castellanos-Gonzalez, A., Yancey, L.S., Wang, H.C., Pantenburg, B., Liscum, K.R., Lewis, D.E., White, A.C., Jr., 2008. Cryptosporidium infection of human intestinal epithelial cells increases expression of osteoprotegerin: a novel mechanism for evasion of host defenses. J Infect Dis 197, 916-923.

Cawthorn, W.P., Sethi, J.K., 2008. TNF-alpha and adipocyte biology. FEBS Lett 582, 117-131.

Chokshi, N.K., Guner, Y.S., Hunter, C.J., Upperman, J.S., Grishin, A., Ford, H.R., 2008. The role of nitric oxide in intestinal epithelial injury and restitution in neonatal necrotizing enterocolitis. Semin Perinatol 32, 92-99.

Durán, W.N., 2008. The double-edge sword of TNF-α in ischemia-reperfusion injury. Am J Physiol Heart Circ Physiol 295, H2221-H2222.

Estensoro, I., Bermúdez, R., Losada, A.P., Quiroga, M.I., Pérez-Sánchez, J., Álvarez-Pellitero, P., Sitjà-Bobadilla, A., 2009. Effect of *Enteromyxum leei* (Myxozoa) on gastrointestinal neuromodulators and cell apoptosis of gilthead sea bream (*Sparus aurata*), 14th EAFP International Conference, Prague, Czech Republic.

Estensoro, I., Benedito-Palos, L., Palenzuela, O., Kaushik, S., Sitjà-Bobadilla, A., Pérez-Sánchez, J., 2011. The nutritional background of the host alters the disease course in a fish–myxosporean system. Vet Parasitol 175, 141-150.

Fleming, A., Jankowski, J., Goldsmith, P., 2010. In vivo analysis of gut function and disease changes in a zebrafish larvae model of inflammatory bowel disease: a feasibility study. Inflamm Bowel Dis 16, 1162-1172.

Fleurance, R., Sauvegrain, C., Marques, A., Le Breton, A., Guereaud, C., Cherel, Y., Wyers, M., 2008. Histopathological changes caused by *Enteromyxum leei* infection in farmed sea bream *Sparus aurata*. Dis Aquat Organ 79, 219-228.

García-Castillo, J., Chaves-Pozo, E., Olivares, P., Pelegrín, P., Meseguer, J., Mulero, V., 2004. The tumor necrosis factor alpha of the bony fish seabream exhibits the in vivo proinflammatory and proliferative activities of its mammalian counterparts, yet it functions in a species-specific manner. Cell Mol Life Sci 61, 1331-1340.

Goetz, F.W., Planas, J.V., MacKenzie, S., 2004. Tumor necrosis factors. Dev Comp Immunol 28, 487-497.

Grayfer, L., Walsh, J.G., Belosevic, M., 2008. Characterization and functional analysis of goldfish (*Carassius auratus* L.) tumor necrosis factor-alpha. Dev Comp Immunol 32, 532-543.

Gunther, C., Neumann, H., Neurath, M.F., Becker, C., 2013. Apoptosis, necrosis and necroptosis: cell death regulation in the intestinal epithelium. Gut 62, 1062-1071.

Hehlgans, T., Pfeffer, K., 2005. The intriguing biology of the tumour necrosis factor/tumour necrosis factor receptor superfamily: players, rules and the games. Immunology 115, 1-20.

Heinecke, R.D., Buchmann, K., 2013. Inflammatory response of rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) larvae against *Ichthyophthirius multifiliis*. Fish Shellfish Immunol 34, 521-528.

Hino, K., Nakamura, O., Yoshiura, Y., Suetake, H., Suzuki, Y., Watanabe, T., 2006. TNF induces the growth of thymocytes in rainbow trout. Dev Comp Immunol 30, 639-647.

Ishimatsu, A., Hayashi, M., Nakane, M., Sameshima, M., 2007. Pathophysiology of cultured Tiger Puffer *Takifugu rubripes* suffering from the Myxosporean Emaciation Disease. Fish Pathol 42, 211-217.

Lam, F.W., Wu, S.Y., Lin, S.J., Lin, C.C., Chen, Y.M., Wang, H.C., Chen, T.Y., Lin, H.T., Lin, J.H., 2011. The expression of two novel orange-spotted grouper (*Epinephelus coioides*) TNF genes in peripheral blood leukocytes, various organs, and fish larvae. Fish Shellfish Immunol 30, 618-629.

Leppkes, M., Roulis, M., Neurath, M.F., Kollias, G., Becker, C., 2014. Pleiotropic functions of TNF-alpha in the regulation of the intestinal epithelial response to inflammation. Int Immunol 26, 509-515.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402-408.

Losada, A.P., Bermúdez, R., Faílde, L.D., Quiroga, M.I., 2012. Quantitative and qualitative evaluation of iNOS expression in turbot (*Psetta maxima*) infected with Enteromyxum scophthalmi. Fish Shellfish Immunol 32, 243-248.

Losada, A.P., Bermúdez, R., Faílde, L.D., de Ocenda, M.V.R., Quiroga, M.I., 2014a. Study of the distribution of active caspase-3-positive cells in turbot, *Scophthalmus maximus* (L.), enteromyxosis. J Fish Dis 37, 21-32.

Losada, A.P., Bermúdez, R., Faílde, L.D., Di Giancamillo, A., Domeneghini, C., Quiroga, M.I., 2014b. Effects of *Enteromyxum scophthalmi* experimental infection on the neuroendocrine system of turbot, *Scophthalmus maximus* (L.). Fish Shellfish Immunol 40, 577-583.

Marcora, S.M., Chester, K.R., Mittal, G., Lemmey, A.B., Maddison, P.J., 2006. Randomized phase 2 trial of anti-tumor necrosis factor therapy for cachexia in patients with early rheumatoid arthritis. Am J Clin Nutr 84, 1463-1472.

Marchiando, A.M., Shen, L., Graham, W.V., Edelblum, K.L., Duckworth, C.A., Guan, Y., Montrose, M.H., Turner, J.R., Watson, A.J., 2011. The epithelial barrier is maintained by in vivo tight junction expansion during pathologic intestinal epithelial shedding. Gastroenterology 140, 1208-1218 e1201-1202.

Millán, A., Gómez-Tato, A., Pardo, B.G., Fernández, C., Bouza, C., Vera, M., Alvarez-Dios, J.A., Cabaleiro, S., Lamas, J., Lemos, M.L., Martínez, P., 2011. Gene expression profiles of the spleen, liver, and head kidney in turbot (*Scophthalmus maximus*) along the infection process with *Aeromonas salmonicida* using an immune-enriched oligo-microarray. Mar Biotechnol (NY) 13, 1099-1114.

Mladineo, I., Block, B.A., 2010. Expression of cytokines IL-1beta and TNF-alpha in tissues and cysts surrounding Didymocystis wedli (Digenea, Didymozoidae) in the Pacific bluefin tuna (*Thunnus orientalis*). Fish Shellfish Immunol 29, 487-493.

Monteiro, H.P., Silva, E.F., Stern, A., 2004. Nitric oxide: a potential inducer of adhesion-related apoptosis--anoikis. Nitric Oxide 10, 1-10.

Montes, A., Figueras, A., Novoa, B., 2010. Nodavirus encephalopathy in turbot (*Scophthalmus maximus*): inflammation, nitric oxide production and effect of anti-inflammatory compounds. Fish Shellfish Immunol 28, 281-288.

Morley, J.E., Thomas, D.R., Wilson, M.M., 2006. Cachexia: pathophysiology and clinical relevance. Am J Clin Nutr 83, 735-743.

Mudter, J., Neurath, M.F., 2007. Apoptosis of T cells and the control of inflammatory bowel disease: therapeutic implications. Gut 56, 293-303.

Nandi, J., Saud, B., Zinkievich, J.M., Yang, Z.-J., Levine, R., 2010. TNF-α modulates iNOS expression in an experimental rat model of indomethacin-induced jejunoileitis. Mol Cell Biochem 336, 17-24.

Ordás, M.C., Costa, M.M., Roca, F.J., López-Castejón, G., Mulero, V., Meseguer, J., Figueras, A., Novoa, B., 2007. Turbot TNFalpha gene: molecular characterization and biological activity of the recombinant protein. Mol Immunol 44, 389-400.

Panaro, M.A., Cianciulli, A., Mitolo, V., Mitolo, C.I., Acquafredda, A., Brandonisio, O., Cavallo, P., 2007. Caspase-dependent apoptosis of the HCT-8 epithelial cell line induced by the parasite *Giardia intestinalis*. FEMS Immunol Med Microbiol 51, 302-309.

Pennacchi, Y., Leef, M.J., Crosbie, P.B., Nowak, B.F., Bridle, A.R., 2014. Evidence of immune and inflammatory processes in the gills of AGD-affected Atlantic salmon, Salmo salar L. Fish Shellfish Immunol 36, 563-570.

Pérez-Cordón, G., Estensoro, I., Benedito-Palos, L., Calduch-Giner, J.A., Sitjà-Bobadilla, A., Pérez-Sánchez, J., 2014. Interleukin gene expression is strongly modulated at the local level in a fish–parasite model. Fish Shellfish Immunol 37, 201-208.

Quiroga, M.I., Redondo, M.J., Sitjà-Bobadilla, A., Palenzuela, O., Riaza, A., Macías, A., Vázquez, S., Pérez, A., Nieto, J.M., Álvarez-Pellitero, P., 2006. Risk factors associated with *Enteromyxum scophthalmi* (Myxozoa) infection in cultured turbot, *Scophthalmus maximus* (L.). Parasitology 133, 433-442.

Rauta, P.R., Nayak, B., Das, S., 2012. Immune system and immune responses in fish and their role in comparative immunity study: a model for higher organisms. Immunol Lett 148, 23-33.

Redondo, M.J., Palenzuela, O., Riaza, A., Macías, A., Álvarez-Pellitero, P., 2002. Experimental transmission of *Enteromyxum scophthalmi* (Myxozoa), an enteric parasite of turbot *Scophthalmus maximus*. J Parasitol 88, 482-488.

Redondo, M.J., Palenzuela, O., Álvarez-Pellitero, P., 2004. Studies on transmission and life cycle of *Enteromyxum scophthalmi* (Myxozoa), an enteric parasite of turbot *Scophthalmus maximus*. Folia Parasitol (Praha) 51, 188-198.

Reid, M.B., Li, Y.P., 2001. Tumor necrosis factor-alpha and muscle wasting: a cellular perspective. Respir Res 2, 269-272.

Robledo, D., Ronza, P., Harrison, P.W., Losada, A.P., Bermúdez, R., Pardo, B.G., Redondo, M.J., Sitjà-Bobadilla, A., Quiroga, M.I., Martínez, P., 2014a. RNA-seq analysis reveals significant transcriptome changes in turbot (*Scophthalmus maximus*) suffering severe enteromyxosis. BMC Genomics 15, 1149.

Robledo, D., Hernández-Urcera, J., Cal, R.M., Pardo, B.G., Sánchez, L., Martínez, P., Viñas, A., 2014b. Analysis of qPCR reference gene stability determination methods and a practical approach for efficiency calculation on a turbot (*Scophthalmus maximus*) gonad dataset. BMC Genomics 15:648.

Roca, F.J., Mulero, I., López-Munoz, A., Sepulcre, M.P., Renshaw, S.A., Meseguer, J., Mulero, V., 2008. Evolution of the inflammatory response in vertebrates: fish TNF-alpha is a powerful activator of endothelial cells but hardly activates phagocytes. J Immunol 181, 5071-5081.

Ronza, P., Losada, A.P., Bermúdez, R., Sitjà-Bobadilla, A., Quiroga, M.I., 2013. Puesta a punto de técnicas inmunohistoquímicas para el estudio de la disfunción de la barrera intestinal en la enteromixosis, XXV Reunión de la Sociedad Española de Anatomía Patológica Veterinaria, Toledo, Spain.

Ronza, P., Losada, A.P., Villamarín, A., Bermúdez, R., Quiroga, M.I., 2015. Immunolocalization of tumor necrosis factor alpha in turbot (*Scophthalmus maximus*, L.) tissues. Fish Shellfish Immunol. 45, 470-476.

Sánchez-Cordón, P.J., Núnez, A., Salguero, F.J., Pedrera, M., Fernandez de Marco, M., Gómez-Villamandos, J.C., 2005. Lymphocyte apoptosis and thrombocytopenia in spleen during classical swine fever: role of macrophages and cytokines. Vet Pathol 42, 477-488.

Schuettpelz, L., Link, D., 2013. Regulation of hematopoietic stem cell activity by inflammation. Front Immunol 4.

Schwenteit, J.M., Breithaupt, A., Teifke, J.P., Koppang, E.O., Bornscheuer, U.T., Fischer, U., Gudmundsdottir, B.K., 2013. Innate and adaptive immune responses of Arctic charr (*Salvelinus alpinus*, L.) during infection with *Aeromonas salmonicida* subsp. *achromogenes* and the effect of the AsaP1 toxin. Fish Shellfish Immunol 35, 866-873.

Sitjà-Bobadilla, A., Redondo, M.J., Bermúdez, R., Palenzuela, O., Ferreiro, I., Riaza, A., Quiroga, I., Nieto, J.M., Álvarez-Pellitero, P., 2006. Innate and adaptive immune responses of turbot, *Scophthalmus maximus* (L.), following experimental infection with *Enteromyxum scophthalmi* (Myxosporea: Myxozoa). Fish Shellfish Immunol 21, 485-500.

Sitjà-Bobadilla, A., Palenzuela, O., Riaza, A., Macias, M.A., Álvarez-Pellitero, P., 2007. Protective acquired immunity to *Enteromyxum scophthalmi* (Myxozoa) is related to specific antibodies in *Psetta maxima* (L.) (Teleostei). Scand J Immunol 66, 26-34.

Sitjà-Bobadilla, A., Calduch-Giner, J., Saera-Vila, A., Palenzuela, O., Álvarez-Pellitero, P., Pérez-Sánchez, J., 2008. Chronic exposure to the parasite *Enteromyxum leei* (Myxozoa: Myxosporea) modulates the immune response and the expression of growth, redox and immune relevant genes in gilthead sea bream, *Sparus aurata* L. Fish Shellfish Immunol 24, 610-619.

Sitjà-Bobadilla, A., Palenzuela, O., 2012. Enteromyxum Species, in: Woo, P.T.K., Buchmann, K. (Eds.), Fish Parasites: Pathobiology and Protection. CABI publishing, UK, pp. 163-176.

Tanekhy, M., Matsuda, S., Itano, T., Kawakami, H., Kono, T., Sakai, M., 2010. Expression of cytokine genes in head kidney and spleen cells of Japanese flounder (*Paralichthys olivaceus*) infected with *Nocardia seriolae*. Vet Immunol Immunopathol 134, 178-183.

Truyens, C., Torrico, F., Angelo-Barrios, A., Lucas, R., Heremans, H., Baetselier, P.D., Carlier, Y., 1995. The cachexia associated with *Trypanosoma cruzi* acute infection in mice is attenuated by anti-TNF-a, but not by anti-IL-6 or anti-IFN-7 antibodies. Parasite Immunol 17, 561-568.

van Schaik, T., Maljaars, J.P.W., Roopram, R.K., Verwey, M.H., Ipenburg, N., Hardwick, J.C.H., Veenendaal, R.A., van der Meulen-de Jong, A.E., 2014. Influence of combination therapy with immune modulators on anti-TNF trough levels and antibodies in patients with IBD. Inflamm Bowel Dis 20, 2292-2298.

Waters, J.P., Pober, J.S., Bradley, J.R., 2013. Tumour necrosis factor in infectious disease. J Pathol 230, 132-147.

Watson, A.J., Hughes, K.R., 2012. TNF-alpha-induced intestinal epithelial cell shedding: implications for intestinal barrier function. Ann N Y Acad Sci 1258, 1-8.

ARTÍCULO III: RNA-SEQ ANALYSIS REVEALS SIGNIFICANT TRANSCRIPTOME CHANGES IN TURBOT (*SCOPHTHALMUS MAXIMUS*) SUFFERING SEVERE ENTEROMYXOSIS

Robledo, D.[1*]; Ronza, P.[2*]; Harrison, P.W.[3]; Losada, A.P.[2]; Bermúdez, R.[4]; G. Pardo, B.[5]; Redondo, M.J.[6]; Sitjà-Bobadilla, A.[6]; Quiroga, M.I.[2]; Martínez, P.[3,5]

[1]Departamento de Genética, Facultad de Biología (CIBUS), Universidade de Santiago de Compostela, 15782 Santiago de Compostela.

[2]Departamento de Ciencias Clínicas Veterinarias, Facultad de Veterinaria, Universidade de Santiago de Compostela, 27002 Lugo.

[3]Department of Genetics, Evolution and Environment, University College London, London, United Kingdom.

[4]Departamento de Anatomía y Producción Animal, Facultad de Veterinaria, Universidade de Santiago de Compostela, 27002 Lugo.

[5]Departamento de Genética, Facultad de Veterinaria, Universidade de Santiago de Compostela, 27002 Lugo.

[6]Instituto de Acuicultura Torre de la Sal (IATS-CSIC), 12595 Ribera de Cabanes, Castellón.

*Primera autoría compartida.

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RESEARCH ARTICLE Open Access

RNA-seq analysis reveals significant transcriptome changes in turbot (*Scophthalmus maximus*) suffering severe enteromyxosis

Diego Robledo[1†], Paolo Ronza[2†], Peter W Harrison[3], Ana Paula Losada[2], Roberto Bermúdez[4], Belén G Pardo[5], María José Redondo[6], Ariadna Sitjà-Bobadilla[6], María Isabel Quiroga[2*] and Paulino Martínez[3,5]

Nota: El material suplementario asociado a este trabajo está disponible en el CD adjunto al ejemplar de Tesis.

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Abstract

Enteromyxosis caused by the intestinal myxozoan parasite *Enteromyxum scophthalmi* is a serious threat for turbot (*Scophthalmus maximus*, L.) aquaculture, causing severe catarrhal enteritis leading to a cachectic syndrome, with no therapeutic options available. There are still many aspects of host-parasite interaction and disease pathogenesis that are yet to be elucidated, and to date, no analysis of the transcriptomic changes induced by *E. scophthalmi* in turbot organs has been conducted. In this study, RNA-seq technology was applied to head kidney, spleen and pyloric caeca of severely infected turbot with the aim of furthering our understanding of the pathogenetic mechanisms and turbot immune response against enteromyxosis. A huge amount of information was generated with more than 23,000 identified genes in the three organs, amongst which 4,762 were differently expressed (DE) between infected and control fish. Associate gene functions were studied based on gene ontology terms and available literature, and the most interesting DE genes were classified into five categories: 1) immune and defence response; 2) apoptosis and cell proliferation; 3) iron metabolism and erythropoiesis; 4) cytoskeleton and extracellular matrix and 5) metabolism and digestive function. The analysis of down-regulated genes of the first category revealed evidences of a connexion failure between innate and adaptive immune response, especially represented by a high number of DE interferon-related genes in the three organs. Furthermore, we found an intense activation of local immune response at intestinal level that appeared exacerbated, whereas in kidney and spleen genes involved in adaptive immune response were mainly down-regulated. The apoptotic machinery was only clearly activated in pyloric caeca, while kidney and spleen showed a marked depression of genes related to erythropoiesis, probably related to disorders in iron homeostasis. The genetic signature of the causes and consequences of cachexia was also demonstrated by the down-regulation of the genes encoding structural proteins and those involved in the digestive metabolism. This transcriptomic study has enabled us to gain a better understanding of the pathogenesis of enteromyxosis and identify a large number of DE target genes that bring us closer to the development of strategies designed to effectively combat this pathogen.

Keywords: RNA-seq, transcriptome, turbot, Enteromyxum scophthalmi, enteromyxosis, immune response, apoptosis, erythropoiesis, cytoskeleton, digestive function.

1. Introduction

Turbot (*Scophthalmus maximus*, L.) is a marine flatfish which has been intensively cultured in Europe for more than 25 years. Turbot aquaculture production currently accounts for over 10,000 tons/year in Europe, and has rapidly increased in China during the last decade, reaching more than 60,000 tons in 2011 (APROMAR, 2013). Although its entire life cycle is routinely carried out at farm facilities, major challenges related to growth rate and disease outbreaks are the main concerns for the turbot industry (Hermida *et al.* 2013; Losada 2013; Ribas *et al.* 2013; Zhi-Hui *et al.* 2014).

The myxozoan genus *Enteromyxum* includes three intestinal species, which all cause serious problems for seawater aquaculture. *Enteromyxum*‘s virulence varies depending on the specific

parasite-host interaction, ranging from highly pathogenic in some species pairs, such as *E. scophthalmi* infection of turbot, to chronic (*E. leei* in gilthead sea bream, *Sparus aurata*) or subclinical (*E. fugu* in tiger puffer, *Takifugu rubripes*) disease signs in others. *E. scophthalmi* is a serious threat for cultured turbot, that spreads rapidly in farm facilities due to direct fish-to-fish transmission, and causes a cachectic syndrome which eventually leads to death (Sitjà-Bobadilla and Palenzuela 2012). Diseased fish present anorexia, anaemia, weight loss, poor conversion rates and delayed growth, with mortality rates reaching up to 100% in many cases (Branson *et al.* 1999; Sitjà-Bobadilla and Palenzuela 2012). The main histological changes are catarrhal enteritis and lymphohaematopoietic depletion of spleen and kidney, the severity of which increases with the progression of the infection (Bermúdez *et al.* 2010). Although some drugs have been able to lower the mortality rates (Palenzuela *et al.* 2009), currently there is no effective treatment for enteromyxosis.

Understanding the disease pathogenesis through the study of host-parasite interaction and turbot immune response is critical in order to develop effective treatments and apply preventive measures. Numerous recent studies have been focused on elucidating these processes, mainly through histopathological, immunoenzymatic and serological assays (Bermúdez *et al.* 2006, 2007; Sitjà-Bobadilla *et al.* 2006; Redondo *et al.* 2008; Redondo and Álvarez-Pellitero, 2010; Losada *et al.* 2012, 2014). Nevertheless, while PCR-array and microarray based molecular profiling of gilthead sea bream response to *E. leei* has been recently published (Davey *et al.* 2011; Pérez-Cordón *et al.* 2014), gene expression characterization of *E. scophthalmi*-infected turbot is lacking. Transcriptome analysis is an invaluable tool for the elucidation of the biological processes behind host-parasite interactions, and in the last decade this approach, mainly based upon microarrays, has been extensively used in fish immunology and pathology (Qian *et al.* 2014), and specifically in turbot for analyzing furunculosis and scuticociliatosis (Millán *et al.* 2011; Pardo *et al.* 2012). Additionally, the identification of relevant immune gene variants conferring tolerance to parasites is essential in order to develop marker assisted selection programmes that can lead to increased resistance (Davey *et al.* 2011; Rodriguez-Ramilo *et al.* 2013). RNA-seq is a powerful technique for the analysis of gene expression due to its higher sensitivity and specificity in comparison to microarrays, along with its ability to detect new genes, rare transcripts, alternative splice isoforms, and novel SNPs which can be used for association studies (Marioni *et al.* 2008; Morozova *et al.* 2009; Nielsen *et al.* 2011). For these reasons it is rapidly becoming the technology of choice for transcriptomic studies (Wang *et al.* 2009; Qian *et al.* 2014).

In this study, a gene expression analysis of *E. scophthalmi*-infected turbot was carried out using RNA-seq on the two major lymphohaematopoietic organs, head kidney and spleen, and also on the pyloric caeca, the target intestinal region where the parasite infection starts in this species (Redondo *et al.* 2004). Fish categorised with a severe infection were used with the aim of capturing the gene expression signatures associated with advanced stages of the disease as a first reference to investigate the genetic mechanisms underlying the pathogenesis of enteromyxosis. Our findings constitute the basis of future studies aimed at investigating

resistance-related genes and associated genetic variants that could be applied in breeding programmes. This is the first study to tackle the molecular basis of lesion development and the immune response underlying enteromyxosis in turbot.

2. Materials and methods

2.1. Experimental design and animal sampling

Turbot (150 g mean weight) were obtained from an *E. scophthalmi*-free farm in northwestern Spain and kept in the facilities of the Instituto de Acuicultura de Torre la Sal (IATS, Cabanes, Castellón, Spain). Animals were divided into 55 recipient (RCPT) and 65 control (CTRL) fish, and were acclimated for two weeks under identical conditions before starting the trial in two 500 L tanks per group with 5µm-filtered and UV-irradiated open flow sea water (37.5‰ salinity) at 19±1ºC.

The experimental infection was carried out by oral route, as described by Redondo *et al.* (2004). Briefly, RCPT fish received 1 ml of intestinal scraping homogenates in Hank's Balanced Salt Solution (HBSS) from 20 donor fish, containing *E. scophthalmi* live parasites, whereas CTRL fish were inoculated with the same amount of HBSS alone. Donor turbot came from an experimentally infected stock maintained at IATS.

Fifteen RCPT and 10 CTRL fish were sampled at 7, 24 and 42 days post-inoculation (DPI) in order to obtain a representative time-course that included different levels of infection for analysis. At each sampling point, fish were euthanized under benzocaine anaesthesia (3-aminobenzoic acid ethyl ester, 100 mg/ml) (Sigma, St. Louis, MO, USA) and necropsied to obtain samples from head kidney, spleen and digestive tract.

The experiment was carried out in accordance with national (Royal Decree RD1201/2005, for the protection of animals used in scientific experiments) and institutional regulations (CSIC, IATS Review Board). Animals were treated according to the Directive 2010/63/UE of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for experimentation and other scientific purposes. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Santiago de Compostela (Spain).

2.2. Histopathology

Tissue samples were fixed in Bouin's fluid at 4ºC for 12 hours and then stored in 70% ethanol until being processed for paraffin-embedding. Thin sections (3 µm) were stained with H&E and toluidine blue for microscopic evaluation. The healthy status of CTRL fish was confirmed while RCPT fish showed a variable level of infection irrespective of the day of sampling, suggesting different resistance to the parasite or variation associated to the infection protocol. Consequently, fish were classified according to the lesional degree in three groups (slight, moderate and severe), as described by Bermúdez *et al.* (2010).

2.3. RNA extraction and sample preparation for RNA-seq

Samples from the three organs (head kidney, spleen and pyloric caeca) were collected in cold RNAlater (Qiagen), kept at 4ºC overnight and then transferred to -20ºC. RNA extraction was performed using the RNeasy mini kit (Qiagen) with DNase treatment following manufacturer's instructions. RNA quality and quantity were evaluated in a Bioanalyzer (Bonsai Technologies) and in a NanoDrop® ND-1000 spectrophotometer (NanoDrop® Technologies Inc), respectively. Prior to sequencing, RNA aliquots from three CTRL fish were pooled by organ, while samples from three RCPT fish were analyzed individually, resulting in three RCPT and one CTRL sample per organ. The three RCPT fish were chosen among those fish graded as severely infected in histopathology assessment and were collected at the same time, thus, both RCPT and CTRL fish belonged to the 42 DPI sampling point.

The 12 samples were barcoded and prepared for sequencing by the Wellcome Trust Centre for Human Genetics, Oxford, using standard protocols. Sequencing was conducted on an Illumina HiSeq 2000 as 100bp paired-end reads.

The quality of the sequencing output was assessed using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/; version 0.10.1). Quality filtering and removal of residual adaptor sequences was conducted on read pairs using Trimmomatic (Lohse *et al.* 2012) (version 0.30). Specifically, residual Illumina specific adaptors were clipped from the reads, leading and trailing bases with a Phred score less than 4 were removed and the read trimmed if a sliding window average Phred score over four bases was less than 15. Only reads where both pairs had a length greater than 36bp post-filtering were retained, leaving on average more than 15 million mappable paired-end reads per sample.

The recently assembled turbot genome (Figueras *et al.* unpublished data), was used as a reference for read mapping. The genome consists of 16,493 scaffolds with an N50 of 4,268,014 bp and N90 of 462,971 bp. Filtered reads were mapped to the genome using Tophat2 (Kim *et al.* 2013) (version 2.09) that leverages the short read aligner Bowtie2 (Langmead and Salzberg 2012) (version 2.1.0) with a maximum intron length of 10kb. HTSeq-count (http://www-huber.embl.de/users/anders/HTSeq/doc/overview.html) was used to extract the raw reads from the mapping files.

To account for differences in the mass composition of the RNA-Seq samples, we conducted trimmed mean of M-values (tmm) normalisation of expression values using EdgeR (Robinson and Oshlack 2010) for each organ. Differential expression between infected and control samples was calculated using EdgeR and resulting *p*-values corrected for false discovery rate (FDR). Differentially expressed (DE) genes were defined as showing an FDR corrected *p*-value < 0.05, a $\log_2$ fold fold change (FC) > 1 and a minimum length of 200bp. The DE genes were identified and annotated using Blast2GO (version 2.7.0) with an E-value cutoff of E^{-6}. Enriched GO terms for each tissue were identified by comparing the DE genes against the full turbot transcriptome using Blast2GO Fisher's exact test ($p < 0.05$, FDR corrected). The correct classification of the samples as either infected treatments (clustering together) or uninfected controls (as outgroups) was confirmed with hierarchical clustering implemented in

the R package "pvclust" (Suzuki and Shimodaira 2006), using complete Euclidean distance with 1000 bootstrap replicates.

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar *et al.* 2002) and are accessible through GEO Series accession number GSE63911 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE63911).

3. Results

3.1. Histopathology

Experimentally-infected (RCPT) fish selected for RNA-seq analysis presented catarrhal enteritis characterized by severe parasitic load along the entire gastrointestinal tract associated with moderate to severe inflammatory infiltrates and lining epithelium detachment. In most gut segments, apoptotic figures in both epithelium and lamina propria were observed, while signs of epithelium regeneration were sporadically annotated. Spleen and head kidney showed moderate to severe cellular depletion, with occasional observation of indicators of apoptosis. There were no significant histological changes in the other organs of RCPT fish neither in any samples from CTRL fish.

3.2. RNA-seq

A total of ~170 million 100 bp pair-end reads were sequenced, accounting for on average 15 million reads per sample post-filtering. Filtered reads were mapped to the turbot genome identifying a total of 54,864 transcripts and 23,063 genes in the three organs. The average number of raw, filtered and mapped reads for the samples of each organ are shown in Table 1. For each organ the control and infected samples were hierarchically clustered according to their transcript expression (Figure 1) confirming the correct classification of infected and control samples.

Table 1. RNA-Seq sample statistics.

Sample	Raw reads	Trimmed reads	Aligned reads
Head kidney	17,431,026	15,929,258	14,321,389
Spleen	16,393,159	14,705,171	13,124,477
Pyloric caeca	17,094,324	15,341,340	9,963,567

Average raw reads obtained by Illumina sequencing, average trimmed reads remaining after filtering and average reads aligned to the turbot genome per sample are shown for each organ.

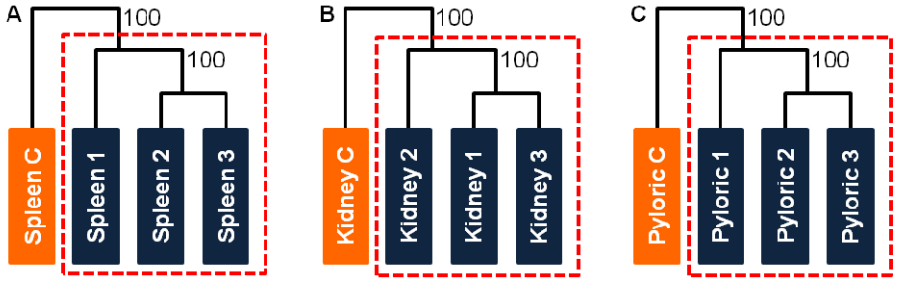

Figure 1. Samples hierarchical clustering by organ. Hierarchical clustering of all diseased and control samples for A Spleen, B Head kidney and C Pyloric caeca. Approximately unbiased *p*-values, computed by multi-scale bootstrap resampling, are displayed on branch nodes and clusters of samples with an approximately unbiased *p*-value > 0.95 are indicated with a dashed red box, indicating strong support.

3.3. Differential expression analysis

A total of 4,762 DE genes were identified across the three organs when comparing RCPT and CTRL fish. The number of DE genes (up- and down-regulated) for each organ and those shared between organs is shown in Figure 2. A high number of down-regulated genes (3,062) were detected, 68.5% more than up-regulated (1,817). Pyloric caeca showed the highest amount of DE genes, almost double that of the other two organs. The percentage of DE annotated transcripts was similar in the three organs: 44.1% in head kidney, 181 up-regulated and 400 down-regulated; 42.3% in spleen, 229 up-regulated and 353 down-regulated; and 46.9% in pyloric caeca, 562 up-regulated and 851 down-regulated. Log_2 FC values ranged from 11.26 to -11.18 in head kidney, from 13.29 to -12.83 in spleen, and from 12.13 to -15.18 in pyloric caeca.

3.3.1. Common DE genes between the three organs

A group of 117 DE genes were shared between the three organs. Among them, 48 were successfully annotated: 11 up- and 26 down-regulated, and 11 either up- or down-regulated depending on the organ (Supplementary Table S1).

Amongst the shared up-regulated genes, some were involved in innate immune response and antigen presentation such as *IL4I1*, involved in the lysosomal processing and presentation of antigens; *ALOXE3*, an enzyme that participates in leukotrienes metabolism; and *MASP3b*, which plays a prominent role in the activation of the lectin complement pathway.Amongst the down-regulated genes, two sets were of particular interest, one directly involved in immune response and the other related to cell and tissue structure disruption. The first group included several interferon (IFN)-related genes involved in antiviral immune response, such as *IRF7*, *Gig2*, *IFIT-1*, *HERC5* and *IFI44*. Other two interesting genes were related to major histocompatibility complex class I molecules (MHC-I), involved in the presentation of intracellular-derived antigens, and *CAT*, which encodes an essential antioxidant enzyme for cell protection against oxidative damage. The second set included several genes related to cytoskeleton: *FMNL1* (cytoskeletal organization and cell morphology and motility); *TMOD4* (geometry of the membrane skeleton); *GRXCR1* (architecture of actin filament-rich structures); and *SNPH* (microtubule-associated protein). *COL1A2*, involved in extracellular matrix structure and organization, was also found in this group.

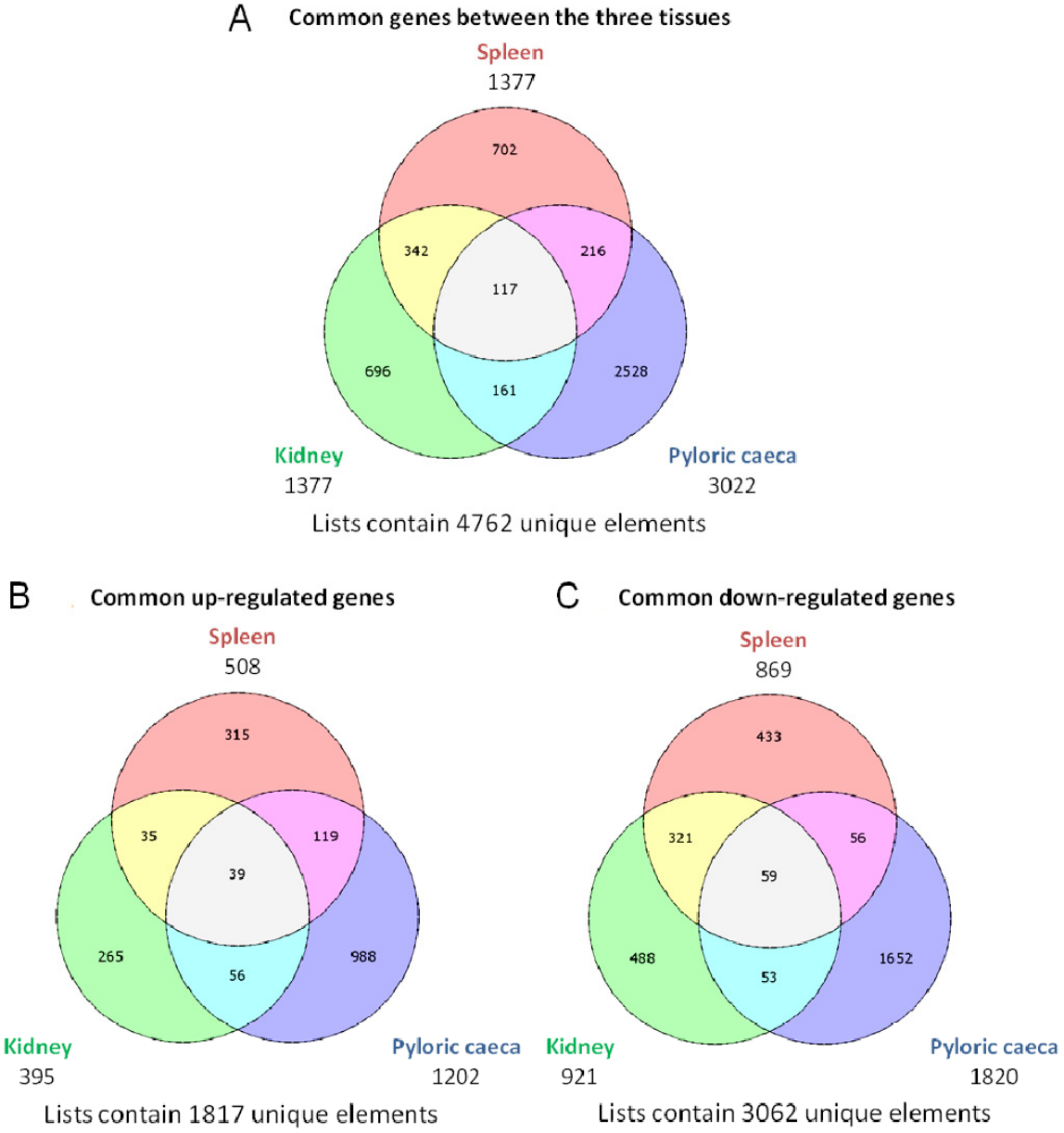

Figure 2. DE genes Venn diagrams. Venn diagrams of A) all DE genes, B) up-regulated DE genes, C) down-regulated DE genes in the three organs (head kidney, spleen and pyloric caeca) are shown. The total number of DE expressed genes in each tissue and the number of unique and common genes between them is displayed.

A group of 12 up-regulated genes in pyloric caeca, but down-regulated in head kidney and spleen, included three genes which promote apoptosis (*KCNN3*, *EGR* and *TNFRSF10B*) and the immunoglobulin light chain, which plays a key role in the adaptive immune response. The only up-regulated gene in pyloric caeca and head kidney, but down-regulated in spleen was the complement component C3, essential for the activation of the complement pathway. Finally, the only gene up-regulated in spleen and head kidney and down-regulated in pyloric caeca was aminopeptidase n, which encodes an enzyme that participates in the final digestion of peptides, but also in processing other peptide molecules such as hormones, neuropeptides and MHC class II- bound antigen peptides.

3.3.2. DE genes classification and organ-specific expression

We inspected the list of organ-specific DE transcripts and made a selection of the most interesting genes, which were grouped in five key broad functional categories based on GO term characterisation and on the current literature in the field. The chosen categories were 1) immune and defence response, 2) apoptosis and cell proliferation, 3) iron metabolism and erythropoiesis and 4) metabolism and digestive function and 5) cytoskeleton and extracellular matrix. The selected genes and the group to which they belong to are listed in Supplementary Table S2 for head kidney, S3 for spleen and S4 for pyloric caeca. A selection of the 50 most relevant DE genes from these five categories is presented in Table 2 and their expression shown in a heatmap (Figure 3).

Immune and defence response. A large number of genes related to this function were found in the three organs, but, while the number of down-regulated genes was comparable, pyloric caeca showed the most numerous group of up-regulated genes. This included chemokines, chemokine receptors, immunoglobulin chains, interleukins and several other genes involved in both innate and adaptive immune response. In particular, a broad representation of components of the inflammatory reaction pathway like *G-CSFR*, immune-responsive gene-1, p- and e-selectin, as well as the transcription factors *AP-1* and *CEBPB*, was detected. Moreover, genes such as *LITAF*, which promotes the expression of the pro-inflammatory cytokine TNF-α, and *CYBB*, a superoxide-generating enzyme of phagocytes, were up-regulated in pyloric caeca but down-regulated in spleen and head kidney, respectively. Lymphoid organs, spleen and head kidney, showed a similar number of up- and down-regulated genes involved in inflammation and acute-phase response, including shared up-regulated genes like hepcidin, heat shock proteins and prostaglandin synthases. On the other hand, several genes related to B and T cells (such as those encoding immunoglobulin molecules and the T cell-related proteins *CD4*, *TCRB*, granzyme and perforin) were down-regulated in these organs. Moreover, in spleen there was a depression of two genes considered to be markers for dendritic cells, *CD83* and *CD209*, which were, conversely, up-regulated in pyloric caeca. Spleen and pyloric caeca, in turn, showed a common up-regulation of the C-type lectin *MRC1,* while other two members of this family were up-regulated, but only in spleen (*MASP1*) or in pyloric caeca (*CLEC4M*).

Noticeably, more genes related to IFNs were identified among down-regulated genes, like *Gig1*, *IFNGR1* and *IFR3* in head kidney and spleen or *GVINP1* and *IRF4* in spleen and pyloric caeca. Moreover, the *TLR9*, also involved in defence against intracellular pathogens, was down-regulated in spleen and head kidney. Also, some regulated genes involved in Th17 cells response were detected, particularly the down-regulation of Th17 cytokines: *IL22* in head kidney and pyloric caeca and *IL17* and its receptor in pyloric caeca. In this organ, it was also remarkable the down-regulation of four genes related to anti-oxidant defence (*MRSA*, *SOD*, *GSTT1* and *TXNDC17*) and the up-regulation of several genes involved in tissue remodelling and repair (e.g. *MMP13*, *PLAT*, *FGF10*, *VNT*). Spleen and head kidney, as well, showed up-regulation of *HGF*, a cytokine acting in tissue repair but also in modulation of immune response, and of two *HGF*-related genes (*HGFR* in spleen and *HGFAC* in head kidney).

Table 2. Selection of the 50 most relevant DE genes.

Gene	Short name	Log-FC HK	Log-FC SP	Log-FC PC	CAT	Associated function
MHC class I antigen	MHC I	-6.3	-4.9	-5.4	1	Antigen processing and presentation
Interferon regulatory factor 7	IFR7	-1.8	-1.6	-3.6	1	Positive regulation of interferons production
Interferon-induced protein 44	IFI44	-3.2	-3.6	-2.2	1	Interferon-inducible protein
Interferon-induced protein with tetratricopeptide repeats 1	IFIT-1	-4.7	-5.0	-2.1	1	Interferon-inducible protein
Gig2-like protein	Gig2	-10.4	-12.8	-7.4	1	Interferon-inducible protein
Interferon gamma receptor alpha chain	IFNGR1	-2.7	-4.4	--	1	Regulation of interferon-gamma-mediated signalling pathway
Interferon regulatory factor 3	IFR3	-1.8	-2.1	--	1	Positive regulation of interferons production
Toll-like receptor 9	TLR9	-3.0	-3.0	--	1	Innate immune response; Positive regulation of interferons production
Mannose-binding lectin-associated serine protease-3b	MASP3b	7.6	4.9	4.5	1	Complement activation
Epidermis-type lipoxygenase 3	ALOXE3	5,7	6.8	10.3	1	Leukotrienes metabolic process
L-amino-acid oxidase	IL4I1	4.8	5.9	9.5	1	Innate immune response
Interleukin-17a f-1	IL-17A/F-1	--	--	-3.8	1	Inflammatory response
Interleukin-22	IL22	-4.5	--	-1.7	1	Inflammatory response
CD83 antigen	CD83	--	-1.8	2.5	1	Defence response
CD209 antigen	CD209	--	-4.3	2.2	1	Innate immune response
Cytochrome b-245 heavy chain	CYBB	-4.0	--	3.7	1	Inflammatory response
CC chemokine	CCL	--	1.9	4.0	1	Inflammatory response
Interleukin-1 receptor accessory protein	IL1RAP	--	-4.2	2.6	1	Inflammatory response
Lipopolysaccharide-induced tumor necrosis factor-alpha factor	LITAF	--	-1.6	6.2	1,2	Regulation of cytokine production-Apoptotic process
Immunoglobulin light chain	IGlc	-1.8	-2.0	2.7	1	Antigen binding
T-cell surface glycoprotein cd4	CD4	--	-1.6	--	1	T cell receptor signalling pathway
T-cell receptor beta chain	TCRB	--	-2.0	--	1	T cell receptor signalling pathway
Perforin-1	PRF1	-3.2	--	--	1	T cell-mediated cytolysis
Granzyme A/K	GZM-A/K	-1.7	--	--	1	T cell-mediated cytolysis
Catalase	CAT	-2.0	-1.8	-2.5	1	Hydrogen peroxide catabolic process
Superoxide dismutase	SOD	--	--	-2.1	1	Removal of superoxide radicals
Glutathione s-transferase theta-1	GSTT1	--	--	-2.0	1	Oxidation-reduction process
Caspase-3-like	CASP3	--	--	5.5	2	Positive regulation of apoptotic process
Cytochrome c	CYTC	2.5	--	4.3	2	Apoptotic DNA fragmentation
TNF receptor-associated factor 2-like	TRAF2	--	--	3.6	2	Regulation of apoptotic process

Statistically significant fold changes (Log-FC) are shown for each organ for 50 relevant genes associated with enteromyxosis. Non significant differences have been marked as "--". HK = head kidney, SP = spleen, PC = pyloric caeca. CAT = Functional category: 1) Immune and defence response; 2) Apoptosis and cell proliferation; 3) Iron metabolism and erythropoiesis; 4) Metabolism and digestive function; 5) Cytoskeleton and extracellular matrix.

Table 2. Selection of the 50 most relevant DE genes (Continued).

Gene	Short name	Log-FC HK	Log-FC SP	Log-FC PC	CAT	Associated function
Tumor necrosis factor receptor superfamily member 10b-like	TNFRSF10B	-3.2	-2.2	2.6	2	Regulation of apoptotic process
Hemoglobin subunit alpha-d	HBAD	-5.2	-3.5	--	3	Oxygen transport
Hemoglobin subunit beta-2	HBB2	-4.7	-3.8	--	3	Oxygen transport
Hemoglobin subunit beta-1	HBB1	-4.6	-3.2	--	3	Oxygen transport
Band 3 anion exchange protein	SLC4A1	-6.6	-4.5	--	3	Erythrocytes differentiation
Gata-binding factor 2	GATA2	-3.5	-2.2	--	3	Erythrocytes differentiation
Mitoferrin-1	SLC25A37	-3.6	-3.5	--	3	Erythrocytes maturation
T-cell acute lymphocytic leukemia protein 1	TAL1	-2,8	-1,6	--	3	Erythrocytes differentiation-Erythrocytes maturation
Hepcidin	HEPC	2.7	2.0	--	3	Iron metabolism
Aminopeptidase n	ANPEP	2.2	7.3	-9.2	4	Protein metabolic process
Intestinal-type alkaline phosphatase 1	ALPI	--	--	-11.7	1,4	Metabolic process
Acidic mammalian chitinase	CHIA	--	--	-14.1	4	Carbohydrate metabolic process
Apolipoprotein a-iv precursor	APOA4	--	--	-6.5	4	Lipoprotein metabolic process
Gastric inhibitory polypeptide	GIP	--	--	-3.1	4	Response to nutrient levels
Cocaine- and amphetamine-regulated transcript	CART	--	--	6.7	4	Negative regulation of appetite
Gastrin cholecystokinin-like	GAST-CCK	--	--	-4.4	4	Digestion
Collagen alpha-2 chain	COL1A2	-2.0	-1.5	-2.4	5	Extracellular matrix structural constituent
Tropomodulin 4	TMOD4	-5.6	-3.9	-2.3	5	Tropomyosin binding
Formin-like protein 1	FMNL1	-4.0	-3.0	-2.8	5	Actin cytoskeleton organization
Alpha actin	ACTA	-3.9	--	-2.7	5	Skeletal muscle fiber development

Statistically significant fold changes (Log-FC) are shown for each organ for 50 relevant genes associated with enteromyxosis. Non significant differences have been marked as "--". HK = head kidney, SP = spleen, PC = pyloric caeca. CAT = Functional category: 1) Immune and defence response; 2) Apoptosis and cell proliferation; 3) Iron metabolism and erythropoiesis; 4) Metabolism and digestive function; 5) Cytoskeleton and extracellular matrix.

Finally, a few genes known to be induced under hypoxic and/or oxidative stress conditions were found to be up-regulated, including the angiopoietin related proteins showing an increase between 4 and 7.4 FC in the three organs, and *HIGD1A*, the adrenomedullin genes and a cytochrome c oxidase mitochondrial subunit in pyloric caeca and head kidney.

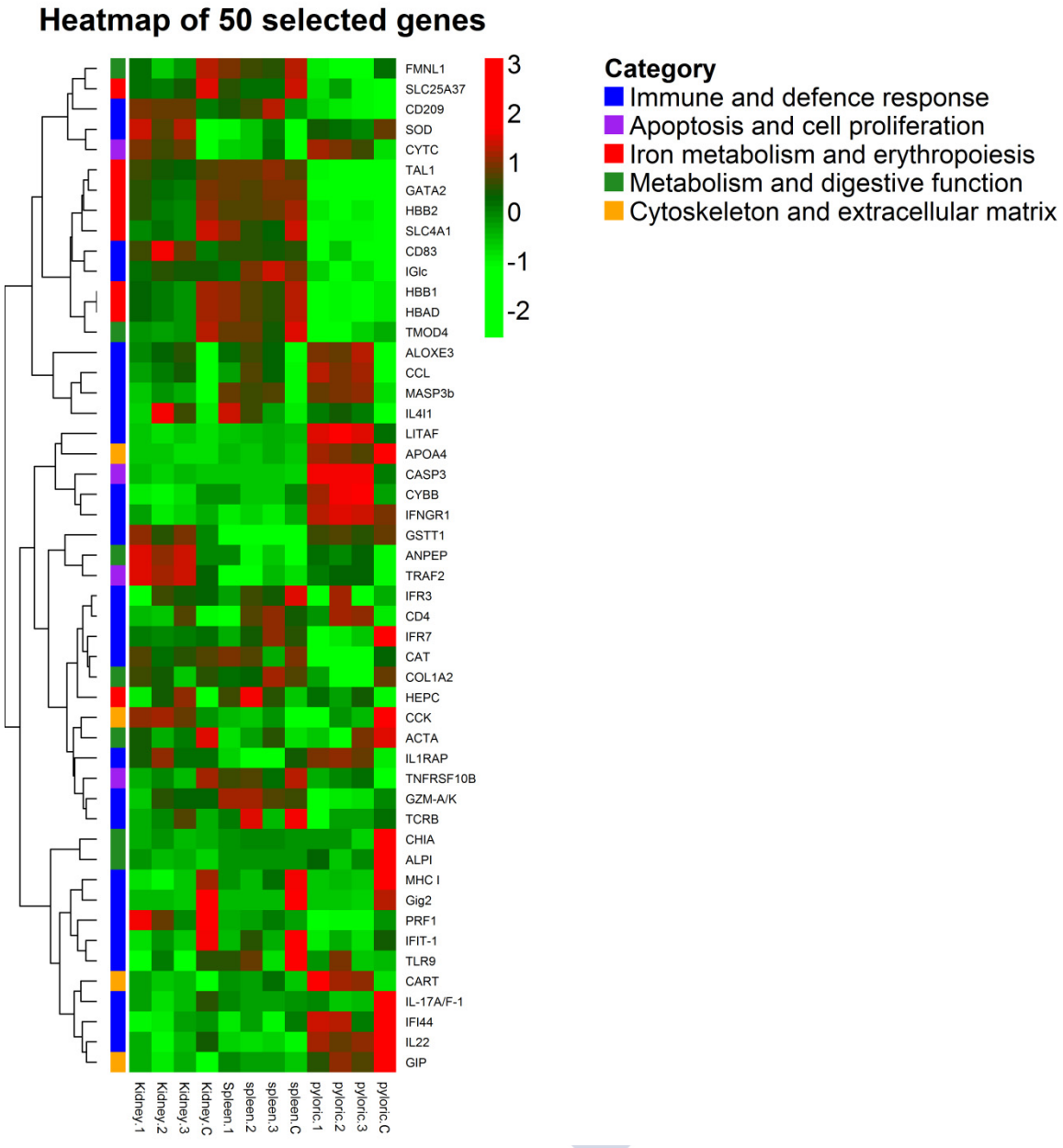

Figure 3. Heatmap of 50 selected genes. Heatmap of the fifty selected genes presented in Table 2. Displayed are EdgeR (Robinson and Oshlack 2010) normalized counts for each sample and gene. Sample names are displayed at the bottom of the figure whilst gene symbols are shown to the right and have been hierarchically clustered according to their Pearson correlation. The category assigned to each gene is also shown with a colour code.

Apoptosis and cell proliferation. Apoptosis and cell proliferation DE genes were found mainly in pyloric caeca. Several genes participating in the apoptotic process, especially the caspase-3 (FC = 5.5) and cytochrome c (FC = 4.3), which are essential players in the execution phase of apoptosis, were up-regulated. In general, in this organ we found more pro- than anti-apoptotic genes, but also other genes involved in cell proliferation, such as the *PCNA*, *FGF10* and cyclins b1 and a2, were up-regulated. In head kidney and spleen a few genes belonging to this group, like the pro-apoptotic cytochrome c in head kidney and clusterin in spleen were up-regulated.

Iron metabolism and erythropoiesis. In head kidney and spleen, the main lymphohaematopoietic organs in teleosts, we observed down-regulation of several genes related to haematopoiesis. The expression of genes involved in erythrocyte maturation and differentiation, like *TAL1*, *GATA2* and mitoferrin-1, was depressed in both organs, and we also observed a dramatic decrease in the expression of genes related to oxygen transport. For example, haemoglobin subunit alpha-d and haemoglobin subunit beta-2 showed a -5.2 and a -4.7 FC, respectively, in head kidney, and a -3.5 and -3.8 in spleen. The band 3 anion transport protein gene, the major glycoprotein of the erythrocyte membrane, also suffered a notable down-regulation in head kidney (FC = -6.6) and spleen (FC = -4.6). On the other hand, two genes related to iron homeostasis, hepcidin (up-) and ferritin heavy subunit (down-), were regulated in both organs.

Metabolism and digestive function. This group of genes was analyzed in pyloric caeca to evaluate intestinal function during enteromyxosis, and were mainly down-regulated. Most of these genes showed high expression in the control sample, while its expression was practically undetectable in infected individuals. That was the case of the digestive enzymes *CHIA* (-14.1), *ALPI* (-11.7), *CYP7A1* (-9.1) or *CPO* (-6.0). Also the *FABP2* and *APOA4* genes, involved in lipid metabolism, showed very highly expression in control samples, but -4.1 and -6.5 FCs, respectively, in infected samples. Moreover, there was a depression of genes induced by food intake (*GIP*, *CCK2*, gastrin-cholecystokinin-like peptide) and of the gene coding for the galanin type I receptor, an orexigenic petide. On the other hand, two anorexigenic genes (*CART* and *CGRP*) were up-regulated.

Cytoskeleton and extracellular matrix. Several myosin, collagen, actin, tubulin, coronin and spectrin genes, were down-regulated in the three organs. Pyloric caeca and head kidney exhibited the highest number of down-regulated genes. Of particular interest were collagen alpha-1, alpha actin and the different myosin genes, that were abundantly expressed in control samples, showing FCs ranging from -6.1 (myosin heavy chain) to -1.8 (collagen alpha-1). Likewise, the *TPM4*, which was highly expressed in spleen and pyloric caeca of CTRL fish, was down-regulated in RCPT samples.

3.4. GO enrichment analysis

The full transcriptome of the three organs was annotated and GO terms for each sequence were obtained. A Fisher exact test (FDR corrected *p*-value = 0.05) was used to compare DE sequences with the background transcriptome to obtain the enriched GO terms for each organ (Figure 4). Oxygen binding was clearly overrepresented in both spleen and head kidney of RCPT fish, likely indicating alterations in the erythrocyte machinery, as mentioned earlier. Lipid metabolism and catalytic activity were enriched categories in pyloric caeca, which might evidence problems in the digestive function. Extracellular space or extracellular region GO terms were present in all three organs. GO enrichment was also performed for up-regulated and down-regulated genes separately, obtaining an additional up-regulated GO category in spleen, peptidase activity, and in pyloric caeca, cell cycle.

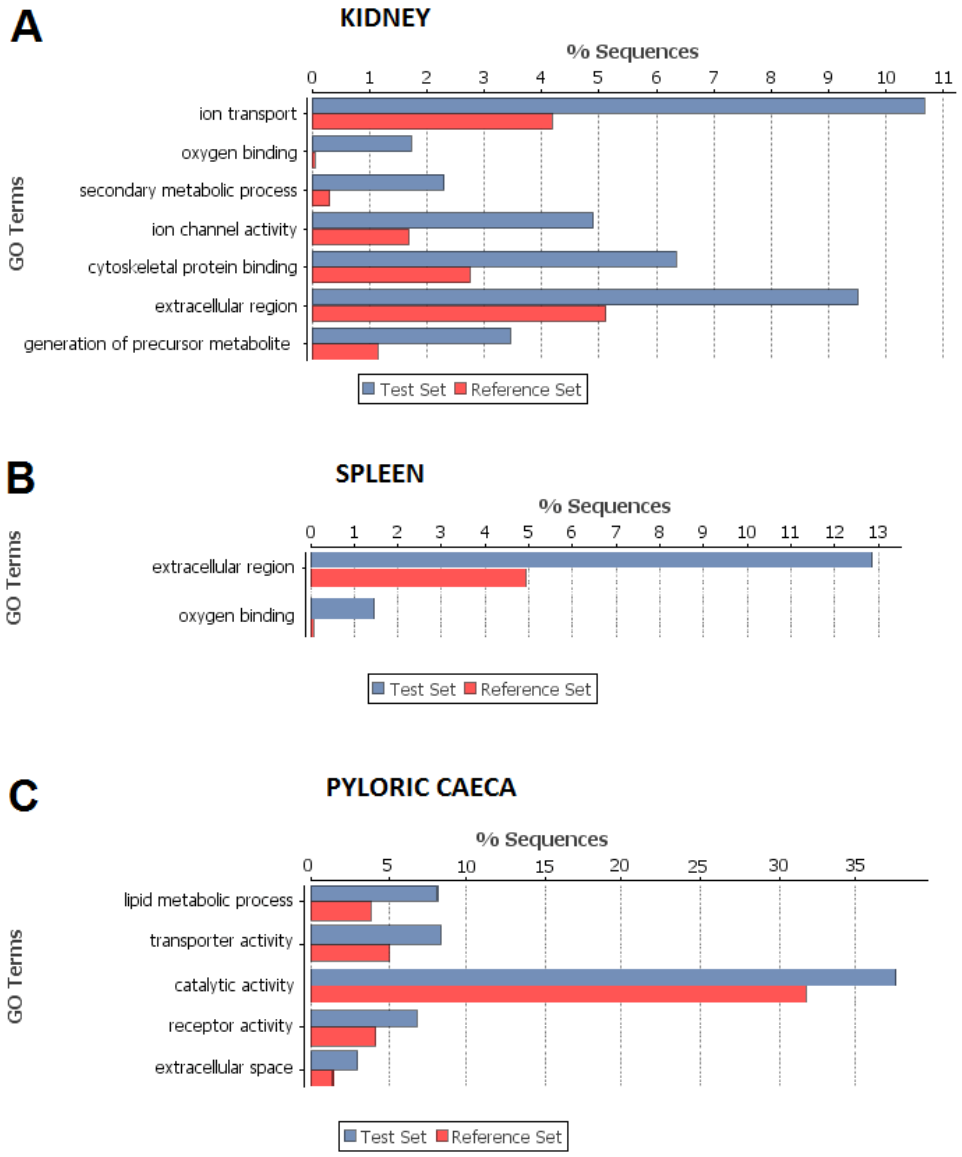

Figure 4. GO terms enrichment. GO enrichment ($p < 0.01$ FDR corrected) for DE genes in a) head kidney, b) spleen and c) pyloric caeca. The percentage of sequences with the associated GO term present in the full organ transcriptome is shown in blue, while the percentage of sequences with the GO term in the DE gene group is shown in red.

4. Discussion

This is, to our knowledge, the first report of a RNA-seq transcriptomic analysis applied to the study of a fish-parasite model. We investigated turbot at an advanced enteromyxosis stage, selected on the basis of histopathological evaluation. This approach allows the analysis of fish with a more uniform health status, minimizing interindividual variation, and consequently, enabling a more consistent identification of regulated genes on a reduced number of animals. This study advances our understanding of how the *E. scophthalmi* infection develops and the determination of the clinical signs and lesions characteristic of infection in turbot. Figure 5 depicts the cascade of events leading to severe enteromyxosis in turbot considering in particular the transcriptomic changes found in the current study.

We found far more DE genes in pyloric caeca (3022) than in either kidney or spleen (1316 and 1377, respectively). This is perhaps not that surprising since the intestine is the target tissue of *Enteromyxum* spp. infection and the lesions caused by the disease are mostly restricted to the gastrointestinal tract. Additionally, the most characteristic clinical signs of the disease, such as weight loss and anorexia, are attributable to the alteration of the normal physiology of the digestive system. Furthermore, spleen and kidney shared 321 down-regulated genes, an interesting result which can be attributed to the common lymphohaematopoietic functions and the cellular depletion observed in both organs in late stages of the disease (Bermúdez *et al.* 2010).

4.1. Immune and defence response

The defence response of turbot against *E. scophthalmi* was characterized by the activation of the innate immune response, but it seems that different elements acting in bridging innate and adaptive response are failing, and an inadequate onset of the adaptive immune response was noticed. The transcriptomic changes detected were especially intense in pyloric caeca, corresponding, and likely contributing, to the severe histological changes observed at tissue level.

Numerous regulated genes belonging to immune and defence response were found in the three organs analyzed. The number of up-regulated genes in pyloric caeca far exceeded the down-regulated ones, while this is not observed in kidney and spleen. Among the three DE genes related to the innate immune response shared by the three organs (*ALOXE3*, *IL4I1* and *MASP3b*), the overexpression of *MASP3b*, which mediates the activation of the complement lectin pathway, is of particular interest (Dahl *et al.* 2001; Hajela *et al.* 2002; Gadjeva *et al.* 2004). This is also the function of *MASP1*, overexpressed in spleen. The complement lectin pathway is considered the most ancient route of complement activation (Fujita *et al.* 2004; Kania *et al.* 2010), with a well-demonstrated role in several parasitic diseases (Gruden-Movsesijan *et al.* 2003; Ambrosio and De Messias-Reason 2005; Evans-Osses *et al.* 2010; Cestari *et al.* 2013). Interestingly, the third component of the complement system, C3, which plays a central role supporting the activation of the three complement pathways (Sahu and Lambris 2001) was up-regulated in head kidney and pyloric caeca, but down-regulated in spleen. This could be related to the changes also observed in components of the classical pathway (overexpression of C1q receptor in spleen and pyloric caeca, but depression of C1q-like protein 4 and protein 2 in pyloric caeca), probably as a rebound effect after a previous increase, or due to a progressive exhaustion of the complement system, hypothesized in late stages of enteromyxosis in turbot and gilthead sea bream (Cuesta *et al.* 2006; Sitjà-Bobadilla *et al.* 2006; Davey *et al.* 2011).

The overexpression of *MASP3b* in the three organs suggests the involvement of lectin complement pathway in the immune response and of C-type lectins as pattern recognition receptors (PRRs) for *E. scophthalmi*. The latter would be also confirmed by the regulation of three genes encoding this type of lectins: *MRC1*, up-regulated in spleen and pyloric caeca; *CLEC4M* up-regulated in pyloric caeca and *CD209*, up-regulated in pyloric caeca and down-regulated in spleen. These results support the hypothesis on *E. scophthalmi* recognition by

turbot advanced in previous studies, which investigated the presence of carbohydrate terminals in the parasite (Redondo *et al.* 2008) and the effect of lectins on the attachment and invasion of intestinal epithelium (Redondo and Álvarez-Pellitero 2010).

C-type lectins receptors located on antigen-presenting cells play a major role in pathogen recognition and induction of immune response (van Vliet *et al.* 2008; Vázquez-Mendoza *et al.* 2013), and *CD209*, in particular, is considered a marker for dendritic cells (Geijtenbeek *et al.* 2000), key linkers between innate and adaptive immunity. Additionally, our study revealed the same expression pattern (up-regulation in pyloric caeca and down-regulation in spleen) of another dendritic cell marker, *CD83*. The presence and function of these cells in teleosts is largely unknown (Lugo-Villarino *et al.* 2010) and our findings provide additional evidence of their involvement in disease response in turbot (Hu *et al.* 2010). The down-regulation of these genes in a main antigen-processing organ, like the spleen in teleost (Rauta *et al.* 2012), together with previous observations (Bermúdez *et al.* 2006; Sitjà-Bobadilla *et al.* 2006, 2007) and other findings of this work, suggests that there might be a failure in the connection between innate and adaptive immune response during turbot enteromyxosis.

On this regard, it was remarkable to observe the down-regulation of several IFN-related genes in the three organs. Several type I IFN-induced genes and type II IFN receptors were depressed, and *PRDM1a*, a repressor of type II IFN (Keller and Maniatis 1991), showed a five-fold increase in pyloric caeca. Interferons play a major role in signalling between innate and adaptive immune response (Le Bon and Tough 2002; Schroder *et al.* 2004), and there are multiple lines of evidence that suggest their involvement in anti-parasitic response and resistance (McCall and Sauerwein 2010; Davey *et al.* 2011; Zou and Secombes 2011). Notably, head kidney and spleen showed down-regulation of *TLR9*, a PRR which induces the expression of type I interferon via the action *IFR3* and *IFR7* (likewise down-regulated in these organs), and enhances MHC-I antigen (down-regulated in all organs) cross processing (Zou and Secombes 2011). Although depression of MHC-I, IFNs and *TLR9* genes is commonly associated with immune system evasion by viruses (Rappocciolo *et al.* 2003; Vincent *et al.* 2011; Gainey *et al.* 2012; Song *et al.* 2013), the importance of this mechanism in parasite infections is becoming increasingly evident (Babu *et al.* 2005; Asteal 2011; Srivastava *et al.* 2013). In teleost, Young *et al.* (2008) established the connection between the coordinated down-regulation of MHC-I and IFN-related genes in amoebic gill disease-affected Atlantic salmon and the inhibition of acquired immunity development and high susceptibility of this species to the disease. On the other hand, in gilthead sea bream chronically exposed to *E. leei*, IFNs and IFN-stimulated genes were hypothesized as markers for pathogen resistance due to their up-regulation in exposed but not parasitized fish (Davey *et al.* 2011).

Interestingly, our RNA-seq analysis also found a down-regulation of *IL22* and *IL17* that could reflect a decrease of their major producers, Th17 cells. Besides, *SOSCS*3, an inhibitor of *IL17* expression, was up-regulated in spleen. Th17 cells also coordinate innate and adaptive immune response (Peck and Mellins 2010) and are described as critical for mucosal and epithelial host defence against extracellular pathogens (van de Veerdonk *et al.* 2009). The

balance between the protective and harmful effects of Th17 cells through cytokines *IL17* and *IL22* is extremely delicate and there are reports supporting their contribution to host defence and others highlighting their inflammatory damage when the infection persists over time (Fujino *et al.* 2003; Kelly *et al.* 2005; Zenewicz *et al.* 2008; van de Veerdonk *et al.* 2009). A deeper understanding of the genes underlying the activation or depression of the different T helper subsets responses is considered of great significance for resistance and tolerance of livestock species (Glass 2012).

Nowadays, it is becoming evident that in a specific adaptive response is essential to deal with infection and to acquire resistance in teleost (Sitjà-Bobadilla *et al.* 2007; Wynne *et al.* 2008; Young *et al.* 2008). However these data points towards a possible failure or slowness in the activation of the adaptive response, as previously suggested for turbot enteromyxosis based on the evidence of delayed or even undetectable production of specific antibodies against *E. scophthalmi* by turbot (Sitjà-Bobadilla *et al.* 2006, 2007).

In this work, we found several down-regulated genes related to B and T cell activity in lymphohaematopoietic organs, also in accordance with previous work describing lymphocyte depletion, reduced Ig^+ cells and marked lymphohaematopoietic depletion in spleen and head kidney of turbot in advanced stages of the disease (Bermúdez *et al.* 2006, 2010; Sitjà-Bobadilla *et al.* 2006). Globally, spleen and head kidney showed a depression of genes related to acquired immune response, which may reflect the incapability of the immune system to stop the infection. On the other hand, several immunoglobulin-related genes were up-regulated in pyloric caeca, which is consistent with the results of Bermúdez *et al.* (2006), who found a progressive increase in IgM^+ cells in the intestine of *E. scophthalmi*-infected turbot, reaching the maximum at 78 days post-exposure, suggesting that it may reflect a local reaction against the parasite. The importance of the local immune response has been recently highlighted for *E. leei*-infected gilthead sea bream (Pérez-Cordón *et al.* 2014), where significant changes in the expression of interleukins (ILs) and IL receptors were found in the intestine but not in head kidney nor in spleen of infected fish. In this organ, a switch from an early pro-inflammatory IL expression profile to an anti-inflammatory pattern in later stages of disease was also reported.

In our study, a remarkable number of up-regulated genes involved in immune and defence response were found in pyloric caeca, including several innate immune components involved in promoting the inflammatory reaction, which does not seem to be in a resolution phase. Also, intestinal-type alkaline phosphatase, an essential enzyme in controlling gut microflora and maintaining epithelial integrity, showed a -11.7 FC in infected turbot. The role of this enzyme in preventing intestinal inflammation has been demonstrated in zebrafish (Bates *et al.* 2007) and there is evidence of the beneficial effect of its administration in conditions of severe intestinal epithelial damage (Bol-Schoenmakers *et al.* 2010; Lee *et al.* 2014). This supports the hypothesis of an exacerbated local immune response of turbot against the parasite and its products (Bermúdez *et al.* 2007; Losada *et al.* 2012), which can explain the development of the observed lesions. The prolonged inflammation of pyloric caeca is likely to create an oxidative environment at the intestinal level, and the action of the inflammatory

mediators and the oxidative stress can be responsible for the desquamation of the intestinal epithelial lining, typical of the disease in turbot, as seen in other conditions characterized by an exacerbated immune response (Keklikoglu *et al.* 2008; Hansen *et al.* 2010; Marchiando *et al.* 2011; Watson and Hughes 2012). In this context, beyong the up-regulation of two genes realted to oxidative stress response (calcipressin 1 and adrenomedullin 5), the antioxidant defences appear to be failing, as revealed by the depression of the antioxidants *SOD*, *CAT*, *MSRA*, *GSTT1* and *TXNDC17*. In chronic *E. leei*-infected gilthead sea bream, Davey *et al.* (2011) found several genes encoding antioxidant enzymes up-regulated in response to the high reactive oxygen species production, so the opposite pattern reported here could also be one of the factors contributing to the higher susceptibility of turbot to enteromyxosis.

4.2. Apoptosis and cell proliferation

Apoptosis is an essential biological process induced in response to many extrinsic stimuli (like inflammatory reaction and oxidative stress) and it can be considered as part of the host innate immune response during infection but also, in some cases, as an infection-associated immunopathology (Lovegrove *et al.* 2007; Panaro *et al.* 2007; Bienvenu *et al.* 2010; Wyllie 2010). The ability of pathogen microorganisms, including intestinal parasites, to modulate apoptosis in their hosts has been widely documented (Castellanos-González *et al.* 2008; James and Green 2004; Panaro *et al.* 2007). We observed several DE genes related to apoptotic cell death in pyloric caeca of infected fish, including a considerable up-regulation of caspase-3. This is in accordance with previous histological observations describing increased number of cells with apoptotic features (Bermúdez *et al.* 2010) and with the increase of active caspase-3 occurring in both lining epithelium and lamina propria of turbot intestine during enteromyxosis (Losada *et al.* 2014). It has been suggested that this could be a mechanism used by the parasite to spread (Bermúdez *et al.* 2010), though the observations of apoptotic cells between the inflammatory infiltrates of lamina propria have also been related to a strategy for immune evasion (Losada *et al.* 2014). Our data also suggests that this process plays a prominent role in the pathogenesis of turbot enteromyxosis.

The up-regulation of some anti-apoptotic or apoptosis-induced genes (apoptosis inhibitor 5, *TNFRSF11B* and *HIGD1A*) and depression of some pro-apoptotic genes (*TRAIL* and *DEDD2*) was also detected at intestinal level, suggesting that complex adjustments in apoptotic signals may occur during enteromyxosis, as reported for *E. leei* (Davey *et al.* 2011). These findings may be related to different requirements of the parasite to induce or inhibit apoptosis depending on the developmental stage, as hypothesized for human cryptosporidiosis (Castellanos-González *et al.* 2008). Another possible explanation is that these genes may be counterbalancing the effects of the exacerbated immune response, also consistent with the activation of some tissue repair (up-regulation of *MMP13*, *PLAT* and *FGF10*) and cell proliferation (*PCNA* and cyclins) related genes observed in this organ.

Apoptotic cell death has also been involved in the lymphohaematopoietic depletion observed in infected turbot, either directly on cell components of these organs or indirectly as a result of the increment of leukocyte apoptosis in the intestine (Bermúdez *et al.* 2010; Losada *et al.* 2014). In the current study, very few genes related to apoptosis are differently expressed in

head kidney and spleen, displaying a substantial balance between cell death/survival signals. Concerning cell proliferation, the up-regulation in both organs of *HGF*, a pleiotropic cytokine that plays a major role in tissue regeneration, but also with potent anti-inflammatory properties, is a relevant observation, as it is involved in interfering the function of dendritic cells and $CD4^+$ and $CD8^+$ T cells (Okunishi *et al.* 2005; Benkhoucha *et al.* 2010, 2013). Our results showed that CD4 and the dendritic cell markers (CD83, CD209) are down-regulated in spleen. Furthermore, *HGF* affects $CD8^+$ cytotoxic cells by down-regulating IFN-gamma, granzyme and perforin (Benkhoucha *et al.* 2013), consistent with our results in head-kidney. The role of *HGF* in teleost is largely unknown, and in this case its activation may be an attempt to counterbalance the loss of cell population or to modulate the deleterious effects of an exacerbated immune response. These considerations need further research, in particular by focusing on the events occurring at earlier stages of the disease regarding cell-mediated immune response and the activation of interferon pathways at the site of infection as well as in lymphoid organs. In addition, more information about the changes in cell death/ proliferation balance along the course of the disease will help to clarify the role of apoptosis in lymphoid depletion, and the conflict between host and parasite induced functions.

4.3. Iron metabolism and erythropoiesis

Oxygen binding seems to be altered in both spleen and head kidney as shown by the gene-enrichment analysis performed, which strongly support alterations in the erythrocyte function and haemoglobin production. Both spleen and head kidney showed up-regulation of hepcidin, a peptidic hormone initially known by its antimicrobial activity, but later recognized as the principal regulator of iron homeostasis (Ganz 2011; Neves *et al.* 2011; Pereiro *et al.* 2012). It acts as acute-phase protein to induce iron sequestration during infections and is considered the main gene responsible for the so called "anaemia of chronic disease" or "anaemia of infection" (Ganz 2002, 2011). Hepcidin determines a decreased absorption of iron in the intestine and sequestration of iron in macrophages, so limiting its availability for haemoglobin synthesis in maturing erythrocytes (Tizard 2009; Ganz 2011). RNA-seq analysis showed that in the lymphohaematopoietic organs several genes related to haemoglobin and erythrocytes maturation are markedly down-regulated as well as ferritin, the main iron-storage protein. This group of is genes is tightly clustered in the heatmap (Figure 3), revealing a strong common regulation. The depression of this group of genes can explain the decrease in hematocrit and haemoglobin values seen in *Enteromyxum*-infected fish (Sitjà-Bobadilla and Palenzuela 2012) and the activation of genes related to the response to hypoxia found in this study (*HIGD1A*, cytochrome c oxidase and angiopoietin-related). The reduction in iron availability, however, may be explained both by the infection-related iron sequestration and the probable restricted iron intake by diet, due to the anorexia and the impaired intestinal absorptive function shown by fish suffering enteromyxosis (Estensoro *et al.* 2011; Sitjà-Bobadilla and Palenzuela 2012).

4.4. Metabolism and digestive function

Enteromyxum-infected turbot have significantly lower weight and poorer condition at advanced stages of the disease (Sitjà-Bobadilla *et al.* 2006), which is likely due to the

reduction of food intake due to anorexia and intestinal damage (Branson *et al.* 1999; Bermúdez *et al.* 2010). Effects of starvation have been shown to affect blood as well as immune function, besides the expected alterations in nutrient metabolism (Salem *et al.* 2007; Pérez-Jiménez *et al.* 2012; Antonopoulou *et al.* 2013), as observed for the enriched GO category "lipid metabolism" in pyloric caeca in our study. Actually, in infected turbot we have found a remarkable decrease in the expression of several digestive enzymes in this organ, probably related to a general loss of the intestinal function as recently reported in cunner (*Tautogolabrus adspersus,* Walbaum) subjected to acute or long-term fasting (Hayes and Volkoff 2014).

Moreover, we observed changes in the expression of genes that encode for peptide hormones, which act in feeding behaviour. Gastrin cholecystokinin-like peptide and its related receptor *CCK2*, *GIP* and galanin receptor 1, were all down-regulated. Gastrin and cholecystokinin are structurally and functionally related hormones that act in response to food intake to stimulate different digestion processes. Similarly, gastric inhibitory polypeptide (*GIP*) belongs to the incretin family of gastrointestinal hormones whose main function is to induce insulin secretion in response to the increase in the glucose blood level after food ingestion. The depression of these genes can be explained by the overall reduction of digestive function caused by food deprivation, as postulated by Hayes & Volkoff (2014) for cholecystokinin decreased expression seen in fasting cunner. On the other hand, galanin is an orexigenic hormone and its decrease can be related to the parallel increase of the anorexigenic *CART* and *CGRP*.

Anorexia is a quite common clinical sign observed in parasitic infection but the causes and significance that underlie this behaviour, despite being investigated for many years, are still controversial (Kyriazakis *et al.* 1998; Barber 2007; Colditz 2008). In *E. leei*-infected gilthead sea bream, Estensoro *et al.* (2011) found evidence that anorexia is the main cause of body mass loss, hypothesizing the involvement of cachectic cytokines, gastrointestinal peptides and growth factors in the voluntary reduction of food intake. The results of our study are consistent with these considerations, although further investigation of the mechanisms underlying anorexia in enteromyxosis should be addressed.

4.5. Cytoskeleton and extracellular matrix

RNA-Seq analysis revealed that the three studied organs shared common depression of numerous genes encoding cytoskeletal and structural proteins. Protein synthesis is an energy-demanding process and can be affected by the detrimental effects of a prolonged reduction in food intake, further exacerbated if it is associated with impaired intestinal absorption (Wykes *et al.* 1996; Lenaerts *et al.* 2006). These changes can therefore be considered as indicative of a progressive overall tissue wasting which leads to the lethal outcome of turbot enteromyxosis.

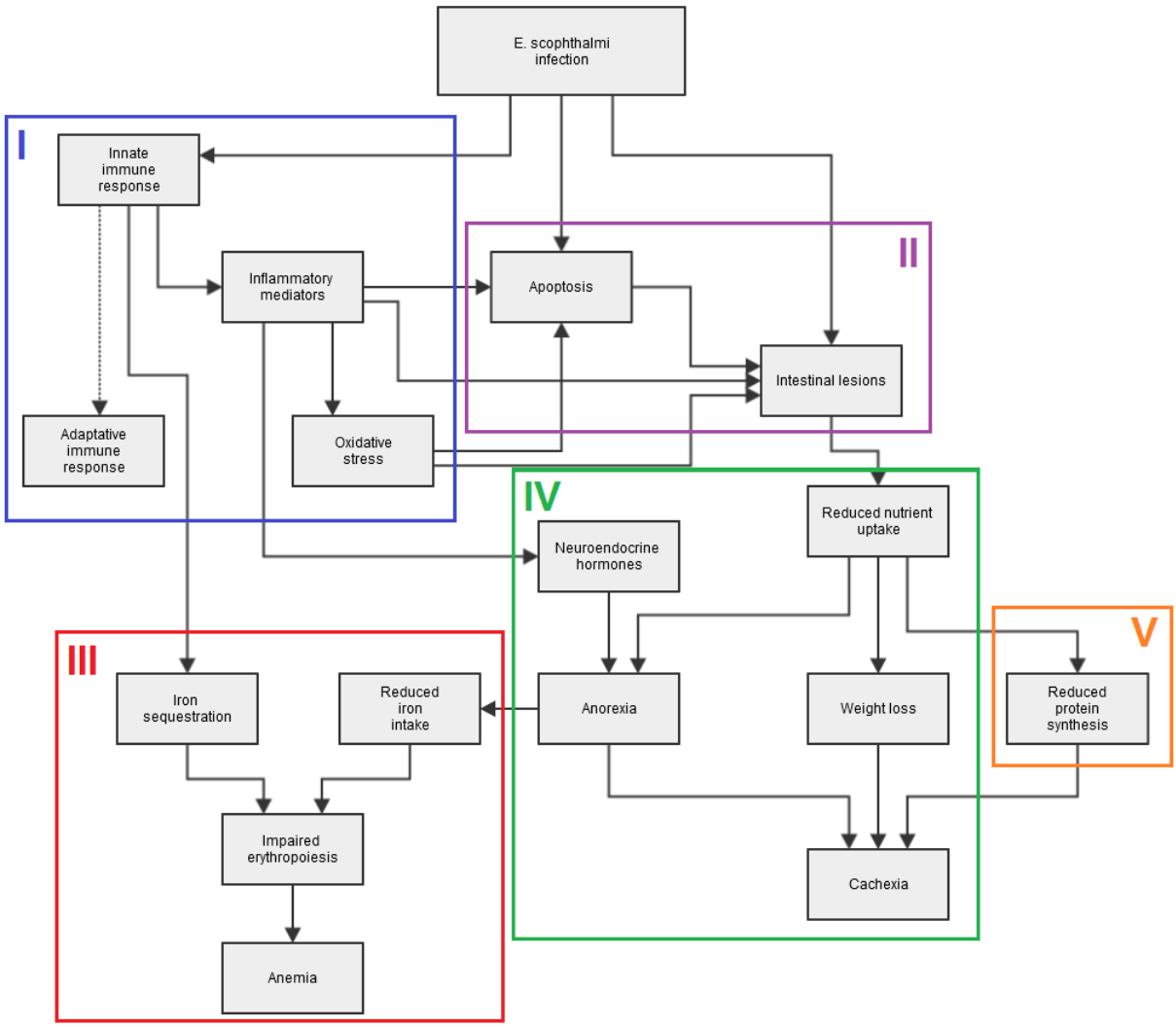

Figure 5. Enteromyxosis flowchart. Flowchart showing the main processes involved in severe turbot enteromyxosis. The flowchart has been divided in five sections according to the most representative processes occurring during Enteromyxum infection: I, blue, immune and defence response; II, purple, apoptosis and cell proliferation; III, red, iron metabolism and erythropoiesis; IV, green, metabolism and digestive function; V, orange, cytoskeleton and extracellular matrix.

5. Conclusions

This is the first application of RNA-seq technology to the study of turbot transcriptomic response and particularly to the analysis of *E. scophthalmi* infection. This experiment has greatly enriched our knowledge on the major turbot biological processes and responses against this disease. The results obtained point towards the presence of an exacerbated local immune response associated with an inadequate activation of the adaptive immunity, probably related to the failure of some components acting in bridging innate and adaptive immune response. Additionally, the involvement of C-type lectins as PRR for the parasite and of apoptosis in the pathogenic mechanism is highly plausuble. The transcriptomic analysis has also revealed details on the genetic basis underlying the characteristic clinical signs and lesions associated with the progression of this disease, like cachexia and anaemia. This knowledge is essential to investigate the pathogenetic mechanisms and the differences in

species-specific susceptibility to enteromyxosis with the aim of identifying resistance-related genes. This information will be useful for the derivation of new therapeutic treatments and to exploit genetic variation associated with these key genes in order to achieve more resistant broodstock through breeding programmes. Further analyses should focus on transcriptomic changes at earlier stages of the disease and on comparative studies with other affected species.

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List of gene symbols in alphabetical order

ALOXE3: epidermis-type lipoxygenase 3; *AP-1*: transcription factor ap-1; *CART*: cocaine- and amphetamine-regulated transcript; *CAT*: catalase; *CCK2*: cholecystokinin B receptor; *CEBPB*: ccaat enhancer-binding protein beta; *CGRP*: calcitonin gene-related peptide; *CLEC4M:* C-type lectin domain family 4, member M*; COL1A2*: collagen alpha-2 chain; *CYBB*: cytochrome b-245 heavy chain; *DEDD2*: DNA-binding death effector domain-containing protein 2; *EGR*: early growth response; *FGF10*: fibroblast growth factor 10; *FMNL1*: formin-like protein 1; *GATA2*: gata-binding factor 2; *G-CSFR*: granulocyte colony-stimulating factor receptor; *Gig1* and *-2*: grass carp reovirus-induced gene 1, -2; *GIP*: gastric inhibitory polypeptide; *GRXCR1*: Glutaredoxin domain-containing cysteine-rich protein 1; *GSTT1*: glutathione s-transferase theta-1; *GVINP1*: interferon-induced very large gtpase 1; *HERC5*: e3 isg15--protein ligase herc5; *HGF*: hepatocyte growth factor; *HGFAC*: hepatocyte growth factor activator; *HGFR*: hepatocyte growth factor receptor; *HIGD1A*: hig1 domain family member 1a; *IFI44*: interferon-induced protein 4; *IFIT-1*: interferon-induced protein with tetratricopeptide repeats 1; *IFNGR1*: interferon gamma receptor alpha chain; *IL22, -17*: interleukin 22, 17; *IL4I1*: L-amino-acid-oxidase; *IRF3, -4* and *-7*: interferon regulatory factor 3; 4 and 7; *KCNN3*: small conductance calcium-activated potassium channel protein 3; *LITAF*: lipopolysaccharide-induced tumor necrosis factor-alpha factor; *MASP1* and *-3b:* mannose-binding lectin-associated serine protease 1 and 3b; *MMP13*: collagenase 3; *MRC1:* macrophage mannose receptor 1*; MSRA*: peptide methionine sulfoxide reductase; *PCNA*: proliferating cell nuclear antigen; *PLAT*: tissue-type plasminogen activator; *PRDM1a*: *PR* domain containing 1a, with ZNF domain; *SNPH*: syntaphilin-like isoform x1; *SOSCS*3: suppressor of cytokine signalling 3; *SOD*: superoxide dismutase*; TAL1*: t-cell acute lymphocytic leukemia protein 1; *TCRB*: t-cell receptor beta chain; *TLR9*: toll-like receptor 9; *TMOD4*: tropomodulin 4; *TNFRSF10B* and *-11B*: tumor necrosis factor receptor superfamily, member 10b and member 11B; *TPM4*: tropomyosin alpha-4 chain; *TRAIL*: TNF-related apoptosis-inducing ligand; *TXNDC17*: thioredoxin domain-containing protein 17; *VNT*: vitronectin.

Supplementary files

Supplementary Table S1. List of shared differentially expressed genes between the three organs. Common differentially expressed genes between kidney, spleen and pyloric caeca. Fold change by tissue, fragments per kilobase of exon per million fragments mapped (FPKM) in control and infected samples by tissue and GO terms for each gene are shown.

Supplementary Table S2. List of differentially expressed genes in head kidney. Differentially expressed genes in kidney. Fold change, fragments per kilobase of exon per million fragments mapped (FPKM) in control and infected samples and associated functional group for each gene are shown.

Supplementary Table S3. List of differentially expressed genes in spleen. Differentially expressed genes in spleen. Fold change, fragments per kilobase of exon per million fragments mapped (FPKM) in control and infected samples and associated functional group for each gene are shown.

Supplementary Table S4. List of differentially expressed genes in pyloric caeca. Differentially expressed genes in pyloric caeca. Fold change, fragments per kilobase of exon per million fragments mapped (FPKM) in control and infected samples and associated functional group for each gene are shown.

References

Ambrosio, A.R., De Messias-Reason, I.J., 2005. *Leishmania (Viannia) braziliensis*: interaction of mannose-binding lectin with surface glycoconjugates and complement activation. An antibody-independent defence mechanism. Parasite Immunol 27, 333-340.

Antonopoulou, E., Kentepozidou, E., Feidantsis, K., Roufidou, C., Despoti, S., Chatzifotis, S., 2013. Starvation and re-feeding affect Hsp expression, MAPK activation and antioxidant enzymes activity of European Sea Bass (*Dicentrarchus labrax*). Comp Biochem Phys A:165, 79-88.

APROMAR, 2013. La Acuicultura en España 2013. Available online at http://www.apromar.es/content/informesanuales.

Asteal, F., 2011. A study of immune responses to *L. mexicana* antigens and immunogenicity of *L. donovani* centrin-3. PhD Thesis, School of Science and Technology. Nottingham Trent University, Notthingam, United Kingdom.

Babu, S., Blauvelt, C.P., Kumaraswami, V., Nutman, T.B., 2005. Diminished Expression and Function of TLR in Lymphatic Filariasis: A Novel Mechanism of Immune Dysregulation. J Immunol 175, 1170-1176.

Barber, I., 2007. Parasites, behaviour and welfare in fish. Appl Anim Behav Sci 104, 251-264.

Bates, J.M., Akerlund, J., Mittge, E., Guillemin, K., 2007. Intestinal alkaline phosphatase detoxifies lipopolysaccharide and prevents inflammation in zebrafish in response to the gut microbiota. Cell Host Microbe 2, 371-382.

Benkhoucha, M., Santiago-Raber, M.-L., Schneiter, G., Chofflon, M., Funakoshi, H., Nakamura, T., Lalive, P.H., 2010. Hepatocyte growth factor inhibits CNS autoimmunity by inducing

tolerogenic dendritic cells and CD25+Foxp3+ regulatory T cells. Proc Natl Acad Sci USA 107, 6424-6429.

Benkhoucha, M., Molnarfi, N., Schneiter, G., Walker, P., Lalive, P., 2013. The neurotrophic hepatocyte growth factor attenuates CD8+ cytotoxic T-lymphocyte activity. J Neuroinflammation 10, 154.

Bermúdez, R., Vigliano, F., Marcaccini, A., Sitjà-Bobadilla, A., Quiroga, M.I., Nieto, J.M., 2006. Response of Ig-positive cells to *Enteromyxum scophthalmi* (Myxozoa) experimental infection in turbot, *Scophthalmus maximus* (L.): A histopathological and immunohistochemical study. Fish Shellfish Immunol 21, 501-512.

Bermúdez, R., Vigliano, F., Quiroga, M.I., Nieto, J.M., Bosi, G., Domeneghini, C., 2007. Immunohistochemical study on the neuroendocrine system of the digestive tract of turbot, *Scophthalmus maximus* (L.), infected by *Enteromyxum scophthalmi* (Myxozoa). Fish Shellfish Immunol 22, 252-263.

Bermúdez, R., Losada, A.P., Vázquez, S., Redondo, M.J., Álvarez-Pellitero, P., Quiroga, M.I., 2010. Light and electron microscopic studies on turbot *Psetta maxima* infected with *Enteromyxum scophthalmi*: histopathology of turbot enteromyxosis. Dis Aquat Organ 89, 209-221.

Bienvenu, A.-L., Gonzalez-Rey, E., Picot, S., 2010. Apoptosis induced by parasitic diseases. Parasit Vectors 3, 106.

Bol-Schoenmakers, M., Fiechter, D., Raaben, W., Hassing, I., Bleumink, R., Kruijswijk, D., Maijoor, K., Tersteeg-Zijderveld, M., Brands, R., Pieters, R., 2010. Intestinal alkaline phosphatase contributes to the reduction of severe intestinal epithelial damage. Eur J Pharmacol 633, 71-77.

Branson, E., Riaza, A., Álvarez-Pellitero, P., 1999. Myxosporean infection causing intestinal disease in farmed turbot, *Scophthalmus maximus* (L.), (Teleostei: Scophthalmidae). J Fish Dis 22, 395-399.

Castellanos-González, A., Yancey, L.S., Wang, H.C., Pantenburg, B., Liscum, K.R., Lewis, D.E., White, A.C., Jr., 2008. *Cryptosporidium* infection of human intestinal epithelial cells increases expression of osteoprotegerin: a novel mechanism for evasion of host defenses. J Infect Dis 197, 916-923.

Cestari, I., Evans-Osses, I., Schlapbach, L.J., de Messias-Reason, I., Ramirez, M.I., 2013. Mechanisms of complement lectin pathway activation and resistance by trypanosomatid parasites. Mol Immunol 53, 328-334.

Colditz, I.G., 2008. Six costs of immunity to gastrointestinal nematode infections. Parasite Immunol 30, 63-70.

Cuesta, A., Muñoz, P., Rodríguez, A., Salinas, I., Sitjà-Bobadilla, A., Álvarez-Pellitero, P., Esteban, M.A., Meseguer, J., 2006. Gilthead seabream (*Sparus aurata* L.) innate defence against the parasite *Enteromyxum leei* (Myxozoa). Parasitology 132, 95-104.

Dahl, M.R., Thiel, S., Matsushita, M., Fujita, T., Willis, A.C., Christensen, T., Vorup-Jensen, T., Jensenius, J.C., 2001. MASP-3 and its association with distinct complexes of the mannan-binding lectin complement activation pathway. Immunity 15, 127–135.

Davey, G.C., Calduch-Giner, J.A., Houeix, B., Talbot, A., Sitjà-Bobadilla, A., Prunet, P., Pérez-Sánchez, J., Cairns, M.T., 2011. Molecular profiling of the gilthead sea bream (*Sparus aurata* L.) response to chronic exposure to the myxosporean parasite *Enteromyxum leei*. Mol Immunol 48, 2102-2112.

Edgar, R., Domrachev, M., Lash, A.E., 2002. Gene Expression Omnibus: NCBI gene expression and hybridization data repository. Nucleic Acids Res 30, 207–210.

Estensoro, I., Benedito-Palos, L., Palenzuela, O., Kaushik, S., Sitjà-Bobadilla, A., Pérez-Sánchez, J., 2011. The nutritional background of the host alters the disease course in a fish–myxosporean system. Vet Parasitol 175, 141-150.

Evans-Osses, I., Ansa-Addo, E.A., Inal, J.M., Ramirez, M.I., 2010. Involvement of lectin pathway activation in the complement killing of *Giardia intestinalis*. Biochem Biophys Res Commun 395, 382-386.

Fujino, S., Andoh, A., Bamba, S., Ogawa, A., Hata, K., Araki, Y., Bamba, T., Fujiyama, Y., 2003. Increased expression of interleukin 17 in inflammatory bowel disease. Gut 52, 65-70.

Fujita, T., Matsushita, M., Endo, Y., 2004. The lectin-complement pathway--its role in innate immunity and evolution. Immunol Rev 198, 185-202.

Hajela, K., Kojima, M., Ambrus, G., Wong, K.H., Moffatt, B.E., Ferluga, J., Hajela, S., Gal, P., Sim, R.B., 2002. The biological functions of MBL-associated serine proteases (MASPs). Immunobiology 205, 467–475.

Gadjeva, M., Takahashi, K., Thiel, S., 2004. Mannan-binding lectin–a soluble pattern recognition molecule. Mol Immunol 41, 113–121.

Gainey, M.D., Rivenbark, J.G., Cho, H., Yang, L., Yokoyama, W.M., 2012. Viral MHC class I inhibition evades CD8+ T-cell effector responses in vivo but not CD8+ T-cell priming. Proc Natl Acad Sci USA 109, E3260–E3267.

Ganz, T., 2002. The role of hepcidin in iron sequestration during infections and in the pathogenesis of anemia of chronic disease. Isr Med Assoc J 4, 1043-1045.

Ganz, T., 2011. Hepcidin and iron regulation, 10 years later. Blood 117, 4425-4433.

Geijtenbeek, T.B., Torensma, R., van Vliet, S.J., van Duijnhoven, G.C., Adema, G.J., van Kooyk, Y., Figdor, C.G., 2000. Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. Cell 100, 575-585.

Glass, E.J., 2012. The molecular pathways underlying host resistance and tolerance to pathogens. Front Genet 3, 263.

Gruden-Movsesijan, A., Petrovic, M., Sofronic-Milosavljevic, L., 2003. Interaction of mannan-binding lectin with *Trichinella spiralis* glycoproteins, a possible innate immune mechanism. Parasite Immunol 25, 545-552.

Hansen, R., Thomson, J.M., El-Omar, E.M., Hold, G.L., 2010. The role of infection in the aetiology of inflammatory bowel disease. J Gastroenterol 45, 266-276.

Hayes, J., Volkoff, H., 2014. Characterization of the endocrine, digestive and morphological adjustments of the intestine in response to food deprivation and torpor in cunner, Tautogolabrus adspersus. Comp Biochem Phys A 170, 46-59.

Hermida, M., Bouza, C., Fernández, C., Sciara, A.A., Rodríguez-Ramilo, S.T., Fernández, J., Martínez, P., 2013. Compilation of mapping resources in turbot (*Scophthalmus maximus*): A new integrated consensus genetic map. Aquaculture 414–415, 19-25.

Hu, Y.-h., Zhang, M., Sun, L., 2010. Expression of *Scophthalmus maximus* CD83 correlates with bacterial infection and antigen stimulation. Fish Shellfish Immunol 29, 608-614.

James, E.R., Green, D.R., 2004. Manipulation of apoptosis in the host parasite interaction. Trends Parasitol 20, 280-287.

Kania, P.W., Sorensen, R.R., Koch, C., Brandt, J., Kliem, A., Vitved, L., Hansen, S., Skjodt, K., 2010. Evolutionary conservation of mannan-binding lectin (MBL) in bony fish: identification, characterization and expression analysis of three bona fide collectin homologues of MBL in the rainbow trout (*Onchorhynchus mykiss*). Fish Shellfish Immunol 29, 910-920.

Keklikoglu, N., Koray, M., Kocaelli, H., Akinci, S., 2008. iNOS expression in oral and gastrointestinal tract mucosa. Dig Dis Sci 53, 1437-1442.

Keller, A.D., Maniatis, T., 1991. Identification and characterization of a novel repressor of beta-interferon gene expression. Genes Dev 5, 868-879.

Kelly, M.N., Kolls, J.K., Happel, K., Schwartzman, J.D., Schwarzenberger, P., Combe, C., Moretto, M., Khan, I.A., 2005. Interleukin-17/interleukin-17 receptor-mediated signaling is important for generation of an optimal polymorphonuclear response against *Toxoplasma gondii* infection. Infect Immun 73, 617-621.

Kim, D., Pertea, G., Trapnell, C., Pimentel, H., Kelley, R., Salzberg, S., 2013. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol 14, R36.

Kyriazakis, I., Tolkamp, B.J., Hutchings, M.R., 1998. Towards a functional explanation for the occurrence of anorexia during parasitic infections. Anim Behav 56, 265-274.

Langmead, B., Salzberg, S.L., 2012. Fast gapped-read alignment with Bowtie 2. Nat Meth 9, 357-359.

Le Bon, A., Tough, D.F., 2002. Links between innate and adaptive immunity via type I interferon. Curr Opin Immunol 14, 432-436.

Lee, C., Chun, J., Hwang, S.W., Kang, S.J., Im, J.P., Kim, J.S., 2014. The effect of intestinal alkaline phosphatase on intestinal epithelial cells, macrophages and chronic colitis in mice. Life Sci 100, 118-124.

Lenaerts, K., Sokolovic, M., Bouwman, F.G., Lamers, W.H., Mariman, E.C., Renes, J., 2006. Starvation induces phase-specific changes in the proteome of mouse small intestine. J Proteome Res 5, 2113-2122.

Lohse, M., Bolger, A.M., Nagel, A., Fernie, A.R., Lunn, J.E., Stitt, M., Usadel, B., 2012. RobiNA: a user-friendly, integrated software solution for RNA-Seq-based transcriptomics. Nucleic Acids Res 40, W622-W627.

Losada, A.P., Bermúdez, R., Faílde, L.D., Quiroga, M.I., 2012. Quantitative and qualitative evaluation of iNOS expression in turbot (*Psetta maxima*) infected with *Enteromyxum scophthalmi*. Fish Shellfish Immunol 32, 243-248.

Losada, A.P., 2013. Enteromixosis del rodaballo. Esclarecimiento de mecanismos patogénicos y rutas de respuesta inmunitaria. PhD Thesis, Ciencias Clínicas Veterinarias. Universidad de Santiago de Compostela.

Losada, A.P., Bermúdez, R., Faílde, L.D., de Ocenda, M.V.R., Quiroga, M.I., 2014. Study of the distribution of active caspase-3-positive cells in turbot, *Scophthalmus maximus* (L.), enteromyxosis. J Fish Dis 37, 21-32.

Lovegrove, F.E., Gharib, S.A., Patel, S.N., Hawkes, C.A., Kain, K.C., Liles, W.C., 2007. Expression microarray analysis implicates apoptosis and interferon-responsive mechanisms in susceptibility to experimental cerebral malaria. Am J Pathol 171, 1894-1903.

Lugo-Villarino, G., Balla, K.M., Stachura, D.L., Bañuelos, K., Werneck, M.B.F., Traver, D., 2010. Identification of dendritic antigen-presenting cells in the zebrafish. Proc Natl Acad Sci USA 107, 15850-15855.

Marchiando, A.M., Shen, L., Graham, W.V., Edelblum, K.L., Duckworth, C.A., Guan, Y., Montrose, M.H., Turner, J.R., Watson, A.J., 2011. The epithelial barrier is maintained by in vivo tight junction expansion during pathologic intestinal epithelial shedding. Gastroenterology 140, 1208-1218 e1201-1202.

Marioni, J.C., Mason, C.E., Mane, S.M., Stephens, M., Gilad, Y., 2008. RNA-seq: An assessment of technical reproducibility and comparison with gene expression arrays. Genome Res 18, 1509-1517.

McCall, M.B.B., Sauerwein, R.W., 2010. Interferon-γ—central mediator of protective immune responses against the pre-erythrocytic and blood stage of malaria. J Leukoc Biol 88, 1131-1143.

Millán, A., Gómez-Tato, A., Pardo, B.G., Fernández, C., Bouza, C., Vera, M., Álvarez-Dios, J.A., Cabaleiro, S., Lamas, J., Lemos, M.L., Martínez, P., 2011. Gene expression profiles of the spleen, liver, and head kidney in turbot (*Scophthalmus maximus*) along the infection process with *Aeromonas salmonicida* using an immune-enriched oligo-microarray. Mar Biotechnol (NY) 13, 1099-1114.

Morozova, O., Hirst, M., Marra, M.A., 2009. Applications of new sequencing technologies for transcriptome analysis. Annu Rev Genom Hum Genet 10, 135-151.

Neves, J.V., Caldas, C., Wilson, J.M., Rodrigues, P.N.S., 2011. Molecular mechanisms of hepcidin regulation in sea bass (*Dicentrarchus labrax*). Fish Shellfish Immunol 31, 1154-1161.

Nielsen, R., Paul, J.S., Albrechten, A., Song, Y.S., 2011. Genotype and SNP calling from next-generation sequencing data. Nat Rev Genet, 12, 443–51.

Okunishi, K., Dohi, M., Nakagome, K., Tanaka, R., Mizuno, S., Matsumoto, K., Miyazaki, J., Nakamura, T., Yamamoto, K., 2005. A novel role of hepatocyte growth factor as an immune regulator through suppressing dendritic cell function. J Immunol 175, 4745-4753.

Palenzuela, O., López -Grandal, E., Zarza, C., Álvarez-Pellitero, P., 2009. Treatment of turbot enteromyxosis with antiparasitic drugs and bioactive natural extracts-supplemented feeds, 14th International Conference of the European Association of Fish Pathologists (EAFP) on Diseases of Fish and Shellfish, Prague, Czech Republic, pp. 142-143.

Panaro, M.A., Cianciulli, A., Mitolo, V., Mitolo, C.I., Acquafredda, A., Brandonisio, O., Cavallo, P., 2007. Caspase-dependent apoptosis of the HCT-8 epithelial cell line induced by the parasite *Giardia intestinalis*. FEMS Immunol Med Microbiol 51, 302-309.

Pardo, B.G., Millán, A., Gómez-Tato, A., Fernández, C., Bouza, C., Álvarez-Dios, J.A., Cabaleiro, S., Lamas, J., Leiro, J.M., Martínez, P., 2012. Gene expression profiles of spleen, liver, and head kidney in turbot (*Scophthalmus maximus*) along the infection process with *Philasterides dicentrarchi* using an immune-enriched oligo-microarray. Mar Biotechnol (NY) 14, 570-582.

Peck, A., Mellins, E.D., 2010. Precarious balance: Th17 cells in host defense. Infect Immun 78, 32-38.

Pereiro, P., Figueras, A., Novoa, B., 2012. A novel hepcidin-like in turbot (*Scophthalmus maximus* L.) highly expressed after pathogen challenge but not after iron overload. Fish Shellfish Immunol 32, 879-889.

Pérez-Cordón, G., Estensoro, I., Benedito-Palos, L., Calduch-Giner, J.A., Sitjà-Bobadilla, A., Pérez-Sánchez, J., 2014. Interleukin gene expression is strongly modulated at the local level in a fish–parasite model. Fish Shellfish Immunol 37, 201-208.

Pérez-Jiménez, A., Cardenete, G., Hidalgo, M.C., García-Alcázar, A., Abellán, E., Morales, A.E., 2012. Metabolic adjustments of *Dentex dentex* to prolonged starvation and refeeding. Fish Physiol Biochem 38, 1145-1157.

Qian, X., Ba, Y., Zhuang, Q., Zhong, G., 2014. RNA-Seq technology and its application in fish transcriptomics. OMICS 18, 98-110.

Rappocciolo, G., Birch, J., Ellis, S.A., 2003. Down-regulation of MHC class I expression by equine herpesvirus-1. J Gen Virol 84, 293-300.

Rauta, P.R., Nayak, B., Das, S., 2012. Immune system and immune responses in fish and their role in comparative immunity study: a model for higher organisms. Immunol Lett 148, 23-33.

Redondo, M.J., Palenzuela, O., Álvarez-Pellitero, P., 2004. Studies on transmission and life cycle of *Enteromyxum scophthalmi* (Myxozoa), an enteric parasite of turbot *Scophthalmus maximus*. Folia Parasitol 51, 188-198.

Redondo, M.J., Cortadellas, N., Palenzuela, O., Álvarez-Pellitero, P., 2008. Detection of carbohydrate terminals in the enteric parasite *Enteromyxum scophthalmi* (Myxozoa) and possible interactions with its fish host *Psetta maxima*. Parasitol Res 102, 1257-1267.

Redondo, M.J., Álvarez-Pellitero, P., 2010. The effect of lectins on the attachment and invasion of *Enteromyxum scophthalmi* (Myxozoa) in turbot (*Psetta maxima* L.) intestinal epithelium in vitro. Exp Parasitol 126, 577-581.

Ribas, L., Pardo, B.G., Fernández, C., Álvarez-Dios, J.A., Gómez-Tato, A., Quiroga, M.I., Planas, J.V., Sitjà-Bobadilla, A., Martínez, P., Piferrer, F., 2013. A combined strategy involving Sanger and 454 pyrosequencing increases genomic resources to aid in the management of reproduction, disease control and genetic selection in the turbot (*Scophthalmus maximus*). BMC Genomics 14, 180.

Robinson, M., Oshlack, A., 2010. A scaling normalization method for differential expression analysis of RNA-seq data. Genome Biology 11, R25.

Rodríguez-Ramilo, S.T., Fernández, J., Toro, M.A., Bouza, C., Hermida, M., Fernández, C., Pardo, B.G., Cabaleiro, S., Martínez, P., 2013. Uncovering QTL for resistance and survival time to *Philasterides dicentrarchi* in turbot (*Scophthalmus maximus*). Anim Genet 44, 149-157.

Sahu, A., Lambris, J.D., 2001. Structure and biology of complement protein C3, a connecting link between innate and acquired immunity. Immunol Rev 180, 35-48.

Salem, M., Silverstein, J., Rexroad, C.E., 3rd, Yao, J., 2007. Effect of starvation on global gene expression and proteolysis in rainbow trout (*Oncorhynchus mykiss*). BMC Genomics 8, 328.

Schroder, K., Hertzog, P.J., Ravasi, T., Hume, D.A., 2004. Interferon-gamma: an overview of signals, mechanisms and functions. J Leukoc Biol 75, 163-189.

Sitjà-Bobadilla, A., Redondo, M.J., Bermúdez, R., Palenzuela, O., Ferreiro, I., Riaza, A., Quiroga, I., Nieto, J.M., Álvarez-Pellitero, P., 2006. Innate and adaptive immune responses of turbot, *Scophthalmus maximus* (L.), following experimental infection with *Enteromyxum scophthalmi* (Myxosporea: Myxozoa). Fish Shellfish Immunol 21, 485-500.

Sitjà-Bobadilla, A., Palenzuela, O., Riaza, A., Macias, M.A., Álvarez-Pellitero, P., 2007. Protective acquired immunity to *Enteromyxum scophthalmi* (Myxozoa) is related to specific antibodies in *Psetta maxima* (L.) (Teleostei). Scand J Immunol 66, 26-34.

Sitjà-Bobadilla, A., Palenzuela, O., 2012. Enteromyxum Species, in: Woo, P.T.K., Buchmann, K. (Eds.), Fish Parasites: Pathobiology and Protection. CABI publishing, UK, pp. 163-176.

Song, W., Kao, W., Zhai, A., Qian, J., Li, Y., Zhang, Q., Zhao, H., Hu, Y., Li, H., Zhang, F., 2013. Borna disease virus nucleoprotein inhibits type I interferon induction through the interferon regulatory factor 7 pathway. Biochem Biophys Res Commun 438, 619-623.

Srivastava, S., Pandey, S.P., Jha, M.K., Chandel, H.S., Saha, B., 2013. *Leishmania* expressed lipophosphoglycan interacts with Toll-like receptor (TLR)-2 to decrease TLR-9 expression and reduce anti-leishmanial responses. Clin Exp Immunol 172, 403-409.

Suzuki, R., Shimodaira, H., 2006. Pvclust: an R package for assessing the uncertainty in hierarchical clustering. Bioinformatics 22, 1540–1542.

Tizard, I.R., 2009. Veterinary Immunology: An Introduction, 8th Edition. CBS Publishers & Distributors, India.

van de Veerdonk, F.L., Gresnigt, M.S., Kullberg, B.J., van der Meer, J.W., Joosten, L.A., Netea, M.G., 2009. Th17 responses and host defense against microorganisms: an overview. BMB Rep 42, 776-787.

van Vliet, S.J., García-Vallejo, J.J., van Kooyk, Y., 2008. Dendritic cells and C-type lectin receptors: coupling innate to adaptive immune responses. Immunol Cell Biol 86, 580-587.

Vázquez-Mendoza, A., Carrero, J.C., Rodríguez-Sosa, M., 2013. Parasitic infections: a role for C-type lectins receptors. Biomed Res Int 2013, 456352.

Vincent, I.E., Zannetti, C., Lucifora, J., Norder, H., Protzer, U., Hainaut, P., Zoulim, F., Tommasino, M., Trépo, C., Hasan, U., Chemin, I., 2011. Hepatitis B virus impairs TLR9 expression and function in plasmacytoid dendritic cells. PLoS One 6, e26315.

Wang, Z., Gerstein, M., Snyder, M., 2009. RNA-Seq: a revolutionary tool for transcriptomics. Nat Rev Genet 10, 57-63.

Watson, A.J., Hughes, K.R., 2012. TNF-alpha-induced intestinal epithelial cell shedding: implications for intestinal barrier function. Ann N Y Acad Sci 1258, 1-8.

Wykes, L.J., Fiorotto, M., Burrin, D.G., Del Rosario, M., Frazer, M.E., Pond, W.G., Jahoor, F., 1996. Chronic low protein intake reduces tissue protein synthesis in a pig model of protein malnutrition. J Nutr 126, 1481-1488.

Wyllie, A.H., 2010. "Where, o death, is thy sting?" A brief review of apoptosis biology. Mol Neurobiol.42, 4-9.

Wynne, J.W., O'Sullivan, M.G., Cook, M.T., Stone, G., Nowak, B.F., Lovell, D.R., Elliott, N.G., 2008. Transcriptome analyses of amoebic gill disease-affected Atlantic salmon (*Salmo salar*) tissues reveal localized host gene suppression. Mar Biotechnol (NY) 10, 388-403.

Young, N.D., Cooper, G.A., Nowak, B.F., Koop, B.F., Morrison, R.N., 2008. Coordinated down-regulation of the antigen processing machinery in the gills of amoebic gill disease-affected Atlantic salmon (*Salmo salar* L.). Mol Immunol 45, 2581-2597.

Zenewicz, L.A., Yancopoulos, G.D., Valenzuela, D.M., Murphy, A.J., Stevens, S., Flavell, R.A., 2008. Innate and adaptive interleukin-22 protects mice from inflammatory bowel disease. Immunity 29, 947-957.

Zhi-Hui, H., Ai-Jun, M., Xin-An, W., Ji-Lin, L., The interaction of temperature, salinity and body weight on growth rate and feed conversion rate in turbot (*Scophthalmus maximus*). Aquaculture 432, 237-242.

Zou, J., Secombes, C.J., 2011. Teleost fish interferons and their role in immunity. Dev Comp Immunol 35, 1376-1387.

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ARTÍCULO IV: RNA-SEQ ANALYSIS OF EARLY ENTEROMYXOSIS IN TURBOT (*SCOPHTHALMUS MAXIMUS*): NEW INSIGHTS INTO PARASITE INVASION AND IMMUNE EVASION STRATEGIES

Ronza, P.[1*]; Robledo, D.[2*]; Bermúdez, R.[3]; Losada, A.P.[1]; G. Pardo, B.[4]; Sitjà-Bobadilla, A.[5]; Quiroga, M.I.[1]; Martínez, P.[4]

[1]Departamento de Ciencias Clínicas Veterinarias, Facultad de Veterinaria, Universidade de Santiago de Compostela, 27002 Lugo.

[2]Departamento de Genética, Facultad de Biología (CIBUS), Universidade de Santiago de Compostela, 15782 Santiago de Compostela.

[3]Departamento de Anatomía y Producción Animal, Facultad de Veterinaria, Universidade de Santiago de Compostela, 27002 Lugo.

[4]Departamento de Genética, Facultad de Veterinaria, Universidade de Santiago de Compostela, 27002 Lugo.

[5]Instituto de Acuicultura Torre de la Sal (IATS-CSIC), 12595 Ribera de Cabanes, Castellón.

*Primera autoría compartida.

Nota: El material suplementario asociado a este trabajo está disponible en el CD adjunto al ejemplar de Tesis.

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Abstract

Enteromyxum scophthalmi, an intestinal myxozoan parasite, is the causative agent of a threatening disease for turbot (*Scophthalmus maximus*, L.) aquaculture. The colonization of the digestive tract by this parasite leads to a cachectic syndrome associated with high morbidity and mortality rates. This myxosporidiosis has a long pre-patent period and the first detectable clinical and histopathological changes are subtle. The pathogenic mechanisms acting in the early stages of infection are still far from being fully understood. Further information on the host-parasite interaction is needed to assist in finding efficient preventive and therapeutic measures. Here, a RNA-seq-based transcriptome analysis of head kidney, spleen and pyloric caeca from experimentally-infected and control turbot was performed. Only infected fish with early signs of infection, determined by histopathology and immunohistochemical detection of *E. scophthalmi*, were selected. The RNA-seq analysis revealed, as expected, less intense transcriptomic changes than those previously found at late stages of the disease. Several genes involved in interferon-related pathways were up-regulated in the three organs, suggesting that interferon-mediated immune response plays a main role in this phase of the disease. Interestingly, an opposite expression pattern had been found in a previous study on severely infected turbot. In addition, possible strategies for immune system evasion were suggested by the down-regulation of different genes encoding complement components and acute phase proteins. At the site of infection (pyloric caeca), modulation of genes related to different structural proteins was detected and the expression profile indicated the inhibition of cell proliferation and differentiation. These transcriptomic changes provide indications regarding the parasite's mechanisms of host attachment and invasion. The current results contribute to a better knowledge of the events that characterize the early stages of turbot enteromyxosis and provide valuable information to identify molecular markers for early detection and control of this important parasitosis.

Keywords: RNA-seq, transcriptome, turbot, Enteromyxum scophthalmi, Myxozoa, pathogenesis

1. Introduction

Turbot (*Scophthalmus maximus*, L.) is an appreciated cultured marine flatfish, whose production in 2013 accounts for over 77,000 tons, being China (67,000 tons in 2013) and the European Union (7,700 tons in 2013, 11,000 in 2014) the main producers (APROMAR 2015). Enteromyxosis caused by *Enteromyxum scophthalmi* (Myxozoa) is a serious threat for turbot aquaculture, currently without effective therapeutic measures (Sitjà-Bobadilla and Palenzuela 2012). The target site of this myxozoan parasite is the gastrointestinal tract, where it proliferates and spreads from the anterior intestine and pyloric caeca to other gut regions (Redondo *et al.* 2004). The infection leads to severe catarrhal gastroenteritis associated to a cachectic syndrome, with reduction of growth performance and high mortality rates (Bermúdez *et al.,* 2010; Sitjà-Bobadilla and Palenzuela 2012). Under culture conditions, the trophozoites are transmitted directly from fish to fish, which leads to a fast spread of the disease in infected tanks and facilities (Redondo *et al.* 2002; Quiroga *et al.* 2006; Sitjà-

Bobadilla and Palenzuela 2012). However, the disease shows a long pre-patent period, being the parasite detectable in the digestive tract by histology only after several weeks in natural infections (Redondo *et al.* 2004; Quiroga *et al.* 2006;). In experimental infections by effluent transmission or cohabitation, the parasite is firstly observed at around 20 days post-exposure and at around 8 days after experimental *per os* transmission (Redondo *et al.* 2004; Bermúdez *et al.* 2006; Sitjà-Bobadilla *et al.* 2006; Losada *et al.* 2014a). Experimental infection by oral route results in a very high and quick prevalence of infection and homogeneous lesions in recipient fish. In addition, the ingestion of trophozoites released from infected fish is thought to be the main infection route occurring in fish farm (Redondo *et al.* 2002, 2004). In the early stages of infection there are no external clinical signs, histological lesions are very subtle, and the parasite is hard to detect in conventional histological sections of the digestive tract (Quiroga *et al.* 2006; Bermúdez *et al.* 2010). *In vitro*, *E. scophthalmi* is able to penetrate the intestinal epithelium from the lumen as well as via the basement membrane, and the report of parasitic stages in blood smears suggests the existence of a haematic route of spread (Redondo *et al.* 2003, 2004; Redondo and Álvarez-Pellitero 2010). However, a detailed comprehension of entry routes and epithelial invasion strategies is lacking. We are still far from a full knowledge of the host-parasite interaction and further investigation is needed to clarify the pathogenetic mechanisms of enteromyxosis (Sitjà-Bobadilla and Palenzuela 2012; Robledo *et al.* 2014), especially those acting at early infection.

Whole-transcriptome analysis using RNA-seq is a suitable approach for the identification of the genes and pathways involved in host-pathogen interaction, and it is of major support for understanding the pathogenesis of human and veterinary diseases (Costa *et al.* 2013; Qian et *et al.* 2014; Li *et al.* 2015). This is an essential starting point for the development of control measures, therapeutic options and genetic breeding programs. An RNA-seq analysis of turbot experimentally infected by oral route was previously addressed, investigating the advanced stages of the disease, by studying specimens at 42 days post-inoculation (dpi). That work enabled a better understanding of the genetic basis of the clinical signs and lesions which characterize the infection (Robledo *et al.* 2014). In this study, using a similar methodological approach, we performed a transcriptome analysis of turbot showing very early signs of infection aimed to contribute to the current understanding of incipient enteromyxosis.

2. Materials and methods

2.1. Experimental design

The experimental setup and sampling were as previously described (Robledo *et al.* 2014). Briefly, infection was achieved by oral route (Redondo *et al.* 2002) and tissue samples were collected in Bouin's fluid and RNAlater for histological techniques and RNA-seq, respectively. A histological evaluation was performed, and infected turbot were classified into three groups (slightly, moderately and severely infected) according to the histopathological grading described by Bermúdez *et al.* (Bermúdez *et al.* 2010). For RNA-seq analysis, spleen, head kidney and pyloric caeca from 3 control (CTRL) and 3 *E. scophthalmi*-infected (recipient, RCPT) fish at 24 dpi were used. The 3 RCPT fish were selected by histology

among those graded as slightly infected and numbered (infected turbot 1, 2 and 3). RNA aliquots from the samples of RCPT fish were sequenced individually, while samples from CTRL fish were pooled by organ, resulting in 3 RCPT and 1 CTRL sample per organ.

2.2. Immunohistochemistry

Immunohistochemical detection of *E. scophthalmi* was performed on sections from different regions of the digestive tract (oesophagus, stomach, pyloric caeca, anterior, middle and posterior intestine) to ensure the presence of the parasite. Thin sections (3 µm) were placed on slides treated with silane to improve section adherence and dried overnight at 37 ºC. After deparaffination (two 5 min-washes in xylene) and rehydration (graded alcohol series), the endogenous peroxidase activity was inhibited by incubating the slides with peroxidase-blocking solution (Dako) during 40 min. A 2 h incubation at room temperature was performed with a polyclonal antibody against *E. scophthalmi* (Estensoro *et al.* 2014) (diluted 1: 50 000). The secondary antibody conjugated with peroxidase was the anti-rabbit EnVision+ System Labelled Polymer-HRP (Dako) for 30 min, followed by developing with diaminobenzidine (Dako). All incubations were performed in humid chambers and three 5 min-washes with 0.01 M phosphate-buffered saline (PBS) were carried out between all subsequent steps. Sections of severely infected turbot were employed as positive controls. In the sections included as negative controls, the primary antibody was replaced by antibody diluents.

2.3. RNA-seq and differential expression analysis

Some of the procedures and methodologies employed were previously described (Robledo *et al.* 2014). Briefly, RNA extraction was performed using the RNeasy mini kit (Qiagen) with DNase treatment and RNA quality and quantity were evaluated in a Bioanalyzer (Bonsai Technologies) and in a NanoDrop® ND-1000 spectrophotometer (NanoDrop® Technologies Inc), respectively. The samples were barcoded and prepared for sequencing by the Wellcome Trust Centre for Human Genetics, Oxford, and sequenced on an Illumina HiSeq 2000 as 100 bp paired-end reads. All the data files have been deposited in the NCBI Short Read Archive (SRA) database under the project ID PRJNA300347, as well as the generated transcriptome sequences and their annotation have been deposited in Mendeley Data (https://data.mendeley.com/) and can be accessed using doi: 10.17632/3vhc8py3cv.2. Quality filtering and removal of residual adaptor sequences was conducted using Trimmomatic v.0.32 (Bolger *et al.* 2014). The recently assembled turbot genome (Figueras *et al.* 2016) was used as a reference for read mapping. Filtered reads were mapped to the genome using Tophat2 v.2.0.11 (Kim *et al.* 2013) that leverages the short read aligner Bowtie2 v.2.2.3 (Langmead and Salzberg, 2012) with a maximum intron length of 20 kb. HTSeq-count (http://www-huber.embl.de/users/anders/HTSeq/doc/overview.html webcite) was used to extract the raw reads from the mapping files and differentially expressed genes were obtained using EdgeR (Robinson and Oshlack 2010) with a False Discovery Rate (FDR) corrected *p*-value of 0.05. The differentially expressed (DE) genes were identified and annotated using Blast2GO v.2.7.0 (Conesa *et al.* 2005) with an E-value cutoff of E^{-6}. Enriched GO terms for each organ were identified by comparing the DE genes against the full transcriptome using Blast2GO Fisher's exact test ($p < 0.05$, FDR corrected). Furthermore, in this study, KEGG enrichment was

assessed using KOBAS 2.0 (Wu et al., 2006) ($p < 0.05$, FDR corrected) with the draft turbot genome annotation as background. Those reads from pyloric caeca samples which did not align against the genome, both from turbot of this study (at 24 dpi) and from a previous study with parasitized turbot at 42 dpi (Robledo *et al.* 2014), were extracted and *de novo* transcriptome assembly was carried out using ABySS (version 1.3.7; Simpson *et al.* 2009) with a 64 k-mer size, scaffolding and contig options on, and remaining parameters by default. Expression values were individually estimated for each pyloric caeca sample by counting reads for each transcript after aligning the genome-unaligned reads of the sample against the reconstructed *de novo* transcriptome using RSEM v.1.2.17 (Li and Dewey 2011). Differential expression between confronted and control groups was estimated using EdgeR (FDR corrected *p*-value < 0.05; Robinson and Oshlack 2010).

3. Results and discussion

3.1. Histopathology revealed minor tissue alterations and *E. scophthalmi* presence

The histological evaluation of RCPT turbot revealed minor alterations at intestinal level. Slight inflammatory infiltrates, mostly composed of mononuclear cells, were occasionally detected in the lamina propria-submucosa or at the basis of the lining epithelium of pyloric caeca and anterior intestine (Figure 1a, b, d). In these areas, basophilic structures consistent with early stages of the parasite were observed (Figure 1a, b). The specimen labelled as "infected turbot 2" also presented some trophozoites in the hindgut, and was the only fish which sporadically showed more advanced developmental stages (ST2 or ST3, Redondo et al., 2004) of *E. scophthalmi* (Figure 1d, e). The histological features of the 3 RCPT fish were in accordance with the "slight infection" degree described by Bermúdez et al. (2010). No significant changes were detected in the remaining examined organs, neither in CTRL fish. The presence of parasitic stages was then confirmed by immunohistochemistry (Figure 1c, f), supporting the histological observations.

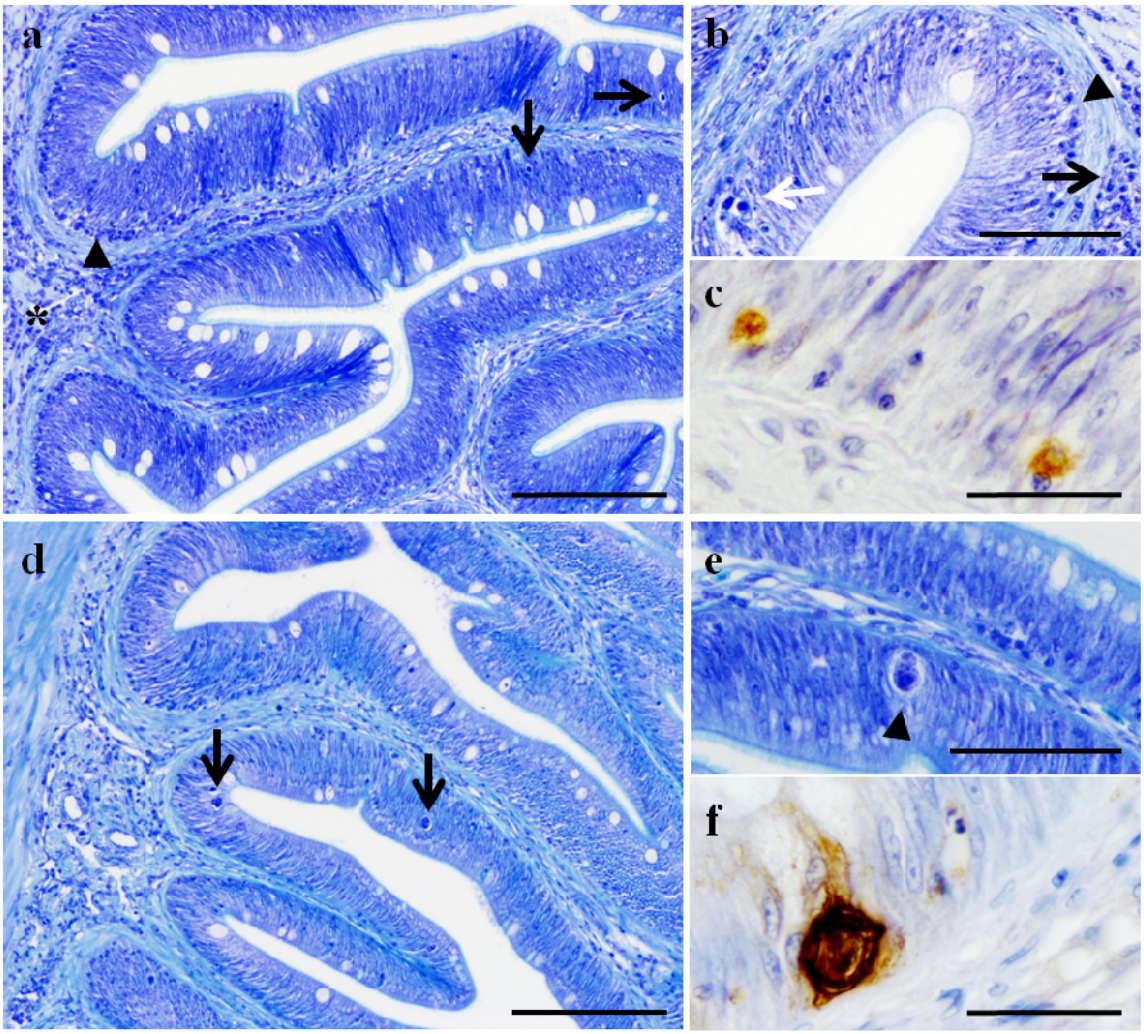

Figure 1. Pyloric caeca histopathology (a, b, d, e; stained with toluidine blue) and immunohistochemistry (c, f). a) Note the slight inflammatory infiltration at the basal part of the lining epithelium (arrowhead) and in the lamina propria-submucosa (asterisk). Also, round basophilic structures can be appreciated into the lining epithelium of an intestinal fold (black arrows). Scale bar = 100 µm. b) Higher magnification of pyloric caeca showing the infiltration of mononuclear cells in the basal part of the epithelium (arrowhead) and the lamina propria-submucosa (black arrow). Note the round basophilic structures near the basement membrane of the lining epithelium (white arrow), consistent with early stages of *E. scophthalmi*. Scale bar = 50 µm. c) Immunohistochemical detection of two early stages of *E. scophthalmi* (brown colored) in the basal part of the epithelium. Scale bar = 20 µm. d, e, f) Histological section from the pyloric caeca of the infected turbot 2. d) Note the presence of two parasitic structures in the lining epithelium, associated to a very mild inflammatory infiltration. Scale bar = 100 µm. e) Higher magnification showing a trophozoite (arrowhead), consistent with a developmental stage 3 of *E. scophthalmi*. Scale bar = 50 µm. f) Immunostaining of a parasitic structure with the polyclonal antibody against *E. scophthalmi*. Scale bar = 20 µm.

3.2. Pyloric caeca showed the higher percentages of unaligned reads

A total of ~170 million 100 bp pair-end reads were sequenced, the same amount as in the previous work with severely infected turbot, accounting on average for 13.3 million reads post-filtering per sample, slightly below the 15 million reads per sample formerly obtained (Robledo *et al.* 2014). A total of 138 million (86.5%) of the filtered reads (~160 million) were mapped to the turbot genome. A notable difference was found between this and the previous study when comparing the result of the alignments in pyloric caeca. In slightly infected turbot, 90% of the trimmed reads aligned to the genome, while in severely infected turbot only 65%

aligned (Robledo *et al*. 2014). The unaligned reads of both 24 and 42 dpi samples from pyloric caeca were used for reconstructing a *de novo* transcriptome (a brief comparison between genome-guided and *de novo* assemblies is shown in Table 1), and differential expression analysis between infected and control samples was carried out. No sequences annotated to *Enteromyxum* spp. were detected among differentially expressed (DE) genes at 24 dpi, but six transcripts, annotated as *Enteromyxum scophthalmi* 18S subunit ribosomal gene, and two more as *Enteromyxum leei* 28s subunit ribosomal gene, were found DE at 42 dpi. The fact that no DE *Enteromyxum* sequences were found at early stages of the disease suggests that the concentration of the parasite in pyloric caeca at that stage is low. On the other hand, another 422 up-regulated sequences were found in pyloric caeca samples at 42 dpi, showing 0 or 1 read in the control, and annotated to invertebrate, plant, bacteria or fungi sequences (Supplementary Table S1). Some of these sequences may correspond to new Enteromyxum sequences and constitute a resource for exploring host-parasite interactions in future studies, while others, especially those annotated to bacteria, may probably reflect gut microbiota alterations caused by the disease.

Table 1. Comparison between genome-guided and *de novo* transcriptomes.

	Genome-guided	***De novo***
Number of reads	160 million	-
Reads mapped to the genome	138 million	-
Reads for *de novo* assembly	-	18 million*
Total transcripts	56,321	328,480
N50	5073	1510

N50= length for which the collection of all transcripts of that length or longer contains at least half of the sum of the lengths of all transcripts. *Total unaligned reads from pyloric caeca samples at 24 and 42 dpi.

3.3. Transcriptomic changes are subtle at early infection

The aligned reads resulted in a total of 56,321 transcripts from 36,356 genes. Samples were hierarchically clustered for each organ according to their transcript expression (Figure 2). Samples corresponding to the infected turbot 2 always clustered closest to the control samples, suggesting a less intense response to infection. This is a remarkable result considering that this was the specimen presenting more advanced stages of *E. scophthalmi* and more extended, and may suggest the silencing of host response at some stages of the infection or/and interindividual response variation. On the other hand, infected turbot 1 and 3 constituted a different cluster only in pyloric caeca, likely related to the stronger effect of the infection in this organ.

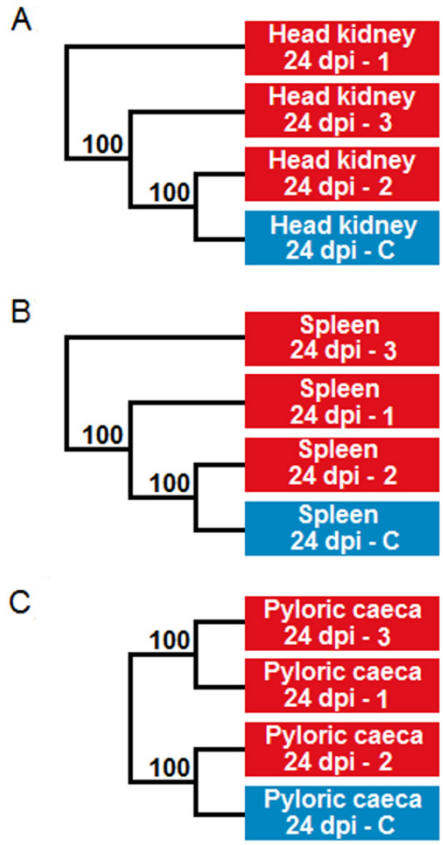

Figure 2. Hierarchical clustering of all infected and control samples for A) head kidney, B) spleen and C) pyloric caeca. Approximately unbiased *p*-values, computed by multi-scale bootstrap resampling, are displayed on branch nodes.

A total of 287, 211 and 187 DE genes were detected in head kidney, spleen and pyloric caeca, highlighting the huge transcriptomic changes between the early and the advanced stage of the disease, where these figures were, respectively, 1,316, 1,377 and 3,022 (Robledo *et al.* 2014). As previously described (Robledo *et al.* 2014), relevant DE genes were grouped in five key broad functional categories: immune and defence response, apoptosis and cell proliferation, cytoskeleton and extracellular matrix, iron metabolism and erythropoiesis, and metabolism and digestive function. Yet, in this study, DE genes related to cell differentiation were included in the category "apoptosis and cell proliferation", being renamed as "apoptosis, cell proliferation and differentiation" (Supplementary Table S2). Heatmaps of selected DE genes (Figure 3), overrepresented GO terms (Figure 4) and Venn diagrams comparing the total number of DE genes between the early and advanced stages (24 and 42 dpi, Figure 5 and Supplementary Table S3) are presented.

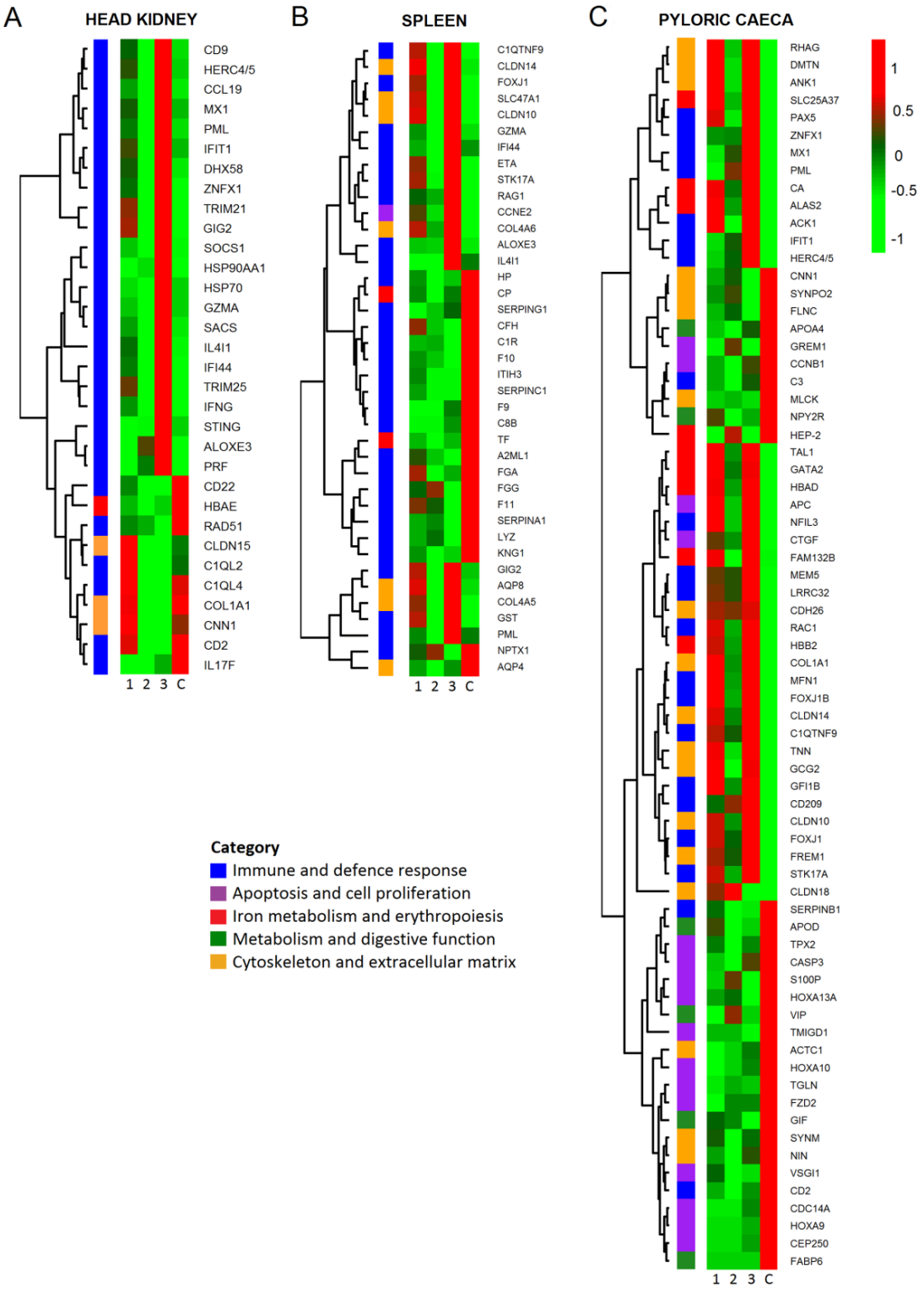

Figure 3. Heatmaps of genes of interest. Heatmaps for A) head kidney, B) spleen and C) pyloric caeca showing the expression of several genes of interest, labelling their functional category. Genes were hierarchically clustered according to their gene expression using correlation as a distance measure. 1, 2, 3 = infected turbot 1, 2, 3; C = control. Gene full names are shown in Supplementary Table S2.

3.4. Immune and defence response: possible strategies for immune evasion and activation of interferon-related pathways

Some mechanisms of innate immunity were active during both early and late stages of the disease (Robledo *et al.* 2014), like the up-regulation in kidney and spleen of *ALOXE3* (gene full names are shown in Supplementary Table S2), acting in the metabolism of leukotrienes, and *IL4I,* which participates in antigen processing. Also, *CD209*, a C-type lectin considered a marker of antigen-presenting cells, was up-regulated in pyloric caeca as in the previous study, adding new evidences about the role of this molecule in recognizing *E. scophthalmi*. In spleen, up-regulation of genes related to endothelin, a vasoconstrictor peptide and also chemoattractant of macrophages, was also found in both studies. In early enteromyxosis, other up-regulated genes acting in innate immunity were *C1QTNF9* and *GF1B* in spleen and pyloric caeca, involved in inflammatory response, and *CCL19* in head kidney*,* a chemokine with chemotactic properties on lymphocytes and dendritic cells. On the other hand, up-regulated genes associated with inhibition of immune response were also detected, like *ZNFX1* in kidney and pyloric caeca, the transcription factor *FOXJ1* in pyloric caeca and spleen and *FOXJ1B* only in pyloric caeca. In the latter organ, these genes were related to the overrepresented GO terms associated with negative regulation of immune-related processes (Figure 4).

Some complement-related genes were down-regulated in the three organs. The most remarkable result was detected in spleen, where the KEGG pathway "complement and coagulation cascades" resulted enriched due to the wide down-regulation (Supplementary Figure S1). Many products of these genes are considered acute phase proteins (APP), like the same complement components and different antiproteases. Also, other APP genes, like haptoglobin, transferrin and ceruloplasmin, related to iron metabolism and antioxidant capacity, were down-regulated in spleen. The acute phase response is an evolutionary conserved immune mechanism activated in teleost by several infectious agents, including parasites (Bayne and Gerwick 2001; Gerwick *et al.* 2002; Peatman *et al.* 2007; Khoo *et al.* 2012; Kovacevic *et al.* 2015). By contrast, in the current parasite model, the opposite pattern was detected. This may reflect a parasite-induced down-regulation as a strategy for immune system evasion or may be a temporary exhaustion of this pathway following a previous activation. The first hypothesis would agree with the pathogens targeting the complement system and host antiproteases as immune evasion strategies (Armstrong 2006; Zipfel *et al.* 2007), as well as with mechanisms for iron acquisition from host cells (Ben-Othman *et al.* 2014; Leon-Sicairos *et al.* 2015). The second hypothesis would be in accordance with previous observations in turbot exposed to *E. scophthalmi* by cohabitation (a slower infection model), where serum complement activity by the alternative pathway was slightly increased in infected fish at 20 days post exposure (dpe), but later on (40 dpe) decreased in comparison to naïve fish (Sitjà-Bobadilla *et al.* 2006). Since liver is the main producer of complement components and APP, a time series study of hepatic gene expression profile would help to clarify this response during enteromyxosis.

Another result that strongly characterized this functional category was the up-regulation of several genes related to interferon (IFN)-mediated immune response. *PML* gene, which positively regulates type I interferon response by promoting transcription of interferon-stimulated genes (ISGs) (Kim and Ahn 2015), was up-regulated in the three organs. Head kidney showed the highest number of DE genes related to IFN signalling, with an increased expression of IFN-γ, and sharing up-regulation of interferon-induced Mx protein, *HERC4/5* and *IFIT1* with pyloric caeca and of *IFI44* and interferon-inducible protein gig2 with spleen. All in all, these results point towards a response mediated by both type I and II IFNs, as observed in early stages of several mammalian protozoan infections (Beiting 2014). Also, IFN-mediated immune response was demonstrated to play a major role in teleosts parasitized by amoebae and myxozoan parasites, with implications in fish resistance or susceptibility to the disease (Young *et al.* 2008; Davey *et al.* 2011; Bjork *et al.* 2014). At advanced stages of turbot enteromyxosis, IFN-related genes were markedly down-regulated in the same organs, possibly indicating the association between the exhaustion of IFN-mediated response and the high susceptibility of turbot to enteromyxosis (Robledo *et al.* 2014). On the other hand, this opposite pattern may also suggest that the immune response to *E. scophthalmi* is differently elicited in the two stages of infection, perhaps depending on a change in the localization of the parasite during the infection.

In this sense, the up-regulation of *STING* (also called *MITA*), *DHX58* and *TRIM25* observed in head kidney and *MFN1* in pyloric caeca suggests the activation of the RIG-I-like receptors (RLRs) pathway. This pathway triggers the innate immune response against intracellular pathogens, promoting the production of type I IFNs, ISGs and proinflammatory cytokines (Dixit and Kagan 2013). RLRs activation involves the participation of mitochondria, signalled through the mitochondrial antiviral signalling protein (MAVS, also called IPS1) (Castanier *et al.* 2010; Koshiba 2013). *MFN1* mediates mitochondria fusion and encodes for a protein associated with MAVS on the outer membrane of mitochondria, being both necessary for signal transduction in the RLRs pathway through the regulation of mitochondria dynamics (Castanier *et al.* 2010; Onoguchi *et al.* 2010). This pathway is mainly known for viral recognition, but some evidence is emerging about type I IFNs production promoted by parasite-activated RLRs (Melo *et al.* 2013; Beiting 2014). Little is known about the pre-patent phase of enteromyxosis, but intracellular parasitic stages have been sporadically described (Redondo *et al.* 2003, 2004; Quiroga *et al.* 2006), so it may be hypothesized that an intracellular phase occurs during *E. scophthalmi* infection where the parasite is recognized by RLRs. Head kidney also showed increased expression of *TRIM21*, described as an intracellular antibody receptor and regulator of IFN pathways acting in viral infections (McEwan *et al.* 2013; Vaysburd *et al.* 2013; Manocha *et al.* 2014), and of *SOCS1*, which plays an evolutionary conserved inhibitory role of IFN signalling pathway (Nie *et al.* 2014). The amount of DE genes classically involved in antiviral defence showed by kidney is reflected by the correspondent enriched GO term found together with other immune-related categories (Figure 4). Both types of IFNs are also related to antigen presentation to cytotoxic cells via the major histocompatibility complex class I (MHC-I) in teleosts (Zou and Secombes 2011). In head kidney, we found up-regulation of *HSP70* and *HSP90*, which participate in the

antigen presentation via MHC-I pathway, as do calreticulin and the MHC-I genes, whose expression was significantly increased in one of the three infected fish (data not shown). Besides, the activation of natural killer (NK) and cytotoxic T-cells was reflected by the up-regulation of *GZMA* in kidney and spleen, and *PRF1* in kidney, both codifying for cytolytic proteins found in granules of these cell types. MHC-I and T-cells-related genes also showed an opposite expression pattern in the lymphohaematopoietic organs of turbot with advanced enteromyxosis (Robledo *et al.* 2014).

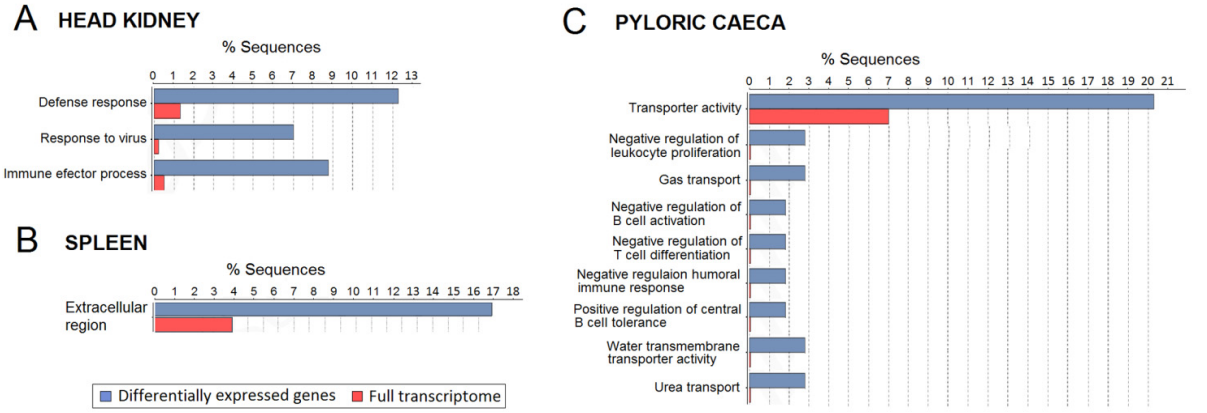

Figure 4. GO term enrichment. GO term enrichment among the differentially expressed (DE) genes for A) head kidney, B) spleen and C) pyloric caeca.

In pyloric caeca, two up-regulated genes related to T-cells were detected, *LRRC32*, a T-reg specific receptor (Tran *et al.* 2009), and *NFIL3*, a transcription factor with several important roles in immune response, like NK cells function development, interleukin 3 transcription in T-cells and regulation of Th2 cells response (Zhang *et al.* 1995; Kashiwada *et al.* 2011). On the other hand, the gene for the T-cell surface antigen CD2 was down-regulated in kidney and pyloric caeca. This molecule is present on T and NK cells, where it plays a role in cell adhesion and acts as co-stimulatory molecule for these cells. Decreased expression of *CD2* has been found associated to the infection by *Leishmania donovani* in human, causing impaired $CD4^+$ T-cell function and protective IFN-γ production (Bimal *et al.* 2008).

Concerning adaptive immunity, *RAG1*, a key gene for rearrangement and recombination of immunoglobulin and T-cell receptor molecules during the VDJ recombination process, was up-regulated in spleen. Nonetheless, little evidences were found about the activation of B cells, in accordance in previous observation about a delayed humoral response (Bermúdez *et al.* 2006; Sitjà-Bobadilla *et al.* 2006). Only pyloric caeca showed up-regulation of *PAX5*, a transcription factor with a major role in B cells differentiation, and of the immunoglobulin-related gene Ig heavy chain Mem5. Other interesting up-regulated genes in pyloric caeca, the target organ of the parasite, were *RAC1*, a member of the small GTPase family, and *ACK1*, a downstream effector of another member of this family, *CDC42*. These genes act in the c-Jun N-terminal protein kinases (JNK) pathway and are involved in actin cytoskeleton remodelling induced by extracellular signals (Chen *et al.* 2004). They have been implicated in host cell invasion by different pathogens, included protozoan parasites (Gruenheid and Finlay 2003; Chen *et al.* 2004; Lodge and Descoteaux 2006).

3.5. Cytoskeleton and extracellular matrix: unrevealing mechanisms of parasite attachment and invasion

Different genes encoding for components of the intracellular cytoskeleton (e.g. *FLNC*, *SYNM* and *SYNPO2*) were down-regulated in pyloric caeca suggesting parasite-induced cytoskeleton remodelling of intestinal cells. Host cytoskeleton is a recognized early target of several pathogens that infect epithelia for invasion of the host (Gruenheid and Finlay 2003; Xu *et al.* 2008; Radhakrishnan and Splitter 2012), a mechanism also observed in teleost skin, gill and digestive tract (Li *et al.* 2012, 2013; Sun *et al.* 2012). Another interesting adjustment in pyloric caeca was the up-regulation of genes encoding for extracellular matrix (ECM) components, like *COL1A1*, *TNN* and *FREM1*. The expression changes in genes related to ECM proteins may be of difficult interpretation, because they may reflect either an early attempt of tissue repair by the host or the pathogen manipulation and infection (Li *et al.* 2013). In fact, ECM proteins are often targeted by many invasive pathogens, including parasites, for adhesion and invasion of the host (Mittal *et al.* 2008; Nde *et al.* 2012; Singh *et al.* 2012). Interestingly, *FREM1* has been recently postulated as a novel candidate gene involved in HIV-1 infection (Luo *et al.* 2012). *E. scophthalmi* is capable of attaching and penetrating the intestinal epithelium both from the surface and the basal part, as shown by *in vitro* studies with intestinal explants (Redondo *et al.* 2004; Redondo and Álvarez-Pellitero 2010). In addition, in different experimental infections it was observed that a longer time is needed to detect the parasite in intestinal histological sections than in blood smears, being blood a hypothesized dispersion route (Redondo *et al.* 2003, 2004). Hence, the possibility of epithelial invasion through the lamina propria-submucosa involving an interaction with ECM proteins cannot be ruled out. Finally, a group of up-regulated DE genes in this location were related to cell-cell junctions, in particular three genes encoding for the tight junction proteins claudins (*CLDN10*, *CLDN14* and *CLDN18*) and for the adhesion protein *CDH26*. These results are in accordance with the increasing expression trend found by qPCR for E-cadherin (CDH1) in turbot with incipient infection (authors' unpublished data). The junctional complexes are essential to maintain the homeostasis of the intestinal barrier (Suzuki 2013; Peterson and Artis 2014) and this expression profile may be indicative of early repair mechanisms in response to the parasite invasion of the lining epithelium. In advanced infection, it is plausible that the extension of lesions, the severe inflammation and the prolonged fasting suffered by fish (Bermúdez *et al.* 2010; Robledo *et al.* 2014) hinder an efficient activation and functioning of tissue repair at intestinal level.

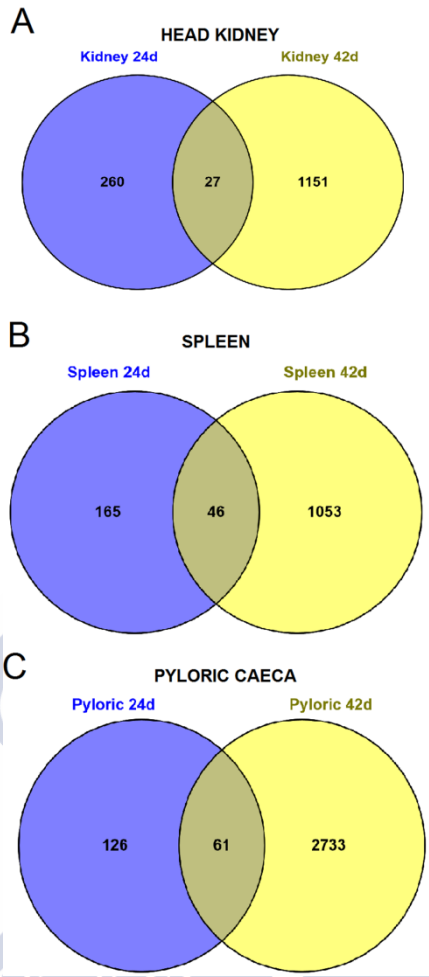

Figure 5. Shared genes between 42 and 24 dpi. Venn diagrams showing shared and specific DE genes between slightly and severely infected turbot, corresponding to 24 and 42 dpi sampling points, for A) head kidney, B) spleen and C) pyloric caeca.

3.6. Apoptosis, cell proliferation and differentiation: effects of *E. scophthalmi* on intestinal renewal

In addition to epithelial integrity, the constant renewal of the epithelium is a main defence mechanism of the intestine against pathogens, and consequently a target for microbial circumvention strategies (Kim *et al.* 2010). Accelerating the epithelial turnover has been described as a host mechanism for parasite expulsion (Cliffe *et al.* 2005; Cortes *et al.* 2015), but several mucosal pathogens can put in place stratagems to avoid their removal and successfully colonize the lining epithelium (Iwai *et al.* 2007; Mimuro *et al.* 2007). In pyloric caeca of *E. scophthalmi*-infected fish, it was remarkable the down-regulation of numerous genes related to cell proliferation (e.g. *CCNB1*, *TPX2* and *CDC14A*) and differentiation (e.g. *HOXA9 HOXA10*, *VSIG1*). Furthermore, *APC* and *TLX1*, which by contrast act as repressors of cell proliferation and differentiation, showed an increased expression. Also *CASP3*, involved in apoptosis, was down-regulated, unlike that observed in severely-infected turbot, which presented up-regulation of this and other pro-apoptotic genes (Robledo *et al.* 2014). A biphasic modulation of apoptotic pathways, consisting in early inhibition and late moderate promotion, was documented in human infection by the intestinal parasite *Cryptosporidium*

parvum (Liu *et al.* 2009). All in all, DE genes of this functional category suggest that inhibitory mechanisms of epithelial renewal occur during incipient enteromyxosis, which may facilitate the parasite entrance and colonization of the digestive tract. At later infection stages, the pathological changes observed in the intestinal epithelium, including the increased apoptotic rate and enterocyte detachment, may be invoked by the exacerbated local immune response (Bermúdez *et al.* 2010; Losada *et al.* 2012, 2014a; Robledo *et al.* 2014) and/or induced by the parasite as a spreading strategy (Bermúdez *et al.* 2010). The only up-regulated gene promoting cell proliferation was *CTGF*, a major connective tissue mitoattractant also involved in ECM secretion, a finding in accordance with the aforementioned up-regulation of different ECM-related genes.

3.7. Iron metabolism and erythropoiesis

Several genes related to haemoglobin (*HBB2* and *HBAD*), iron homeostasis and heme biosynthesis (*FAM123B*; *SLC25A37* and *ALAS2*), and erythrocyte maturation and differentiation (*GATA2* and *TAL1*) showed an increased expression in pyloric caeca. Genes related to erythrocyte structural (*DMTN*, *ANK1* and *RHAG*) and enzymatic (*CA*) components were also up-regulated. These findings point towards an increased presence of red blood cells in the intestine of infected turbot, consistent with hyperaemia. Hyperaemia is one of the first vascular changes occurring after an inflammatory stimulus (McGavin and Zachary 2006), a scenario consistent with the early infection of the digestive tract. On the other hand, a gene related to hepcidin (*HEP-2*), a main regulator of iron metabolism, was down-regulated. In turbot, the existence of two hepcidin genes has been reported (*HEP-1* and *HEP-2*), both showing antimicrobial properties and modulated expression in response to bacterial and viral challenges (Pereiro *et al.* 2012; Zhang *et al.* 2014). Nevertheless, a major role for *HEP-1* in body iron homeostasis has been previously suggested, given that *HEP-2* expression did not change in liver in response to iron overload (Pereiro *et al.* 2012). In our case, the down-regulation of *HEP-2* in pyloric caeca may be related to the requirement of iron for heme biosynthesis, a hypothesis also supported by the contemporary up-regulation of *FAM123B*. The product of this gene is known as erythroferrone, an iron-regulatory hormone with a potent suppressor action on hepcidin mRNA expression in mice (Kautz *et al.* 2014). Under the hypotheses of a main role for *HEP-2* in innate response and considering the expression profile of APP-related genes in the spleen, its down-regulation should also be interpreted in the context of a global modulation of the immune response. Further research is needed to clarify the role of APP and iron metabolism regulation during the infection by *E. scophthalmi*.

3.8. Metabolism and digestive function: a diminished feeding activity

DE genes involved in digestive function were mostly down-regulated, like *VIP*, *NPY2R* and *APOA4*. The only exception was the up-regulation of *GCG2*, a paralog of the glucagon gene, which promotes hydrolysis of glycogen and lipids so increasing blood sugar level (Moon 1998). In previous immunohistochemical studies on the digestive tract of turbot with advanced enteromyxosis, both VIP- and glucagon-immunoreactive cells showed decreased figures (Bermúdez *et al.* 2007; Losada *et al.* 2014b). Also, *APOA4*, involved in lipids metabolism, was down-regulated in the pyloric caeca of severe-infected turbot analyzed by

RNA-Seq (Robledo *et al.* 2014). The down-regulation of a receptor of neuropeptide Y (*NPY2R*), a main regulator of appetite which stimulates food intake (Zhou *et al.* 2013), may indicate that the changes leading to anorexia in fish suffering enteromyxosis (Sitjà-Bobadilla and Palenzuela 2012) are early induced during the infection. The down-regulation of other genes acting in the digestive process, although not numerous, also points towards a diminished feeding activity and, in this sense, the up-regulation of *GCG2* may reflect the effort in maintaining euglycemia. Finally, *VIP* product has been also recognized as an immunomodulatory peptide with immunosuppressive function (Delgado *et al.* 2004), so its reduced expression may be also due to the defence response, as previously suggested (Bermúdez *et al.* 2007).

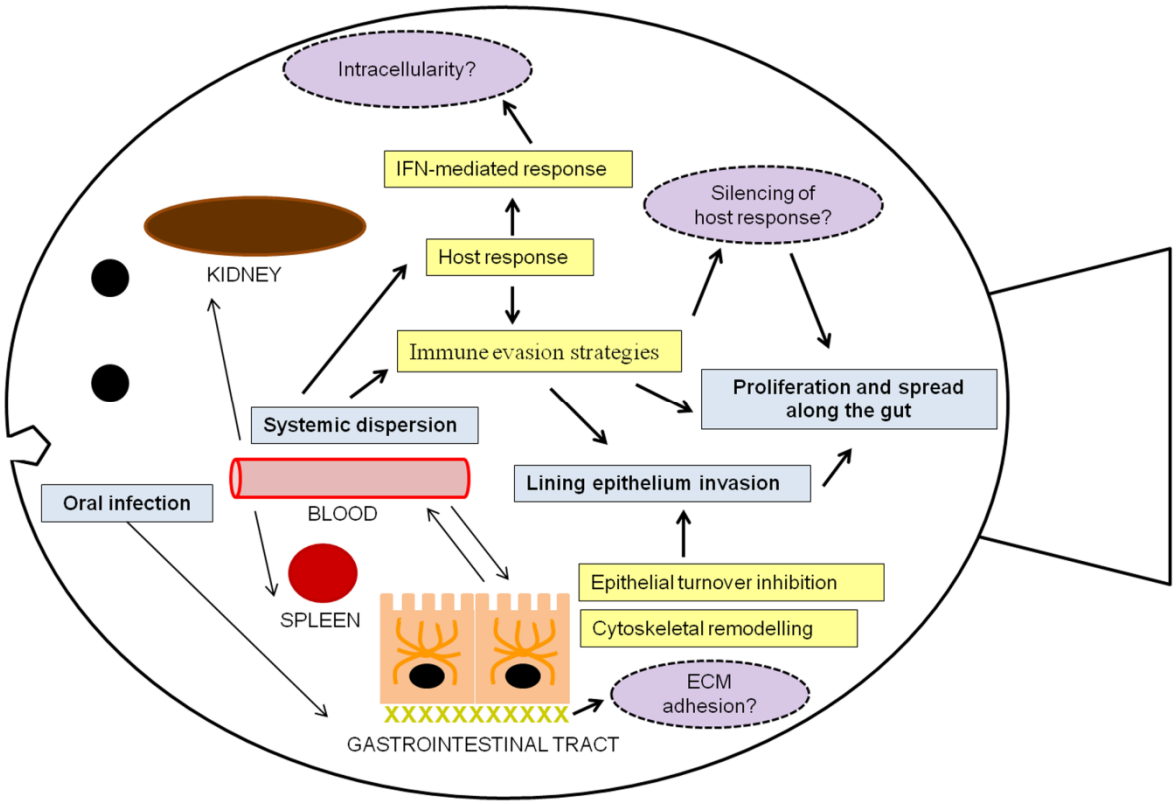

Figure 6. Schematic diagram showing the main events involved at early enteromyxosis inferred from the results of this study. ECM = extra-cellular matrix; IFN = interferon.

4. Conclusions

The pathogenesis of enteromyxosis still shows many unknown features, especially those related to the incipient phase of infection. The parasite in the pre-patent period circumvents the host response and successfully reaches and penetrates the intestinal lining epithelium. The findings of this work constitute a basis for deciphering the mechanisms acting in this phase. A schematic diagram summarizing the main results is presented in Figure 6. Turbot immune response at early enteromyxosis is chiefly characterized by signalling pathways involving IFNs, in contrast to that observed in advanced infection, and only some mechanisms of innate

immunity are shared between both stages. Some evidences arise about the possible targets for parasite immune system evasion, like complement components and APP, which possibly hinder a proper acute phase response. At intestinal level, the invasion and colonization strategies of *E. scophthalmi* appear to involve the cytoskeleton remodelling of the host cells and inhibition of the epithelial renewal. Also, it is noteworthy that one of the fish analyzed, which presented more mature and spreading stages of the myxozoan, showed less intense transcriptomic changes. Further studies using more individuals or families are required to ascertain the consistency and causes of this observation, although it suggests a silencing of the host response, which would allow the early proliferation and colonization of vast areas of the gastrointestinal lining epithelium by *E. scophthalmi*. Likely, when the parasite load and the related tissue damage become important, the immune response is triggered. However, as pointed out by different studies, this delayed response, which is exacerbated at local level contributing to the severe intestinal lesions, is ineffective. The transcriptomic analysis performed here has brought novel and suggestive information about host-parasite interaction in enteromyxosis. The identification of the molecular actors and their roles may speed the development of early detection, control and therapeutic strategies, and even to identify targets for breeding programs.

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List of gene symbols in alphabetical order

ACK1: activated cdc42 kinase 1-like; *ALAS2*: 5-aminolevulinate erythroid- mitochondrial; *ALOXE3*: epidermis-type lipoxygenase 3; *ANK1*: ankyrin-1; *APC*: adenomatous polyposis coli; *APOA4*: apolipoprotein a-iv; *CA*: carbonic anhydrase; *CASP3*: caspase-3; *C1QTNF9*: complement c1q and tumor necrosis factor-related protein 9a; *CCL19*: c-c motif chemokine 19; *CCNB1*: g2 mitotic-specific cyclin-b1; *CD209*: CD209 antigen; *CDC14A*: dual specificity protein phosphatase cdc14a; *CDC42*: cell division control protein 42; *CDH26*: cadherin-like protein 26; *CLDN10, -14* and *-18*: claudin 10, 14 and 18; *COL1A1*: collagen alpha-1 chain; *CTGF*: connective tissue growth factor; *DHX58*: probable atp-dependent rna helicase dhx58; *DMTN*: dematin; *FAM123B*: protein fam132b; *FLNC*: filamin-c; *FOXJ1A* and *-J1B*: forkhead box protein j1A and jiB; *FREM1*: fras1-related extracellular matrix protein 1; *GATA2*: gata-

binding factor 2; *GCG2*: glucagon-2; *GF1B* zinc finger protein gfi-1b; *GZMA*: granzyme a; *HBB2*: hemoglobin subunit beta-2; *HBAD*: hemoglobin subunit alpha-d; *HEP-1* and *-2*: hepcidin-1 and -2; *HERC4/5*: e3 isg15--protein ligase herc5; *HOXA9, -A10*: homeobox protein hox-a9 and hox-a10; *HSP70* and -90: heat shock protein 70 and 90; *IFI44*: interferon-induced protein 44; *IFIT1*: interferon-induced protein with tetratricopeptide repeats 1; *IL4I1*: L-amino-acid-oxidase; *LRRC32*: leucine-rich repeat-containing protein 32; *MFN1*: mitofusin 1; *NFIL3*: nuclear factor interleukin-3-regulated; *NPY2R*: neuropeptide y receptor type 2; *PAX5*: transcription factor pax5; *PML*: promyelocytic leukemia; *PRF1*: perforin-1; *RAC1*: ras-related c3 botulinum toxin substrate 1; *RAG1*: v j recombination-activating protein 1; *RHAG*: ammonium transporter rh type a; *SLC25A37*: mitoferrin-1; *SOCS1:* suppressor of cytokine signaling 1; *STING*: stimulator of interferon genes; *SYNM*: synemin; *SYNPO2*: synaptopodin-2; *TAL1*: t-cell acute lymphocytic leukemia protein 1; *TLX1*: t-cell leukemia homeobox protein 1; *TNN*: tenascin-n; *TPX2*: targeting protein for xklp2-a; *TRIM21* and *-25*: e3 ubiquitin isg15 ligase trim21 and trim25; *VIP*: vasoactive intestinal peptide; *VSIG1*: v-set and immunoglobulin domain-containing protein 1; *ZNFX1*: nfx1-type zinc finger-containing protein 1.

Supplementary files

Supplementary Figure S1. The complement and coagulation cascades KEGG pathway is statistically enriched (FDR < 0.05) among the differentially expressed (DE) genes in spleen. DE genes belonging to this pathway are highlighted in red.

Supplementary Table S1: Up-regulated transcripts from unaligned reads in pyloric caeca of infected fish at 42 dpi (de novo transcriptome reconstructed with the unaligned reads from pyloric caeca samples of turbot at 24 and 42 dpi). Annotation against NCBI's non-redundant protein database, blast E value, blast identity, log fold change between infected and control samples, average log counts per million for all samples, false discovery rate corrected p-value, read counts for all the sample, taxonomic group of the annotated transcript species and fasta format sequence of the transcript are shown.

Supplementary Table S2: List of selected differentially expressed genes in head kidney, spleen and pyloric caeca. Fold change, fragments per kilobase of exon per million fragments mapped (FPKM) in control and infected samples and associated functional group for each gene are shown.

Supplementary Table S3: List of common genes between 24 and 42 dpi for head kidney, spleen and pyloric caeca. Annotation, fold change, counts per million, false discovery rate corrected *p*-value and fragments per kilobase of exon per million fragments mapped (FPKM) are shown.

References

Armstrong, P.B., 2006. Proteases and protease inhibitors: a balance of activities in host-pathogen interaction. Immunobiology 211, 263-281.

APROMAR, 2015. La Acuicultura en España 2015. Available online at http://www.apromar.es/content/informes-anuales

Bayne, C.J., Gerwick, L., 2001. The acute phase response and innate immunity of fish. Dev Comp Immunol 25, 725-743.

Beiting, D.P., 2014. Protozoan parasites and type I interferons: a cold case reopened. Trends Parasitol 30, 491-498.

Ben-Othman, R., Flannery, A.R., Miguel, D.C., Ward, D.M., Kaplan, J., Andrews, N.W., 2014. *Leishmania*-mediated inhibition of iron export promotes parasite replication in macrophages. PLoS Pathog 10, e1003901.

Bermúdez, R., Vigliano, F., Marcaccini, A., Sitjà-Bobadilla, A., Quiroga, M.I., Nieto, J.M., 2006. Response of Ig-positive cells to *Enteromyxum scophthalmi* (Myxozoa) experimental infection in turbot, *Scophthalmus maximus* (L.): A histopathological and immunohistochemical study. Fish Shellfish Immunol 21, 501-512.

Bermúdez, R., Vigliano, F., Quiroga, M.I., Nieto, J.M., Bosi, G., Domeneghini, C., 2007. Immunohistochemical study on the neuroendocrine system of the digestive tract of turbot, *Scophthalmus maximus* (L.), infected by *Enteromyxum scophthalmi* (Myxozoa). Fish Shellfish Immunol 22, 252-263.

Bermúdez, R., Losada, A.P., Vázquez, S., Redondo, M.J., Álvarez-Pellitero, P., Quiroga, M.I., 2010. Light and electron microscopic studies on turbot *Psetta maxima* infected with *Enteromyxum scophthalmi*: histopathology of turbot enteromyxosis. Dis Aquat Organ 89, 209-221.

Bimal, S., Singh, S.K., Sinha, S., Pandey, K., Sinha, P.K., Ranjan, A., Bhattacharya, S.K., Das, P., 2008. Leishmania donovani: role of CD2 on CD4+ T-cell function in Visceral Leishmaniasis. Exp Parasitol 118, 238-246.

Bjork, S.J., Zhang, Y.A., Hurst, C.N., Alonso-Naveiro, M.E., Alexander, J.D., Sunyer, J.O., Bartholomew, J.L., 2014. Defenses of susceptible and resistant Chinook salmon (*Oncorhynchus tshawytscha*) against the myxozoan parasite *Ceratomyxa shasta*. Fish Shellfish Immunol 37, 87-95.

Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30, 2114-2120.

Castanier, C., Garcin, D., Vazquez, A., Arnoult, D., 2010. Mitochondrial dynamics regulate the RIG-I-like receptor antiviral pathway. EMBO Rep 11, 133-138.

Cliffe, L.J., Humphreys, N.E., Lane, T.E., Potten, C.S., Booth, C., Grencis, R.K., 2005. Accelerated intestinal epithelial cell turnover: a new mechanism of parasite expulsion. Science 308, 1463-1465.

Conesa, A., Gotz, S., Garcia-Gomez, J.M., Terol, J., Talon, M., Robles, M., 2005. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21, 3674-3676.

Cortes, A., Munoz-Antoli, C., Martin-Grau, C., Esteban, J.G., Grencis, R.K., Toledo, R., 2015. Differential alterations in the small intestine epithelial cell turnover during acute and chronic infection with *Echinostoma caproni* (Trematoda). Parasit Vectors 8, 334.

Costa, V., Aprile, M., Esposito, R., Ciccodicola, A., 2013. RNA-Seq and human complex diseases: recent accomplishments and future perspectives. Eur J Hum Genet 21, 134-142.

Chen, X.M., Huang, B.Q., Splinter, P.L., Orth, J.D., Billadeau, D.D., McNiven, M.A., LaRusso, N.F., 2004. Cdc42 and the actin-related protein/neural Wiskott-Aldrich syndrome protein network mediate cellular invasion by *Cryptosporidium parvum*. Infect Immun 72, 3011-3021.

Davey, G.C., Calduch-Giner, J.A., Houeix, B., Talbot, A., Sitjà-Bobadilla, A., Prunet, P., Pérez-Sánchez, J., Cairns, M.T., 2011. Molecular profiling of the gilthead sea bream (*Sparus aurata* L.) response to chronic exposure to the myxosporean parasite *Enteromyxum leei*. Mol Immunol 48, 2102-2112.

Delgado, M., Pozo, D., Ganea, D., 2004. The significance of vasoactive intestinal peptide in immunomodulation. Pharmacol Rev 56, 249-290.

Dixit, E., Kagan, J.C., 2013. Intracellular pathogen detection by RIG-I-like receptors. Adv Immunol 117, 99-125.

Estensoro, I., Redondo, M.J., Álvarez-Pellitero, P., Sitjà-Bobadilla, A., 2014. Immunohistochemical characterization of polyclonal antibodies against *Enteromyxum leei* and *Enteromyxum scophthalmi* (Myxozoa: Myxosporea), intestinal parasites of fish. J Fish Dis 37, 785-796.

Figueras, A., Robledo, D., Corvelo, A., Hermida, M., Pereiro, P., Rubiolo, J.A., Gómez-Garrido, J., Carreté L., Bello, X., Gut, M., Gut, I.G., Marcet-Houben, M., Forn-Cuní, G., Abal-Fabeiro, J.L., Pardo, B.G., Taboada, X., Fernández, C., Vlasova, A., Hermoso-Pulido, A., Guigó, R., Álvarez-Dios, J.A., Gómez-Tato, A., Viñas, A., Maside, X., Gabaldón, T., Novoa, B., Bouza, C., Alioto, T., Martínez, P., 2016. Whole genome sequencing of turbot (*Scophthalmus maximus*; Pleuronectiformes): a fish adapted to demersal life. DNA Res, in press.

Gerwick, L., Steinhauer, R., Lapatra, S., Sandell, T., Ortuno, J., Hajiseyedjavadi, N., Bayne, C.J., 2002. The acute phase response of rainbow trout (*Oncorhynchus mykiss*) plasma proteins to viral, bacterial and fungal inflammatory agents. Fish Shellfish Immunol 12, 229-242.

Gruenheid, S., Finlay, B.B., 2003. Microbial pathogenesis and cytoskeletal function. Nature 422, 775-781.

Iwai, H., Kim, M., Yoshikawa, Y., Ashida, H., Ogawa, M., Fujita, Y., Muller, D., Kirikae, T., Jackson, P.K., Kotani, S., Sasakawa, C., 2007. A bacterial effector targets Mad2L2, an APC inhibitor, to modulate host cell cycling. Cell 130, 611-623.

Kashiwada, M., Cassel, S.L., Colgan, J.D., Rothman, P.B., 2011. NFIL3/E4BP4 controls type 2 T helper cell cytokine expression. EMBO J 30, 2071-2082.

Kautz, L., Jung, G., Valore, E.V., Rivella, S., Nemeth, E., Ganz, T., 2014. Identification of erythroferrone as an erythroid regulator of iron metabolism. Nat Genet 46, 678-684.

Khoo, C.-K., Abdul-Murad, A.M., Kua, B.-C., Mohd-Adnan, A., 2012. *Cryptocaryon irritans* infection induces the acute phase response in *Lates calcarifer*: A transcriptomic perspective. Fish Shellfish Immunol 33, 788-794.

Kim, D., Pertea, G., Trapnell, C., Pimentel, H., Kelley, R., Salzberg, S.L., 2013. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol 14, R36.

Kim, M., Ogawa, M., Mimuro, H., Sasakawa, C., 2010. Reinforcement of epithelial cell adhesion to basement membrane by a bacterial pathogen as a new infectious stratagem. Virulence 1, 52-55.

Kim, Y.E., Ahn, J.H., 2015. Positive role of promyelocytic leukemia protein in type I interferon response and its regulation by human cytomegalovirus. PLoS Pathog 11, e1004785.

Koshiba, T., 2013. Mitochondrial-mediated antiviral immunity. Biochim Biophys Acta 1833, 225-232.

Kovacevic, N., Hagen, M.O., Xie, J., Belosevic, M., 2015. The analysis of the acute phase response during the course of *Trypanosoma carassii* infection in the goldfish (*Carassius auratus* L.). Dev Comp Immunol 53, 112-122.

Langmead, B., Salzberg, S.L., 2012. Fast gapped-read alignment with Bowtie 2. Nat Methods 9, 357-359.

Leon-Sicairos, N., Reyes-Cortes, R., Guadrón-Llanos, A.M., Madueña-Molina, J., Leon-Sicairos, C., Canizalez-Román, A., 2015. Strategies of intracellular pathogens for obtaining iron from the environment. Biomed Res Int 2015, 17.

Li, B., Dewey, C.N., 2011. RSEM: accurate transcript quantification from RNA-seq data with or without a reference genome. BMC Bioinformatics 12, 323.

Li, C., Zhang, Y., Wang, R., Lu, J., Nandi, S., Mohanty, S., Terhune, J., Liu, Z., Peatman, E., 2012. RNA-seq analysis of mucosal immune responses reveals signatures of intestinal barrier disruption and pathogen entry following *Edwardsiella ictaluri* infection in channel catfish, *Ictalurus punctatus*. Fish Shellfish Immunol 32, 816-827.

Li, C., Beck, B., Su, B., Terhune, J., Peatman, E., 2013. Early mucosal responses in blue catfish (*Ictalurus furcatus*) skin to *Aeromonas hydrophila* infection. Fish Shellfish Immunol 34, 920-928.

Li, Q., Freeman, L.M., Rush, J.E., Huggins, G.S., Kennedy, A.D., Labuda, J.A., Laflamme, D.P., Hannah, S.S., 2015. Veterinary medicine and multi-omics research for future nutrition targets: metabolomics and transcriptomics of the common degenerative mitral valve disease in dogs. OMICS 19, 461-470.

Liu, J., Deng, M., Lancto, C.A., Abrahamsen, M.S., Rutherford, M.S., Enomoto, S., 2009. Biphasic modulation of apoptotic pathways in *Cryptosporidium parvum*-infected human intestinal epithelial cells. Infect Immun 77, 837-849.

Lodge, R., Descoteaux, A., 2006. Phagocytosis of *Leishmania donovani* amastigotes is Rac1 dependent and occurs in the absence of NADPH oxidase activation. Eur J Immunol 36, 2735-2744.

Losada, A.P., Bermúdez, R., Faílde, L.D., Quiroga, M.I., 2012. Quantitative and qualitative evaluation of iNOS expression in turbot (*Psetta maxima*) infected with *Enteromyxum scophthalmi*. Fish Shellfish Immunol 32, 243-248.

Losada, A.P., Bermúdez, R., Faílde, L.D., de Ocenda, M.V.R., Quiroga, M.I., 2014a. Study of the distribution of active caspase-3-positive cells in turbot, *Scophthalmus maximus* (L.), enteromyxosis. J Fish Dis 37, 21-32.

Losada, A.P., Bermúdez, R., Faílde, L.D., Di Giancamillo, A., Domeneghini, C., Quiroga, M.I., 2014b. Effects of *Enteromyxum scophthalmi* experimental infection on the neuroendocrine system of turbot, *Scophthalmus maximus* (L.). Fish Shellfish Immunol 40, 577-583.

Luo, M., Sainsbury, J., Tuff, J., Lacap, P.A., Yuan, X.Y., Hirbod, T., Kimani, J., Wachihi, C., Ramdahin, S., Bielawny, T., Embree, J., Broliden, K., Ball, T.B., Plummer, F.A., 2012. A genetic polymorphism of FREM1 is associated with resistance against HIV infection in the Pumwani sex worker cohort. J Virol 86, 11899-11905.

Manocha, G.D., Mishra, R., Sharma, N., Kumawat, K.L., Basu, A., Singh, S.K., 2014. Regulatory role of TRIM21 in the type-I interferon pathway in Japanese encephalitis virus-infected human microglial cells. J Neuroinflammation 11, 24.

McEwan, W.A., Tam, J.C.H., Watkinson, R.E., Bidgood, S.R., Mallery, D.L., James, L.C., 2013. Intracellular antibody-bound pathogens stimulate immune signaling via the Fc receptor TRIM21. Nat Immunol 14, 327-336.

McGavin, D., Zachary, J.F., 2006. Pathologic Basis of Veterinary Disease. Elsevier Health Sciences.

Melo, M.B., Nguyen, Q.P., Cordeiro, C., Hassan, M.A., Yang, N., McKell, R., Rosowski, E.E., Julien, L., Butty, V., Darde, M.L., Ajzenberg, D., Fitzgerald, K., Young, L.H., Saeij, J.P., 2013. Transcriptional analysis of murine macrophages infected with different *Toxoplasma* strains identifies novel regulation of host signaling pathways. PLoS Pathog 9, e1003779.

Mimuro, H., Suzuki, T., Nagai, S., Rieder, G., Suzuki, M., Nagai, T., Fujita, Y., Nagamatsu, K., Ishijima, N., Koyasu, S., Haas, R., Sasakawa, C., 2007. Helicobacter pylori dampens gut epithelial self-renewal by inhibiting apoptosis, a bacterial strategy to enhance colonization of the stomach. Cell Host Microbe 2, 250-263.

Mittal, K., Welter, B.H., Temesvari, L.A., 2008. *Entamoeba histolytica*: lipid rafts are involved in adhesion of trophozoites to host extracellular matrix components. Exp Parasitol 120, 127-134.

Moon, T.W., 1998. Glucagon: from hepatic binding to metabolism in teleost fish. Comp Biochem Phys B 121, 27-34.

Nde, P.N., Lima, M.F., Johnson, C.A., Pratap, S., Villalta, F., 2012. Regulation and use of the extracellular matrix by *Trypanosoma cruzi* during early infection. Front Immunol 3, 337.

Nie, L., Xiong, R., Zhang, Y.S., Zhu, L.Y., Shao, J.Z., Xiang, L.X., 2014. Conserved inhibitory role of teleost SOCS-1s in IFN signaling pathways. Dev Comp Immunol 43, 23-29.

Onoguchi, K., Onomoto, K., Takamatsu, S., Jogi, M., Takemura, A., Morimoto, S., Julkunen, I., Namiki, H., Yoneyama, M., Fujita, T., 2010. Virus-infection or 5'ppp-RNA activates antiviral signal through redistribution of IPS-1 mediated by MFN1. PLoS Pathog 6, e1001012.

Peatman, E., Baoprasertkul, P., Terhune, J., Xu, P., Nandi, S., Kucuktas, H., Li, P., Wang, S., Somridhivej, B., Dunham, R., Liu, Z., 2007. Expression analysis of the acute phase response in channel catfish (*Ictalurus punctatus*) after infection with a Gram-negative bacterium. Dev Comp Immunol 31, 1183-1196.

Pereiro, P., Figueras, A., Novoa, B., 2012. A novel hepcidin-like in turbot (*Scophthalmus maximus* L.) highly expressed after pathogen challenge but not after iron overload. Fish Shellfish Immunol 32, 879-889.

Peterson, L.W., Artis, D., 2014. Intestinal epithelial cells: regulators of barrier function and immune homeostasis. Nat Rev Immunol 14, 141-153.

Qian, X., Ba, Y., Zhuang, Q., Zhong, G., 2014. RNA-Seq technology and its application in fish transcriptomics. OMICS 18, 98-110.

Quiroga, M.I., Redondo, M.J., Sitjà-Bobadilla, A., Palenzuela, O., Riaza, A., Macías, A., Vázquez, S., Pérez, A., Nieto, J.M., Álvarez-Pellitero, P., 2006. Risk factors associated with *Enteromyxum scophthalmi* (Myxozoa) infection in cultured turbot, *Scophthalmus maximus* (L.). Parasitology 133, 433-442.

Radhakrishnan, G.K., Splitter, G.A., 2012. Modulation of host microtubule dynamics by pathogenic bacteria. Biomol Concepts 3, 571-580.

Redondo, M.J., Palenzuela, O., Riaza, A., Macías, A., Álvarez-Pellitero, P., 2002. Experimental transmission of *Enteromyxum scophthalmi* (Myxozoa), an enteric parasite of turbot *Scophthalmus maximus*. J Parasitol 88, 482-488.

Redondo, M.J., Quiroga, M.I., Palenzuela, O., Nieto, J.M., Álvarez-Pellitero, P., 2003. Ultrastructural studies on the development of *Enteromyxum scophthalmi* (Myxozoa), an enteric parasite of turbot (*Scophthalmus maximus* L.). Parasitol Res 90, 192-202.

Redondo, M.J., Palenzuela, O., Álvarez-Pellitero, P., 2004. Studies on transmission and life cycle of *Enteromyxum scophthalmi* (Myxozoa), an enteric parasite of turbot *Scophthalmus maximus*. Folia Parasitol 51, 188-198.

Redondo, M.J., Álvarez-Pellitero, P., 2010. The effect of lectins on the attachment and invasion of *Enteromyxum scophthalmi* (Myxozoa) in turbot (*Psetta maxima* L.) intestinal epithelium in vitro. Exp Parasitol 126, 577-581.

Robinson, M.D., Oshlack, A., 2010. A scaling normalization method for differential expression analysis of RNA-seq data. Genome Biol 11, R25.

Robledo, D., Ronza, P., Harrison, P.W., Losada, A.P., Bermúdez, R., Pardo, B.G., Redondo, M.J., Sitjà-Bobadilla, A., Quiroga, M.I., Martínez, P., 2014. RNA-seq analysis reveals significant transcriptome changes in turbot (*Scophthalmus maximus*) suffering severe enteromyxosis. BMC Genomics 15, 1149.

Simpson, J.T., Wong, K., Jackman, S.D., Schein, J.E., Jones, S.J.M., Birol, I., 2009. ABySS: A parallel assembler for short read sequence data. Genome Res 19, 1117-1123.

Singh, B., Fleury, C., Jalalvand, F., Riesbeck, K., 2012. Human pathogens utilize host extracellular matrix proteins laminin and collagen for adhesion and invasion of the host. FEMS Microbiol Rev 36, 1122-1180.

Sitjà-Bobadilla, A., Redondo, M.J., Bermúdez, R., Palenzuela, O., Ferreiro, I., Riaza, A., Quiroga, I., Nieto, J.M., Álvarez-Pellitero, P., 2006. Innate and adaptive immune responses of turbot, *Scophthalmus maximus* (L.), following experimental infection with *Enteromyxum scophthalmi* (Myxosporea: Myxozoa). Fish Shellfish Immunol 21, 485-500.

Sitjà-Bobadilla, A., Palenzuela, O., 2012. *Enteromyxum* Species, in: Woo, P.T.K., Buchmann, K. (Eds.), Fish Parasites: Pathobiology and Protection. CABI publishing, UK, pp. 163-176.

Sun, F., Peatman, E., Li, C., Liu, S., Jiang, Y., Zhou, Z., Liu, Z., 2012. Transcriptomic signatures of attachment, NF-kappaB suppression and IFN stimulation in the catfish gill following columnaris bacterial infection. Dev Comp Immunol 38, 169-180.

Suzuki, T., 2013. Regulation of intestinal epithelial permeability by tight junctions. Cell Mol Life Sci 70, 631-659.

Tran, D.Q., Andersson, J., Wang, R., Ramsey, H., Unutmaz, D., Shevach, E.M., 2009. GARP (LRRC32) is essential for the surface expression of latent TGF-beta on platelets and activated FOXP3+ regulatory T cells. Proc Natl Acad Sci USA 106, 13445-13450.

Vaysburd, M., Watkinson, R.E., Cooper, H., Reed, M., O'Connell, K., Smith, J., Cruickshanks, J., James, L.C., 2013. Intracellular antibody receptor TRIM21 prevents fatal viral infection. Proc Natl Acad Sci USA 110, 12397-12401.

Wu, J., Mao, X., Cai, T., Luo, J., Wei, L., 2006. KOBAS server: a web-based platform for automated annotation and pathway identification. Nucleic Acids Res 34, W720-W724.

Xu, J.H., Qin, Z.H., Liao, Y.S., Xie, M.Q., Li, A.X., Cai, J.P., 2008. Characterization and expression of an actin-depolymerizing factor from *Eimeria tenella*. Parasitol Res 103, 263-270.

Young, N.D., Cooper, G.A., Nowak, B.F., Koop, B.F., Morrison, R.N., 2008. Coordinated down-regulation of the antigen processing machinery in the gills of amoebic gill disease-affected Atlantic salmon (*Salmo salar* L.). Mol Immunol 45, 2581-2597.

Zhang, J., Yu, L.P., Li, M.F., Sun, L., 2014. Turbot (*Scophthalmus maximus*) hepcidin-1 and hepcidin-2 possess antimicrobial activity and promote resistance against bacterial and viral infection. Fish Shellfish Immunol 38, 127-134.

Zhang, W., Zhang, J., Kornuc, M., Kwan, K., Frank, R., Nimer, S.D., 1995. Molecular cloning and characterization of NF-IL3A, a transcriptional activator of the human interleukin-3 promoter. Mol Cell Biol 15, 6055-6063.

Zhou, Y., Liang, X.-F., Yuan, X., Li, J., He, Y., Fang, L., Guo, X., Liu, L., Li, B., Shen, D., 2013. Neuropeptide Y stimulates food intake and regulates metabolism in grass carp, *Ctenopharyngodon idellus*. Aquaculture 380–383, 52-61.

Zipfel, P.F., Wurzner, R., Skerka, C., 2007. Complement evasion of pathogens: common strategies are shared by diverse organisms. Mol Immunol 44, 3850-3857.

Zou, J., Secombes, C.J., 2011. Teleost fish interferons and their role in immunity. Dev Comp Immunol 35, 1376-1387.

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Discusión general

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DISCUSIÓN GENERAL

El objetivo general de esta Tesis Doctoral ha sido profundizar en la patogenia de la enteromixosis del rodaballo, una enfermedad con una importante repercusión en la acuicultura de esta especie, a través de un mayor conocimiento de la interacción parásito-hospedador.

Desde el diseño de la infección experimental se abordó el estudio de forma multidisciplinar, tomando muestras de los tejidos de los peces utilizados en paralelo, para la aplicación de técnicas histopatológicas y moleculares. El estudio histológico y la clasificación de los peces en función del grado lesional hizo posible una selección previa de ejemplares con lesiones similares para la aplicación de estas técnicas. Este diseño permitió reducir el número de peces empleados, a la par que aumentó la cantidad y calidad de información obtenida.

En esta Tesis Doctoral se analizó por primera vez la modulación de la expresión génica derivada de la enfermedad. Por un lado, se emplearon de forma complementaria la Q-PCR y la inmunohistoquímica para el estudio del TNFα. Por otra parte, se aplicó la tecnología RNA-seq, una potente herramienta transcriptómica, con el fin de obtener un cuadro exhaustivo de la modulación transcripcional y analizar su relación con la patogenia de la enteromixosis.

Inicialmente, se realizó la puesta a punto de una técnica inmunohistoquímica para el marcaje del TNFα en tejidos de rodaballo (Artículo I), que posteriormente sería empleada para la evaluación del papel de esta citoquina en el desarrollo de la enfermedad (Artículo II). Los ensayos con un anticuerpo comercial anti-TNFα humano se llevaron a cabo en diferentes tejidos de rodaballos sanos y se evaluó la especificidad del anticuerpo mediante ensayos de Western Blot (Artículo I). Por primera vez se estudió la inmunolocalización del TNFα en rodaballo, observando positividad principalmente en dos tipos celulares: los monocitos/macrófagos y las células rodlet. El marcaje predominante en monocitos y macrófagos confirmó que, como en otros teleósteos y en mamíferos (Goetz y col. 2004; Parameswaran y Patial 2010), estas células son las principales productoras de esta citoquina. La distribución del marcaje coincidía con la presencia de fagocitos residentes en diferentes órganos, con localización estratégica en áreas de estimulación, reconocimiento y procesamiento antigénico (Artículo I). Asimismo, la positividad en las células rodlet fue un resultado muy interesante. Estas células han sido escasamente caracterizadas, pero diversos autores coinciden en afirmar que desempeñan un papel en la respuesta inmunitaria, también por su localización predominante en los epitelios, compatible con una función de células centinela (Reite y Evensen 2006; Dezfuli y col. 2007; Mazon y col. 2007; Schultz y col. 2014). Además, la abundante positividad observada en los órganos linfohematopoyéticos, sobre todo en el riñón, abrió nuevas hipótesis sobre las posibles funciones fisiológicas en las que el TNFα puede estar involucrado en esta especie. En este sentido, esta molécula interviene en la linfohematopoyesis y la diferenciación celular en mamíferos, así como en el mantenimiento, la organización espacial y el movimiento de los leucocitos en los órganos linfoides (Witsell y Schook 1992; Zhang y col. 1995; Sedgwick y col. 2000; Dybedal y col. 2001; Tumanov y col. 2010). Sin embargo, estas funciones del TNFα aún están muy poco estudiadas en teleósteos.

La técnica inmunohistoquímica demostró una buena especificidad, siendo una herramienta válida para el estudio del TNFα en rodaballo. Esta técnica, complementada con el análisis en paralelo de la expresión génica de la citoquina mediante Q-PCR, se aplicó para evaluar el papel del TNFα en la enteromixosis (Artículo II). Los peces con infección moderada mostraron un aumento de células inmunorreactivas en la lámina propia-submucosa, coincidiendo con la observación histológica de infiltrados inflamatorios mononucleares a este nivel. Asimismo, se produjo un incremento de células rodlet positivas en el epitelio de revestimiento intestinal, de acuerdo con la mayor densidad apreciada histológicamente en esa fase de la enfermedad (Bermúdez y col. 2010). Este hallazgo sugiere que la actividad del TNFα en la respuesta local también podría estar promovida por ese tipo celular. En los rodaballos que presentaban enteromixosis avanzada también se apreció esa correlación entre las observaciones histológicas y el inmunomarcaje del TNFα. La mayor severidad del infiltrado inflamatorio en lámina propia-submucosa se correspondió con una mayor densidad de células TNFα-positivas. En cambio, se observaron escasas células rodlet inmunorreactivas, posiblemente debido a su disminución por los daños en el epitelio, que resulta incapaz de sostener este tipo celular (Bermúdez y col. 2010).

Los resultados indicaron que el tracto gastrointestinal sufre una intensa y prolongada exposición a la citoquina a lo largo de la enfermedad, una condición que en mamíferos se ha descrito como predisponente al desarrollo de lesiones (Leppkes y col. 2014). Además, la acción pro-inflamatoria del TNFα incluye promover la producción de óxido nítrico (Bradley 2008, Nandi y col. 2010), hecho observado también en rodaballo (Ordás y col. 2007). A este propósito, Losada y col. (2012) describieron el incremento de inmunomarcaje frente a la sintasa de óxido nítrico (iNOS) en rodaballos parasitados por *E. scophthalmi*. En mamíferos, la inflamación prolongada, mediante la acción de mediadores como el propio TNFα y el óxido nítrico, se ha relacionado con el incremento de la apoptosis y el desprendimiento del epitelio de revestimiento, resultando en la disfunción de la barrera intestinal (Panaro y col. 2007; Chokshi y col. 2008; Bienvenu y col. 2010; Watson y Hughes 2012; Leppkes y col. 2014).

La aplicación de RNA-seq en tejidos de rodaballos con enfermedad severa (Artículo III) proporcionó un mayor soporte a la hipótesis de la implicación de una respuesta inflamatoria local exacerbada en el desarrollo de los daños epiteliales. Se evidenció la sobreexpresión de numerosos genes pro-inflamatorios, mientras que la expresión disminuida de diferentes genes relacionados con enzimas antioxidantes indicó que el estrés oxidativo consiguiente a la inflamación no estaba siendo contrarrestado. Los ciegos pilóricos también presentaron sobreexpresión de varios genes pro-apoptóticos, incluida la caspasa-3, que había sido objeto de un estudio inmunohistoquímico en rodaballos con enteromixosis (Losada y col. 2014a). En ese trabajo, se detectó un incremento de enterocitos inmunorreactivos en la fase avanzada de la enfermedad. La apoptosis, sin embargo, podría no estar implicada únicamente en las lesiones epiteliales, ya que Losada y col. (2014a) también reportaron la presencia de numerosas células apoptóticas en los infiltrados inflamatorios. Esta elevada tasa de muerte celular provocaría una mayor exigencia de reclutamiento de leucocitos desde los órganos

linfohematopoyéticos, pudiendo relacionarse con el desarrollo de otra lesión característica de la enteromixosis severa, la depleción leucocitaria en el bazo y el riñón (Losada y col. 2014a).

La participación de los principales órganos linfohematopoyéticos en la respuesta frente a la enteromixosis fue demostrada por el incremento en el riñón y el bazo de células positivas a IgM (Bermúdez y col. 2006a) e iNOS (Losada y col. 2012). El TNFα puede estar involucrado en esa respuesta, dado su papel central en la regulación de la activación, proliferación y migración de los leucocitos (Goetz y col. 2004; Ordás y col. 2007; Parameswaran y Patial 2010; Waters y col. 2013). En el presente trabajo (Artículo II) se ha demostrado una importante actividad de la citoquina en esos órganos, en los peces con infección moderada, reflejada por el aumento del número de células inmunorreactivas, así como de su expresión génica. La activación de esta respuesta resultaría en la migración de leucocitos hacia el tracto intestinal, tejido diana de la infección (Bermúdez y col. 2006a; Artículo II). Sin embargo, mientras la respuesta local sigue muy activa en las fases más avanzadas de la enfermedad, en bazo y en riñón se produce una disminución de células IgM-positivas (Bermúdez y col. 2006a), TNFα-positivas (Artículo II) e infraexpresión de numerosos genes relacionados con la respuesta adaptativa (Artículo III).

Globalmente, la respuesta frente a *E. scophthalmi* muestra ser inefectiva, y un posible fallo en la conexión entre los mecanismos de respuesta innata y adquirida había sido sugerido en estudios previos (Sitjà-Bobadilla y col. 2006). El análisis transcriptómico de los peces con enfermedad severa (Artículo III) evidenció la infraexpresión de distintos genes propios de elementos que juegan un papel crucial en la coordinación de esos mecanismos, cómo las células dendríticas, los linfocitos Th17 y los interferones. El detrimento de la respuesta inmunitaria durante la enteromixosis avanzada se ha relacionado en varias ocasiones con la importante depleción celular que sufren el riñón y el bazo (Bermúdez y col. 2006a; Sitjà-Bobadilla y col. 2006; Artículo II y Artículo III). El reclutamiento de leucocitos en el tracto intestinal, aunque exacerbado por la apoptosis que los mismos sufren a este nivel, parece una de las posibles causas de esta lesión pero no la única (Bermúdez y col. 2006a; Losada y col. 2014a). Estudios histológicos previos también habían sugerido que la muerte celular por apoptosis podría estar contribuyendo de forma directa a la depleción celular, habiéndose detectado un incremento de figuras apoptóticas en bazo y riñón (Bermúdez y col. 2006a; Sitjà-Bobadilla y col. 2006). Sin embargo, el inmunomarcaje con caspasa-3 activa (Losada y col. 2014a) y los resultados obtenidos con RNA-seq (Artículo III) no aportaron evidencias sólidas que soporten esta hipótesis.

Además de la infraexpresión de genes relacionados con el sistema inmunitario, el bazo y el riñón de los peces con enteromixosis severa también mostraron una infraexpresión de numerosos genes relacionados con la eritropoyesis (Artículo III). Este resultado se relacionó con una reducida disponibilidad de hierro, reflejada por la baja expresión de la ferritina y la sobreexpresión de la hepcidina, considerada la principal hormona reguladora del metabolismo del hierro y responsable de la llamada anemia de las enfermedades crónicas (Ganz 2002, 2011). Asimismo, la anorexia y los graves daños intestinales sufridos por los rodaballos en esa fase de la enfermedad (Bermúdez y col. 2010; Sitjà-Bobadilla y Palenzuela 2012), con la

consiguiente afectación de la función absortiva, podrían contribuir a la escasa disponibilidad de hierro. Por otro lado, en mamíferos también se observó que las rutas de señalización que se activan en las inflamaciones crónicas pueden afectar la hematopoyesis, actuando directamente sobre la capacidad de autorrenovación y repoblación a partir de las células madres hematopoyéticas (Schuettpelz y col. 2013). Todos estos mecanismos podrían estar involucrados, en distintos grados, en la depleción del tejido linfohematopoyético asociada a la enteromixosis. A tal propósito, la posible implicación del TNFα merece ser estudiada en profundidad, ya que reviste un papel importante en la linfohematopoyesis, pudiendo actuar como regulador positivo o negativo (Schuettpelz y col. 2013; Waters y col. 2013). Es interesante resaltar que las doradas parasitadas por *E. leei* no suelen presentar depleción celular, y tampoco muestran una elevada actividad del TNFα ni en bazo ni en riñón durante la enfermedad (Sitjà-Bobadilla y col. 2008; Pérez-Cordón y col. 2014), a diferencia de lo que se ha observado en el rodaballo (Artículo II).

Por otra parte, en la enteromixosis de la dorada también se ha descrito una importante respuesta local a nivel intestinal, incluido un aumento de expresión del TNFα (Davey y col. 2011; Pérez-Cordón y col. 2014). La menor entidad de las lesiones intestinales en esta especie podría ser debida a la activación de mecanismos antiinflamatorios (Sitjà-Bobadilla y col. 2008; Davey y col. 2011; Pérez-Cordón y col. 2014), lo que no parece ocurrir en el rodaballo (Artículo III). Sin embargo, en ambas especies aparecen signos clínicos propios de un síndrome caquectizante tales como anorexia, pérdida de peso y atrofia muscular (Sitjà-Bobadilla y Palenzuela 2012). En su desarrollo se ha postulado la participación de la respuesta inmunitaria y sus interacciones con el sistema neuroendocrino mediante la acción de los péptidos intestinales (Bermúdez y col. 2007; Estensoro y col. 2009, 2011; Losada y col. 2014b). La acción de las citoquinas proinflamatorias tiene demostrados efectos caquectizantes en mamíferos, siendo el TNFα una de las principales moléculas involucradas. Estas moléculas actúan tanto a nivel periférico como central, y, alterando la producción de hormonas y neuromoduladores, repercuten finalmente sobre el metabolismo y el apetito, siendo los principales responsables de la anorexia, la pérdida de peso y el desgaste tisular (Morley y col. 2006; Tizard 2008; Grossberg y col. 2010; Freeman 2012). El bloqueo farmacológico del TNFα ha mostrado efectos beneficiosos en distintos casos de caquexia, incluidos los derivados de enfermedades parasitarias (Truyens y col. 1995; Marcora y col. 2006; Morley y col. 2006; Cawthorn y Sethi 2008; Argilés y col. 2011). Los resultados obtenidos en esta Tesis Doctoral apuntan a la implicación de estos mecanismos patogénicos en el desarrollo del síndrome caquectizante asociado a la enteromixosis. Se evidenció una intensa reacción inflamatoria a nivel intestinal y modulación de la expresión de los genes relacionados con la función digestiva, incluidos los codificantes para neuropéptidos orexigénicos y anorexigénicos (Artículo II y III). Los cambios en el transcriptoma inducidos por la enfermedad resultaron indicativos de la instauración de un estado anoréxico, así como reflejaron el desgaste tisular característico de caquexia, disminuyendo notablemente la actividad de numerosos genes relacionados con las proteínas estructurales en los tres tejidos estudiados (Artículo III).

En el primer análisis transcriptómico realizado (Artículo III) uno de los objetivos fue testar la potencia de la tecnología RNA-seq para el estudio del perfil de expresión génica relacionado con las alteraciones evidentes de la enfermedad avanzada. La aplicación de esta herramienta se reveló de gran utilidad para la investigación de la enteromixosis, proporcionando una gran cantidad de datos valiosos. Se corroboraron observaciones histopatológicas previas y se esclarecieron las bases genéticas de la fisiopatología de la enfermedad avanzada. Además, se abrieron nuevos enfoques para el estudio de los mecanismos patogénicos. A raíz de estas consideraciones, se planteó un trabajo similar sobre la fase inicial de la enteromixosis (Artículo IV), con el fin de esclarecer las incógnitas existentes respecto a las primeras interacciones parásito-hospedador. Para este análisis se seleccionaron rodaballos con signos muy incipientes de enfermedad, constituidos por alteraciones muy sutiles a nivel histológico, tales como la presencia de los primeros estadios de desarrollo del parásito e inflamación leve de los ciegos pilóricos y/o del intestino anterior.

Se observó, como se esperaba, una notable diferencia a nivel cuantitativo en los cambios transcriptómicos asociados a las dos fases de la enfermedad. Los genes diferencialmente expresados en la fase incipiente fueron 287, 211 y 187 en riñón, bazo y ciegos pilóricos, respectivamente (Artículo IV), contra los 1.316, 1.377 y 3.022 encontrados en la enteromixosis severa (Artículo III). Sin embargo, analizando las funciones asociadas se obtuvieron resultados muy interesantes, como una modulación común de algunos genes en las dos etapas de enfermedad. Por ejemplo, en ambos estudios se detectó la sobreexpresión del gen CD209 en los ciegos pilóricos. El producto de este gen es un receptor de lectinas tipo C, un antígeno de superficie característico de las células dendríticas, que actúa en el reconocimiento de agentes patógenos (Osorio y Reis e Sousa 2011; Shao y col. 2015). El papel de las lectinas en el reconocimiento de *E. scophthalmi* se había postulado anteriormente (Redondo y col. 2008; Redondo y Álvarez-Pellitero 2010b) y esta hipótesis se vio reforzada por los resultados de esta memoria (Artículos III y IV).

A nivel intestinal, el estudio de la enteromixosis temprana (Artículo IV), también mostró cambios de expresión posiblemente relacionados con los mecanismos de adhesión y penetración del epitelio por parte de *E. scophthalmi*. Éstos incluyen la remodelación del citoesqueleto de las células del hospedador y la inhibición de la renovación fisiológica del epitelio de revestimiento intestinal (Artículo IV). Estas estrategias son comunes a otros patógenos, incluidos parásitos intestinales como *Cryptosporiudium parvum*, capaz de inducir una modulación bifásica de la apoptosis (Liu y col. 2009). Una situación similar puede observarse en la enteromixosis, donde tanto la proliferación celular como la apoptosis aparecieron reprimidas en la fase inicial (Artículo IV), mientras que la muerte celular está claramente aumentada en las fases más avanzadas de la infección (Losada y col. 2014a; Artículo III). La inhibición de la renovación epitelial podría facilitar el éxito en la invasión y proliferación en el tracto gastrointestinal por parte del parásito, mientras que el incremento de la apoptosis en la fase tardía contribuiría a la dispersión del parásito y su supervivencia en el medio (Redondo y col. 2003b; Bermúdez y col. 2010).

A los mecanismos de infección, también hay que sumar las posibles estrategias que *E. scophthalmi* pueda desarrollar para evadir la respuesta inmunitaria. Muchos agentes parasitarios, incluidos algunas especies de mixozoos en peces (Lom y Dyková 2006; Sitjà-Bobadilla y col. 2015), se benefician de presentar una localización intracelular. Este aspecto sigue siendo controvertido en el caso de *E. scophthalmi*, aunque se ha descrito esporádicamente la localización intracelular de los primeros estadios de desarrollo (Palenzuela y col. 2002; Redondo y col. 2003a, 2004). Los resultados obtenidos investigando la enfermedad incipiente (Artículo IV) proporcionaron soporte a esta hipótesis, ya que se detectaron evidencias de activación de la ruta relacionada con los receptores intracelulares tipo RIG (gen inducible por ácido retinoico). La activación de esos receptores induce una respuesta inmunitaria mediada por los interferones, con sobreexpresión de los genes estimulados por esas moléculas (ISG, genes estimulados por interferón) (Dixit & Kagan 2013; Nie y col. 2015). Ese perfil de expresión se observó en los tres órganos estudiados, junto a algunas evidencias de activación de los linfocitos T, también compatible con una respuesta mediada por los interferones frente a un antígeno intracelular. Asimismo, éstos resultados también corroborarían la existencia de una fase de dispersión por vía hemática del parásito, previamente sugerida por la observación de estadios de *E. scophthalmi* en frotis de sangre (Redondo y col. 2003a, 2004).

En este punto, es interesante resaltar que en el trabajo sobre la enteromixosis avanzada (Artículo III), se detectó un patrón opuesto, con infraexpresión de genes relacionados con las rutas de los interferones en los tres órganos. Esto podría indicar que se desarrolla un tipo de respuesta inmunitaria diferente, lo que, entre otras razones, podría deberse a un cambio de localización del parásito de intracelular a extracelular. Por otra parte, este perfil de expresión podría relacionarse con un fallo en el desarrollo de la respuesta inmunitaria, posiblemente un mecanismo de evasión del patógeno, como a menudo se ha descrito en enfermedades víricas de mamíferos (Gale y Sen 2009; Taylor y Mossman 2012; Song y col. 2013). Además, tanto en mamíferos como en peces, distintas investigaciones han destacado la importancia de la respuesta inmunitaria mediada por interferones frente a enfermedades parasitarias (Álvarez-Pellitero 2008b; McCall y Sauerwein 2010; Beiting 2014; Sitjà-Bobadilla y col. 2015). En teleósteos, su inhibición ha sido relacionada con una mayor susceptibilidad a enfermedad amebiana de las branquias en salmón (Young y col. 2008). En cambio, la sobreexpresión de distintos ISG en doradas expuestas a *E. leei* que no desarrollaron la infección sugirió una correlación con la resistencia a la enfermedad (Davey y col. 2011). En el caso del rodaballo, se puede postular una correlación entre la regulación negativa de esos genes en la enteromixosis avanzada y la elevada susceptibilidad de la especie a la enfermedad.

En la fase incipiente de la enteromixosis, también se detectaron cambios de expresión indicativos de inhibición de la respuesta inmunitaria (Artículo IV). Si por un lado se evidenció la activación de la respuesta mediada por interferones, por otro se encontró en riñón la sobreexpresión de un gen (SOCS1) con acción inhibitoria sobre las vías de señalización de esas moléculas (Skjesol y col. 2014). Asimismo, en riñón y ciegos pilóricos estaba infraexpresado el CD2, gen que codifica para un antígeno de superficie de los linfocitos T y

las células NK (natural killer) (Artículo IV). La infraexpresión del CD2 se ha encontrado también asociada a la parasitación por *Leishmania donovani* en humanos, donde se ha relacionado con trastornos en la función de los linfocitos T (Bimal y col. 2008). Esos resultados indican que, aunque haya activación de ciertos mecanismos para la eliminación del parásito, éstos podrían ser contrarrestados y no desarrollarse adecuadamente.

Otras evidencias sobre las posibles estrategias de evasión del sistema inmune por parte de *E. scophthalmi* se encontraron con respecto a la respuesta de fase aguda (Artículo IV). Numerosos genes codificantes para proteínas de fase aguda mostraron una expresión disminuida, un resultado llamativo para la fase temprana de la infección. Esas proteínas incluyeron diferentes componentes del sistema complemento, antiproteasas y proteínas relacionadas con el metabolismo del hierro. En el bazo se encontró enriquecida la ruta "complemento y cascada de la coagulación" (base de datos KEGG) que reúne muchos de esos genes (Artículo IV). Ese perfil de expresión indica que *E. scophthalmi* podría ser capaz de eludir las defensas innatas del hospedador, mediante la inhibición de sus actores principales. El sistema complemento y el metabolismo del hierro son dianas conocidas de diferentes agentes patógenos (Zipfel y col. 2007; Ben-Othman y col. 2014; Leon-Sicairos y col. 2015), y, del mismo modo, la inhibición de las enzimas antiproteasas del hospedador reforzaría la virulencia del parásito (Armstrong 2006; Gómez y col. 2014; Sitjà-Bobadilla y col. 2015).

Estos hallazgos apuntan a una posible inhibición de la respuesta del hospedador, que favorecería la proliferación y la diseminación del parásito en el tracto gastrointestinal. La hipótesis concordaría con el largo periodo de prepatencia que presenta la enteromixosis y estaría soportada por otras observaciones de este estudio (Artículo IV). En particular, se observó que el perfil de expresión génica de los tres órganos pertenecientes a uno de los peces infectados se agrupó con el grupo control, sugiriendo una escasa respuesta frente al parásito. El grado de infección de este ejemplar también había sido evaluado como leve, dada la ausencia de lesiones y la escasa carga parasitaria. Sin embargo, este pez presentaba algún estadio de desarrollo de *E. scophthalmi* más avanzado, y presencia de formas parasitarias en el intestino posterior, lo que sugiere un estadio de infección ligeramente más avanzado. Estas observaciones podrían indicar que la respuesta del hospedador está silenciada en alguna fase de la enfermedad, y necesitan ser profundizadas en futuros estudios.

En resumen, el conjunto de resultados obtenidos en esta Tesis Doctoral permite postular una posible secuencia de eventos que caracterizan la infección del rodaballo por *E. scophthalmi*. El mixozoo en las fases incipientes de la infección utilizaría diferentes mecanismos para la inhibición y evasión del primer ataque del sistema inmunitario del hospedador (Artículo IV). Esto le permitiría alcanzar su diana, el tracto gastrointestinal, e iniciar la invasión del epitelio de revestimiento. El éxito de la infección, con la proliferación y diseminación del parásito a lo largo del tubo digestivo, estarían favorecidos por la inhibición de la renovación del epitelio intestinal y el silenciamiento de la respuesta del hospedador (Artículo IV). Con el aumento de la carga parasitaria empezarían a producirse daños en la mucosa, desencadenado la reacción inflamatoria (Artículo II). La respuesta inmunitaria suscitada, sin embargo, resultaría disfuncional y exacerbada, siendo inefectiva para frenar la infección, pero estaría involucrada

en el desarrollo de los signos clínicos y las lesiones típicos de la enfermedad (Artículos II y III).

Los estudios realizados sugieren que el diseño de terapias inmunomoduladoras podría ser beneficioso para frenar el desarrollo de las lesiones y el curso de la enteromixosis en rodaballo. Por otra parte, sería más deseable la implementación de medidas de prevención o herramientas de control de la enfermedad en su fase incipiente. A este respecto, la identificación de los mecanismos moleculares involucrados en la patogenia y, en particular, de genes candidatos como marcadores de resistencia y/o de infección, es una base esencial para poder desarrollar programas de selección genética así como para encontrar posibles dianas terapéuticas. La investigación en patología se ha enriquecido con nuevas y potentes herramientas, que pueden conllevar avances significativos en el conocimiento de los mecanismos patogénicos. Para ese fin, se requiere una aproximación multidisciplinar que permita obtener una información genómica de calidad y un análisis e integración adecuados sobre su relevancia patológica en el contexto del organismo y de la enfermedad.

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Conclusiones/Conclusions

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CONCLUSIONES

- **La técnica inmunohistoquímica desarrollada con un anticuerpo anti-TNFα humano presenta una adecuada especificidad para la detección del TNFα en tejidos de rodaballo**, constituyendo una herramienta útil para el estudio de esta citoquina en esta especie.
- **En condiciones fisiológicas, el rodaballo presenta células inmunorreactivas a TNFα en localizaciones estratégicas para la entrada y el procesamiento de antígenos**. Estas células son, en su mayoría, morfológicamente compatibles con monocitos/macrófagos, por lo que se hipotetiza este tipo celular como principal productor de TNFα en rodaballo.
- **Las células rodlet están implicadas en la respuesta inmunitaria del rodaballo**, dada la discreta presencia de células rodlet TNFα-positivas en condiciones fisiológicas y su aumento en los ejemplares con enteromixosis moderada.
- **El TNFα podría tener un papel relevante en la fisiología de los órganos linfohematopoyéticos del rodaballo**, dada la elevada positividad encontrada en ejemplares sanos.
- **El TNFα está involucrado en el desarrollo de la respuesta inmunitaria frente a la enteromixosis**. La elevada activada actividad del TNFα en los órganos linfohematopoyéticos de rodaballos con infección moderada y el aumento progresivo de células TNFα-positivas en el tracto gastrointestinal, sugieren el papel de esa citoquina en la inducción de la migración de leucocitos al sitio de infección.
- **La tecnología RNA-seq resulta una herramienta de gran utilidad para el estudio de la enteromixosis**, maximizándose su potencial mediante una meticulosa interpretación morfofisiopatológica de los datos obtenidos.
- ***E. scophthalmi* podría inhibir la renovación fisiológica del epitelio intestinal** durante las primeras fases de la enteromixosis para asegurar el éxito de la infección. Asimismo, hay evidencias de que la remodelación del citoesqueleto de las células del hospedador está involucrada en los mecanismos de invasión del epitelio por el mixozoo.
- ***E. scophthalmi* resiste el ataque del sistema inmunitario del rodaballo**, durante la fase incipiente de la enfermedad, posiblemente inhibiendo diferentes componentes de la respuesta de fase aguda.
- **Las lectinas tipo C y los receptores RIG se postulan como moléculas de reconocimiento de *E. scophthalmi.*** Las evidencias de activación de los receptores RIG, junto con la sobreexpresión de los genes relacionados con los interferones en la enfermedad incipiente, apoyan la hipótesis de la localización intracelular de los primeros estadios de *E. scophthalmi*.
- **Las rutas mediadas por los interferones revisten un importante papel en la respuesta inmunitaria del rodaballo frente a la enteromixosis**. Son el principal mecanismo activado en la respuesta temprana a la parasitación, aunque no parecen capaces de frenarla. Por otra parte, su inactivación en las fases más avanzadas de la enfermedad podría estar relacionada con una inadecuada conexión entre respuesta

innata y adaptativa. Asimismo, su regulación negativa podría reflejar un cambio de localización del parásito según la fase de la enfermedad, que induciría un tipo de respuesta diferente.

- **Las lesiones intestinales asociadas a la enteromixosis se relacionan con una respuesta inflamatoria exacerbada a nivel local**. El tubo digestivo sufre una prolongada exposición a los mediadores de la respuesta inflamatoria, incluido el TNFα, sin que haya evidencias de activación de mecanismos antiinflamatorios. A este cuadro se asocia un incremento de muerte celular por apoptosis en las fases avanzadas de la enfermedad.
- **La depleción celular de los órganos linfohematopoyéticos observada en la enteromixosis severa se asocia a la infraexpresión de genes involucrados en la respuesta inmunitaria y en la hematopoyesis**. La prolongada reacción inflamatoria, mediante la acción del TNFα, es una de las posibles causas del desarrollo de esta lesión.
- **La inhibición de la hematopoyesis podría tener un origen multifactorial**, relacionándose también con trastornos en el metabolismo del hierro. Esto contribuiría a instaurar el estado anémico de los rodaballos en la enteromixosis severa.
- **La anorexia que sufren los rodaballos enfermos se refleja en el detrimento de la función digestiva y en la modulación de los genes que codifican los neuropéptidos intestinales**. La acción de las citoquinas pro-inflamatorias y de estos péptidos sería la responsable de este signo clínico tan característico de la enteromixosis.
- **En la fase avanzada de la enteromixosis se produce una infraexpresión generalizada de los genes codificantes para diferentes proteínas estructurales**. Esto refleja el desgaste tisular propio de un síndrome caquéctico, y es indicativo de la baja disponibilidad de energía y nutrientes en los peces parasitados.

CONCLUSIONS

- **The immunohistochemical technique set up with an anti-human TNFα antibody exhibits a proper specificity for detecting TNFα in turbot tissues**, being a useful tool to study this cytokine.
- **Under physiological conditions, turbot presents immunoreactive cells to TNFα, which appear to be strategically located in the areas of antigen stimulation and processing.** Mostly of these cells are morphologically consistent with monocytes/macrophages, postulated as the main TNFα producer cells in turbot.
- **Rodlet cells are involved in turbot immune response**, given the amount of TNFα-positive cells found under physiological conditions and their increased numbers in moderately *E. scophthalmi*-infected fish.
- **TNFα may present a main role in the physiology of turbot lymphohaematopoietic organs**, given the wide immunoreactivity to this cytokine detected in healthy specimens.
- **TNFα is involved in turbot immune response against the parasitization by *E. scophthalmi*.** The enhanced gene expression and TNFα-positive cells density in the lymphohaematopoietic organs and the progressive increase TNFα-positive cells in the digestive tract points toward a role for turbot TNFα in inducing leukocyte migration to the infection site.
- **RNA-seq technology proves to be a very useful tool for investigating enteromyxosis**, maximizing its potential through a meticulous morphophysiopathological interpretation of the resulting data.
- ***E. scophthalmi* possibly inhibits the physiological renewal of the host intestinal epithelium** in early stages of enteromyxosis to ensure a successful infection. As well, cytoskeletal remodelling of host cells appear to be involved as a myxozoan strategy for epithelial invasion.
- ***E. scophthalmi* survives the first attack by turbot immune system** possibly inhibiting of different components of the acute phase response.
- **C-type lectins and RIG receptors are postulated as recognition molecules for *E. scophthalmi*.** The evidences of RIG receptors activation along with the up-regulation of interferon-related genes in early enteromixosis support the hypothesis of an intracellular localization of the first developmental stages of *E. scophthalmi*.
- **Interferon-mediated pathways play an important role in the immune response to enteromixosis.** These are the main activated mechanism in the early response to parasitization, although it does not seem capable of stopping it. On the other hand, their inactivation in later stages of the disease may be related to the failure in the connexion between innate and adaptive immunity. Also, the down-regulation of those pathways may reflect a different response depending on the disease stage, due to a change in parasite localization.
- **The intestinal lesions associated to enteromyxosis are related to an exacerbated local inflammatory response.** The digestive tract suffers a prolonged exposure to

inflammatory mediators, included TNFα, and there is no evidence of an efficient activation of anti-inflammatory mechanisms. This condition is associated with increased rates of apoptotic cell death in late stages of the disease.

- **Cell depletion in the lymphohaematopoietic organs of severely infected turbot is related to the down-regulation of several genes involved in immune response and haematopoiesis.** A prolonged inflammatory response, through the action TNFα, is a possible cause for the development of this lesion.
- **Inhibition of haematopoiesis may have a multifactorial origin**, being also related to iron metabolism disorders. This would contribute to the instauration of anaemia during severe enteromyxosis.
- **The anorexia suffered by diseased turbot is reflected by the detriment of digestive function and modulation of genes encoding intestinal neuropeptides.** The effects of neuropeptides and pro-inflammatory cytokines are probably at the basis of the development of this clinical sign characteristic of enteromyxosis.
- **In late enteromyxosis, turbot present a wide down-regulation of genes encoding structural proteins,** which reflects the typical tissue wasting of a cachectic syndrome and denotes the low availability of energy and nutrients in parasitized fish.

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REFERENCIAS BIBLIOGRÁFICAS DE INTRODUCCIÓN Y DISCUSIÓN GENERAL

Adriano, E.A., Carriero, M.M., Maia, A.A.M., Silva, M.R.M., Naldoni, J., Ceccarelli, P.S., Arana, S., 2012. Phylogenetic and host–parasite relationship analysis of *Henneguya multiplasmodialis* n. sp. infecting *Pseudoplatystoma* spp. in Brazilian Pantanal wetland. Veterinary Parasitology 185, 110-120.

Alonso, M., Lago, F.C., Gómez-Reino, M., Fernández Casal, J., Martín Varela, I., Vieites, J.M., Espiñeira, M., 2015. Non-invasive fast real-time PCR assay for detection of the enteric parasite *Enteromyxum scophthalmi* in cultured turbot (*Scophthalmus maximus* L.). Aquaculture Research 46, 2104-2115.

Álvarez-Pellitero, P., Palenzuela, O., Sitjà-Bobadilla, A., 2008a. Histopathology and cellular response in *Enteromyxum leei* (Myxozoa) infections of *Diplodus puntazzo* (Teleostei). Parasitology International 57, 110-120.

Álvarez-Pellitero, P., 2008b. Fish immunity and parasite infections: from innate immunity to immunoprophylactic prospects. Veterinary Immunology and Immunopathology 126, 171-198.

APROMAR, 2015. La Acuicultura en España 2015. Disponible en línea en http://www.apromar.es/content/informes-anuales.

Argilés, J.M., Busquets, S., López-Soriano, F.J., 2011. Anti-inflammatory therapies in cancer cachexia. European Journal of Pharmacology 668 Suppl 1, S81-86.

Armstrong, P.B., 2006. Proteases and protease inhibitors: a balance of activities in host-pathogen interaction. Immunobiology 211, 263-281.

Beiting, D.P., 2014. Protozoan parasites and type I interferons: a cold case reopened. Trends in Parasitology 30, 491-498.

Ben-Othman, R., Flannery, A.R., Miguel, D.C., Ward, D.M., Kaplan, J., Andrews, N.W., 2014. *Leishmania*-mediated inhibition of iron export promotes parasite replication in macrophages. PLoS Pathogens 10, e1003901.

Berman, D.M., Bosenberg, M.W., Orwant, R.L., Thurberg, B.L., Draetta, G.F., Fletcher, C.D.M., Loda, M., 2012. Investigative pathology: leading the post-genomic revolution. Laboratory Investigation 92, 4-8.

Bermúdez, R., Vigliano, F., Marcaccini, A., Sitjà-Bobadilla, A., Quiroga, M.I., Nieto, J.M., 2006a. Response of Ig-positive cells to *Enteromyxum scophthalmi* (Myxozoa) experimental infection in turbot, *Scophthalmus maximus* (L.): A histopathological and immunohistochemical study. Fish & Shellfish Immunology 21, 501-512.

Bermúdez, R., Alemañ, N., Vigliano, F., Vázquez, S., Quiroga, M.I., Nieto, J.M., 2006b. Effects of symmetric triazinone (toltrazuril) on developmental stages of *Enteromyxum scophthalmi* parasitizing turbot (*Scophthalmus maximus* L.): A light and electron microscopic study. Aquaculture 254, 65-71.

Bermúdez, R., Vigliano, F., Quiroga, M.I., Nieto, J.M., Bosi, G., Domeneghini, C., 2007. Immunohistochemical study on the neuroendocrine system of the digestive tract of

turbot, *Scophthalmus maximus* (L.), infected by *Enteromyxum scophthalmi* (Myxozoa). Fish & Shellfish Immunology 22, 252-263.

Bermúdez, R., Losada, A.P., Vázquez, S., Redondo, M.J., Álvarez-Pellitero, P., Quiroga, M.I., 2010. Light and electron microscopic studies on turbot *Psetta maxima* infected with *Enteromyxum scophthalmi*: histopathology of turbot enteromyxosis. Diseases of Aquatic Organisms 89, 209-221.

Bienvenu, A.L., Gonzaléz-Rey, E., Picot, S., 2010. Apoptosis induced by parasitic diseases. Parasites & Vectors 3, 106.

Bimal, S., Singh, S.K., Sinha, S., Pandey, K., Sinha, P.K., Ranjan, A., Bhattacharya, S.K., Das, P., 2008. *Leishmania donovani*: role of CD2 on CD4+ T-cell function in visceral leishmaniasis. Experimental Parasitology 118, 238-246.

Bjork, S.J., Bartholomew, J.L., 2010. Invasion of *Ceratomyxa shasta* (Myxozoa) and comparison of migration to the intestine between susceptible and resistant fish hosts. International Journal for Parasitology 40, 1087-1095.

Bradley, J.R., 2008. TNF-mediated inflammatory disease. The Journal of Pathology 214, 149-160.

Branson, E., Riaza, A., Álvarez-Pellitero, P., 1999. Myxosporean infection causing intestinal disease in farmed turbot, *Scophthalmus maximus* (L.), (Teleostei: Scophthalmidae). Journal of Fish Diseases 22, 395-399.

Bruno, D.W., Noguera, P.A., Poppe T.T., 2013. A colour atlas of salmonid diseases. Springer, London.

Buchmann, K., 2014. Evolution of innate immunity: clues from invertebrates via fish to mammals. Frontiers in Immunology 5, 459.

Canning, E.U., Tops, S., Curry, A., Wood, T.S., Okamura, B., 2002. Ecology, development and pathogenicity of *Buddenbrockia plumatellae* Schroder, 1910 (Myxozoa, Malacosporea) (syn. *Tetracapsula bryozoides*) and establishment of *Tetracapsuloides* n. gen. for *Tetracapsula bryosalmonae*. The Journal of Eukaryotic Microbiology 49, 280-295.

Cawthorn, W.P., Sethi, J.K., 2008. TNF-alpha and adipocyte biology. FEBS Letters 582, 117-131.

Costa, V., Aprile, M., Esposito, R., Ciccodicola, A., 2013. RNA-Seq and human complex diseases: recent accomplishments and future perspectives. European Journal of Human Genetics 21, 134-142.

Chokshi, N.K., Guner, Y.S., Hunter, C.J., Upperman, J.S., Grishin, A., Ford, H.R., 2008. The role of nitric oxide in intestinal epithelial injury and restitution in neonatal necrotizing enterocolitis. Seminars in Perinatology 32, 92-99.

Davey, G.C., Calduch-Giner, J.A., Houeix, B., Talbot, A., Sitjà-Bobadilla, A., Prunet, P., Pérez-Sánchez, J., Cairns, M.T., 2011. Molecular profiling of the gilthead sea bream (*Sparus aurata* L.) response to chronic exposure to the myxosporean parasite *Enteromyxum leei*. Molecular Immunology 48, 2102-2112.

Dezfuli, B.S., Giari, L., Shinn, A.P., 2007. The role of rodlet cells in the inflammatory response in *Phoxinus phoxinus* brains infected with *Diplostomum*. Fish & Shellfish Immunology 23, 300-304.

Diamant, A., 1992. A new pathogenic histozoic *Myxidium* (Myxosporea) in cultured gilthead sea bream *Sparus aurata* L. Bulletin of the European Association of Fish Pathologists 12, 64-66.

Diamant, A., 1997. Fish-to-fish transmission of a marine myxosporean. Disease of Aquatic Organism 30, 99–105.

Diamant, A., Ram, S., Paperna, I., 2006. Experimental transmission of *Enteromyxum leei* to freshwater fish. Diseases of Aquatic Organisms 72, 171-178.

Dixit, E., Kagan, J.C., 2013. Intracellular pathogen detection by RIG-I-like receptors. Advances in Immunology 117, 99-125.

Dyková, I. & Lom, J., 1988. Review of pathogenic myxosporeans in intensive culture of carp (*Cyprinus carpio*) in Europe. Folia Parasitologica 35, 289-307.

Dyková, I., de Buron, I., Roumillat, W.A., Fiala, I., 2011. *Henneguya cynoscioni* sp. n. (Myxosporea: Bivalvulida), an agent of severe cardiac lesions in the spotted seatrout, *Cynoscion nebulosus* (Teleostei: Sciaenidae). Folia Parasitologica 58, 169-177.

Estensoro, I., Bermúdez, R., Losada, A.P., Quiroga, M.I., Pérez-Sánchez, J., Álvarez-Pellitero, P., Sitjà-Bobadilla, A., 2009. Effect of *Enteromyxum leei* (Myxozoa) on gastrointestinal neuromodulators and cell apoptosis of gilthead sea bream (*Sparus aurata*), 14th International Conference of the European Association of Fish Pathologists (EAFP) on Diseases of Fish and Shellfish, Prague, Czech Republic, Libro de Actas, p. 264.

Estensoro, I., Álvarez-Pellitero, P., Sitjà-Bobadilla, A., 2013. Antigenic characterization of *Enteromyxum leei* (Myxozoa: Myxosporea). Diseases of Aquatic Organisms 106, 149-162.

Estensoro, I., Redondo, M.J., Álvarez-Pellitero, P., Sitjà-Bobadilla, A., 2014. Immunohistochemical characterization of polyclonal antibodies against *Enteromyxum leei* and *Enteromyxum scophthalmi* (Myxozoa: Myxosporea), intestinal parasites of fish. Journal of Fish Diseases 37, 785-796.

Feng, J.M., Xiong, J., Zhang, J.Y., Yang, Y.L., Yao, B., Zhou, Z.G., Miao, W., 2014. New phylogenomic and comparative analyses provide corroborating evidence that Myxozoa is Cnidaria. Molecular Phylogenetics and Evolution 81, 10-18.

Figueras, A., Robledo, D., Corvelo, A., Hermida, M., Pereiro, P., Rubiolo, J.A., Gómez-Garrido, J., Carreté L., Bello, X., Gut, M., Gut, I.G., Marcet-Houben, M., Forn-Cuní, G., Abal-Fabeiro, J.L., Pardo, B.G., Taboada, X., Fernández, C., Vlasova, A., Hermoso-Pulido, A., Guigó, R., Álvarez-Dios, J.A., Gómez-Tato, A., Viñas, A., Maside, X., Gabaldón, T., Novoa, B., Bouza, C., Alioto, T., Martínez, P., 2016. Whole genome sequencing of turbot (*Scophthalmus maximus*; Pleuronectiformes): a fish adapted to demersal life. DNA Research, in press.

Fleurance, R., Sauvegrain, C., Marques, A., Le Breton, A., Guereaud, C., Cherel, Y., Wyers, M., 2008. Histopathological changes caused by *Enteromyxum leei* infection in farmed sea bream *Sparus aurata*. Diseases of Aquatic Organisms 79, 219-228.

Freeman, L.M., 2012. Cachexia and sarcopenia: emerging syndromes of importance in dogs and cats. Journal of Veterinary Internal Medicine 26, 3-17.

Gale, M., Sen, G.C., 2009. Viral evasion of the interferon system. Journal of Interferon & Cytokine Research 29, 475-476.

Ganz, T., 2002. The role of hepcidin in iron sequestration during infections and in the pathogenesis of anemia of chronic disease. The Israel Medical Association Journal 4, 1043-1045.

Ganz, T., 2011. Hepcidin and iron regulation, 10 years later. Blood 117, 4425-4433.

García, J.C., 1999. Estudio anatomopatológico del síndrome de la cabeza hundida del rodaballo. Memoria de licenciatura. Universidade de Santiago de Compostela.

García-Castillo, J., Chaves-Pozo, E., Olivares, P., Pelegrín, P., Meseguer, J., Mulero, V., 2004. The tumor necrosis factor alpha of the bony fish seabream exhibits the in vivo proinflammatory and proliferative activities of its mammalian counterparts, yet it functions in a species-specific manner. Cellular and Molecular Life Sciences 61, 1331-1340.

Goetz, F.W., Planas, J.V., MacKenzie, S., 2004. Tumor necrosis factors. Developmental and Comparative Immunology 28, 487-497.

Gómez, D., Bartholomew, J., Sunyer, J.O., 2014. Biology and mucosal immunity to myxozoans. Developmental and Comparative Immunology 43, 243-256.

Grayfer, L., Walsh, J.G., Belosevic, M., 2008. Characterization and functional analysis of goldfish (*Carassius auratus* L.) tumor necrosis factor-alpha. Developmental and Comparative Immunology 32, 532-543.

Grossberg, A.J., Scarlett, J.M., Marks, D.L., 2010. Hypothalamic mechanisms in cachexia. Physiology & Behavior 100, 478-489.

Gunter, N., Adlard, R., 2010. The demise of *Leptotheca* Thélohan, 1895 (Myxozoa: Myxosporea: Ceratomyxidae) and assignment of its species to *Ceratomyxa* Thélohan, 1892 (Myxosporea: Ceratomyxidae), *Ellipsomyxa* Køie, 2003 (Myxosporea: Ceratomyxidae), *Myxobolus* Bütschli, 1882 and *Sphaerospora* Thélohan, 1892 (Myxosporea: Sphaerosporidae). Systematic Parasitology 75, 81-104.

Hasanuzzaman, A.F.M., Robledo, D., Gómez-Tato, A., Álvarez-Dios, J.A., Harrison, P.W., Cao, A., Fernández-Boo, S., Villalba, A., Pardo, B.G., Martínez, P., 2016. *De novo* transcriptome assembly of *Perkinsus olseni* trophozoite stimulated in vitro with Manila clam (*Ruditapes philippinarum*) plasma. Journal of Invertebrate Pathology 135, 22-33.

Hehlgans, T., Pfeffer, K., 2005. The intriguing biology of the tumour necrosis factor/tumour necrosis factor receptor superfamily: players, rules and the games. Immunology 115, 1-20.

Kamekura, R., Nava, P., Feng, M., Quiros, M., Nishio, H., Weber, D.A., Parkos, C.A., Nusrat, A., 2015. Inflammation-induced desmoglein-2 ectodomain shedding compromises the mucosal barrier. Molecular Biology of the Cell 26, 3165-3177.

Katharios, P., Rigos, G., Divanach, P., 2011. *Enteromyxum leei* (Myxozoa), a lethal intruder of tropical pet fish: first case in humphead wrasse, *Cheilinus undulatus* (Rüppell, 1835). Journal of Exotic Pet Medicine 20, 138-143.

Katharios, P., Kokkari, C., Sterioti, A., Smyrli, M., Kalatzis, P.G., 2014. *Enteromyxum leei* infection in parrotfish, *Sparisoma cretense*: histopathological, morphological and molecular study. Veterinary Parasitology 199, 136-143.

KEGG PATHWAY Database. Disponible en línea en http://www.genome.jp/kegg/pathway.html

Kent, M.L., Andree, K.B., Bartholomew, J.L., El-Matbouli, M., Desser, S.S., Devlin, R.H., Feist, S.W., Hedrick, R.P., Hoffmann, R.W., Khattra, J., Hallett, S.L., Lester, R.J., Longshaw, M., Palenzeula, O., Siddall, M.E., Xiao, C., 2001. Recent advances in our knowledge of the Myxozoa. The Journal of Eukaryotic Microbiology 48, 395-413.

Koppang, E.O., Fischer, U., Satoh, M., Jirillo, E., 2007. Inflammation in fish as seen from a morphological point of view with special reference to the vascular compartment. Current Pharmaceutical Design 13, 3649-3655.

Kumar, G., Abd-Elfattah, A., Saleh, M., El-Matbouli, M., 2013. Fate of *Tetracapsuloides bryosalmonae* (Myxozoa) after infection of brown trout *Salmo trutta* and rainbow trout *Oncorhynchus mykiss*. Diseases of Aquatic Organisms 107, 9-18.

Lam, F.W., Wu, S.Y., Lin, S.J., Lin, C.C., Chen, Y.M., Wang, H.C., Chen, T.Y., Lin, H.T., Lin, J.H., 2011. The expression of two novel orange-spotted grouper (*Epinephelus coioides*) TNF genes in peripheral blood leukocytes, various organs, and fish larvae. Fish & Shellfish Immunology 30, 618-629.

Le Breton, A., Marques, A., 1995. Occurrence of a histozoic *Myxidium* infection in two marine cultured species: *Puntazzo puntazzo* C. and *Pagrus major*. Bulletin of the European Association of Fish Pathologists 15, 210-212.

Leon-Sicairos, N., Reyes-Cortes, R., Guadrón-Llanos, A.M., Madueña-Molina, J., Leon-Sicairos, C., Canizalez-Román, A., 2015. Strategies of intracellular pathogens for obtaining iron from the environment. BioMed Research International 2015, 17.

Leppkes, M., Roulis, M., Neurath, M.F., Kollias, G., Becker, C., 2014. Pleiotropic functions of TNF-alpha in the regulation of the intestinal epithelial response to inflammation. International Immunology 26, 509-515.

Levsen, A., Jørgensen, A., Mo, T.A., 2008. Occurrence of postmortem myoliquefactive kudoosis in Atlantic mackerel, *Scomber scombrus* L., from the North Sea. Journal of Fish Diseases 31, 601-611.

Li, C., Zhang, Y., Wang, R., Lu, J., Nandi, S., Mohanty, S., Terhune, J., Liu, Z., Peatman, E., 2012. RNA-seq analysis of mucosal immune responses reveals signatures of intestinal barrier disruption and pathogen entry following *Edwardsiella ictaluri* infection in channel catfish, *Ictalurus punctatus*. Fish & Shellfish Immunology 32, 816-827.

Liu, J., Deng, M., Lancto, C.A., Abrahamsen, M.S., Rutherford, M.S., Enomoto, S., 2009. Biphasic modulation of apoptotic pathways in *Cryptosporidium parvum*-infected human intestinal epithelial cells. Infection and Immunity 77, 837-849.

Lom, J., Dyková, I., 2006. Myxozoan genera: definition and notes on taxonomy, life-cycle terminology and pathogenic species. Folia Parasitologica 53, 1-36.

Losada, A.P., Bermúdez, R., Faílde, L.D., Quiroga, M.I., 2012. Quantitative and qualitative evaluation of iNOS expression in turbot (*Psetta maxima*) infected with *Enteromyxum scophthalmi*. Fish & Shellfish Immunology 32, 243-248.

Losada, A.P., Bermúdez, R., Faílde, L.D., de Ocenda, M.V.R., Quiroga, M.I., 2014a. Study of the distribution of active caspase-3-positive cells in turbot, *Scophthalmus maximus* (L.), enteromyxosis. Journal of Fish Diseases 37, 21-32.

Losada, A.P., Bermúdez, R., Faílde, L.D., Di Giancamillo, A., Domeneghini, C., Quiroga, M.I., 2014b. Effects of *Enteromyxum scophthalmi* experimental infection on the neuroendocrine system of turbot, *Scophthalmus maximus* (L.). Fish & Shellfish Immunology 40, 577-583.

Lovy, J., Goodwin, A., Speare, D., Wadowska, D., Wright, G., 2011. Histochemical and ultrastructural analysis of pathology and cell responses in gills of channel catfish affected with proliferative gill disease. Diseases of Aquatic Organisms 94, 125-134.

Marcora, S.M., Chester, K.R., Mittal, G., Lemmey, A.B., Maddison, P.J., 2006. Randomized phase 2 trial of anti-tumor necrosis factor therapy for cachexia in patients with early rheumatoid arthritis. The American Journal of Clinical Nutrition 84, 1463-1472.

Marioni, J.C., Mason, C.E., Mane, S.M., Stephens, M., Gilad, Y., 2008. RNA-seq: An assessment of technical reproducibility and comparison with gene expression arrays. Genome Research 18, 1509-1517.

Martínez-Gómez, P., Sánchez-Pérez, R., Rubio, M., 2012. Clarifying omics concepts, challenges, and opportunities for *Prunus* breeding in the postgenomic era. OMICS: A Journal of Integrative Biology 16, 268-283.

Matsukane, Y., Sato, H., Tanaka, S., Kamata, Y., Sugita-Konishi, Y., 2011. *Kudoa iwatai* and two novel *Kudoa* spp., *K. trachuri* n. sp. and *K. thunni* n. sp. (Myxosporea: Multivalvulida), from daily consumed marine fish in western Japan. Parasitology Research 108, 913-926.

Mazon, A.F., Huising, M.O., Taverne-Thiele, A.J., Bastiaans, J., Verburg-van Kemenade, B.M., 2007. The first appearance of rodlet cells in carp (*Cyprinus carpio* L.) ontogeny and their possible roles during stress and parasite infection. Fish & Shellfish Immunology 22, 27-37.

McCall, M.B.B., Sauerwein, R.W., 2010. Interferon-γ—central mediator of protective immune responses against the pre-erythrocytic and blood stage of malaria. Journal of Leukocyte Biology 88, 1131-1143.

Moran, J.D.W., Whitaker, D.J. & Kent, M.L., 1999. A review of the myxosporean genus *Kudoa* Meglitsch, 1947, and its impact on the international aquaculture industry and commercial fisheries. Aquaculture 172, 163-196.

Morley, J.E., Thomas, D.R., Wilson, M.M., 2006. Cachexia: pathophysiology and clinical relevance. The American Journal of Clinical Nutrition 83, 735-743.

Morozova, O., Hirst, M., Marra, M.A., 2009. Applications of new sequencing technologies for transcriptome analysis. Annual Review of Genomics and Human Genetics 10, 135-151.

Morris, D.J., 2012. A new model for myxosporean (Myxozoa) development explains the endogenous budding phenomenon, the nature of cell within cell life stages and evolution of parasitism from a cnidarian ancestor. International Journal for Parasitology 42, 829-840.

Mulero, I., Pilar Sepulcre, M., Roca, F.J., Meseguer, J., García-Ayala, A., Mulero, V., 2008. Characterization of macrophages from the bony fish gilthead seabream using an antibody against the macrophage colony-stimulating factor receptor. Developmental and Comparative Immunology 32, 1151-1159.

Naldoni, J., Arana, S., Maia, A.A.M., Ceccarelli, P.S., Tavares, L.E.R., Borges, F.A., Pozo, C.F., Adriano, E.A., 2009. *Henneguya pseudoplatystoma* n. sp. causing reduction in epithelial area of gills in the farmed pintado, a South American catfish: Histopathology and ultrastructure. Veterinary Parasitology 166, 52-59.

Nandi, J., Saud, B., Zinkievich, J.M., Yang, Z.-J., Levine, R., 2010. TNF-α modulates iNOS expression in an experimental rat model of indomethacin-induced jejunoileitis. Molecular and Cellular Biochemistry 336, 17-24.

Nie, L., Zhang, Y.-S., Dong, W.-R., Xiang, L.-X., Shao, J.-Z., 2015. Involvement of zebrafish RIG-I in NF-κB and IFN signaling pathways: Insights into functional conservation of RIG-I in antiviral innate immunity. Developmental & Comparative Immunology 48, 95-101.

Nesnidal, M.P., Helmkampf, M., Bruchhaus, I., El-Matbouli, M., Hausdorf, B., 2013. Agent of whirling disease meets orphan worm: phylogenomic analyses firmly place Myxozoa in Cnidaria. PloS One 8, e54576.

Nookaew, I., Papini, M., Pornputtpong, N., Scalcinati, G., Fagerberg, L., Uhlén, M., Nielsen, J., 2012. A comprehensive comparison of RNA-Seq-based transcriptome analysis from reads to differential gene expression and cross-comparison with microarrays: a case study in *Saccharomyces cerevisiae*. Nucleic Acids Research, 1-14.

Ordás, M.C., Costa, M.M., Roca, F.J., López-Castejón, G., Mulero, V., Meseguer, J., Figueras, A., Novoa, B., 2007. Turbot TNFalpha gene: molecular characterization and biological activity of the recombinant protein. Molecular Immunology 44, 389-400.

Osorio, F., Reis e Sousa, C., 2011. Myeloid C-type lectin receptors in pathogen recognition and host defense. Immunity 34, 651-664.

Padrós, F., Palenzuela, O., Hispano, C., Tosas, O., Zarza, C., Crespo, S., Álvarez-Pellitero, P., 2001. *Myxidium leei* (Myxozoa) infections in aquarium-reared Mediterranean fish species. Diseases of Aquatic Organisms 47, 57-62.

Palenzuela, O., López-Grandal, E., Zarza, C., Álvarez-Pellitero, P., 2009. Treatment of turbot enteromyxosis with antiparasitic drugs and bioactive natural extracts-supplemented feeds, 14th International Conference of the European Association of Fish Pathologists

(EAFP) on Diseases of Fish and Shellfish, Prague, Czech Republic, Libro de Actas, pp. 142-143.

Palenzuela, O., Redondo, M.J., Álvarez-Pellitero, P., 2002. Description of *Enteromyxum scophthalmi* gen. nov., sp. nov. (Myxozoa), an intestinal parasite of turbot (*Scophthalmus maximus* L.) using morphological and ribosomal RNA sequence data. Parasitology 124, 369-379.

Palenzuela, O., Redondo, M.J., López, E., Álvarez-Pellitero, P., 2007. Cultured sole, Solea senegalensis is susceptible to *Enteromyxum scophthalmi*, the myxozoan parasite causing turbot emaciative enteritis, Parassitologia 49, 73.

Palenzuela, O., Sitjà-Bobadilla, A., Álvarez-Pellitero, P., 1997. *Ceratomyxa sparusaurati* (Protozoa:Myxosporea) infections in cultured gilthead sea bream *Sparus aurata* (Pisces:Teleostei) from Spain: aspects of the host-parasite relationship. Parasitology Research 83, 539-548.

Palmer, J.M., Greenwood-Van Meerveld, B., 2001. Integrative neuroimmunomodulation of gastrointestinal function during enteric parasitism. Journal of Parasitology 87, 483-504.

Panaro, M.A., Cianciulli, A., Mitolo, V., Mitolo, C.I., Acquafredda, A., Brandonisio, O., Cavallo, P., 2007. Caspase-dependent apoptosis of the HCT-8 epithelial cell line induced by the parasite *Giardia intestinalis*. FEMS Immunology and Medical Microbiology 51, 302-309.

Parameswaran, N., Patial, S., 2010. Tumor necrosis factor-alpha signaling in macrophages. Critical Reviews in Eukaryotic Gene Expression 20, 87-103.

Peatman, E., Li, C., Peterson, B.C., Straus, D.L., Farmer, B.D., Beck, B.H., 2013. Basal polarization of the mucosal compartment in *Flavobacterium columnare* susceptible and resistant channel catfish (*Ictalurus punctatus*). Molecular Immunology 56, 317-327.

Pérez-Cordón, G., Estensoro, I., Benedito-Palos, L., Calduch-Giner, J.A., Sitjà-Bobadilla, A., Pérez-Sánchez, J., 2014. Interleukin gene expression is strongly modulated at the local level in a fish–parasite model. Fish & Shellfish Immunology 37, 201-208.

Peterson, L.W., Artis, D., 2014. Intestinal epithelial cells: regulators of barrier function and immune homeostasis. Nature Reviews. Immunology 14, 141-153.

Piazzon, C., Mallo, N., Martín, I., Fernández-Casal, J., Sanmartín, M.L., Lamas, J., Leiro, J.M., 2012. Development of a real-time PCR assay for detection and quantification of *Enteromyxum scophthalmi* parasites in turbot intestinal samples. Aquaculture 366–367, 122-128.

Qian, X., Ba, Y., Zhuang, Q., Zhong, G., 2014. RNA-Seq technology and its application in fish transcriptomics. OMICS: a Journal of Integrative Biology 18, 98-110.

Quiroga, M.I., Redondo, M.J., Sitjà-Bobadilla, A., Palenzuela, O., Riaza, A., Macías, A., Vázquez, S., Pérez, A., Nieto, J.M., Álvarez-Pellitero, P., 2006. Risk factors associated with *Enteromyxum scophthalmi* (Myxozoa) infection in cultured turbot, *Scophthalmus maximus* (L.). Parasitology 133, 433-442.

Ramos-Vara, J.A., Kiupel, M., Baszler, T., Bliven, L., Brodersen, B., Chelack, B., Czub, S., Del Piero, F., Dial, S., Ehrhart, E.J., Graham, T., Manning, L., Paulsen, D., Valli,

V.E., West, K., 2008. Suggested guidelines for immunohistochemical techniques in veterinary diagnostic laboratories. Journal of Veterinary Diagnostic Investigation 20, 393-413.

Randelli, E., Buonocore, F., Scapigliati, G., 2008. Cell markers and determinants in fish immunology. Fish & Shellfish Immunology 25, 326-340.

Redondo, M.J., Palenzuela, O., Riaza, A., Macías, A., Álvarez-Pellitero, P., 2002. Experimental transmission of *Enteromyxum scophthalmi* (Myxozoa), an enteric parasite of turbot *Scophthalmus maximus*. The Journal of Parasitology 88, 482-488.

Redondo, M.J., Quiroga, M.I., Palenzuela, O., Nieto, J.M., Álvarez-Pellitero, P., 2003a. Ultrastructural studies on the development of *Enteromyxum scophthalmi* (Myxozoa), an enteric parasite of turbot (*Scophthalmus maximus* L.). Parasitology Research 90, 192-202.

Redondo, M.J., Palenzuela, O., Álvarez-Pellitero, P., 2003b. In vitro studies on viability and proliferation of *Enteromyxum scophthalmi* (Myxozoa), an enteric parasite of cultured turbot *Scophthalmus maximus*. Diseases of Aquatic Organisms 55, 133-144.

Redondo, M.J., Palenzuela, O., Álvarez-Pellitero, P., 2004. Studies on transmission and life cycle of *Enteromyxum scophthalmi* (Myxozoa), an enteric parasite of turbot *Scophthalmus maximus*. Folia Parasitologica 51, 188-198.

Redondo, M.J., Cortadellas, N., Palenzuela, O., Álvarez-Pellitero, P., 2008. Detection of carbohydrate terminals in the enteric parasite *Enteromyxum scophthalmi* (Myxozoa) and possible interactions with its fish host *Psetta maxima*. Parasitology Research 102, 1257-1267.

Redondo, M.J., Álvarez-Pellitero, P., 2010a. Carbohydrate patterns in the digestive tract of *Sparus aurata* L. and *Psetta maxima* (L.) (Teleostei) parasitized by *Enteromyxum leei* and *E. scophthalmi* (Myxozoa). Parasitology International 59, 445-453.

Redondo, M.J., Álvarez-Pellitero, P., 2010b. The effect of lectins on the attachment and invasion of *Enteromyxum scophthalmi* (Myxozoa) in turbot (*Psetta maxima* L.) intestinal epithelium in vitro. Experimental Parasitology 126, 577-581.

Reite, O.B., Evensen, O., 2006. Inflammatory cells of teleostean fish: a review focusing on mast cells/eosinophilic granule cells and rodlet cells. Fish & Shellfish Immunology 20, 192-208.

Rigos, G., Grigorakis, K.,Christophilogannis, P., Nengas, I., Alexis, M., 1997. *Ceratomyxa* spp. (Myxosporea) infection in cultured common dentex from Greece. Bulletin of the European Association of Fish Pathologists 17, 174.

Rigos, G., Katharios, P., 2010. Pathological obstacles of newly-introduced fish species in Mediterranean mariculture: a review. Reviews in Fish Biology and Fisheries 20, 47-70.

Roca, F.J., Mulero, I., López-Munoz, A., Sepulcre, M.P., Renshaw, S.A., Meseguer, J., Mulero, V., 2008. Evolution of the inflammatory response in vertebrates: fish TNF-alpha is a powerful activator of endothelial cells but hardly activates phagocytes. Journal of Immunology 181, 5071-5081.

Ronza, P., Coscelli, G., Losada, A. P., Bermúdez, R. and Quiroga, M. I., 2013a. Changes in splenic melanomacrophage centres of turbot, *Psetta maxima* (L.), infected

experimentally with *Enteromyxum scophthalmi* (Myxozoa). Journal of Comparative Pathology 148, 92–92.

Ronza, P., Losada, A.P., Bermúdez, R., Sitjà-Bobadilla, A., Quiroga, M.I., 2013b. Puesta a punto de técnicas inmunohistoquímicas para el estudio de la disfunción de la barrera intestinal en la enteromixosis, XXV Reunión de la Sociedad Española de Anatomía Patológica Veterinaria, Toledo, Spain, Libro de Actas, p. 80.

Sarker, S., Kallert, D., Hedrick, R., El-Matbouli, M., 2015. Whirling disease revisited: pathogenesis, parasite biology and disease intervention. Diseases of Aquatic Organisms 114, 155-175.

Schuettpelz, L., Link, D., 2013. Regulation of hematopoietic stem cell activity by inflammation. Frontiers in Immunology 4, 204.

Schultz, A.G., Jones, P.L., Toop, T., 2014. Rodlet cells in Murray cod, *Maccullochella peelii peelii* (Mitchell), affected with chronic ulcerative dermatopathy. Journal of Fish Diseases 37, 219-228.

Shao, T., Zhu, L.-Y., Nie, L., Shi, W., Dong, W.-R., Xiang, L.-X., Shao, J.-Z., 2015. Characterization of surface phenotypic molecules of teleost dendritic cells. Developmental & Comparative Immunology 49, 38-43.

Skjesol, A., Liebe, T., Iliev, D.B., Thomassen, E.I.S., Tollersrud, L.G., Sobhkhez, M., Lindenskov Joensen, L., Secombes, C.J., Jørgensen, J.B., 2014. Functional conservation of suppressors of cytokine signaling proteins between teleosts and mammals: Atlantic salmon SOCS1 binds to JAK/STAT family members and suppresses type I and II IFN signaling. Developmental & Comparative Immunology 45, 177-189.

Sinicropi, D., Qu, K., Collin, F., Crager, M., Liu, M.-L., Pelham, R.J., Pho, M., Rossi, A.D., Jeong, J., Scott, A., Ambannavar, R., Zheng, C., Mena, R., Esteban, J., Stephans, J., Morlan, J., Baker, J., 2012. Whole transcriptome RNA-Seq analysis of breast cancer recurrence risk using formalin-fixed paraffin-embedded tumor tissue. PLoS One 7, e40092.

Sitjà-Bobadilla, A., Redondo, M.J., Macias, M.A., Ferreiro, I., Riaza, A., Álvarez-Pellitero, P., 2004. Development of immunohistochemistry and enzyme-linked immunosorbent assays for the detection of circulating antibodies against *Enteromyxum scophthalmi* (Myxozoa) in turbot (*Scophthalmus maximus* L.). Fish & Shellfish Immunology 17, 335-345.

Sitjà-Bobadilla, A., Redondo, M.J., Bermúdez, R., Palenzuela, O., Ferreiro, I., Riaza, A., Quiroga, I., Nieto, J.M., Álvarez-Pellitero, P., 2006. Innate and adaptive immune responses of turbot, *Scophthalmus maximus* (L.), following experimental infection with *Enteromyxum scophthalmi* (Myxosporea: Myxozoa). Fish & Shellfish Immunology 21, 485-500.

Sitjà-Bobadilla, A., Palenzuela, O., Riaza, A., Macias, M.A., Álvarez-Pellitero, P., 2007. Protective acquired immunity to *Enteromyxum scophthalmi* (Myxozoa) is related to specific antibodies in *Psetta maxima* (L.) (Teleostei). Scandinavian Journal of Immunology 66, 26-34.

Sitjà-Bobadilla, A., Calduch-Giner, J., Saera-Vila, A., Palenzuela, O., Álvarez-Pellitero, P., Pérez-Sánchez, J., 2008. Chronic exposure to the parasite *Enteromyxum leei* (Myxozoa: Myxosporea) modulates the immune response and the expression of growth, redox and immune relevant genes in gilthead sea bream, *Sparus aurata* L. Fish & Shellfish Immunology 24, 610-619.

Sitjà-Bobadilla, A., Palenzuela, O., 2012. *Enteromyxum* Species, in: Woo, P.T.K., Buchmann, K. (Eds.), Fish Parasites: Pathobiology and Protection. CABI publishing, UK, pp. 163-176.

Sitjà-Bobadilla, A., Schmidt-Posthaus, H., Wahli, T., Holland, J., Secombes, C., 2015. Fish immune responses to Myxozoa, in: Okamura, B., Gruhl, A., Bartholomew, J.L. (Eds.), Myxozoan Evolution, Ecology and Development. Springer International Publishing, pp. 253-280.

Song, W., Kao, W., Zhai, A., Qian, J., Li, Y., Zhang, Q., Zhao, H., Hu, Y., Li, H., Zhang, F., 2013. Borna disease virus nucleoprotein inhibits type I interferon induction through the interferon regulatory factor 7 pathway. Biochemical and Biophysical Research Communications 438, 619-623.

Sutherland, G.T., Janitz, M., Kril, J.J., 2011. Understanding the pathogenesis of Alzheimer's disease: will RNA-Seq realize the promise of transcriptomics? Journal of Neurochemistry 116, 937-946.

Taylor, K.E., Mossman, K.L., 2013. Recent advances in understanding viral evasion of type I interferon. Immunology 138, 190-197.

Tizard, I., 2008. Sickness behavior, its mechanisms and significance. Animal Health Research Reviews 9, 87-99.

Truyens, C., Torrico, F., Angelo-Barrios, A., Lucas, R., Heremans, H., Baetselier, P.D., Carlier, Y., 1995. The cachexia associated with *Trypanosoma cruzi* acute infection in mice is attenuated by anti-TNF-a, but not by anti-IL-6 or anti-IFN-7 antibodies. Parasite Immunology 17, 561-568.

Tun, T., Ogawa, K., Wakabayashi H., 2002. Pathological changes induced by three myxosporeans in the intestine of cultured tiger puffer, *Takifugu rubripes* (Temminck and Schlegel). Journal of Fish Diseases 25, 63-72.

Valenzuela-Miranda, D., Boltaña, S., Cabrejos, M.E., Yáñez, J.M., Gallardo-Escárate, C., 2015. High-throughput transcriptome analysis of ISAV-infected Atlantic salmon *Salmo salar* unravels divergent immune responses associated to head-kidney, liver and gills tissues. Fish & Shellfish Immunology 45, 367-377.

Wang, Z., Gerstein, M., Snyder, M., 2009. RNA-Seq: a revolutionary tool for transcriptomics. Nature Reviews. Genetics 10, 57-63.

Waters, J.P., Pober, J.S., Bradley, J.R., 2013. Tumour necrosis factor in infectious disease. The Journal of Pathology 230, 132-147.

Watson, A.J., Hughes, K.R., 2012. TNF-alpha-induced intestinal epithelial cell shedding: implications for intestinal barrier function. Annals of the New York Academy of Sciences 1258, 1-8.

Williams, J.M., Duckworth, C.A., Burkitt, M.D., Watson, A.J., Campbell, B.J., Pritchard, D.M., 2015. Epithelial cell shedding and barrier function: a matter of life and death at the small intestinal villus tip. Veterinary Pathology 52, 445-455.

Wolf, K. & Markiw, M.E., 1984. Biology contravenes taxonomy in the Myxozoa: New discoveries show alternation of invertebrate and vertebrate hosts. Science 225, 1449-1452.

Yanagida, T., Nomura, Y., Kimura, T., Fukuda, Y., Yokoyama, H., Ogawa, K., 2004. Molecular and morphological redescriptions of enteric myxozoans, *Enteromyxum leei* (formerly *Myxidium* sp. TP) and *Enteromyxum fugu* comb. n. (syn. *Myxidium* fugu) from cultured tiger puffer. Fish Pathology 39, 137-143.

Yanagida, T., Sameshima, M., Nasu, H., Yokoyama, H., Ogawa, K., 2006. Temperature effects on the development of *Enteromyxum* spp. (Myxozoa) in experimentally infected tiger puffer, *Takifugu rubripes* (Temminck & Schlegel). Journal of Fish Diseases 29, 561-567.

Yasuda, H., Ooyama, T., Iwata, K., Tun, T., Yokoyama, H., Ogawa, K., 2002. Fish-to-fish transmission of *Myxidium* spp. (Myxozoa) in cultured tiger puffer suffering from emaciation disease. Fish Pathology 37, 29-33.

Yokoyama, H., Kawakami, H., Yasuda, H. & Tanaka, S., 2003. *Henneguya lateolabracis* sp.n. (Myxozoa: Myxosporea), the causative agent of cardiac henneguyosis in Chinese sea bass *Lateolabrax* sp. Fisheries Science 69, 1116-1120.

Yokoyama, H., Whipps, C.M., Kent, M.L., Mizuno K. & Kawakami. H., 2004. *Kudoa thyrsites* from Japanese flounder and *Kudoa lateolabracis* n. sp. from Chinese sea bass: causative agent of post-mortem myoliquefaction. Fish Pathology 39, 79-85.

Yokoyama, H., Itoh, N. & Tanaka, S., 2005a. *Henneguya pagri n.* sp. (Myxozoa: Myxosporea) causing cardiac henneguyosis in red sea bream, *Pagrus major* (Temminck & Schlegel). Journal of Fish Diseases 28, 479-487.

Yokoyama, H., Itoh, N., 2005b. Two multivalvulid myxozoans causing postmortem myoliquefaction: *Kudoa megacapsula* n. sp. from red barracuda (*Sphyraena pinguis*) and *Kudoa thyrsites* from splendid alfonso (*Beryx splendens*). The Journal of Parasitology 91, 1132-1137.

Yokoyama, H., Grabner, D., Shirakashi, S., 2012. Trasmission biology of the Myxozoa. En: Carvalho ED, David GS, Silva RJ (Eds.), Health and environment in aquaculture. InTech, Rijeka, p 3-42.

Young, N.D., Cooper, G.A., Nowak, B.F., Koop, B.F., Morrison, R.N., 2008. Coordinated down-regulation of the antigen processing machinery in the gills of amoebic gill disease-affected Atlantic salmon (*Salmo salar* L.). Molecular Immunology 45, 2581-2597.

Zhang, H., Sun, Z., Wei, W., Liu, Z., Fleming, J., Zhang, S., Lin, N., Wang, M., Chen, M., Xu, Y., Zhou, J., Li, C., Bi, L., Zhou, G., 2014. Identification of serum microRNA biomarkers for tuberculosis using RNA-seq. PLoS One 9, e88909.

Zhu, L.Y., Nie, L., Zhu, G., Xiang, L.X., Shao, J.Z., 2013. Advances in research of fish immune-relevant genes: a comparative overview of innate and adaptive immunity in teleosts. Developmental and Comparative Immunology 39, 39-62.

Zipfel, P.F., Wurzner, R., Skerka, C., 2007. Complement evasion of pathogens: common strategies are shared by diverse organisms. Molecular Immunology 44, 3850-3857.