Tesis Doctoral

Estudio proteómico y transcriptómico comparado de la interfase parásito-hospedador en la fasciolosis

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CERTIFICAN:

Que el presente trabajo titulado **«Estudio proteómico y transcriptómico comparado de la interfase parásito-hospedador en la fasciolosis»** ha sido realizado bajo su dirección por **D. David Becerro Recio** en el Instituto de Recursos Naturales y Agrobiología de Salamanca (IRNASA-CSIC), y reúne, a su juicio, las condiciones de originalidad y calidad científica requeridas para su presentación y defensa para optar al Grado de Doctor por la Universidad de Salamanca.

Y para que así conste a los efectos legales y oportunos, expiden y firman el presente certificado en Salamanca, a 14 de julio de 2023.

Mª Mar Siles Lucas   Javier González Miguel   Mª Ángeles Serrano García

La presente Tesis Doctoral está elaborada en el formato de compendio de artículos/publicaciones según la normativa aprobada por la Comisión de Doctorado y Posgrado de la Universidad de Salamanca el 15 de febrero de 2013 y consta de las siguientes publicaciones:

1. Set up of an *in vitro* model to study early host-parasite interactions between newly excysted juveniles of *Fasciola hepatica* and host intestinal cells using a quantitative proteomics approach

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2. Proteomics coupled with *in vitro* model to study the early crosstalk occurring between newly excysted juveniles of *Fasciola hepatica* and host intestinal cells

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3. Study of the migration of *Fasciola hepatica* juveniles across the intestinal barrier of the host by quantitative proteomics in an *ex vivo* model

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4. Study of the cross-talk between *Fasciola hepatica* juveniles and the intestinal epithelial cells of the host by transcriptomics in an *in vitro* model

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Abreviaturas

µm: Micrómetro

2D-PAGE (*Two-dimensional PolyAcrylamide Gel Electrophoresis*)**:** Electroforesis bidimensional en gel de poliacrilamida

AAM (*Alternatively Activated Macrophage*)**:** Macrófago activado alternativamente

ABZ: Albendazol

ADCC (*Antibody-Dependent Cellular Cytotoxicity*)**:** Citotoxicidad celular mediada por anticuerpos

ARN: Ácido ribonucleico

ARNm: ARN mensajero

CD: Cluster de diferenciación

ELISA (*Enzime-Linked ImmunoSorbent Assay*)**:** Ensayo de inmunoabsorción ligado a enzimas

FABP (*Fatty Acid Binding Protein*)**:** Proteína de unión a ácidos grasos

FITC: Isotiocianato de fluoresceína

G+C: Guanina + Citosina

GALT (*Gut-Associated Lymphoid Tissue*)**:** Tejido linfoide asociado al intestino

GAPDH: Gliceraldehído 3-fosfato deshidrogenasa

Gb: Gigabase

GST: Glutatión S-transferasa

HDM (*Helminth Defense Molecule*)**:** Molécula de defensa helmíntica

HPLC (*High Performance Liquid Chromatography*)**:** Cromatografía líquida de alta resolución

IFN: Interferón

Ig: Inmunoglobulina

IL: Interleuquina

iNOS (*inducible Nitric Oxide Synthase*)**:** Óxido nítrico sintasa inducible

iTRAQ (*isobaric Tag for Absolute and Relative Quantitation*): Marcaje isobárico para la cuantificación absoluta y relativa

Kb: Kilobase

Mb: Megabase

MB: Membrana basal

MEC: Matriz extracelular

MI: Matriz intersticial

miARN: Micro ARN

mm: Milímetro

MPSIEC (*Mouse Primary Small Intestinal Epithelial Cells*): Células epiteliales primarias de intestino delgado de ratón

MS (*Mass Spectrometry*): Espectrometría de masas

NEJ (*Newly Excysted Juvenile*): Juvenil recién excistado

NGS (*Next Generation Sequencing*): Secuenciación de nueva generación

nm: Nanómetro

NTD (*Neglected Tropical Disease*): Enfermedad tropical desatendida

OMS: Organización Mundial de la Salud

PAGE (*PolyAcrilamide Gel Electrophoresis*): Electroforesis en gel de poliacrilamida

PAMP (*Pathogen-Associated Molecular Pattern*): Patrón molecular asociado a patógeno

PCR (*Polymerase Chain Reaction*): Reacción en cadena de la polimerasa

PCR-RFLP (*Polymerase Chain Reaction - Restriction Fragment Length Polymorphism*): Reacción en cadena de la polimerasa con fragmentos de restricción de longitud polimórfica

p.i.: Postinfección

Prx: Peroxirredoxina

RER: Retículo endoplásmico rugoso

RISC (*RNA-Induced Silencing Complex*): Complejo de silenciamiento inducido por ARN

RNA-Seq (*RNA-Sequencing*): Secuenciación de ARN

ROS (*Reactive Oxygen Species*): Especies reactivas del oxígeno

SNP (*Single Nucleotide Polymorphism*): Polimorfismo de un solo nucleótido

SWATH-MS (*Sequential Window Acquisition for All Theoretical Mass Spectra*): Adquisición secuencial en ventanas de todos los espectros de masas teóricos

TCBZ: Triclabendazol

TGF-β (*Transforming Growth Factor β*): Factor de crecimiento transformante β

Th: Linfocito/respuesta T colaborador (*helper*)

TLR (*Toll-Like Receptor*): Receptor de tipo *Toll*

TNF-α (*Tumor Necrosis Factor α*): Factor de necrosis tumoral α

Treg: linfocito/respuesta T regulador

TRITC: Tetrametilrodamina

Resumen

La fasciolosis es una zoonosis parasitaria causada por vermes trematodos del género *Fasciola*, principalmente por las especies *F. hepatica* y *F. gigantica*. Está considerada como la enfermedad de transmisión alimentaria con la distribución geográfica más amplia conocida, con casos documentados en los 5 continentes. El carácter ubicuo de la enfermedad es, en parte, debido a la capacidad de los vermes para establecerse sobre una amplia variedad de hospedadores, tanto intermediarios como definitivos.

La fasciolosis causa anualmente pérdidas millonarias a la industria ganadera en forma de muertes de cabezas de ganado o reducción en la productividad de derivados animales (carne, leche, lana, etc.). Además, los costes indirectos como consecuencia del tratamiento farmacológico o la disminución de la fertilidad como resultado de la infección incrementan aún más la cifra. Por otra parte, aunque los hospedadores más habituales del parásito se identifican con mamíferos rumiantes, el ser humano también puede actuar como hospedador definitivo, por lo que la fasciolosis puede suponer un problema de salud pública, con varias regiones endémicas en todo el mundo.

El control de la enfermedad resulta complicado por la naturaleza inespecífica de los signos y síntomas clínicos, la variabilidad de los mismos y la escasez de alternativas terapéuticas. Además, el tratamiento farmacológico presenta problemas como la persistencia de los fármacos en los tejidos del hospedador, lo que dificulta su aplicación en animales destinados al consumo, la contaminación ambiental, y la aparición de resistencias frente a los principales antihelmínticos conocidos, lo que puede desembocar en la ineficacia del tratamiento.

La fasciolosis ha sido designada por la Organización Mundial de la Salud (OMS) como enfermedad abordable mediante la perspectiva *One Health*. Según este enfoque, el tratamiento eficaz de la enfermedad a nivel humano, veterinario o ambiental redundaría positivamente en los demás niveles, siendo requisito imprescindible aumentar el conocimiento sobre las interacciones que se establecen a nivel molecular entre parásito y hospedador, especialmente en las fases tempranas de la infección. En este sentido, la propuesta de nuevos modelos de laboratorio que permitan replicar el contacto y la relación entre ambos organismos durante estas fases, y su estudio mediante tecnologías -ómicas, indispensables para identificar las moléculas que rigen dichas interacciones, supondrían herramientas de gran utilidad para desentrañar estos importantes mecanismos.

En la presente Tesis Doctoral, se llevó a cabo la puesta a punto de dos modelos de interacción parásito-hospedador que buscan replicar las condiciones de la infección temprana en la fasciolosis, la cual se produce tras la ingesta de las formas de resistencia del parásito, denominadas metacercarias, que liberan en el intestino delgado del hospedador los juveniles recién excistados (NEJ):

- El primer modelo reproduce el primer contacto entre los NEJ y el intestino de su hospedador definitivo, representado por un cultivo primario de células epiteliales intestinales de ratón (MPSIEC), mediante un abordaje *in vitro*. Para ello, los NEJ previamente excistados se pusieron en contacto con un cultivo en placa de MPSIEC, y tras un tiempo de contacto comprendido entre las 3 y las 24 horas se separaron para analizar los cambios a nivel molecular en ambos organismos.

- El segundo modelo recurre a una aproximación *ex vivo* con el fin de recrear el paso de los NEJ a través de la pared intestinal de su hospedador. En dicho modelo, se obtuvieron fragmentos de intestino delgado procedentes de ratones de laboratorio, en cuyo interior se introdujeron los NEJ previamente excistados. Pasadas dos horas y media, los NEJ que lograron atravesar el intestino se recolectaron y analizaron.

La caracterización molecular de los cambios en parásito y hospedador tras su interacción se realizó mediante técnicas proteómicas y transcriptómicas. En concreto, el perfil de expresión diferencial a nivel de proteína en ambos organismos se evaluó mediante las metodologías iTRAQ (*Isobaric Tag for Relative and Absolute Quantitation*) y SWATH-MS (*Sequential Window Acquisition of All Theoretical Mass Spectra*), las cuales hacen uso de la cromatografía líquida de alta resolución (HPLC) acoplada a espectrometría de masas (MS) para la determinación del perfil proteómico de las muestras estimuladas y no estimuladas. Por su parte, los estudios transcriptómicos se realizaron mediante secuenciación de ARN (RNA-Seq) y su posterior mapeo sobre los correspondientes genomas de referencia.

La utilización de estas técnicas permitió identificar un alto número de proteínas y transcritos cuyos niveles de expresión en muestras estimuladas se modificaban significativamente respecto a las no estimuladas. En los NEJ, estos se correspondieron con procesos biológicos importantes para la supervivencia del parásito, tales como la proteolisis y su regulación, la respuesta al estrés oxidativo, el metabolismo energético y el crecimiento o los mecanismos de evasión inmune, mientras que, en las MPSIEC, las

proteínas y genes diferencialmente expresados se relacionaron principalmente con procesos de transporte intra y extracelular, la modulación de la respuesta inmune o el control de la expresión génica, además de incluir un elevado número de pseudogenes.

El análisis e interpretación de los resultados de este trabajo proporcionan una mejor comprensión de la interacción parásito-hospedador a nivel temprano en la fasciolosis, y señalan una serie de antígenos importantes para la misma que podrían permitir la identificación de nuevas dianas terapéuticas y la formulación de nuevas vacunas, con el fin de lograr un control eficaz de la enfermedad en el futuro.

Revisión bibliográfica

1. Biología y ciclo de *Fasciola hepatica*

1.1. Sistemática y filogenia de *Fasciola hepatica*

La fasciolosis es una enfermedad parasitaria de transmisión alimentaria causada por vermes planos pertenecientes al género *Fasciola*, comúnmente conocidos como duelas hepáticas. Las dos especies con mayor implicación como agentes etiológicos de la fasciolosis son *F. hepatica* y *F. gigantica*. Desde un punto de visto taxonómico, el género *Fasciola* se encuadra dentro del filo Platyhelminthes, clase Trematoda, subclase Digenea, familia Fasciolidae, subfamilia Fasciolinae (Andrews *et al*., 2022). En la actualidad se conoce que las dos especies mencionadas divergieron evolutivamente hace unos 5 millones de años, mientras que la separación de otros géneros pertenecientes a la misma familia se produjo hace unos 90 millones de años (Choi *et al*., 2020) (**Figura 1**). Si bien existen otros géneros evolutivamente próximos, responsables de otras enfermedades, es *Fasciola* spp. el que presenta la distribución longitudinal, latitudinal y altitudinal más amplia. Así, mientras que *F. gigantica* se restringe a zonas tropicales de África, Oriente Medio, Europa del Este y el Sudeste Asiático, *F. hepatica* está presente en los cinco continentes, constituyendo un problema considerable de salud tanto a nivel humano como animal y, como consecuencia, se trata del miembro de la familia Fasciolidae más ampliamente estudiado (Mas-Coma *et al*., 2005).

1.2. Ciclo biológico de *Fasciola hepatica*

Desde la primera descripción de *F. hepatica* a mediados del siglo XIV (de Brie, 1379), sucesivas observaciones de las distintas fases de desarrollo del parásito permitieron esclarecer que todas ellas pertenecían a un mismo organismo, determinando de qué manera se sucedían y articulaban en su ciclo biológico (Andrews *et al*., 2022). Aunque todas las fases parasitarias habían sido ya descritas a finales del siglo XIX, no fue hasta inicios del siglo XX cuando se realizó la primera descripción completa del ciclo de *F. hepatica*, y actualmente solo quedan algunos puntos controvertidos por dilucidar, relacionados con la ruta migratoria del parásito por los tejidos del hospedador y su localización definitiva dentro de este (Moazeni y Ahmadi, 2016).
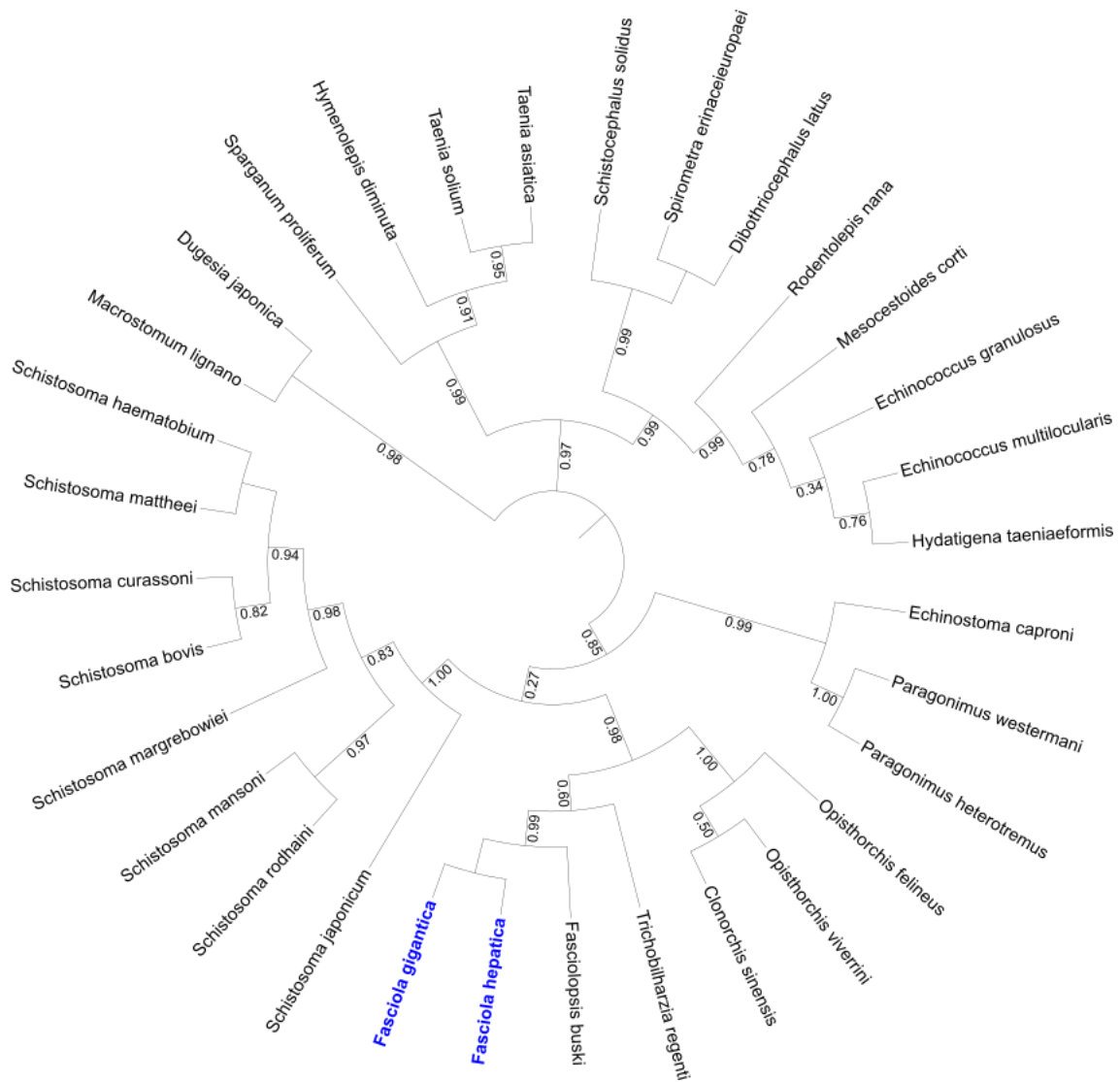
Figura 1 – Árbol filogenético radial de las principales especies de platelmintos elaborado con el software FastTree, realizado a partir de un alineamiento múltiple con Clustal Omega (Sievers *et al*., 2011) de las secuencias de la proteína gliceraldehído 3-fosfato deshidrogenasa (GAPDH), empleando el algoritmo Neighbor Joining (Saitou y Nei, 1987).

Al igual que en otros trematodos, el ciclo biológico de *F. hepatica* involucra a un hospedador intermediario (caracoles de agua dulce) y a otro definitivo (generalmente rumiantes y el hombre), el cual alberga la forma adulta del parásito. Esta tiene unas medidas aproximadas de entre 20-50 mm de largo por 8-10 mm de ancho, si bien en regiones donde la presencia de esta especie se solapa con la de *F. gigantica* pueden encontrarse especímenes híbridos entre ambas, con medidas algo mayores (Mas-Coma *et al*., 2009a). A nivel morfológico, el verme adulto de *F. hepatica* presenta en su parte externa un cuerpo plano con un cono cefálico en la región delantera y un extremo posterior más estrecho. En el extremo anterior se localiza una ventosa oral, bajo la cual se encuentra el poro genital, y una ventosa ventral en la unión del cono con el cuerpo, con un poro excretor en la parte terminal (**Figura 2**). El parásito está recubierto por un

tegumento de estructura sincitial que tiene una gran importancia en la relación de *F. hepatica* con su entorno inmediato. En el interior del verme se localizan diferentes órganos, entre ellos el intestino bifurcado en dos ciegos o el sistema reproductor, donde destacan dos testículos y un único ovario, todos ellos ramificados (Robinson *et al.*, 2022).
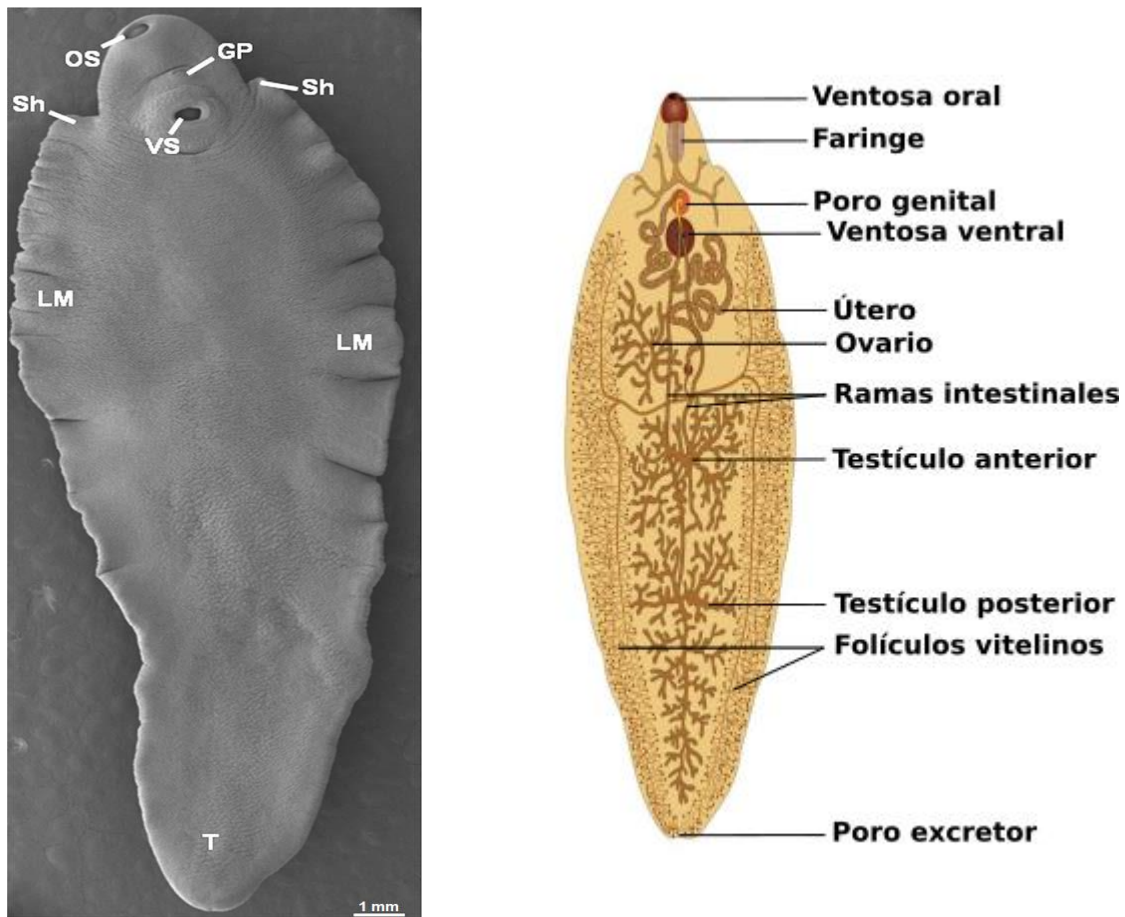
Figura 2 – **Izquierda**: Imagen de microscopía electrónica de barrido de un verme adulto de *F. hepatica* de 12 semanas de edad, en la que puede apreciarse la morfología general del verme y sus partes más representativas: ventosa oral (OS), ventosa ventral (VS), poro genital (GP), hombros (Sh), márgenes laterales (LM) y parte posterior (T). Fuente: McConville *et al.* (2009). **Derecha**: Esquema de un verme adulto de *F. hepatica*, señalando sus órganos principales.

Las formas adultas del parásito ponen huevos que salen al medio exterior tras ser eliminados por el hospedador definitivo junto con las heces. Estas proporcionan no solo un vehículo para salir del organismo, sino también un entorno protegido y húmedo que evita la desecación inmediata de los huevos en ambientes áridos, pudiendo sobrevivir de esta forma durante periodos de tiempo de hasta varios meses (Rowcliffe y Ollerenshaw, 1960). Si bien una gran proporción de los huevos acaban siendo inviables, una parte de los mismos terminan, gracias a diferentes circunstancias (lluvias, transporte por otros seres vivos, etc.), sumergidos en masas de agua dulce poco profundas, donde se produce su maduración y eclosión en un plazo de 2-3 semanas, siempre y cuando se cumplan unas

condiciones ambientales favorables, tales como una temperatura adecuada (Rowcliffe y Ollerenshaw, 1960) y la incidencia de luz de los espectros azul y ultravioleta (Roberts, 1950).

La eclosión libera, a través del opérculo del huevo, el miracidio, un organismo ciliado de unos 130 µm capaz de desplazarse por el agua. Cabe destacar que, incluso bajo las mismas condiciones ambientales, no todos los huevos eclosionan al mismo tiempo, lo cual parece responder a una estrategia evolutiva para favorecer la liberación de miracidios durante un tiempo prolongado. El miracidio es un organismo de metabolismo principalmente anaerobio cuyo tiempo de vida es corto, de unas 24 horas como máximo, por lo que la necesidad de localizar un hospedador en el que desarrollarse es primordial (Smith y Grenfell, 1984).

1.2.1. Desarrollo de *Fasciola hepatica* en el hospedador intermediario

El miracidio de *F. hepatica* infecta gasterópodos pulmonados acuáticos del grupo *Galba*/*Fossaria*, siendo la especie más habitual *Galba truncatula* (anteriormente *Lymnaea truncatula*), si bien en la actualidad se conocen más de 20 especies de caracoles susceptibles a la infección por *F. hepatica* (Correa *et al*., 2010; Siles-Lucas *et al*., 2021).

La penetración en el cuerpo del caracol se produce gracias a la acción mecánica del miracidio, así como a la secreción de enzimas proteolíticas. Tras acceder al interior, el parásito se deshace de su envuelta ciliada para dar lugar al esporocisto, el cual se desplaza a través de los vasos sanguíneos del caracol y se establece, principalmente, en su glándula digestiva. Esta fase de desarrollo consiste a grandes rasgos en una masa de células germinales capaces de crecer y reproducirse asexualmente, de forma que cada célula inicial deriva en una mórula capaz de generar una nueva fase larvaria conocida como redia (Rondelaud *et al*., 2009; **Figura 3**). Las redias, a su vez, también presentan capacidad de división, por lo que si las condiciones del entorno son poco favorables pueden producirse nuevas generaciones de redias hijas. De las células germinales de la redia se producen las cercarias. Estas últimas son una fase evolutiva de unos 250-350 µm de longitud con un apéndice móvil o cola, la cual migra a través de los tejidos y abandona el cuerpo del caracol por la región perianal, llegando de nuevo al agua. La invasión del hospedador intermediario supone un importante paso de amplificación del parásito, de tal forma que a partir de un único miracidio infectante pueden llegar a liberarse más de 3 000

cercarias (Hodgkinson *et al*., 2018). Globalmente, este proceso finaliza unas 4-7 semanas tras la infección, si bien la liberación de las cercarias se produce de manera sostenida en el tiempo, permitiendo al hospedador regenerar sus tejidos mientras se prolonga la llegada de cercarias al medio acuático (Vignoles *et al*., 2006). Tras quedar libres del hospedador intermediario, las cercarias se desplazan por el agua hasta adherirse, gracias a su ventosa ventral, a una superficie sólida (habitualmente hojas de plantas dulceacuícolas comestibles), momento en que empiezan a secretar una cubierta protectora a su alrededor y se desprenden de su cola, generando una nueva fase evolutiva denominada metacercaria (Køie *et al*., 1977). Se trata de una forma de resistencia cuya viabilidad puede superar el año de duración y que constituye la forma infectiva para el hospedador definitivo vertebrado.

1.2.2. Desarrollo de *Fasciola hepatica* en el hospedador vertebrado

Las metacercarias de *F. hepatica* son infectantes principalmente para herbívoros rumiantes y, de manera más concreta, para ovinos, bovinos y caprinos (Robinson *et al*., 2022). El ser humano también constituye un hospedador definitivo de *F. hepatica*, especialmente en regiones donde este se encuentra en contacto estrecho con el ganado, lo que contextualiza a la fasciolosis como una enfermedad zoonótica. En este caso, la infección, al igual que ocurre en otros hospedadores definitivos, se produce mediante la ingesta de plantas acuáticas contaminadas con metacercarias, o bebiendo el agua donde estas se encuentran en suspensión (Reinhardt, 1957) (**Figura 3**).

Las metacercarias recorren el tubo digestivo del hospedador junto con el alimento, y a su paso por el estómago comienza un proceso de activación al entrar en contacto con el medio ácido y las secreciones digestivas de esta cavidad. Más tarde, al acceder al intestino delgado, las metacercarias se ven sometidas a una serie de estímulos fisicoquímicos que promueven la liberación de la larva que contienen, y que incluyen tanto factores extrínsecos (temperatura, pH y presencia de CO_2 y sales biliares) como intrínsecos (secreciones producidas por el verme) (Hernández-González *et al*., 2010). Es precisamente en el duodeno, tras el paso por el colédoco, cuando el tapón ventral de la metacercaria se abre y de su interior emerge la siguiente fase de desarrollo del parásito, denominada juvenil recién excistado o NEJ (por sus siglas en inglés, *Newly Excysted Juvenile*) (Sukhdeo y Sukhdeo, 2002).
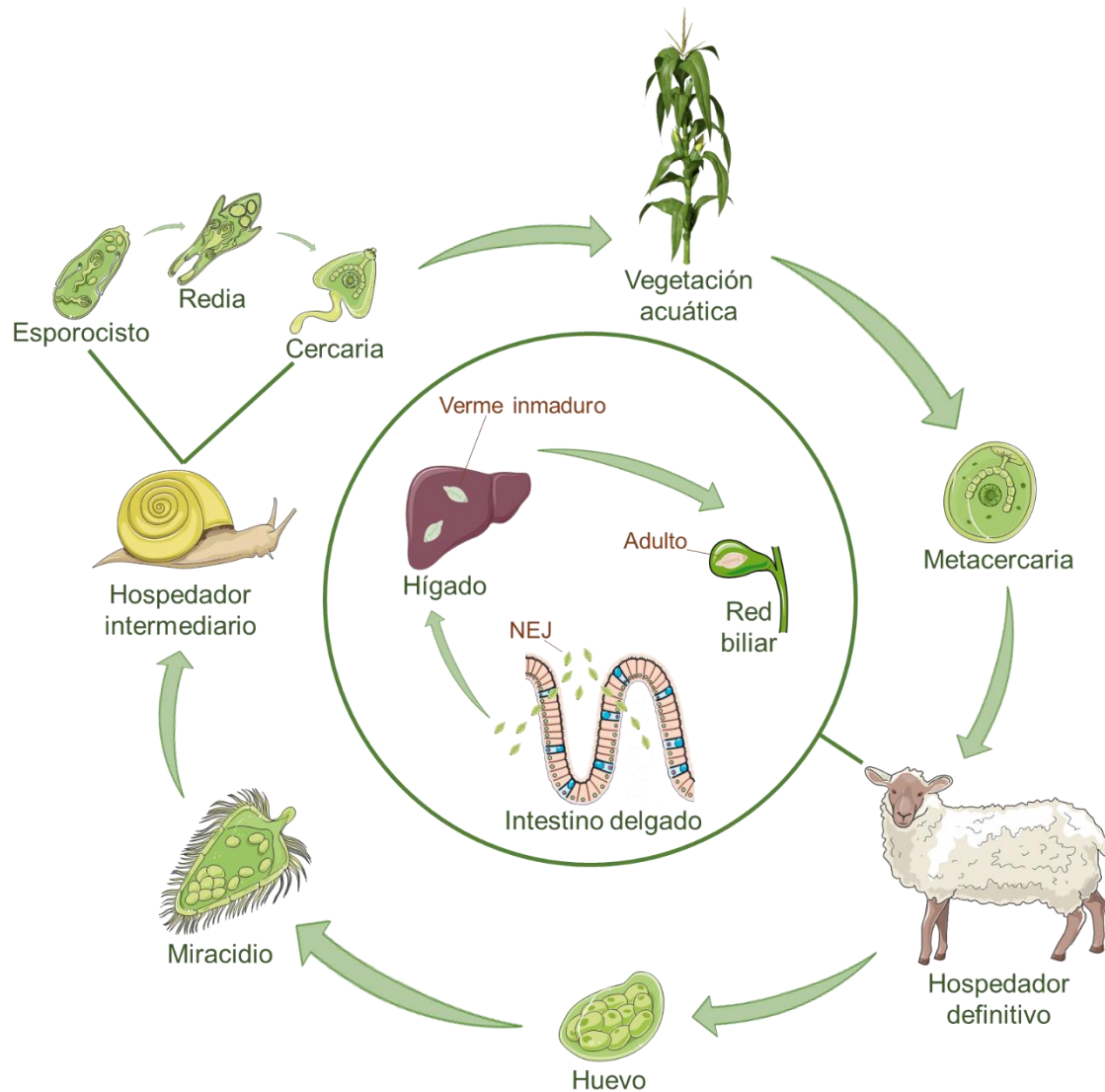
Figura 3 – Ciclo biológico de *F. hepatica*. Tras ser expulsados junto con las heces, los huevos embrionan en el medio acuático y liberan el miracidio, el cual infecta al hospedador intermediario (caracol) y, tras sucesivas divisiones, da lugar a las cercarias. Estas se enquistan como metacercarias en la vegetación acuática o en el agua, y son ingeridas por el hospedador definitivo, liberando los juveniles recién excistados o NEJ (por sus siglas en inglés, *Newly Excysted Juveniles*) a su paso por el intestino delgado. A continuación, los parásitos atraviesan la pared intestinal y migran por el peritoneo y el tejido hepático donde, tras ingresar en los canales biliares, alcanzan el estadio adulto y comienzan a producir huevos que son vertidos al lumen intestinal.

El lumen intestinal constituye un entorno hostil para los NEJ, tanto por la presencia de enzimas digestivas como por la acción surfactante de la bilis, por lo que deben abandonarlo rápidamente para sobrevivir (Tielens *et al*., 1981). Para ello, los vermes juveniles penetran en la mucosa intestinal y, gracias principalmente a la secreción de proteasas y a los propios movimientos del parásito, son capaces de atravesar los diferentes estratos de la pared intestinal hasta alcanzar el peritoneo, tras lo cual los parásitos realizan un proceso de migración en dirección al hígado que concluye pasados unos 4-6 días desde la ingestión de la metacercaria (Valero *et al*., 2006a). Durante este proceso desempeñan un papel fundamental las proteasas secretadas por el verme juvenil,

las cuales cumplen una triple función: en primer lugar, degradan los tejidos del hospedador facilitando los procesos de invasión parasitaria. Esto genera nutrientes simples que el juvenil absorbe y utiliza en su propia nutrición y crecimiento a partir de su llegada al hígado. Finalmente, la secreción de proteasas constituye una estrategia de evasión inmune, ya que estas son capaces de degradar las inmunoglobulinas (Igs) adheridas a la superficie de los NEJ, dificultando las acciones defensivas del sistema inmune del hospedador (Dalton *et al*., 2003).

La llegada al hígado se produce normalmente por el lóbulo izquierdo (por ser el más próximo al peritoneo) atravesando la cápsula de Glisson. Una vez alcanzado este órgano, los vermes inmaduros migran por el parénquima hepático durante 5-6 semanas generando una patología que constituye la fase aguda de la enfermedad. Durante este periodo el parásito crece enormemente en tamaño, evolucionando desde los 0,2 mm de longitud al salir de la metacercaria, hasta alcanzar unos 30 mm de media. Finalmente, a partir de la séptima semana postinfección (p.i.), los vermes realizan una última migración, esta vez hacia los conductos biliares, donde continúan creciendo hasta el estadio adulto, mientras alcanzan la madurez sexual. Una vez dentro de la red biliar, tiene lugar el proceso de reproducción sexual mediante autofecundación, siendo posible también la fecundación cruzada. Esto desencadena la consecuente producción de huevos que son excretados al lumen intestinal a través del colédoco, de tal forma que continúan el recorrido del tubo digestivo hasta ser eliminados junto con las heces, dando continuidad al ciclo de vida del parásito. Los vermes adultos de *F. hepatica* pueden vivir adheridos a la pared interior de las vías biliares durante grandes periodos de tiempo en función del hospedador al que parasitan. Así, se han descrito tiempos de permanencia de hasta 2 años en vacas, 13 en humanos y 20 en ovejas, a lo largo de los cuales cada verme adulto puede producir hasta 25 000 huevos al día (Forbes, 2017). Durante esta etapa, que constituye la fase crónica de la enfermedad, los parásitos se alimentan de la sangre obtenida de los vasos sanguíneos adyacentes a las vías biliares del hospedador. Cabe destacar que, aunque la red biliar constituye la localización definitiva de los vermes adultos del género *Fasciola*, ocasionalmente pueden ocurrir infecciones ectópicas en las que los parásitos inmaduros invaden otras partes del cuerpo, como el páncreas, los pulmones o el cerebro (Mas-Coma *et al*., 2014a).

1.2.3. Importancia de los vermes juveniles en el ciclo de *Fasciola hepatica*

Los vermes juveniles o inmaduros de *F. hepatica* se identifican con la fase larvaria causante de la fasciolosis aguda, ocasionada por la migración del parásito a través de los tejidos del hospedador, y que cursa con dolor abdominal, fiebre y problemas gastrointestinales, entre otros, además de ser la principal causa de mortalidad por esta enfermedad. Los NEJ son la primera forma móvil del parásito que entra en contacto con el hospedador definitivo, por lo que su eliminación permitiría el control de la infección antes de que el parásito pudiese establecerse en un nicho poco accesible para los efectores del sistema inmune, como los canales biliares, evitando además la aparición de la patología más grave asociada a la fasciolosis.

En este sentido, el paso de los NEJ a través de la pared intestinal ha sido recientemente considerado como un «punto de no retorno» en la infección, a partir del cual, el control de la misma se vuelve mucho más complicado, por lo que se trata de una fase de importancia crítica desde el punto de vista clínico y veterinario (González-Miguel *et al*., 2021). A pesar de esto, y desde una perspectiva investigadora, los vermes juveniles de *F. hepatica* han recibido menor atención que las fases adultas del parásito, y en la actualidad existe una información limitada acerca de su fisiología, los mecanismos moleculares que rigen la orientación y migración en las etapas tempranas de la fasciolosis, y las relaciones que establecen con el hospedador vertebrado durante los primeros días de la infección. Además, las estructuras del parásito juvenil desempeñan un papel fundamental en la relación temprana parásito-hospedador en la fasciolosis, por lo que resulta de interés conocerlas en detalle.

1.2.3.1. El tegumento

La consideración de esta estructura en la literatura científica ha evolucionado notablemente con el paso de los años. Aunque históricamente se ha visto como una mera cubierta protectora, en la actualidad existe el consenso de que se trata de una matriz dinámica, biológicamente activa y metabólicamente compleja que se encuentra en contacto estrecho con los tejidos del hospedador definitivo. Por ello, se asume que los cambios tanto funcionales como morfológicos que tienen lugar en el tegumento, permiten

al parásito adaptarse al entorno que lo rodea durante las etapas iniciales de la infección (Bennett y Threadgold, 1975; Wilson *et al*., 2011).
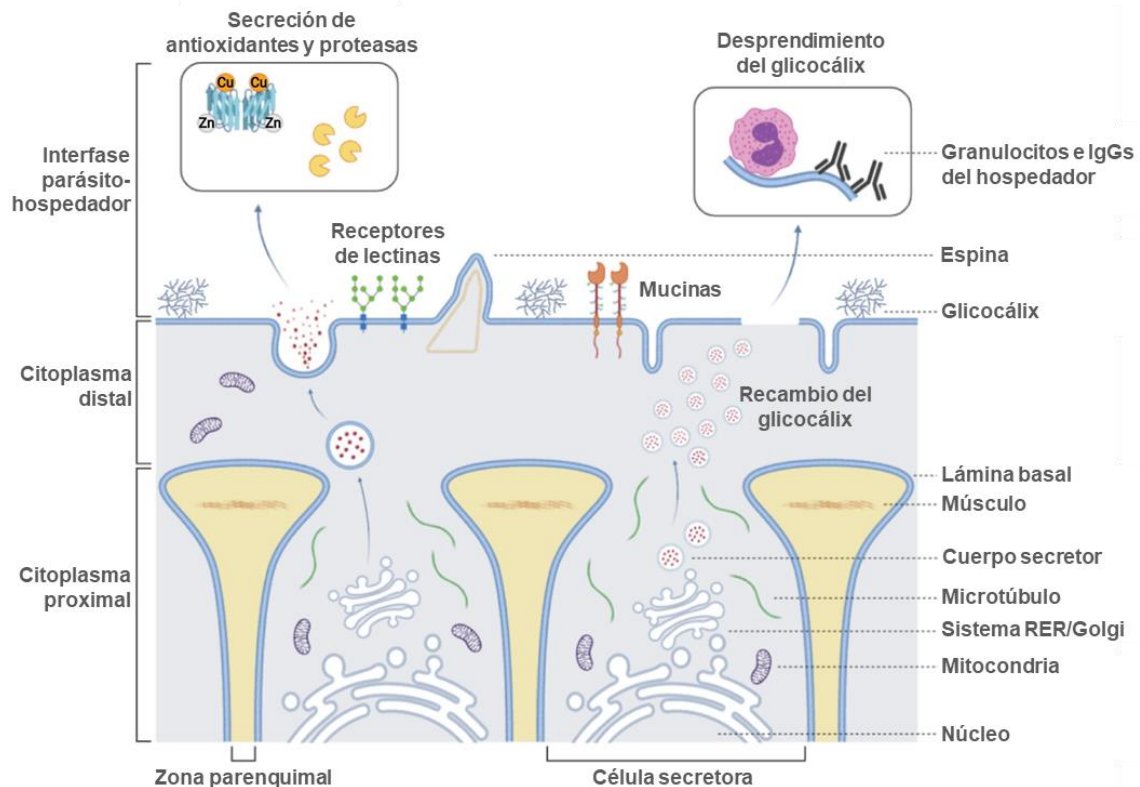
Figura 4 – Esquema de la estructura del tegumento del verme juvenil de *F. hepatica*. Fuente: adaptado de González-Miguel *et al.* (2021).

Desde un punto de vista morfológico, el tegumento es una estructura sincitial que rodea todo el cuerpo del parásito, la cual cuenta con numerosas invaginaciones que incrementan ampliamente su superficie, y está recubierta por un glicocálix en su parte más externa, además de contar con una gran cantidad de mitocondrias en su región basal. El sincitio se encuentra conectado a los núcleos celulares por medio de canales citoplásmicos, de tal forma que entre ambos existe una región de parénquima extracelular recubierta por una lámina basal y en cuyo interior existen capas de músculo en disposición circular y longitudinal. Ancladas a la lámina basal se localizan numerosas espinas de composición proteica, las cuales sobresalen al exterior y juegan un papel importante en el movimiento y el anclaje a las estructuras del hospedador, favoreciendo además la acción erosiva del verme en su migración a través de los tejidos. Por su parte, en los núcleos celulares destaca la presencia de un extenso sistema secretor compuesto por cisternas de retículo endoplásmico rugoso (RER) en estrecha relación con el complejo de Golgi, responsable del procesamiento de vesículas que viajan en dirección al sincitio gracias a la presencia de microtúbulos que conectan ambos compartimentos (González-

Miguel *et al*., 2021). Por último, la superficie del tegumento cuenta con varias papilas sensitivas capaces de percibir estímulos mecánicos y químicos, que resultan importantes en la relación de *F. hepatica* con su entorno (**Figura 4**).

A nivel funcional, el tegumento de *Fasciola* spp. es una estructura indispensable para el mantenimiento de su homeostasis, como concluyen diferentes estudios en los que la disrupción de esta estructura conduce a la muerte del parásito (Devine *et al*., 2011; Bennett *et al*., 2020). De forma general, el tegumento participa en la osmorregulación e incorporación de nutrientes mediante el intercambio de sustancias como agua, electrolitos o moléculas orgánicas con el exterior. Los mecanismos para este intercambio son diversos, ya que algunas sustancias atraviesan la membrana apical del tegumento mediante difusión pasiva o facilitada por transportadores, mientras que otras lo hacen mediante sistemas de transporte activo. No obstante, la función más relevante del tegumento durante las primeras fases de la infección viene dada por la secreción de sustancias al medio externo, las cuales son producidas por las células de la región basal y procesadas mediante el sistema RER-Golgi, para después viajar hacia la cara apical en forma de vesículas extracelulares (Hanna *et al*., 1980a). El contenido de estas vesículas es variado y en él cabe destacar moléculas antioxidantes y sustancias inmunomoduladoras que participan en mecanismos de evasión parasitaria durante la etapa migratoria, así como proteasas, importantes para el movimiento y la nutrición de los NEJ, entre otros procesos (Bennett *et al*., 2020). Los cuerpos secretores juegan además un papel clave en la renovación de la composición proteica del glicocálix, ya que esta estructura está sometida a una renovación continua a medida que el verme juvenil migra a través de los tejidos, y que es más intensa cuanto más rápido es su movimiento. Esto constituye una estrategia defensiva que permite al parásito eliminar sustancias adheridas a su superficie tales como anticuerpos y moléculas del sistema del complemento, evitando así el reconocimiento por células inmunes del hospedador (Hanna *et al*., 1980b).

Además de su interacción inicial con el exterior, el tegumento sufre modificaciones a lo largo de la vida del parásito que le permiten adaptarse a las condiciones cambiantes de su entorno. El cambio más significativo es la transformación de las células secretoras, ya que, aunque los NEJ en un inicio producen únicamente las vesículas denominadas T0, tras los primeros 5 días de infección se produce una transición gradual a cuerpos secretores T1, los cuales terminan desplazando por completo a los T0 cuando el parásito se convierte en adulto. Finalmente, la diferenciación de nuevas células

secretoras permite la producción de cuerpos T2, cuya secreción parece ser un requisito imprescindible para el acceso del verme a los conductos biliares (Hanna *et al*., 1980c). Esto se traduce en cambios en el perfil de un gran número de moléculas, incluyendo proteasas y efectores inmunes secretados por el parásito, así como en una mayor resistencia a las condiciones físico-químicas de las vías biliares (McCusker *et al*., 2016).

1.2.3.2. El soma

Inmediatamente por debajo del tegumento se encuentran el resto de estructuras del parásito, en conjunto denominadas soma, que incluye diferentes tejidos y órganos. El primero de los mismos es el parénquima, un tejido de relleno que se localiza entre los órganos, y está formado tanto por células como por una matriz extracelular (MEC) producida por estas. A pesar de tratarse de un tejido de relleno, participa activamente en el almacenamiento y secreción de vesículas, ya que contiene diferentes tipos celulares con especialización funcional (Pankao *et al*., 2006), así como conexiones con otros tejidos que permiten el intercambio de sustancias (Martínez-Sernández *et al*., 2014). Además, tiene un papel activo en el metabolismo energético durante la primera fase de la infección, al ser responsable de movilizar las reservas de glucógeno, polisacárido que constituye la principal fuente de carbohidratos para los NEJ (Cwiklinski *et al*., 2018).

El músculo se localiza inmediatamente por debajo del tegumento y consta de dos capas: una circular externa y otra interior con disposición longitudinal (Mair *et al*., 1998). La acción conjunta de ambas capas permite el movimiento del parásito, que es muy activo durante la infección temprana (Kumar *et al*., 2003). También destaca la presencia de este tejido en ambas ventosas, siendo claves en el proceso de locomoción del verme juvenil (Bennett, 1975; Sukhdeo *et al*., 1988a).

El sistema nervioso de *F. hepatica* permite la captación de estímulos durante la migración, además de participar en la coordinación y regulación del desarrollo del verme. Este sistema consta de dos ganglios en la región anterior, situados justo por debajo de la ventosa oral, los cuales recorren el cuerpo hasta la parte posterior y se ramifican, contactando con los distintos órganos, así como con las ventosas. Se conocen algunas sustancias que pueden actuar como neurotransmisores, las cuales incluyen la acetilcolina, la serotonina o la dopamina (Magee *et al*., 1989), y más recientemente, la secuenciación

del genoma de *F. hepatica* ha dado a conocer distintos genes cuya secuencia se asemeja a la de neuropéptidos de otras especies (Cwiklinski *et al*., 2015a).

El citoesqueleto se compone de 3 estructuras: microtúbulos, microfilamentos y filamentos intermedios. Aunque estos últimos han sido relativamente poco estudiados en *F. hepatica*, los dos primeros han sido objeto de numerosas investigaciones, y se sabe que participan en el mantenimiento de la morfología celular, la contracción muscular, el transporte de vesículas y la división celular, entre otros procesos (Stitt y Fairweather, 1991; Stitt *et al*., 1995), por lo que tienen una gran importancia durante las etapas iniciales de la infección (Fuchs *et al*., 2013; Cwiklinski *et al*., 2015a). Además, resultan importantes desde el punto de vista clínico, ya que son la diana de diferentes fármacos antihelmínticos (Robinson *et al.*, 2003).

El intestino se divide en dos regiones: un intestino anterior que consta de boca, faringe y esófago, y dos ciegos ramificados. Esta estructura desempeña funciones de absorción y secreción de sustancias, si bien inmediatamente después de la excistación, todas las células del intestino de los NEJ son de tipo secretor, jugando un papel importante en la excistación y la migración, ya que las vesículas producidas en este compartimento contienen la mayoría de proteasas del parásito almacenadas en forma de zimógeno (Cwiklinski *et al*., 2015b) (**Figura 5**). A medida que el verme juvenil se desarrolla, una parte de las células gastrodérmicas adquiere la capacidad de absorber sustancias del lumen intestinal, de tal forma que las células absorbentes y secretoras parecen ser capaces de intercambiar sus funciones de manera cíclica (Robinson y Threadgold, 1975). En cuanto a la nutrición del parásito, el metabolismo de los NEJ es fundamentalmente de tipo aerobio, aunque a medida que el parásito penetra en los tejidos del hospedador y crece en tamaño se reduce la difusión de oxígeno y se produce una transición a un metabolismo de tipo anaerobio facultativo (Cwiklinski *et al*., 2018). Finalmente, es importante reseñar que la superficie interior del intestino no parece estar sometida a un proceso de recambio como sí sucede en el tegumento, por lo que este compartimento ha sido señalado como una interesante diana terapéutica (Hanna *et al*., 1980c).

Al igual que en otros platelmintos, el sistema excretor de *F. hepatica* presenta una estructura protonefridial, con células flamígeras conectadas con una serie de túbulos, los cuales desembocan en un conducto común, y este, a su vez, en un poro excretor situado en la región posterior. Cada célula flamígera presenta una gran cantidad de flagelos

orientados hacia el lumen del túbulo, cuyo movimiento impulsa el fluido excretado y filtra las sustancias de desecho, las cuales incluyen compuestos nitrogenados (urea y diversos aminoácidos) y lípidos. Aunque el sistema excretor de los NEJ es inmaduro, este se desarrolla de forma rápida en las primeras horas p.i. y, en apenas 12 horas, ya es totalmente funcional (Bennett, 1977). Más allá de su función excretora, en los últimos años se ha propuesto que este sistema puede desempeñar también un papel en la secreción de vesículas extracelulares, bien a través del mismo poro excretor o del intestino, gracias a las conexiones que parecen existir entre ambos sistemas (Bennett *et al*., 2020).

Figura 5 – Micrografía confocal de un NEJ de *F. hepatica* en la que se aprecia la morfología intestinal. Tras la fijación, el citoesqueleto de actina (en rojo) se ha teñido con faloidina conjugada con tetrametilrodamina (TRITC), mientras que los ciegos intestinales (en verde) se han marcado con un anticuerpo frente a catepsina L3, conjugado con isotiocianato de fluoresceína (FITC). Fuente: Pritsch *et al*. (2020).

El aparato reproductor de *F. hepatica* presenta una estructura común a la mayoría de trematodos (Robinson *et al*., 2001), con dos testículos ramificados que convergen cerca del poro genital situado entre las ventosas oral y ventral, así como de un ovario también ramificado y dos folículos vitelinos que actúan como precursores de huevos, los cuales convergen con el oviducto para formar el denominado ootipo, del cual surge un conducto que desemboca en el poro genital. El desarrollo de este sistema fue descrito a mediados del pasado siglo (Dawes, 1962), dando a conocer que los NEJ presentan un aparato reproductor rudimentario que madura progresivamente hasta estar completamente formado al inicio de la fase crónica de la infección.

2. La fasciolosis animal y humana

2.1. Epidemiología de la fasciolosis

La fasciolosis exhibe una excepcionalmente amplia distribución altitudinal, longitudinal y latitudinal en todo el mundo, con un incremento notable en el número de casos diagnosticados cada año, aunque los datos de prevalencia disponibles subestimaban en gran medida la magnitud del problema (Mas-Coma *et al*., 2009a). De modo general, *F. hepatica* se encuentra presente en los cinco continentes, demostrando una mayor capacidad de adaptación que *F. gigantica*, cuya distribución se localiza restringida a regiones de Europa, África y Asia en las que la presencia de caracoles del género *Radix* permite su expansión. Uno de los factores clave para explicar la amplia distribución del parásito es su elevada capacidad de adaptación a climas y entornos muy diversos, así como de infectar a un amplio rango de hospedadores, tanto intermediarios como definitivos (Mas-Coma *et al*., 2009a). Por otra parte, factores antropogénicos como la construcción de presas o diques, canales de regadío, etc., dan lugar a acumulaciones de agua dulce y zonas con elevada humedad ambiental, facilitando la colonización de nuevos ecosistemas (Tum *et al*., 2007). El cambio climático es otro elemento que ha contribuido a la expansión de la fasciolosis, ya que el incremento de la temperatura global ha permitido al parásito acceder a regiones que anteriormente eran demasiado frías, acelerando, además, su desarrollo en el hospedador intermediario y durante las etapas de vida libre (Mas-Coma *et al*., 2009b; Rondelaud *et al*., 2013). De hecho, varios modelos climáticos coinciden en que el calentamiento global ha contribuido a la propagación de *Fasciola* spp. en Europa, y se espera que la tendencia continúe en el futuro (Caminade *et al*., 2015). Por todo ello, se considera a la fasciolosis como una enfermedad reemergente en países desarrollados, a la que la Organización Mundial de la Salud (OMS) ha otorgado la denominación de «enfermedad tropical desatendida» (NTD), al ser sus efectos especialmente preocupantes en regiones de escasos recursos situadas en los trópicos (WHO, 2013).

Desde la descripción de la primera epidemia de fasciolosis humana en Europa a mediados del siglo XX (Coudert y Triozon, 1958), los reportes relativos a la enfermedad se han multiplicado, y en la actualidad se han descrito brotes en un total de 51 países de los cinco continentes. Más concretamente, las estimaciones de personas afectadas por el parásito han evolucionado desde unos 2 500 casos a mediados de la década de los 90 hasta

la actualidad, en la que se estima que entre 2,4 y 17 millones de personas padecen la enfermedad, y hasta 90 millones están en riesgo de ser infectadas. No obstante, debido a la dificultad de obtener datos fiables en determinadas regiones del planeta, es posible que esta predicción se aleje aún de los datos reales (Mas-Coma, 2005). Por regiones, las principales zonas donde la fasciolosis humana constituye un problema endémico de salud se identificarían con los países de la Cuenca del Mediterráneo, especialmente Francia, España y Portugal; Oriente Medio, afectando de manera preocupante a Irán y Turquía; Paquistán y Nepal en el Sur de Asia, así como zonas del Sur de China, Vietnam y Laos en el Este del continente asiático; la región del Delta del Nilo, considerada como una zona hiperendémica de África, alcanzando porcentajes de prevalencia de hasta un 19 %; Cuba en el Caribe; diferentes áreas de México en Norteamérica, y en América del Sur, donde se encuentran las principales zonas hiperendémicas de fasciolosis en el mundo asociadas a regiones de elevada altitud en la Cordillera de los Andes. En concreto, en el Altiplano Boliviano se alcanzan prevalencias del 72 y 100 %, en ensayos coprológicos y serológicos de la población, respectivamente. Situaciones similares se encuentran también en zonas de elevada altitud en Perú, Ecuador, Colombia, Venezuela y Argentina (Mas-Coma *et al*., 2019) (**Figura 6**).
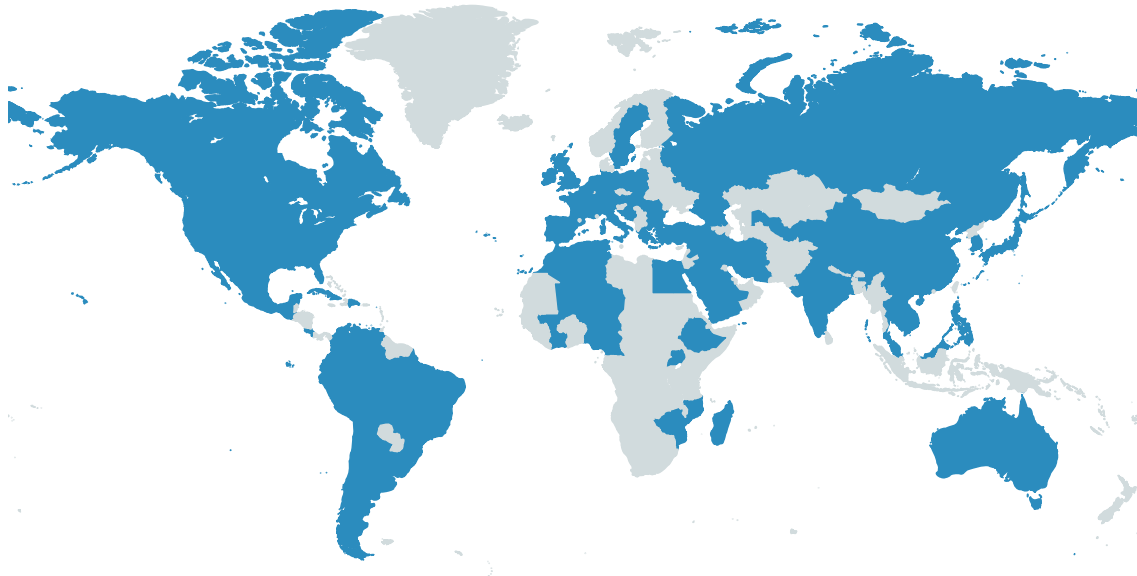
Figura 6 – Distribución de la fasciolosis humana en el mundo. En color azul aparecen representados los países con casos reportados de fasciolosis, de acuerdo con los últimos datos facilitados por la OMS (WHO, 2013). Mapa generado con mapchart.net.

A nivel ganadero, los estudios de prevalencia en la fasciolosis se efectúan mediante la identificación *post mortem* de vermes adultos en hígado o vías biliares, el

recuento de huevos en heces y la determinación de anticuerpos en muestras de suero. A pesar de que la presencia de la enfermedad se conoce desde hace décadas, la aplicación de estas técnicas ha permitido conocer datos concretos de prevalencia en explotaciones ganaderas, los cuales a menudo alcanzan valores muy elevados. Sin embargo, la variabilidad de los métodos empleados en los estudios epidemiológicos llevados a cabo hasta la fecha implica que los datos reportados no sean comparables. No obstante, se puede señalar que la fasciolosis animal es más prevalente en África, Asia y algunos países de América, y comparativamente mayor en países en vías de desarrollo respecto a aquellos más desarrollados dentro de cada continente. En cualquier caso, las altas tasas de prevalencia se relacionan con malas prácticas de manejo y falta de conocimiento entre los ganaderos sobre su control (Mehmood *et al*., 2017). Además, la fasciolosis animal está directamente relacionada con pérdidas económicas a nivel ganadero estimadas en torno a los 3 000 millones de dólares anuales, las cuales se encuentran vinculadas a la muerte de cabezas de ganado, abortos espontáneos y una disminución del rendimiento de subproductos animales como carne, leche o lana (Cwiklinski *et al*., 2016). Asimismo, la fasciolosis también genera pérdidas indirectas como consecuencia de la mayor susceptibilidad a otras patologías o el coste de los fármacos para tratar la enfermedad, por lo que el impacto económico real de la enfermedad resulta difícilmente cuantificable (Rushton *et al*., 2018).

2.2. Patología y aspectos clínicos

Los factores que afectan al desarrollo de la fasciolosis en el hospedador definitivo son variados, e incluyen principalmente la naturaleza del hospedador, la carga parasitaria y la virulencia de la cepa infectante. Existe además una amplia variabilidad entre individuos. La especie hospedadora juega un papel importante, ya que en muchos casos determina la susceptibilidad a la infección, así como la gravedad del cuadro clínico. De esta forma, mientras los rumiantes descritos como hospedadores clásicos (principalmente ovejas, vacas y cabras) presentan una alta susceptibilidad, con una gran proporción de las metacercarias ingeridas capaces de desarrollarse hasta alcanzar la fase adulta, y provocando signos severos e incluso mortalidad (Bosco *et al*., 2015), los mamíferos no rumiantes parecen servir como hospedadores accidentales, en los cuales el éxito de la infección resulta menor y los daños son más leves. El tamaño del hospedador es otro

elemento a tener en cuenta, ya que cuanto más pequeño sea este, el daño relativo causado por el parásito será mayor. De esta forma, mientras en vacas las infecciones con una cantidad de metacercarias inferior a 1 000 suelen pasar inadvertidas (Dow *et al*., 1967), en ratones la administración de 10 metacercarias llega a causar un 100 % de mortalidad (De Paula *et al*., 2010). Del mismo modo, y al igual que sucede en otras trematodiasis, la gravedad del cuadro clínico es proporcional a la carga parasitaria del individuo afectado.

En conjunto, la gravedad de la infección es altamente variable, y la intensidad de sus manifestaciones clínicas abarca desde asintomática a severa, pudiendo incluso llegar a ser mortal (Keiser y Utzinger, 2009). A menudo resulta complicado esclarecer los signos clínicos asociados a la fasciolosis debido a los frecuentes casos de coinfección parasitaria en áreas endémicas y a la alta variabilidad intra e interespecífica. Sin embargo, las infecciones experimentales utilizando modelos animales han contribuido a determinarlos (Valero *et al*., 2017), y la mayoría de autores distinguen 4 fases en la infección por *F. hepatica* (Mas-Coma y Bargues, 1997):

- **Fase prehepática o de incubación**: Abarca desde la ingesta de las metacercarias hasta la llegada de los vermes juveniles al hígado. La duración de esta fase varía en función de factores como la especie y el tamaño del hospedador, pudiendo comprender desde unos días hasta varios meses. Salvo en las infecciones más graves, no suele presentar signos clínicos (Zafra *et al*., 2013).

- **Fase aguda, invasiva o hepática**: Implica la migración de los vermes inmaduros a través del hígado y su desplazamiento hacia las vías biliares. Por lo general, la duración de esta etapa oscila entre 2 y 4 meses (**Figura 7A**).

- **Periodo de latencia**: Esta fase puede prolongarse durante meses o incluso años, y abarca desde el establecimiento de los vermes adultos en los canalículos biliares hasta la observación de huevos en heces y la aparición de los signos típicos de la fase crónica.

- **Fase crónica, obstructiva o biliar**: Esta etapa se inicia una vez que los vermes adultos invaden las vías biliares y comienzan a depositar huevos que son vertidos al lumen intestinal junto con la bilis. La duración y gravedad de esta fase varía ampliamente en función de la especie, pudiendo abarcar desde unos pocos meses hasta más de una década (**Figura 7B**).

Figura 7 – Patología aguda y crónica en la fasciolosis. (**A**) Hígado de cabra a 21 semanas postinfección, en el que se pueden apreciar los tractos migratorios y la fibrosis característicos de la etapa aguda de la enfermedad, especialmente en el lóbulo izquierdo del hígado. Fuente: adaptado de Buffoni *et al*. (2010). (**B**) Hígado de oveja durante la fase crónica de la infección, en el que puede apreciarse engrosamiento e hipertrofia de los ductos biliares (*). Fuente: Stuen y Ersdal, 2022.

Desde el punto de vista clínico, las dos fases más relevantes de la enfermedad son la aguda y la crónica, cada una con unos daños característicos (Chen y Mott, 1990). Los signos de la fase aguda son causados por la destrucción mecánica del tejido hepático asociada a la migración de las formas inmaduras del parásito. Este hecho está provocado por las proteasas del parásito y la acción erosiva de sus espinas y ventosas, así como por la respuesta inmune innata desencadenada por la presencia del verme, e incluye fiebre moderada o alta, dolor abdominal, tos, hemorragia, diarrea, flatulencia y urticaria, entre otros signos. Una exploración del sujeto durante esta fase a menudo revela hepatomegalia y esplenomegalia. Adicionalmente el daño hepático puede causar fibrosis, la cual se traduce en una disminución de la capacidad biosintética del hígado, alterando los niveles enzimáticos o de proteínas plasmáticas, principalmente albúmina (Valero *et al*., 2016). También se ven afectados los niveles de enzimas antioxidantes, principalmente catalasa y glutatión S-transferasa (GST), a la vez que se incrementan los niveles de especies reactivas del oxígeno (ROS), creando un ambiente de estrés oxidativo mantenido (da Silva *et al*., 2017). Es en esta etapa cuando se produce la mayor parte de la mortalidad asociada a la enfermedad, si bien se trata de un fenómeno por lo general poco frecuente, y muy dependiente de la susceptibilidad de la especie hospedadora (Bosco *et al*., 2015).

Después del periodo latente, la fase crónica de la enfermedad asociada a la presencia de los vermes adultos en la red biliar puede manifestarse tras varios meses de infección. La clínica es muy variada, e incluye colangitis (inflamación de la pared de los canalículos biliares), colecistitis (dilatación de la vesícula) y generación de cálculos biliares, así como obstrucción como consecuencia de la inflamación y el taponamiento de

las vías biliares por los vermes o sus huevos. Otros signos incluyen intolerancia a alimentos ricos en ácidos grasos, eosinofilia (alcanzando niveles hasta 4 veces superiores a los de animales sanos), ictericia, calcificación del colédoco o hematobilia (presencia de sangre en la bilis) (Valero *et al*., 2003). En infecciones animales, es frecuente durante esta etapa observar una reducción del aumento de peso (en torno al 6-9 %), así como de la producción de otros derivados, como leche (entre 0,7-2 kg/vaca/día) o lana (hasta un 10 %), la cual puede darse incluso en infecciones subclínicas (Charlier *et al*., 2014; Hayward *et al*., 2021).

La proporción de parásitos que alcanzan esta fase, así como el tiempo de residencia en las vías biliares, es variable en función de la especie hospedadora. En infecciones prolongadas puede desarrollarse anemia como consecuencia de la sangre consumida por los vermes (estimada en 0,2-0,5 ml/verme/día) (Dawes y Hughes, 1964), así como cirrosis biliar (Valero *et al*., 2008). Frecuentemente, también aparecen infecciones bacterianas derivadas de la presencia del parásito, causando sepsis biliar (Valero *et al*., 2006b). Cabe destacar que, en caso de producirse reinfecciones, pueden coexistir simultáneamente los signos agudos y crónicos de la enfermedad, especialmente en regiones donde la enfermedad es endémica (Mas-Coma *et al*., 2019). Además de los daños citados, la infección por *F. hepatica* es responsable de un estado de inmunosupresión/inmunorregulación que favorece la aparición de infecciones concomitantes (Gironès *et al*., 2007). De hecho, es frecuente encontrar coinfecciones tanto parasitarias como causadas por virus o bacterias, a menudo pertenecientes a los géneros *Mycobacterium* (Moreau y Chauvin, 2010), *Escherichia* (Howell *et al*., 2018) o *Clostridium* (Karagulle *et al*., 2022).

Las infecciones ectópicas originadas por el desplazamiento accidental de los vermes a través de otros tejidos causan cuadros clínicos generalmente agudos. Afectan principalmente al tracto gastrointestinal y al tejido subcutáneo, aunque también a otros órganos como pulmones, cerebro, corazón, ojos, bazo o páncreas, y sus signos clínicos dependerán del órgano afectado (Mas-Coma *et al*., 2014a; Taghipour *et al*., 2019).

2.3. Interacción inmune entre *Fasciola hepatica* y su hospedador

Como se acaba de señalar, *F. hepatica*, al igual que otros helmintos parásitos, puede vivir durante tiempos prolongados en el interior de su hospedador dando lugar a procesos patológicos crónicos. Esta situación es consecuencia directa de largos procesos de coevolución que, en el caso particular de *F. hepatica*, han motivado la aparición de mecanismos de evasión e inmunomodulación que facilitan la supervivencia del parásito frente a las diversas respuestas inmunológicas que enfrenta a lo largo de su migración en el interior del animal vertebrado (Corral-Ruiz y Sánchez-Torres, 2020). De hecho, el parásito secreta y expone en su superficie un amplio repertorio de moléculas con cualidades inmunomoduladoras, que en general resultan en una respuesta antiinflamatoria y reguladora mediada por linfocitos T colaboradores (comúnmente denominada T *helper* o Th) de tipo Th2/Treg en fase crónica, que permite la supervivencia de *F. hepatica* y produce un daño controlado al hospedador.

La respuesta inmunitaria que se desarrolla durante las primeras etapas de la fasciolosis es una respuesta mixta Th1/Th2 en la que aumenta la expresión de citoquinas como el interferón (IFN) γ, la interleuquina (IL)-4, IL-10 y TGF-β. A medida que avanza la infección, se amplifica la respuesta Th2 junto con la supresión de la inflamación, lo que permite una infección prolongada caracterizada por una alta producción de IL-4 (O`Neill *et al*., 2000). Además, se ha postulado que TGF-β e IL-10 podrían modular la producción de IL-4 e IFNγ en infecciones agudas y crónicas, respectivamente (Flynn y Mulcahy, 2008). Como la IL-4 es una citoquina crítica a lo largo de la patogénesis de la infección por *F. hepatica*, no sorprende la aparición de una población abundante de macrófagos activados alternativamente (AAM) en etapas tempranas de la infección, que permanecen en el peritoneo hasta 3 semanas después de la misma (Walsh *et al*., 2009).

Se han estudiado algunos de los productos de los vermes adultos de *F. hepatica*, ya sea como extracto total, vesículas extracelulares o moléculas purificadas, para establecer y caracterizar sus mecanismos inmunomoduladores. Estos incluyen antioxidantes, proteasas y péptidos similares a mucinas, así como moléculas de defensa helmíntica (HDM), entre otras, cada una con influencias únicas en la respuesta inflamatoria del hospedador (Ryan *et al*., 2020). El grupo de HDMs es uno de los que ha sido más ampliamente estudiado en cuanto a su capacidad inmunomoduladora. Las

HDMs son un grupo conservado de proteínas descubiertas en el secretoma de *F. hepatica*. Sus propiedades inmunomoduladoras han propiciado la generación de un péptido, denominado FhHDM-1, que ha demostrado amplias capacidades antiinflamatorias (Ryan *et al*., 2020). Al igual que las HDMs, se han descrito otras moléculas del parásito, principalmente de su fase adulta, con capacidad inmunomoduladora, incluyendo las proteínas de unión a ácidos grasos (FABP), las moléculas tipo Kunitz, las GST y las peroxirredoxinas (Prx) (Ryan *et al*., 2020).

Se conoce que la fase en la que los NEJ cruzan la pared intestinal hacia el hígado a través del peritoneo se caracteriza por inflamación y daño hasta que los parásitos llegan a los conductos biliares del hígado, donde maduran hasta convertirse en adultos. Sin embargo, en cuanto a las molécuclas específicas con capacidad inmunomoduladora que se producen por el parásito en sus etapas migratorias juveniles, hay poca o ninguna información (Ryan *et al*., 2020). Asimismo, se tiene un conocimiento muy limitado sobre las interacciones específicas entre estos productos y las células inmunitarias del hospedador, tanto en las fases juveniles como en los parásitos adultos.

2.4. Diagnóstico

Las estrategias para el diagnóstico de la fasciolosis en humanos y animales son diversas y han evolucionado considerablemente durante las últimas décadas. Estas incluyen metodologías tanto directas como indirectas, y en la práctica no se trata de aproximaciones excluyentes, sino complementarias, ya que ninguna cubre todos los posibles escenarios. Aunque lo deseable es que el diagnóstico sea lo más temprano posible para evitar los efectos perjudiciales de la enfermedad, en muchos casos no resulta posible, no solo por la falta de métodos compatibles, sino también por la ausencia de signos clínicos que hagan sospechar que existe infección o por la poca especificidad de los mismos (Mas-Coma *et al*., 2014b).

Dentro de la categoría de técnicas directas, el recuento de huevos en heces constituye una alternativa rápida, fiable y de bajo coste, que permite confirmar la infección y estimar su intensidad, además de requerir escasos medios técnicos para su utilización, por lo que resulta el método más aplicable en zonas con escasos recursos (Ghodsian *et al*., 2019). En este sentido, la OMS recomienda la utilización de la técnica

de Kato-Katz como método de elección para llevar a cabo este tipo de diagnóstico (WHO, 2007). Pese a su fiabilidad, estos métodos presentan una serie de desventajas importantes como su baja sensibilidad, la necesidad de contar con personal con suficiente experiencia, la falta de correlación entre la carga parasitaria y la puesta de huevos o la imposibilidad de distinguir entre diferentes especies de fasciólidos. Además, se trata de técnicas aplicables solo durante la fase crónica de la infección, posteriormente a la oviposición, por lo que no resultan adecuadas para la detección temprana de la enfermedad (Valero *et al*., 2009). Otros métodos directos son la identificación de huevos en líquido duodenal o bilis, los cuales, aunque más fiables y sensibles que el recuento en heces, son difíciles de llevar a cabo debido a su carácter invasivo (Mas-Coma *et al*., 1999).

En contraposición a las técnicas directas, existen diferentes test de base molecular cuyo desarrollo ha hecho posible el diagnóstico de la infección durante la fase aguda, ofreciendo además una mayor sensibilidad. La mayoría de estas técnicas se basan en reacciones de tipo antígeno-anticuerpo, mediante la detección de antígenos parasitarios circulantes o de anticuerpos frente a estos (Espino y Finlay, 1994). No obstante, en la práctica las técnicas basadas en la detección de antígenos son poco utilizadas por su escasa relación señal/ruido (Duménigo *et al*., 2000). En consecuencia, la mayoría de técnicas serológicas se basan en la detección de anticuerpos circulantes frente al parásito, para lo cual se emplean antígenos obtenidos en forma de extractos naturales, fracciones purificadas o proteínas recombinantes, que se enfrentan a muestras de suero del paciente (Espino y Finlay, 1994). Muchos de estos antígenos se han ensayado, reportando tasas de especificidad y sensibilidad superiores al 95 % en ensayos ELISA (*Enzyme-Linked ImmunoSorbent Assay*) (Álvarez-Rojas *et al*., 2014). En este sentido, las catepsinas del parásito han mostrado resultados satisfactorios como biomarcadores altamente sensibles y específicos en el diagnóstico de la fasciolosis y, por ello, varios test han sido desarrollados basándose en la detección de anticuerpos específicos frente a esta familia de proteasas en muestras de suero (Siles-Lucas *et al*., 2021). Un ejemplo exitoso de esta aproximación es el inmunoensayo de flujo lateral SeroFluke^{TM}, el cual muestra una máxima especificidad y sensibilidad (100 %) en la detección de anticuerpos frente a la forma recombinante de la catepsina L1 de *F. hepatica* en muestras de suero o sangre (Martínez-Sernández *et al*., 2011). No obstante, y pese a las ventajas inherentes a las pruebas serológicas, también existen aspectos negativos como la imposibilidad de realizar un seguimiento efectivo de la enfermedad debido a la permanencia de los anticuerpos en

el organismo, la cual dificulta discriminar entre infecciones pasadas o en curso (Valero *et al*., 2009; Munita *et al*., 2019). En este sentido, la disponibilidad de anticuerpos monoclonales frente a las catepsinas L1, L2 y la proteína de tipo Kunitz ha permitido detectar la presencia de estos antígenos de *F. hepatica* en muestras biológicas no invasivas y de fácil acceso como las heces, lo que facilita tanto el diagnóstico, como la monitorización posterior al tratamiento. Disponible comercialmente y basado en esta tecnología, se encuentra el ensayo copro-ELISA BIO K 201™ (Mezo *et al*., 2004; Martínez-Sernández *et al*., 2016).

Ninguna de las técnicas mencionadas anteriormente es capaz de diferenciar entre infecciones causadas por las diferentes especies del género *Fasciola*, por lo que el diagnóstico diferencial en regiones endémicas en las que coexisten *F. hepatica* y *F. gigantica* resulta complicado. Recientemente, se han desarrollado métodos de detección basados en la hibridación de ácidos nucleicos, capaces no solo de detectar la presencia del parásito sino también de determinar la especie causante de la infección, como la aproximación denominada PCR-RFLP (*Polymerase Chain Reaction - Restriction Fragment Length Polymorphism*) (Marcilla *et al*., 2002; Ai *et al*., 2010; Rokni *et al*., 2010). A pesar de su especificidad, estos métodos presentan desventajas como el gran coste de la infraestructura requerida, la falta de estandarización en la metodología analítica o la escasa disponibilidad de marcadores genéticos. En este sentido, la publicación de los borradores del genoma de *F. hepatica* (Cwiklinski *et al*., 2015a) y *F. gigantica* (Pandey *et al*., 2020) se espera que provea de nuevos *loci* que sirvan como potenciales dianas diagnósticas.

2.5. Tratamiento farmacológico

Durante las últimas décadas, las estrategias disponibles para el tratamiento de la fasciolosis han utilizado diferentes grupos de compuestos antihelmínticos que incluyen benzimidazoles (albendazol –ABZ–, mebendazol, triclabendazol –TCBZ–), salicilanilidas (closantel, rafoxanida, oxiclozanida), sulfonamidas (clorsulón), fenoles halogenados (nitroxinil, bitionol) y lactonas macrocíclicas (ivermectina), de forma aislada o en combinación, produciendo en ocasiones un efecto sinérgico cuando los fármacos actúan sobre diferentes dianas (Alvarez *et al*., 2022). Para que un fármaco pueda ejercer su efecto, debe ser capaz de acceder a las estructuras del parásito y acumularse en

la mismas, por lo que su biodisponibilidad y farmacocinética son los principales aspectos a tener en cuenta en su aplicación. En el caso de *F. hepatica*, las principales vías de entrada de los fármacos son a través de la ventosa oral o por difusión transtegumental, de manera que la segunda vía es la predominante en el verme juvenil por su pequeño tamaño, mientras que la primera es más importante en el adulto al tratarse de un organismo hematófago (Moreno *et al*., 2014).

La mayoría de antihelmínticos conocidos afectan al metabolismo energético del parásito al interferir con la cadena de transporte de electrones mitocondrial, produciendo una disrupción de los tejidos altamente demandantes (principalmente el sistema reproductor, el tegumento y el intestino) (Fairweather y Boray, 1999). Aunque eficaces, la mayoría de fármacos solo resultan efectivos contra los vermes adultos, al presentar una buena capacidad de unión a proteínas plasmáticas que permite que sean ingeridos por el verme cuando reside en las vías biliares, pero su escasa capacidad de distribución por otros tejidos los hace poco eficaces contra juveniles (Alvarez *et al*., 2022). En este escenario, el principal antihelmíntico en el tratamiento de la fasciolosis es el TCBZ (**Figura 8**), un benzimidazol halogenado que presenta una excelente eficacia frente a estadios maduros e inmaduros de *F. hepatica* desde la primera semana p.i., empleando dosis estándares de entre 10 y 15 mg/kg de peso, dependiendo de la especie de hospedador parasitado (Turner *et al*., 1984). El fármaco tiene un tiempo de permanencia en el organismo elevado gracias a su capacidad de unión a proteínas plasmáticas (Hennessy *et al*., 1987), lo cual no impide su distribución por otros tejidos, y ayuda a comprender su efectividad frente a los vermes juveniles. Además, la principal vía de excreción del TCBZ es a través de la bilis (Moreno *et al*., 2014) y no de la orina, lo que facilita su entrada en contacto con el adulto.
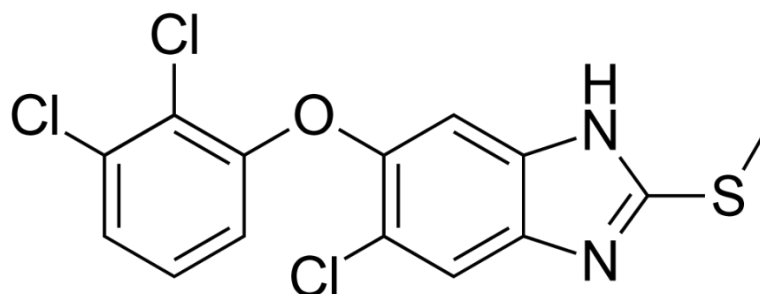
Figura 8 – Estructura molecular del triclabendazol (TCBZ), elaborada con el software Pubchem.

Existe un consenso general de que, a diferencia de otros antihelmínticos, los benzimidazoles (incluyendo el TCBZ) actúan como agentes disruptivos de los microtúbulos del parásito, aunque su mecanismo de acción no es completamente conocido (Fairweather *et al.*, 2020). En la práctica, el TCBZ es capaz de eliminar la totalidad de los vermes de *F. hepatica* empleando una pauta de una o dos administraciones separadas habitualmente por 12 horas (Fairweather y Boray, 1999), si bien en infecciones con una elevada carga parasitaria se recomienda una administración sostenida en el tiempo, puesto que la muerte y el desprendimiento de una gran cantidad de vermes en un corto espacio de tiempo durante la fase crónica puede ocasionar el colapso de las vías biliares (Valero *et al.*, 2016).

Pese a la alta eficacia del TCBZ, a mediados de los años 90 se describió la primera cepa de *F. hepatica* resistente a su acción en Australia (Overend y Bowen, 1995), y desde entonces el problema ha evolucionado hasta convertirse en una prioridad de primer nivel. En la actualidad, se han definido hasta 11 aislados del parásito resistentes a diferentes antihelmínticos (incluyendo 5 cepas resistentes a TCBZ, solo o en combinación con ABZ y/o clorsulón), denunciándose estas situaciones en granjas de todo el mundo (Fairweather *et al.*, 2020). Por ello, y con el objetivo de mejorar el control de la enfermedad, es crucial comprender las bases genéticas y moleculares de los mecanismos de resistencia a antihelmínticos, los cuales se han estudiado principalmente en el caso del TCBZ (Fairweather *et al.*, 2020). En este sentido, se han propuesto diferentes mecanismos para explicar la resistencia a este compuesto, los cuales incluyen la alteración de: 1) la estructura de la β-tubulina mediante una mutación en la proteína que impida su interacción con el TCBZ (Robinson *et al.*, 2002), 2) del transporte del TCBZ, lo cual afectaría a la captación del fármaco por las cepas resistentes mediante una posible alteración en la función de la glicoproteína P, responsable de su transporte al interior de las células (Mottier *et al.*, 2006), y 3) del metabolismo del TCBZ, lo cual está directamente relacionado con la mitigación de los efectos del fármaco durante las fases inmaduras (Robinson *et al.*, 2004). En cualquier caso, y sea cual sea el mecanismo implicado, es necesario plantear alternativas que permitan el tratamiento eficaz de la enfermedad ante la emergencia de resistencias frente al TCBZ. Ya que uno de los factores limitantes en la farmacocinética de los benzimidazoles (y en particular del TCBZ) es su baja solubilidad en medio acuoso, en la actualidad las perspectivas farmacológicas se centran en la obtención de derivados más solubles de estos fármacos (Real *et al.*, 2019) o

en vectorizar su administración para favorecer su entrada en los tejidos diana, mediante estrategias como la conjugación con quitosano (Real *et al*., 2018).

2.6. Prevención y control

Pese a los problemas descritos en referencia al control farmacológico de la fasciolosis, en zonas donde se presentan numerosos casos en humanos se debe considerar la posibilidad de administrar TCBZ como quimioterapia preventiva. Esto se llevaría a cabo en intervenciones poblacionales, para controlar la morbilidad asociada a la fasciolosis mediante la reducción de la carga de helmintos en las personas infectadas (PAHO, sin fecha). Sin embargo, y con el objetivo de reducir la transmisión, es indispensable realizar actividades de educación sanitaria e implantar medidas de salud pública veterinaria (Mas-Coma *et al*., 2018; 2019).

Según las indicaciones de diversos expertos, es recomendable diseñar los programas de control veterinario en base a la información epidemiológica local (Mas-Coma *et al*., 2019). El historial de uso de antiparasitarios, fechas de tratamiento, topografía, tipo de pastos y de establos, carga animal, rotaciones, etc., inciden mucho en la gravedad y, por lo tanto, en las decisiones a tomar. Un control eficiente debe estar basado en la acción sobre los tres componentes del ciclo del parásito: control sobre el parásito en el animal; control de los estadios libres y control de los caracoles. En el primer caso, se suele utilizar el tratamiento farmacológico tanto frente a fases adultas como juveniles de *F. hepatica*, aunque también se debe incidir en medidas de manejo para disminuir la contaminación de pastos por huevos del parásito. La finalidad del tratamiento profiláctico es reducir la contaminación de los pastos con huevos de *F. hepatica*, y disminuir la carga parasitaria de los animales en épocas en las que el número de parásitos por animal sea elevado o cuando exista un estrés nutricional o de gestación. En zonas endémicas se deben aplicar al menos dos tratamientos anuales, uno antes de salir los animales a los pastos para evitar su contaminación, y otro a final de otoño si no ha habido casos agudos antes (Mas-Coma *et al*., 2019).

El control de los estadios libres del parásito se ejerce fundamentalmente a nivel de la excreta de huevos, restringiendo las áreas de pastoreo a los animales susceptibles en épocas críticas. En cuanto al control de los caracoles, el mejor método a largo plazo es el

drenaje. Aunque también se utilizan molusquicidas, se recomienda su uso restringido por su impacto ambiental. El control biológico con competidores del caracol hospedador o con depredadores de los mismos también se utilizan en algunas zonas (Mas-Coma *et al*., 2019).

2.7. Aproximaciones vacunales para el control de la fasciolosis

En la actualidad, las perspectivas globales en el ámbito de la fasciolosis humana son preocupantes dadas sus elevadas prevalencias en regiones endémicas y su creciente incidencia en países desarrollados. Por otra parte, la vertiente animal de la enfermedad se considera de distribución cosmopolita y las pérdidas anuales a nivel ganadero ocasionadas por la fasciolosis son cuantiosas, como ya se ha mencionado. Estas pérdidas podrían ser mayores en el futuro, debido a la expansión que esta parasitosis está experimentando hacia nuevas regiones como consecuencia del calentamiento global. El uso indiscriminado y sin planificación de los fármacos de elección, especialmente en el contexto ganadero, está detrás del reporte recurrente de fenómenos de resistencia. Además, dicha estrategia no protege al hospedador frente a futuras reinfecciones y puede ser la causa de residuos indeseables en productos animales y en el medio ambiente. Por ello, la sustitución de fármacos por otros medios, como la vacunación, para el control de la fasciolosis, debería tener un impacto importante en la mejora de la calidad y seguridad alimentarias, ya que las vacunas recombinantes no dejan residuos en los alimentos de origen animal y tienen un efecto relativamente duradero.

Aunque por el momento no existe ninguna vacuna disponible comercialmente para el tratamiento de la fasciolosis, desde la segunda mitad del siglo XX se han llevado a cabo numerosos ensayos de vacunación en las principales especies de ganado afectadas por *F. hepatica* (vacas, ovejas y cabras), así como en modelos animales de laboratorio, utilizando como antígenos extractos proteicos del parásito, proteínas nativas purificadas o péptidos recombinantes, entre otros (Toet *et al*., 2014; McManus, 2020). En concreto, la mayor parte de los antígenos ensayados hasta el momento se identifican con versiones recombinantes de proteínas procedentes del verme adulto de *F. hepatica*, involucradas en procesos de importancia para la fisiopatología del parásito tales como la proteolisis

(catepsinas y otras proteasas de cisteína, leucina aminopeptidasa), el metabolismo redox (GST, Prx, tiorredoxina glutatión reductasa) o el transporte de biomoléculas (FABP), así como formulaciones que combinan dos o más de estas moléculas (González-Miguel *et al*., 2021). Si bien en algunos casos los porcentajes de protección (expresados como el tanto por ciento de reducción en el número de vermes adultos en comparación con animales control no vacunados) han llegado a ser esperanzadores tanto en ganado bovino (Dalton *et al*., 1996) como en ovino (Piacenza *et al*., 1999), no se ha logrado que dichos resultados sean reproducibles, presentando por lo general una eficacia limitada y existiendo una gran variabilidad incluso entre animales de un mismo ensayo (Dominguez *et al*., 2018).

Por todo ello, en los últimos años se han sugerido diferentes pautas metodológicas basadas en el conocimiento actual de la fasciolosis, destinadas a lograr una mejora palpable en futuros ensayos vacunales (Molina-Hernández *et al*., 2015; Spithill *et al*., 2022). En la práctica, no se cree posible eliminar por completo la fasciolosis en explotaciones ganaderas, pero sí evitar que afecte al bienestar animal y a la productividad, para lo cual se estima que una vacuna comercial debería ser capaz de reducir la carga parasitaria al menos en un 70-80 % de forma consistente. Para lograr dicho objetivo, se ha estimado oportuno identificar unas condiciones óptimas relacionadas con la elección de antígenos, adyuvantes, modelos animales y vías de administración.

En cuanto a la selección de antígenos, los estudios en animales con resistencia adquirida a la infección por *F. hepatica* muestran que la eliminación de los vermes se produce antes de su entrada en las vías biliares, cuando el parásito todavía se encuentra en su fase juvenil (Dalton *et al*., 1996). Esto pone de manifiesto que la mejor estrategia sería dirigir la vacunación frente a antígenos expresados por las formas inmaduras de *F. hepatica*, permitiendo, además, una actuación rápida frente al parásito antes de que se manifiesten los signos clínicos de las fases aguda y crónica. En este sentido, los candidatos vacunales idóneos serían proteínas expresadas en la superficie del parásito (por encontrarse más expuestas) durante las primeras semanas p.i. (Piedrafita *et al*., 2007). Las proteínas transmembrana podrían constituir también buenas dianas al permanecer unidas a la superficie del verme (McCusker *et al*., 2020), así como las proteínas secretadas en vesículas extracelulares (Cwiklinski *et al*., 2015b), por su importancia en la comunicación parásito-hospedador y sus funciones inmunomoduladoras. Una gran proporción de las proteínas con estas características están glicosiladas, por lo que en caso

de producirse en forma recombinante debería emplearse un sistema de expresión capaz de reproducir el patrón de glicosilación de la proteína nativa, ya que puede ser determinante para su antigenicidad (Ravidà *et al*., 2016), y se cree que este puede ser uno de los motivos de la menor eficacia observada en antígenos recombinantes frente a los nativos (Toet *et al*., 2014). En este sentido, las aproximaciones vacunales empleando antígenos del verme juvenil, aunque escasas, han mostrado unos buenos porcentajes de protección en modelos animales con roedores (Chantree *et al*., 2013; Wesołowska *et al*., 2018a), especialmente al utilizar combinaciones de diferentes antígenos, por lo que se plantean como opciones prometedoras de cara a futuros ensayos.

Pese a la gran cantidad de adyuvantes disponibles en el mercado, las mayores tasas de efectividad se han logrado utilizando el adyuvante completo de Freund (Dalton *et al*., 1996; Piacenza *et al*., 1999), si bien los efectos secundarios asociados a este lo hacen inviable para su uso fuera del ámbito de la investigación, por lo que de cara a una formulación comercial se debería determinar cuál de las opciones disponibles proporciona un mejor resultado. Aunque apenas existen estudios en *F. hepatica* que comparen la utilización de diferentes adyuvantes, se ha comprobado que, con un mismo antígeno, la modificación del adyuvante puede dar lugar a diferencias en la tasa de protección de hasta 6 veces (Maggioli *et al*., 2016), por lo que no parece un aspecto que deba ser desdeñado a la hora de diseñar la formulación vacunal. En general, se consideran los adyuvantes más adecuados aquellos que sean capaces de inducir una respuesta inmune de tipo Th1, al ser las que teóricamente permiten una protección más eficaz frente a *F. hepatica* (Molina-Hernández *et al*., 2015).

La elección del modelo animal es otro aspecto clave en el diseño de un ensayo de vacunación, ya que a menudo no hay correlación entre los niveles de protección obtenidos en pequeños mamíferos (Maggioli *et al*., 2011) y en rumiantes (Maggioli *et al*., 2016) utilizando una misma formulación. Esto implica que pueden existir antígenos protectores en animales de laboratorio que no resulten efectivos en el ganado (McCusker *et al*., 2020), y viceversa, por lo que lo ideal sería realizar los ensayos futuros directamente en dichos animales, o recurrir a modelos de cribado *in vitro* que permitan evaluar la eficacia en estos (Piedrafita *et al*., 2007; Sulaiman *et al*., 2016).

Finalmente, aunque la mayoría de ensayos se han realizado empleando vías de administración parenterales (principalmente subcutánea o intramuscular), algunos autores han sugerido que una posible forma de dirigir la respuesta de anticuerpos al verme

juvenil sería administrar la vacuna a través de mucosas (por vía intranasal u oral), ya que la mucosa intestinal es uno de los primeros tejidos con los que los NEJ entran en contacto en las primeras etapas de la infección. En este sentido, algunos estudios apoyan esta hipótesis tras detectar una diferencia significativa en los niveles de protección de una misma vacuna administrada por vía intranasal (40,5 %) e intramuscular (20,9 %) en un modelo ovino (Norbury *et al*., 2018).

Los antecedentes introducidos hasta el momento en la presente Tesis Doctoral ponen de relieve que las fases parasitarias más tempranas durante la infección del hospedador vertebrado en la fasciolosis son poco conocidas, a pesar de representar, potencialmente, una diana importante para el desarrollo de vacunas. En un contexto de preocupante actualidad, debido a la creciente incidencia de la enfermedad, a los daños provocados, a las cuantiosas pérdidas económicas anexas, y a la aparición de resistencias al fármaco de elección, la generación de conocimiento sobre las relaciones parásito-hospedador resulta de urgente necesidad, máxime en aquellas interacciones que rigen los mecanismos moleculares entre ambos organismos en un estadio inicial de la infección. Para ello, resulta fundamental la aplicación en el estudio de la fasciolosis de las últimas técnicas -ómicas acopladas a nuevos modelos que permitan replicar y desentrañar dichas interacciones mientras se identifican las moléculas implicadas en las mismas, tanto de *F. hepatica* como de su hospedador. A continuación, se expone la información disponible en la literatura científica acerca de estos aspectos.

3. La aplicación de las técnicas –ómicas en la fasciolosis

El término «-ómica» hace referencia a un conjunto heterogéneo de metodologías de análisis que incluye, entre otras, la secuenciación y anotación estructural y funcional de los genes (genómica), la determinación de los genes transcritos en un contexto biológico determinado (transcriptómica) y la caracterización de las proteínas sintetizadas en tales situaciones (proteómica).

En lo referente a *F. hepatica*, actualmente existe una cantidad considerable de información -ómica, que incluye ensamblajes genómicos, transcriptomas de diferentes fases de desarrollo del parásito y proteomas de las fracciones somática, secretada y de las vesículas extracelulares (Cwiklinski y Dalton, 2018). Estos datos aportan información muy valiosa acerca del ciclo biológico del parásito, su relación con los diferentes hospedadores o sus mecanismos de virulencia, y han permitido, por ejemplo, profundizar en la comprensión de los mecanismos moleculares de la resistencia a fármacos.

3.1. Aspectos generales sobre el genoma de *Fasciola hepatica*

Gracias al progreso constante de las tecnologías de secuenciación genómica de nueva generación (NGS), en la actualidad existen dos proyectos de secuenciación del genoma de *F. hepatica*, ambos alojados en la plataforma *WormBase ParaSite* (https://parasite.wormbase.org). Uno de ellos procede de un aislado parasitario del Reino Unido (PRJEB6687/PRJEB25283) (Cwiklinski *et al*., 2015a), mientras que el otro se generó a partir de una cepa procedente de Estados Unidos (PRJNA179522) (McNulty *et al*., 2017).

La publicación del primer borrador del genoma de *F. hepatica* (PRJEB6687) (Cwiklinski *et al*., 2015a) marcó el inicio de los estudios -ómicos en esta especie. Dicho genoma está constituido por 10 pares de cromosomas y presenta un tamaño aproximado de 1,2 gigabases (Gb), lo que lo convierte en el mayor genoma de un trematodo documentado hasta la fecha. Dicho tamaño es consecuencia de un alto contenido en elementos repetitivos (Choi *et al*., 2020), aunque su número de genes es similar al de especies cercanas. Esto resulta paradójico desde un punto de vista evolutivo, ya que su

replicación para la producción de huevos por el verme adulto supone un coste energético muy importante, y se ha planteado como un posible mecanismo para mantener la diversidad genética en situaciones de autofecundación y expansión clonal (Noël *et al*., 2017). El proyecto de secuenciación fue actualizado en 2018 y rebautizado como PRJEB25283, mejorando la calidad del ensamblaje, el cual presenta en la actualidad un total de 9 732 secuencias codificantes y 7 098 no codificantes, una proporción de guanina + citosina (G+C) del 44,1 %, un 3,3 % de nucleótidos indeterminados, y unos valores N50 y N90 de 1,9 megabases (Mb) y 518,9 kilobases (Kb), respectivamente. Esto último indica que el 50 y el 90 % de los cóntigos que componen el ensamblaje tienen una longitud superior a dichos valores. Destaca la existencia de un gran número de polimorfismos de un solo nucleótido (SNPs), de los cuales se ha estimado que existe al menos uno en el 48 % de los genes predichos, lo que pone de manifiesto la variabilidad genética de la especie entre cepas, y refleja su adaptación a numerosos ecosistemas y hospedadores (Cwiklinski *et al*., 2015a). Si bien todavía no se trata de un genoma maduro, es de esperar que la introducción de tecnologías de secuenciación de 3ª generación contribuya a lograr un mejor ensamblaje y obtener un tamaño definitivo, reduciendo el número de genes al eliminar falsos positivos y mejorando la anotación.

Hasta el momento, los estudios genómicos han permitido constatar, entre otros aspectos, la expansión de familias de genes importantes para la supervivencia de los parásitos, incluyendo las proteasas de cisteína (International Helminth Genomes Consortium, 2019), receptores acoplados a proteína G (McVeigh *et al*., 2018) o β-tubulina, relevante desde el punto de vista de la resistencia a fármacos (Fairweather *et al*., 2020). Además del genoma nuclear, también se ha documentado la secuenciación del genoma mitocondrial de *F. hepatica* (Le *et al*., 2001), el cual ha proporcionado biomarcadores de especial utilidad en estudios de genética poblacional y de análisis epidemiológico (Ai *et al*., 2011), así como para la identificación basada en criterios moleculares de las especies de *Fasciola* spp. y sus formas híbridas (Liu *et al*., 2014).

3.2. Estudios transcriptómicos y de expresión génica en *Fasciola hepatica*

A diferencia del estudio del genoma, de naturaleza estática, los estudios transcriptómicos permiten caracterizar cuantitativamente la funcionalidad genética de tejidos concretos o de etapas del ciclo de vida de un organismo, así como su comportamiento en respuesta a determinados estímulos, por lo general mediante técnicas de secuenciación del ARN (RNA-Seq).

Las primeras aproximaciones transcriptómicas en *Fasciola* spp. se remontan al principio de la década pasada, en las que se examinó el perfil de expresión génica de los vermes adultos de *F. hepatica* utilizando la plataforma de secuenciación *Roche 454* (Young *et al*., 2010), detectando hasta 160 productos de excreción-secreción previamente descritos a nivel de proteína (Robinson *et al*., 2009), y caracterizando numerosos genes codificantes de proteínas estructurales (Wilson *et al*., 2011). Sin embargo, el principal avance metodológico se produjo con la introducción de la tecnología de secuenciación de *Illumina* (Reuter *et al*., 2015), que gracias a su capacidad de escalado y a la pequeña cantidad de material genético requerida, permitió la caracterización transcriptómica de todas las fases de desarrollo del parásito en el hospedador vertebrado, incluyendo la metacercaria, los NEJ a 1, 3 y 24 horas p.i., y los vermes inmaduros a 21 días p.i. (Cwiklinski *et al*., 2018). Gracias a esto se pudo comprobar, entre otras cosas, que la metacercaria es una estructura metabólicamente muy activa, y que los estadios de desarrollo de *F. hepatica* presentan un perfil de expresión génica bien diferenciado (Cwiklinski *et al*., 2018). Las proteasas de cisteína de tipo catepsina suponen un buen ejemplo al tratarse de una de las familias de genes más extensas en el parásito. De hecho, se han identificado hasta 23 isoformas de catepsinas L y 11 isoformas de catepsinas B, distribuidas en 5 y 2 clados bien diferenciados, respectivamente, los cuales son diferencialmente expresados en los NEJ, los vermes inmaduros y los adultos (Cwiklisnki *et al*., 2019). Asimismo, los inhibidores de proteasas también modifican sus niveles de expresión durante la vida del parásito, ya que se ha documentado que las dos principales familias de inhibidores de proteasas de cisteína (cistatinas, con 3 isoformas, e inhibidores de tipo Kunitz, con 7 isoformas) presentan patrones de expresión claramente diferenciados entre los juveniles y los vermes adultos (Cancela *et al*., 2017; Smith *et al*., 2020).

El metabolismo energético es otro de los procesos que varían enormemente a nivel transcriptómico durante la vida del parásito, evolucionando desde una situación inicial en la que predomina el catabolismo de las reservas de glucógeno, y que se corresponde con la fase de metacercaria y el NEJ en las primeras horas p.i., hasta una situación mixta en la que se combinan la biosíntesis y degradación de glucógeno, en torno a las 24 horas p.i. (Cwiklinski *et al*., 2018). A medida que los vermes crecen, la capacidad de difusión del oxígeno a los tejidos se reduce, por lo que el metabolismo debe adaptarse a una situación de progresiva anaerobiosis, mediante la síntesis de enzimas del ciclo del glioxilato y la ruta del dicarboxilato (Cwiklinski *et al*., 2021). Estos cambios parecen completarse una vez que los parásitos alcanzan el hígado, el cual parece ser además el momento en el que se regula diferencialmente una mayor cantidad de genes, previsiblemente como preparación para la migración hacia la red biliar (Cwiklinski *et al*., 2018).

Los mecanismos de evasión inmune de *F. hepatica* también se han explorado mediante RNA-Seq, habiéndose identificado hasta 64 transcritos con capacidad de interacción con moléculas del sistema inmune del hospedador (Haçariz *et al*., 2015). Dicha aproximación transcriptómica se ha empleado también en el estudio de la resistencia a antihelmínticos. Un primer estudio caracterizó el perfil de expresión de cepas de *F. hepatica* resistentes al TCBZ y al ABZ, resistentes únicamente al ABZ, y susceptibles a ambos fármacos, concluyendo que las cepas resistentes presentaban un nivel global de transcripción más bajo, si bien los niveles de β-tubulina no diferían respecto a los de una cepa susceptible. Asimismo, la cepa resistente al ABZ presentó un perfil de expresión diferente al de las otras dos, lo que sugiere que los mecanismos de resistencia a ambos fármacos tienen una base molecular diferente (Radio *et al*., 2018). Un estudio posterior comprobó el comportamiento de cepas susceptibles y resistentes en respuesta a ambos fármacos, identificando un gran número de transcritos que podrían estar involucrados en la respuesta a estos (Miranda-Miranda *et al*., 2021).

Además de los ARNs codificantes, recientemente se ha hecho hincapié en los micro ARNs (miARNs) expresados por *Fasciola* spp., ya que su importancia en eucariotas superiores es tal, que se estima que cada gen está regulado por al menos una de estas secuencias (Friedman *et al*., 2009). Se trata de fragmentos de ARN de pequeño tamaño capaces de modular la expresión génica al asociarse con el complejo proteico RISC (*RNA-Induced Silencing Complex*), uniéndose a los ARN mensajeros (ARNm) diana y favoreciendo su degradación o bloqueando su acceso a la maquinaria de síntesis

de proteínas (Chong *et al*., 2010). Los primeros estudios basados en la identificación de miARNs de *F. hepatica* se centraron tanto en el verme adulto (Xu *et al*., 2012) como en el NEJ (Fontenla *et al*., 2015), los cuales detectaron un total de 55 y 45 secuencias candidatas a ser consideradas miARNs, respectivamente. Actualmente, en *F. hepatica* se han descrito un total de 77 secuencias que cumplen con los criterios establecidos para ser consideradas como miARN, de las cuales 15 son específicas del NEJ, 26 del adulto, y las 36 restantes se han detectado en ambas fases (Ricafrente *et al*., 2020).

Muchos de los miARNs en *F. hepatica* están conservados, por lo que su función puede inferirse a partir de la descrita en otros organismos. Gracias a esto, se ha comprobado que algunos de los miARNs caracterizados intervienen en la regulación de la proliferación celular y los fenómenos de apoptosis (Fontenla *et al*., 2015), así como en la diferenciación de los tejidos durante el paso a la fase adulta (Ricafrente *et al*., 2020). Del mismo modo, varios miARNs procedentes de *Fasciola* spp. presentan homología con secuencias de mamíferos con funciones inmunomoduladoras, las cuales a menudo forman parte del contenido de vesículas extracelulares (de la Torre-Escudero *et al*., 2019). Recientemente, se ha descrito cómo un miARN de *F. hepatica* puede prevenir la activación de un fenotipo proinflamatorio en macrófagos (Tran *et al*., 2021), por lo que el potencial de estas moléculas como elementos de la evasión inmune se plantea como una interesante vía de estudio.

3.3. Estudios proteómicos en *Fasciola hepatica*

Se entiende como proteómica el estudio simultáneo del conjunto de proteínas presentes en una muestra biológica, el cual permite no solo cuantificar la expresión de las mismas, sino también identificar fenómenos de empalme alternativo y patrones de modificación postraduccional (Sotillo *et al*., 2017). Al igual que en el anterior grupo de técnicas, los avances en proteómica se han visto potenciados en gran medida por el desarrollo tecnológico de métodos de detección altamente sensibles, como la espectrometría de masas (MS) (Scherp *et al*., 2011), capaz de detectar concentraciones en el rango de las partes por billón o incluso inferiores, y con suficiente capacidad de análisis como para permitir la identificación y diferenciación de especies de *Fasciola* spp. de manera rápida (Sy *et al*., 2020).

Al tratarse de una fase de desarrollo más fácilmente obtenible, los vermes adultos de *F. hepatica* han sido objeto de un mayor número de estudios proteómicos. Las aproximaciones anteriores a la publicación de su genoma utilizaron la electroforesis bidimensional (2D-PAGE) o la cromatografía líquida de alta resolución (HPLC) en combinación con la MS para la caracterización de los productos de excreción-secreción de los vermes, identificando diferentes enzimas antioxidantes (superóxido dismutasa, Prx, GST) y FABPs (Jefferies *et al*., 2001), así como una gran cantidad de catepsinas L (Robinson *et al*., 2009). Asimismo, se realizó una caracterización proteómica del tegumento de vermes adultos, detectando un enriquecimiento en proteínas estructurales, transportadores, enzimas antioxidantes y proteínas relacionadas con rutas de secreción respecto a la fracción somática, las cuales podrían desempeñar una función relevante en la patogénesis al formar parte de la interfase parásito-hospedador (Wilson *et al*., 2011). La publicación del primer borrador del genoma de *F. hepatica* (Cwiklinski *et al*., 2015a) permitió una caracterización más profunda del secretoma del parásito, logrando identificar una mayor cantidad de proteínas, responsables de diferentes funciones biológicas, tales como inhibidores de proteasas capaces de actuar sobre las enzimas del parásito y las del propio hospedador (De Marco Verissimo *et al*., 2020; Smith *et al*., 2020).

Asimismo, la mejora en la sensibilidad de las técnicas analíticas facilitó el estudio del repertorio antigénico de los vermes inmaduros (Di Maggio *et al*., 2016). Aunque los estudios centrados en esta fase son más escasos, hace más de una década se realizaron las primeras aproximaciones para caracterizar la fracción somática de los NEJ (Hernández-González *et al*., 2010) y su secretoma, del que se identificaron un total de 29 proteínas, la mayoría de las cuales fueron catalogadas como proteasas, si bien estas presentaban un patrón de expresión distinto al observado en vermes adultos (Robinson *et al*., 2009). Estudios posteriores ampliaron la cantidad de proteínas identificadas en la fracción secretada por los NEJ, corroborando que esta se compone en su mayoría por catepsinas L3 y B3 (las cuales se incluyen entre las 10 proteínas más abundantes, que conjuntamente suponen en torno al 70 % de las proteínas secretadas), y presentando una composición muy diferente al proteoma somático (Cwiklinski *et al*., 2018). Otras estrategias similares se han aplicado al estudio de los vermes inmaduros, entre las que destaca un estudio reciente que caracterizaron las fracciones secretada y somática de vermes de *F. hepatica* a 21 días p.i., concluyendo que el proteoma somático de esta fase está dominado por

proteínas metabólicas, mientras que el secretoma se compone principalmente de catepsinas L2 y L3, junto con inhibidores de peptidasas y moléculas inmunomoduladoras (Cwiklinski *et al*., 2021). Finalmente, aunque mucho más escasos, existen estudios que han analizado la composición proteica de los huevos (Moxon *et al*., 2010) y las fases de desarrollo del parásito que se suceden en el interior del hospedador intermediario (Gourbal *et al*., 2008).

Además de la caracterización de los estadios de desarrollo de *F. hepatica*, las técnicas proteómicas también se han utilizado para determinar las diferencias entre cepas susceptibles y resistentes al TCBZ, detectando niveles más elevados de actina, gelsolina y triosa fosfato isomerasa en cepas susceptibles, mientras que las resistentes presentaban mayores niveles de calreticulina, catepsinas L y enolasa (Morphew *et al*., 2014). Estas proteínas podrían servir como biomarcadores a la hora de caracterizar nuevas cepas de *Fasciola* spp.

Las vesículas extracelulares producidas por *F. hepatica* también han sido objeto de estudios proteómicos debido a su importancia en la interacción con el hospedador, hasta el punto de que la disrupción de dichas vesículas o la prevención de su incorporación por el hospedador se plantean como estrategias terapéuticas viables (Cwiklinski *et al*., 2015b). El genoma de este organismo contiene casi todos los elementos de la maquinaria celular requerida para la producción de exosomas, de los cuales existe evidencia a nivel de proteína (de la Torre-Escudero *et al*., 2019). Además, y a diferencia de los proteomas somáticos, que varían ampliamente entre estadios de desarrollo, las proteínas presentes en vesículas extracelulares tienen una composición más estable en el tiempo (Cwiklinski *et al*., 2015b). Por otra parte, y al contrario de lo que se pensaba inicialmente, el intestino del verme parece ser la fuente principal de vesículas, en lugar del tegumento (Bennett *et al*., 2020). Pese a suponer una pequeña proporción del contenido proteico de los parásitos, las vesículas extracelulares contienen numerosas sustancias inmunomoduladoras (Roig *et al*., 2018) capaces de interactuar con poblaciones concretas del sistema inmune, gracias a las moléculas en su superficie (de la Torre-Escudero *et al*., 2019). En cuanto al contenido de las mismas, la primera aproximación proteómica identificó un total de 79 proteínas, muchas de las cuales habían sido descritas previamente en la fracción secretora del parásito (Marcilla *et al*., 2012). No obstante, los mayores avances se produjeron a partir de la publicación del borrador del genoma de *F. hepatica*, identificando dos grandes poblaciones de vesículas claramente diferenciadas en su tamaño y composición

(Cwiklinski *et al*., 2015b; de la Torre-Escudero *et al*., 2016): las de mayor tamaño (denominadas 15K) presentan un mayor contenido en leucina aminopeptidasa, FABPs y enzimas antioxidantes pertenecientes a las familias de las Prx y tiorredoxinas, mientras que las más pequeñas (denominadas 120K) son más abundantes en GST, ferritinas y saposinas (Murphy *et al*., 2020). Ambas poblaciones contienen además proteasas e inhibidores de proteasas, si bien el perfil es distinto entre ambos grupos (Cwiklinski *et al*., 2015b). Aunque la mayoría de estudios se han enfocado en las vesículas procedentes de vermes adultos, recientemente se describió que las fases tempranas del desarrollo de *F. hepatica* también producen vesículas extracelulares (Sánchez-López *et al*., 2020), en las que se identificaron 23 proteínas en vesículas procedentes de huevos, y 29 en vesículas de juveniles mantenidos en cultivo durante 28 días, cuya composición incluía, entre otras, proteínas citoesqueléticas, enzimas glicolíticas y proteínas de respuesta a estrés, además de carecer aparentemente de proteasas (Trelis *et al*., 2022).

3.4. Análisis transcriptómicos y proteómicos de las respuestas del hospedador en la fasciolosis

Aunque a menudo se ha caracterizado la respuesta celular y molecular en infecciones naturales y experimentales por medio de técnicas clásicas, los experimentos -ómicos que describen la reacción del hospedador a la infección por *Fasciola* spp. son mucho más escasos. En la década pasada, algunas aproximaciones transcriptómicas estudiaron los cambios en la expresión de genes en el hígado de ovejas infectadas, describiendo un incremento global en la tasa de transcripción, particularmente en genes relacionados con la fibrosis y la reparación tisular, así como en la inducción de la respuesta Th2 y la síntesis de hemoglobina (Álvarez-Rojas *et al*., 2015), resultados similares a los observados en ratones (Rojas-Caraballo *et al*., 2015) y en búfalos infectados por *F. gigantica* (Zhang *et al*., 2017). De forma semejante, el análisis mediante RNA-Seq de células sanguíneas periféricas de oveja durante la etapa crónica de la infección mostró una clara supresión de genes relacionados con respuestas Th1 y Th17, junto con un incremento en la expresión de marcadores característicos de respuestas Th2 (Álvarez-Rojas *et al*., 2016; Fu *et al*., 2016). En un modelo de infección bovino se observaron resultados similares, además de un incremento de moléculas del sistema del

complemento y de la señalización mediada por receptores de tipo *Toll* (TLR) (Álvarez-Rojas *et al*., 2019).

A nivel proteómico, cabe destacar el experimento llevado a cabo por Ruiz-Campillo *et al*., (2017), en el que se identificaron un total de 176 proteínas en el líquido peritoneal de ovejas a 18 días p.i., entre las que destaca la presencia de moléculas del sistema del complemento, así como componentes de la MEC hepática, que se asociaron con el daño causado por los vermes a los tejidos.

4. Las relaciones parásito-hospedador en la fasciolosis a nivel intestinal

En el último apartado de la Revisión bibliográfica de esta Tesis Doctoral, resulta pertinente poner en contexto el conocimiento que se tiene actualmente de las relaciones parásito-hospedador en la fasciolosis, principalmente a nivel intestinal, durante las primeras fases de la infección del hospedador vertebrado. Esta información permite contextualizar la formulación de la hipótesis y los objetivos del trabajo, así como poner de manifiesto la importancia del verme juvenil en la biología de *F. hepatica*, su importante papel como diana en el desarrollo de vacunas, así como la falta de información acerca de estas fases, tanto a nivel -ómico como del desarrollo de modelos para el estudio de las relaciones tempranas en la fasciolosis.

4.1. El intestino delgado del hospedador

El tubo digestivo es un sistema destinado a la degradación de alimentos en moléculas simples para su captación, cuya principal función es el transporte de agua, nutrientes y electrolitos del medio externo a los tejidos. Aunque se tiende a pensar en este sistema exclusivamente en términos de su función metabólica, cabe destacar que se trata de la mayor superficie de contacto entre el organismo y el medio exterior, la cual desempeña un importante papel en la lucha frente a agresiones externas. Por ello, debe ser capaz de absorber nutrientes mientras restringe la entrada de agentes patógenos, evitando además causar daño a la microbiota intestinal. En este sentido, el intestino no solo actúa como barrera física, sino que cuenta también con una compleja serie de mecanismos moleculares de protección (Allaire *et al*., 2018). En el caso de la fasciolosis, el intestino delgado constituye la puerta de entrada del parásito al hospedador definitivo, al tratarse del primer tejido del animal vertebrado con el que los NEJ entran en contacto tras la excistación. Por tanto, se trata del compartimento de mayor importancia en el inicio de la infección y, por ello, resulta de capital importancia revisar tanto su anatomía y fisiología, como su papel de barrera frente a agentes patógenos.

4.1.1. Anatomía y fisiología del intestino delgado

El intestino delgado de los mamíferos consta de tres secciones denominadas duodeno, yeyuno e íleon, siendo el duodeno el tramo inicial del intestino, en el que se produce la inserción de los conductos pancreático y biliar. Se trata también de la región donde se reúnen las condiciones necesarias para la excistación de las metacercarias y la liberación de los NEJ. La estructura básica de la pared del estómago y del intestino es similar, aunque con variaciones en la disposición tisular y las interacciones célula-célula. Dicha estructura es bien conocida desde hace décadas (Silverthorn, 2007), y consta de 4 capas bien diferenciadas (**Figura 9**):

-   **Mucosa:** Se trata del revestimiento interno del tubo digestivo, el cual consta a su vez de tres capas: una capa única de epitelio de revestimiento, la lámina propia, constituida por tejido conjuntivo, y la muscular de la mucosa, formada por una delgada capa de músculo liso. La superficie de la mucosa presenta invaginaciones destinadas a incrementar la superficie de contacto con la luz del tubo, las cuales se proyectan tanto hacia arriba, generando microvellosidades, como hacia abajo, dando lugar a las criptas intestinales o de Lieberkühn (Spence *et al*., 2011). La capa más variable es el epitelio, que presenta cambios en su estructura y función en las diferentes regiones. De esta forma, aunque el principal componente de esta capa son los enterocitos, encargados del movimiento de nutrientes, agua e iones entre la luz del tubo y el medio extracelular, también se han identificado células endocrinas/exocrinas y células madre o troncales, las cuales comprenden un tejido indiferenciado de rápida división, encargado de producir nuevo epitelio para suplir el que se va degradando a consecuencia de las condiciones a las que se ve expuesto, ya que su vida media es de apenas unos pocos días (van der Flier y Clevers, 2009). Las uniones intercelulares que mantienen la integridad del epitelio son también un elemento que varía ampliamente ya que, a diferencia del estómago y el intestino grueso, donde forman una estrecha barrera que disminuye la permeabilidad y limita el paso de sustancias, en el intestino delgado dichas uniones no son tan restrictivas, facilitando el intercambio bidireccional (Lee *et al*., 2018). A continuación, la lámina propia es una capa de tejido conectivo que contiene las fibras nerviosas y vasos sanguíneos y linfáticos en los que se produce el intercambio de sustancias, además de contener células del sistema inmune que combaten la presencia de amenazas externas. Adyacentes al epitelio existen

acumulaciones de tejido linfoide denominadas placas de Peyer. Finalmente, la región más externa es la denominada muscular de la mucosa, una delgada capa de músculo liso capaz de modificar el área efectiva de absorción del epitelio al desplazar las vellosidades adelante y atrás.

- **Submucosa:** La región inmediatamente inferior a la mucosa está compuesta por tejido conjuntivo, el cual contiene vasos sanguíneos y linfáticos de mayor tamaño que los de la lámina propia, así como redes nerviosas que se entrelazan formando el plexo submucoso, encargado de coordinar la función digestiva y de inervar la muscular de la mucosa.

- **Muscular:** Esta región consta de dos capas concéntricas de músculo liso, una circular interna y una longitudinal externa. La contracción de la primera permite controlar el diámetro de la luz del tubo, mientras que la actividad de la capa longitudinal lo acorta o elonga. Entre ambas capas se localiza el plexo mientérico, que controla la actividad de ambas capas de músculo.

- **Serosa:** Esta membrana de tejido conectivo constituye la cubierta externa de todo el tubo digestivo, la cual se continúa con la membrana peritoneal que recubre el abdomen y con las hojas del mesenterio, que mantienen el intestino en su lugar.

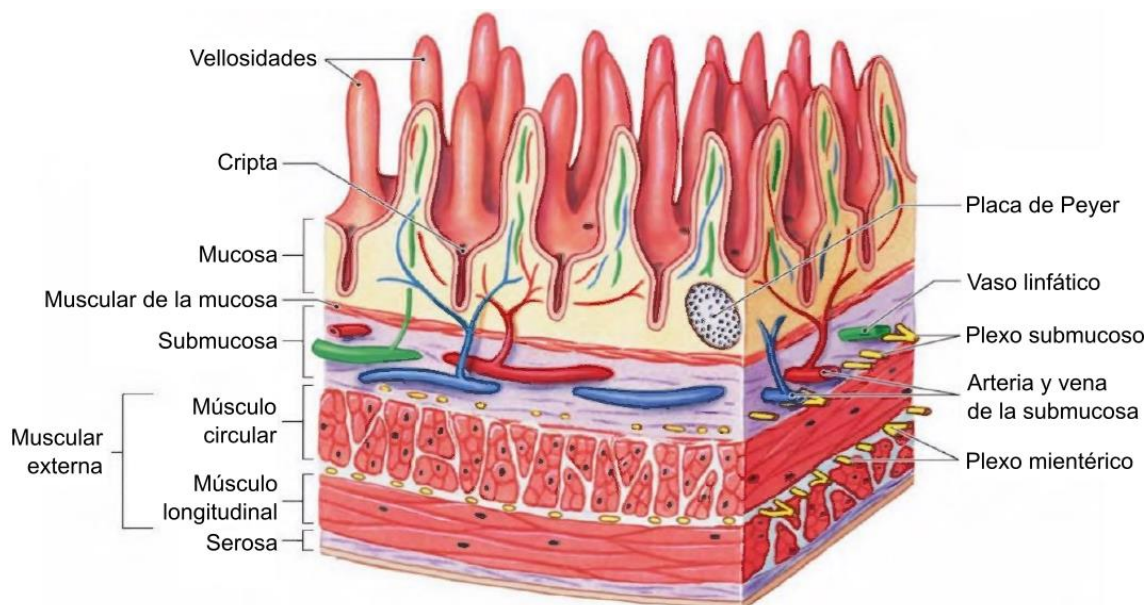**Figura 9** – Estructura tisular del intestino delgado, en la que se aprecian las 4 capas que componen este órgano. Fuente: Silverthorn, 2007.

En este contexto, es importante también la denominada matriz extracelular (MEC; **Figura 10**), puesto que es una estructura de interacción fundamental para los NEJ. La estructura y composición de la MEC ha sido recientemente revisada en profundidad por

Pompili *et al.* (2021). La MEC intestinal, que está en constante renovación por sus células productoras, representa una red compleja de proteínas que forma una estructura de soporte para las células residentes, y es una barrera selectiva para componentes externos. Además, interactúa estrechamente con las células, modulando sus fenotipos y funciones. Dentro de la MEC, se han identificado numerosas moléculas, algunas de ellas con propiedades bioquímicas y funciones biológicas únicas.

La estructura de la MEC incluye la membrana basal (MB) y la matriz intersticial (MI) que están íntimamente interconectadas. La MB se encuentra debajo de las células epiteliales, mientras que la MI se halla en la lámina propia, submucosa, y en las capas muscular y serosa. La MB es una capa de 50 a 100 nm situada entre el epitelio y el mesénquima de la lámina propia, y se compone principalmente de colágeno tipo IV, lamininas, nidógeno y perlecán.
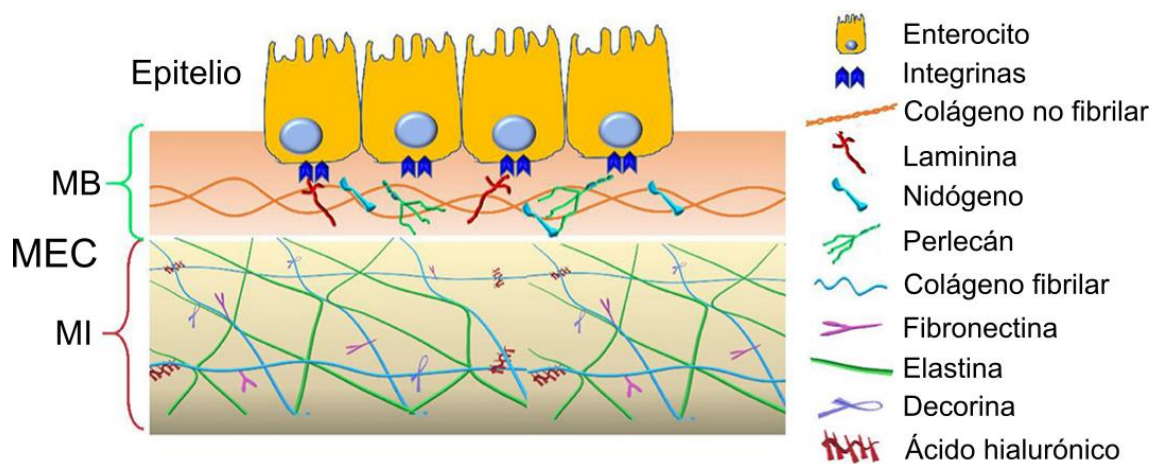
Figura 10 – Compartimentos y composición de la matriz extracelular (MEC) del intestino delgado. MB, membrana basal; MI, matriz intersticial. Fuente: Pompili *et al.* (2021).

La MI se encuentra debajo de la MB, cooperando en la preservación de la integridad estructural y funcional de la pared intestinal. Esta MI está principalmente constituida por fibronectina, colágenos tipo I y III, elastina, decorina y ácido hialurónico.

Además de los componentes de la MB y la MI, se encuentran colágenos transmembrana y proteoglicanos expresados por las células epiteliales, incluido el colágeno tipo XXIII y el sindecán-1. El colágeno tipo XXIII parece estar involucrado en el contacto célula-célula y en la polarización de las células epiteliales, y el sindecán-1, por su parte, modula la adhesión, proliferación y migración de las células epiteliales, y estabiliza las uniones estrechas de dichas células.

4.1.2. El intestino delgado como barrera frente a infecciones por patógenos

Como ya se ha mencionado, el intestino no constituye únicamente una herramienta para la absorción de nutrientes, sino que también supone una barrera que reacciona de forma activa frente a agresiones externas. Para este fin, el epitelio intestinal que recubre la luz del aparato digestivo es asistido por diferentes células especializadas en funciones de protección, las cuales incluyen las células caliciformes, responsables de la secreción de mucus y distintos péptidos (Baska y Norbury, 2022), las células de Paneth, capaces de secretar diferentes péptidos antimicrobianos (Rodríguez-Colman *et al*., 2017) o las células M, que actúan como presentadoras de antígenos luminales (Ohno, 2015). También existen células enteroendocrinas y las denominadas células en penacho, importantes en el reconocimiento y la respuesta a helmintos (Gerbe *et al*., 2016). Finalmente, el intestino delgado cuenta con la mayor acumulación de tejido linfoide del organismo, el denominado tejido linfoide asociado al intestino o GALT (*Gut-Associated Lymphoid Tissue*) (Takahashi *et al*., 2021). Tanto es así, que se estima que en torno al 80 % de los linfocitos del organismo residen en este sistema (Silverthorn, 2007) (**Figura 11**).

Figura 11 – Estructura celular del epitelio intestinal. Fuente: adaptado de Allaire *et al*. (2018).

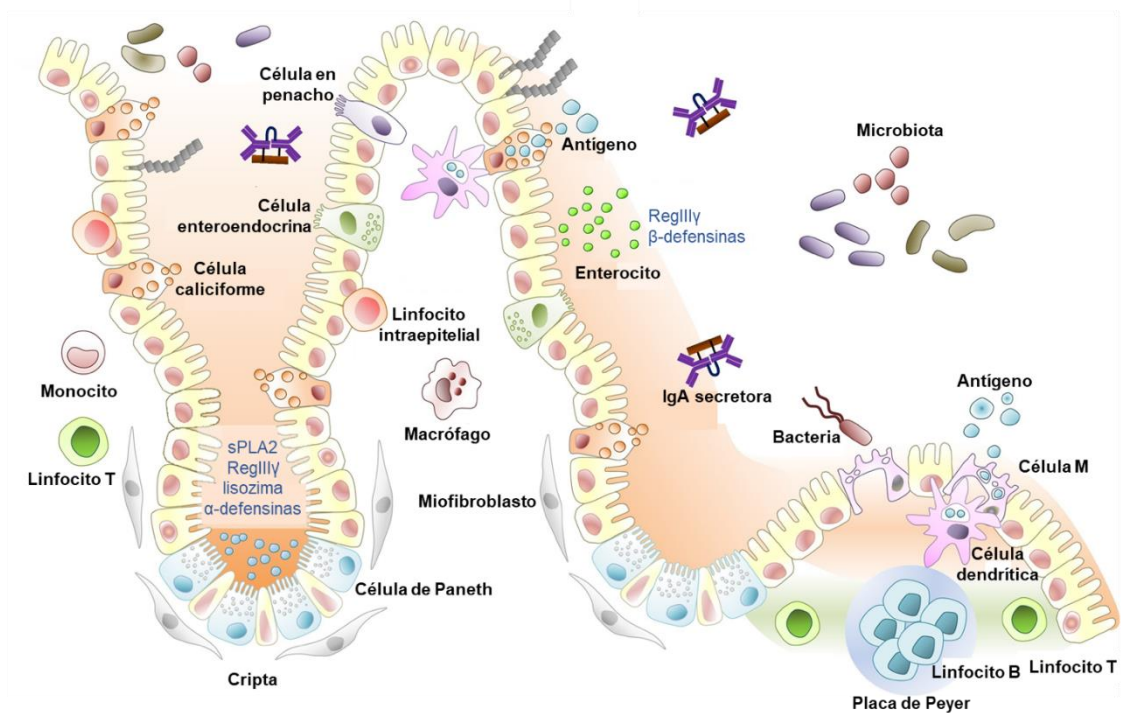La respuesta más habitual frente a microorganismos patógenos viene dada por la interacción de estos con los TLR situados en la región apical o basal del epitelio, cuya estimulación promueve la producción de diferentes citoquinas capaces de activar las células inmunes que se localizan debajo (Abreu, 2010), además de estimular la secreción de péptidos antimicrobianos por las células de Paneth (Gong *et al*., 2010). Los efectores más relevantes incluyen neutrófilos, macrófagos, células dendríticas y linfocitos T, cuya acción es especialmente importante en las placas de Peyer, acúmulos de tejido linfático situados bajo el epitelio, y que constituyen una parte importante del GALT (Ahluwalia *et al*., 2017). La microbiota intestinal, como el conjunto de flora bacteriana que establece una relación de simbiosis con este tejido, juega también un papel importante en la función del epitelio, favoreciendo la proliferación y secreción de sustancias protectoras (Kau *et al*., 2011).

No obstante, aunque se ha descrito que los helmintos pueden interaccionar con los TLR, los patrones moleculares asociados a patógenos (PAMPs) que gobiernan estas interacciones no son bien conocidos (Rajasekaran *et al*., 2017). En su lugar, la principal respuesta intestinal frente a helmintos, y en menor medida frente a protozoos, es la denominada «*weep and sweep*», la cual implica una estrecha coordinación del epitelio intestinal, las células inmunes y las nerviosas, y es desencadenada por la interacción del parásito con las células en penacho, que inician una cascada de señalización caracterizada por la producción de IL-25 e IL-33 (von Moltke *et al*., 2016; Roan *et al*., 2019). Se trata de una respuesta de tipo Th2, caracterizada por un incremento en la producción de mucus por las células caliciformes, el cual actúa como barrera física impidiendo el acceso de los parásitos a los tejidos y dificultando su movimiento. Esta secreción viene acompañada por un aumento en la proliferación del epitelio, el cual, teniendo en cuenta que las células troncales intestinales residen en el fondo de las criptas de Lieberkühn y que los parásitos suelen acumularse también allí, favorece la expulsión de los vermes de nuevo al lumen (Artis y Grencis, 2008). A continuación, tiene lugar un incremento de la motilidad intestinal que permite la expulsión de los parásitos, en lugar de su destrucción. Aunque esta respuesta se ha descrito en varias especies de nematodos que residen en el intestino (Maizels *et al*., 2011; Saracino *et al*., 2020), la información de este proceso en la respuesta a trematodos es escasa, por lo que su relevancia frente a parásitos que solo habitan en este compartimento de forma transitoria como *F. hepatica* requiere de más investigación.

4.2. Los juveniles recién excistados de *Fasciola hepatica* y su interacción con el hospedador

Como se ha venido señalando en la presente Tesis Doctoral, las estrategias de control frente a la fasciolosis se han dirigido generalmente hacia la forma adulta del parásito, aunque su ubicación definitiva en los conductos biliares del hospedador representa un nicho *a priori* poco accesible para la inmunidad del mismo. A pesar de su importancia durante el curso de la infección, los estudios destinados a desentrañar la relación parásito-hospedador, especialmente en las primeras etapas de la fasciolosis, son aún escasos (González-Miguel *et al*., 2021). En los siguientes apartados, se presentan los principales avances logrados en el estudio de los NEJ de *F. hepatica* en su relación con el hospedador, mayoritariamente a nivel intestinal, incluyendo los modelos desarrollados para replicar dichas interacciones en el laboratorio, aspectos relacionados con su invasión y migración, su metabolismo, y la regulación de la respuesta inmune de su hospedador.

4.2.1. Modelos *in vitro*, *ex vivo* e *in vivo* de infección

Puesto que los NEJ deben migrar desde el lumen intestinal al peritoneo, y desde este al hígado, modificando su comportamiento, dieta, metabolismo y fisiología en consonancia a un entorno cambiante, resulta imprescindible disponer de modelos experimentales que permitan caracterizar estos procesos en el laboratorio.

El primer paso para lograrlo implica la excistación *in vitro* de metacercarias, para lo cual se han descrito diferentes metodologías que han hecho posible obtener cantidades significativas de NEJ (McGonigle *et al*., 2008, Robinson *et al*., 2009, Hernández-González *et al*., 2010). La mayoría de métodos incluyen características comunes como la utilización de CO_2 y sales biliares para replicar las condiciones fisicoquímicas del lumen intestinal, y han resultado útiles para el estudio de los productos de excreción-secreción de *F. hepatica* (de la Torre-Escudero y Robinson, 2020). Esto ha permitido mantener *in vitro* durante tiempos cortos (de 1 a 24 horas) a los NEJ, para utilizarlos posteriormente en estudios -ómicos comparativos con otras fases parasitarias obtenidas de modelos *in vivo* de ratón a tiempos en los que los juveniles ya han alcanzado el hígado (21 días p.i.), o con vermes adultos obtenidos de hospedadores naturales (Cwiklinski y Dalton, 2022). Además, hace unos años se desarrolló un sistema *in vitro* que consigue mantener en

cultivo NEJ de *F. hepatica* hasta 29 semanas, permitiendo observar el desarrollo del parásito a lo largo de este tiempo, aunque sin conseguir la madurez completa del verme adulto (McCusker *et al*., 2016). No obstante, todos estos modelos *in vitro* se han realizado sin la presencia del hospedador o de células derivadas del mismo, por lo que no resultan útiles para el estudio de los cambios que se inducen durante la relación parásito-hospedador en ambos organismos, ya que se conoce que los cambios inducidos en los NEJ por el hospedador no se reflejan fielmente en los cultivos axénicos del parásito (González-Miguel *et al*., 2021).

En este sentido, durante las últimas décadas se han desarrollado metodologías que han permitido reproducir en el laboratorio el paso de los NEJ a través de la pared intestinal. En un primer modelo (van Milligen *et al*., 1998), se realizó una aproximación *ex vivo* utilizando ratas a las que se realizó una incisión en el abdomen para extraer un asa intestinal, cuyos extremos se ligaron con pinzas. En los segmentos extraídos se inyectaron NEJ de *F. hepatica* y dichos segmentos se incubaron en medio de cultivo durante un tiempo total de 6 horas, cuantificando el paso de los NEJ al exterior a lo largo del tiempo. Este modelo se utilizó para investigar la importancia de determinadas proteínas del verme en la penetración intestinal, mediante estudios de silenciamiento génico (McGonigle *et al*., 2008). En una segunda aproximación (García-Campos *et al*., 2016a), se diseñó un modelo *in vitro* en el que se obtuvieron fragmentos de intestino delgado procedentes de ratas, los cuales se colocaron alrededor de la apertura de un tubo de centrífuga situado boca abajo sobre un recipiente con medio de cultivo. En el fondo de los tubos se practicaron agujeros por los que se introdujeron los NEJ de *F. hepatica* previamente excistados, monitorizando su paso a través del intestino a diferentes tiempos. Estos modelos permitieron determinar el lapso de tiempo que los NEJ requieren para atravesar la pared intestinal y la tasa de éxito del proceso. Sin embargo, no se han utilizado para analizar mediante técnicas -ómicas los cambios inducidos por esta interacción en ninguno de los actores de la misma.

En cuanto a los modelos *in vivo* para el estudio específico de los cambios en los NEJ tras su interacción con el hospedador, o de los cambios inducidos por los NEJ en este, se han utilizado modelos murinos para obtener fases parasitarias más tardías (juveniles a 21 días p.i.) y utilizarlas en estudios -ómicos (Cwiklinski *et al*., 2021), pero no para estudiar fases más tempranas. También se han utilizado los propios hospedadores naturales para intentar desentrañar los cambios en la respuesta inmune de estos durante

las fases tempranas de la infección, refiriéndose estos estudios exclusivamente al hospedador, pero no al parásito. Los resultados obtenidos en estos últimos se presentan más detalladamente en el apartado 4.2.4 de la presente Tesis Doctoral.

En resumen, los modelos existentes han permitido comenzar a entender los procesos de invasión parasitaria en la fasciolosis, si bien existe la necesidad de desarrollar nuevos modelos que permitan estudiar los cambios tanto en los NEJ como en el hospedador durante el primer contacto de los vermes con el intestino, y su posterior migración a través del mismo, acoplados a técnicas -ómicas de última generación. Este tipo de aproximaciones permitiría comparaciones intra e intercompartimentales de las moléculas del hospedador y del parásito antes y después de la interacción, mediante el uso de enfoques -ómicos cuantitativos de alto rendimiento, lo que facilitaría el estudio de los procesos biológicos subyacentes.

4.2.2. Mecanismos de invasión y migración parasitaria

Como se acaba de evidenciar, la mayor parte de los modelos experimentales señalados en el estudio de la fasciolosis tratan de replicar la migración llevada a cabo por los NEJ a través de los tejidos del hospedador, ya que esta resulta clave para el proceso infeccioso. Dicha migración ocurre unas 2 horas después de la ingestión de las metacercarias. Dentro de este periodo de tiempo, los NEJ son capaces de atravesar la pared intestinal del hospedador e iniciar una ruta migratoria compleja que conduce a los parásitos a su ubicación definitiva dentro de los conductos biliares. Desafortunadamente, y a pesar de ser un proceso de suma importancia en la fisiopatología de la fasciolosis, los mecanismos que rigen la migración de *F. hepatica* no se conocen por completo.

En general, se admite que la presencia de sales biliares es un requisito para la excistación de las metacercarias de *F. hepatica*. Sin embargo, y aunque los vermes adultos viven en las vías biliares, los NEJ no sobreviven en soluciones que contienen bilis. Esta es la razón por la cual la bilis se ha postulado como un estímulo quimiotáctico para que los vermes juveniles penetren a través de la pared intestinal y abandonen rápidamente el intestino después de la excistación (Tielens *et al*, 1981). El hecho de que los NEJ no sobrevivan en presencia de bilis podría explicar por qué migran al hígado a través de la cavidad abdominal, en lugar de tomar una ruta directa a través del conducto biliar principal que comienza en el intestino delgado, como es el caso de otras especies de

trematodos, incluyendo *Opistorchis* y *Clonorchis* spp., cuyas formas juveniles ascienden a través de la ampolla de Vater directamente a los conductos biliares. Aún más intrigante es el hecho de que otra especie dentro de la familia Fasciolidae, *Fasciolopsis buski*, permanezca en el intestino delgado del hospedador definitivo. Esto sugiere una expansión diferencial en términos de genes relevantes asociados con la migración y la invasión de tejidos entre los genomas de *F. hepatica* y *F. buski*, como se ha demostrado recientemente (Choi *et al*., 2020). Además, la bilis contribuye a la movilidad de los NEJ, al producir una estimulación inespecífica de la actividad muscular (Sukhdeo *et al*., 1988b). El proceso de locomoción de los NEJ se ha descrito como una secuencia de ondas del cuerpo del verme, coordinadas con la unión y liberación alternas de las ventosas oral y ventral, y un peristaltismo vermiforme que da como resultado cambios secuenciales en la longitud del parásito entre 100 y 170 μm (Sukhdeo *et al*., 1988b). Además, la presencia de espinas en la superficie del NEJ facilita el avance de los parásitos a través de la pared intestinal gracias a su orientación (Bennett y Threadgold, 1975). Finalmente, y con respecto a la orientación del parásito, se ha sugerido que los NEJ son capaces de realizar un movimiento direccional en respuesta a estímulos específicos (por ejemplo, serotonina) (Sukhdeo y Mettrick, 1986).

Además de los mecanismos de locomoción mecánicos de los NEJ, la migración del parásito se ve facilitada por actividades proteolíticas dependientes de catepsinas. Las catepsinas son proteasas de amplio espectro con capacidad para degradar, entre otros componentes, moléculas de la MEC del hospedador, como colágeno, fibronectina o laminina (Caffrey *et al*., 2018). Además, el silenciamiento génico de las catepsinas B y L en los NEJ se ha relacionado con una reducción de su capacidad de migración a través de la pared intestinal en ratas (McGonigle *et al*., 2008). Estas proteasas se encuentran principalmente en la superficie y las secreciones de los vermes, en forma de vesículas extracelulares o productos de regurgitación (González-Miguel *et al*., 2021). El perfil molecular de las catepsinas de *F. hepatica* sufre numerosos cambios durante la migración, con un estadio inicial en el que predominan las isoformas de catepsina L3 y catepsinas B1/2/3/9 durante la fase intestinal y peritoneal (Cwiklinski *et al*., 2018). A continuación, cuando los vermes inmaduros alcanzan el hígado, la secreción de catepsina L2 se hace cada vez más importante, mientras que la de L3 se reduce y la de catepsinas del grupo B disminuye hasta prácticamente desaparecer (McVeigh *et al*., 2011). Más adelante, cuando los vermes llegan a los conductos biliares, la catepsina L1 se convierte en la isoforma

mayoritaria, destinada principalmente a la degradación de hemoglobina (Lalor *et al*., 2021) (**Figura 12**). Asimismo, existen otras proteasas de cisteína, denominadas legumaínas, que se producen en grandes cantidades durante la fase inicial de la infección, y participan no solo en la degradación de la MEC, sino también en la activación de otras catepsinas del parásito, que son sintetizadas en forma de zimógeno (Robinson *et al*., 2009; Zhang *et al*., 2019). Recientemente, se ha planteado que *F. hepatica* podría hacer uso de otros mecanismos de degradación de proteínas no dependientes de su propio repertorio de proteasas, sino de la maquinaria proteolítica del hospedador (González-Miguel *et al*., 2021), como es el caso del sistema fibrinolítico (Serrat *et al*., 2023), algo que también se ha documentado en otras especies de helmintos (González-Miguel *et al*., 2016).
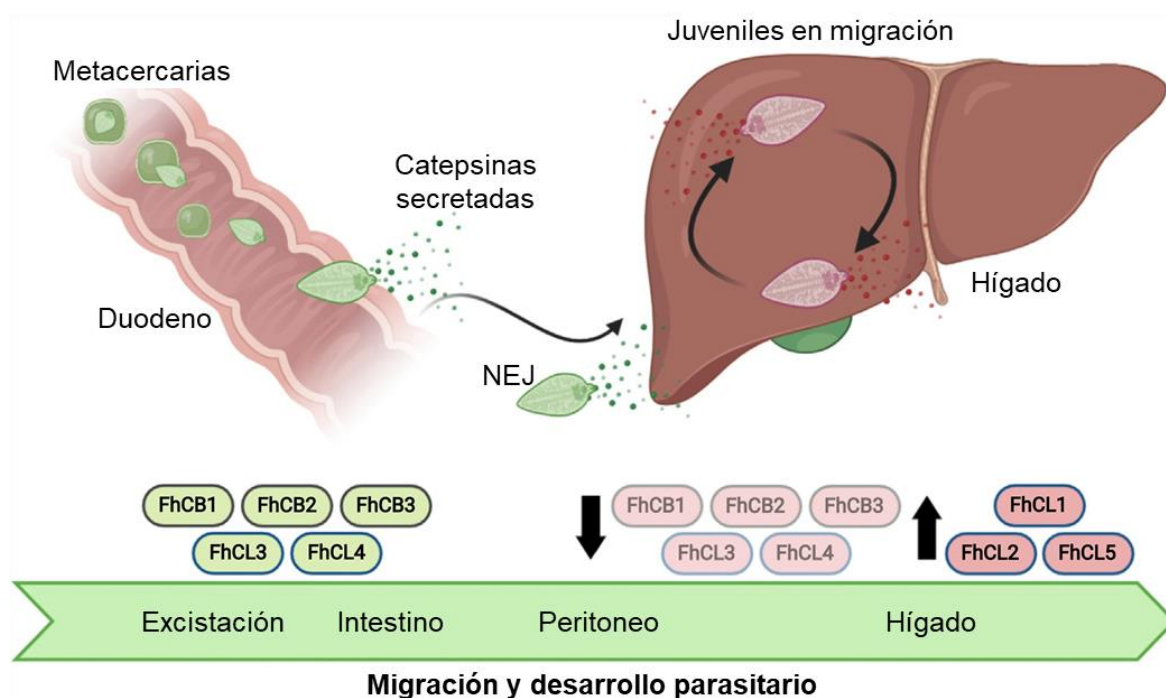
Figura 12 – Evolución del perfil de catepsinas de *F. hepatica* a lo largo de su migración en el hospedador vertebrado. Fuente: adaptada de González-Miguel *et al*. (2021).

Además, otros mecanismos no dependientes de proteínas, como los glicanos y los receptores de unión a lectinas en el tegumento de los NEJ, se han postulado como importantes en la migración del parásito (García-Campos *et al*., 2017). De hecho, el estudio de los cambios que ocurren en el tegumento de los NEJ durante la migración parasitaria y su relación con los tejidos del hospedador es de suma importancia para comprender el progreso de la infección en la fasciolosis (González-Miguel *et al*., 2021). Como ya se ha descrito en el apartado 1.2.3.1 de la presente Tesis Doctoral, los juveniles de *F. hepatica* tienen un glicocálix único que recubre su tegumento, y que se desprende

durante la fase migratoria de la infección. Además, la morfología del tegumento que subyace al glicocálix también cambia durante el desarrollo del parásito, particularmente en relación con el número y el tipo de cuerpos secretores que alberga, responsables de la renovación del glicocálix (González-Miguel *et al*., 2021). Es importante mencionar que estos cambios parecen no ocurrir en el revestimiento glicoproteico del intestino del verme juvenil. En este sentido, varias lectinas con capacidad de unión al tegumento de los NEJ no mostraron unión positiva en las células gastrodérmicas de los mismos, lo que sugiere que la composición de glicanos de la superficie del intestino del verme es diferente a la superficie tegumentaria. Estas propiedades sugieren que el intestino del verme sería un compartimento más adecuado para un ataque inmunitario eficaz que la superficie exterior del mismo (Hanna *et al*., 1980c; de Marco Verissimo *et al*., 2022).

Otro rasgo específico de los NEJ es la elevada síntesis de mucinas. Estas se han descrito como moléculas clave para los mecanismos de evasión inmune de los parásitos, y se sobreexpresan en los NEJ en comparación con los vermes adultos. La PCR a tiempo real confirmó la expresión predominante de mucinas por parte de los NEJ, que parecen ser importantes para la adhesión e invasión de los parásitos (Cancela *et al*., 2015).

4.2.3. Adaptación metabólica

A lo largo del ciclo biológico de *F. hepatica*, las sucesivas transformaciones de los NEJ en vermes inmaduros y, posteriormente, en parásitos adultos, se reflejan en cambios metabólicos durante la migración en respuesta a los ambientes cambiantes que enfrenta dentro de su hospedador vertebrado. De hecho, después de la excistación, los NEJ movilizan sus reservas de energía y ajustan rápidamente sus rutas metabólicas para hacer frente a un entorno nuevo y cada vez más anaerobio (Cwiklinski *et al*., 2018). Los cambios metabólicos y nutricionales del parásito en el hospedador vertebrado han sido revisados a fondo por Tielens y van Hellemond (2022).

El catabolismo de la glucosa es la diferencia metabólica mejor descrita entre los NEJ y los vermes adultos. Los trematodos adultos son completamente anaerobios, y el principal producto de la degradación citosólica de la glucosa en esta etapa es el acetato, mientras que los NEJ utilizan principalmente la ruta aerobia inmediatamente después de su excistación, evolucionando más tarde a un perfil anaerobio facultativo que mantiene rutas aerobias específicas tanto en presencia como en ausencia de oxígeno (González-

Miguel *et al*., 2021). La sustitución del ciclo de Krebs, la principal ruta de producción de energía del verme juvenil, por la formación de acetato aerobio y más tarde por las reacciones de dismutación anaerobia típicas del verme adulto, se lleva a cabo gradualmente. Recientes estudios -ómicos han señalado que los trematodos juveniles mantenidos *in vitro* ajustan sus vías metabólicas al entorno anaerobio mediante la regulación positiva de genes asociados con el catabolismo del glucógeno, entre otros procesos metabólicos y fisiológicos que facilitan el crecimiento, desarrollo y maduración del parásito (Cwiklinski *et al*., 2018). En consecuencia, la transición del metabolismo aerobio a anaerobio de este parásito es un fenómeno que, *a priori*, ocurre independientemente de los estímulos del hospedador.

4.2.4. Respuesta defensiva del hospedador e inmunomodulación parasitaria

Como se ha descrito de manera general en el apartado 2.3 de la presente Tesis Doctoral, la fasciolosis se caracteriza en sus primeras etapas por una respuesta inmune que presenta características mixtas Th1/Th2, evolucionando después hacia una respuesta de tipo Th2 pura junto con una respuesta T reguladora de tipo anérgico (Walsh *et al*., 2009). La respuesta Th2, sin embargo, no se correlaciona con la expulsión de los vermes, sino que parece estar más relacionada con la reparación de los tejidos dañados, mientras que la activación de una respuesta Th1 permitiría una lucha eficaz contra el parásito, como se ha observado en diferentes ensayos vacunales (Hansen *et al*., 1999). No obstante, esta respuesta temprana Th1/Th2 ha sido más estudiada en las fases parasitarias que ya han logrado atravesar la pared intestinal, existiendo grandes vacíos de conocimiento en lo que respecta a los mecanismos involucrados en la invasión intestinal de los NEJ.

En este sentido, la investigación llevada a cabo con ratas y ovejas resistentes a la infección por *F. hepatica* ha demostrado que los antígenos tegumentales de la superficie de los vermes juveniles son el objetivo de mecanismos de citotoxicidad celular mediada por anticuerpos (ADCC), los cuales han demostrado su eficacia contra los NEJ *in vitro* (Gonzalez-Miguel *et al*., 2021). Por el contrario, en hospedadores susceptibles a la infección, las respuestas frente a las fases tempranas de *F. hepatica* son relativamente variables.

En bovinos, los eosinófilos están presentes desde los primeros momentos de la infección en forma de infiltrados en el intestino (McCole *et al*., 1998), siendo capaces de destruir a los parásitos mediante procesos de ADCC (Duffus *et al*., 1980). De forma similar, los macrófagos también pueden destruir eficazmente a los vermes inmaduros mediante este proceso (Nimmerjahn y Ravetch, 2008). Finalmente, en las primeras semanas p.i. se ha descrito la producción de IFNγ por los nódulos linfáticos y los leucocitos, y que dicha producción se reduce una vez que los vermes acceden a la red biliar (Bossaert *et al*., 2000), lo que se interpreta como el inicio de una respuesta proinflamatoria de tipo Th1, que se ve contrarrestada por la respuesta Th2 inducida por el parásito.

En el hospedador ovino, los mecanismos inmunológicos son similares a los descritos en bovinos, aunque en este caso cabe destacar una serie de trabajos donde se evaluaron las respuestas tempranas frente a la infección experimental con *F. hepatica*. Entre ellos, Pacheco *et al*. (2017) estudiaron la producción de IFNγ e IL-4 en ganglios linfáticos hepáticos y en hígado de ovejas experimentalmente infectadas, a distintos tiempos p.i. Estos autores observaron que la expresión de IFNγ disminuye paulatina y significativamente a partir del primer día p.i. en ganglios linfáticos, aumentando la producción de IL-4 en días sucesivos, lo que sugiere una marcada polarización Th2 ya desde los 9 días p.i. Utilizando el mismo modelo experimental, Escamilla *et al*. (2017) señalaron que el porcentaje de leucocitos peritoneales apoptóticos aumenta significativamente a partir del tercer día p.i. Los resultados de este trabajo sugieren la importancia de la inducción de apoptosis para la supervivencia de los parásitos juveniles en las etapas migratorias peritoneales de la infección. Posteriormente, Ruiz-Campillo *et al*. (2018) estudiaron la activación alternativa de macrófagos, lo que propiciaría la detención de su proliferación, a través de la evaluación de la expresión de CD68, CD14, CD206 y óxido nítrico sintasa inducible (iNOS) en células presentes en el líquido peritoneal de ovejas durante las primeras etapas de la infección por *F. hepatica*. Estos autores encontraron un aumento significativo de CD14 y CD206 desde el primer día p.i., lo que sugiere que, efectivamente, la infección con *F. hepatica* induce una activación alternativa de los macrófagos peritoneales desde fases muy tempranas de la infección, lo que podría facilitar la supervivencia del parásito. Aplicando el mismo modelo, Pérez-Caballero *et al*. (2018a; 2018b) estudiaron más a fondo los cambios en las poblaciones celulares inmunocompetentes en líquido peritoneal de ovejas infectadas, encontrando que

las células T CD4$^+$ estaban disminuidas ya desde el primer día p.i., en comparación con los controles no infectados. Asimismo, demostraron que la producción de óxido nítrico por los granulocitos a los 3 días p.i. es mayor en animales infectados, comparados con los no infectados.

Estos estudios se extendieron posteriormente con la evaluación de los niveles de células Treg (Foxp3$^+$) y de las citoquinas reguladoras IL-10 y TGF-β en el hígado de ovejas infectadas (Pacheco *et al*., 2018). Se encontró que estas citoquinas estaban aumentadas desde el primer día p.i., aunque las células Treg Foxp3$^+$ solo aumentaron a partir del día 9 p.i. Estos resultados mostraron que *F. hepatica* es capaz de inducir un fenotipo regulador desde el inicio de la infección, que puede ser importante para la supervivencia del parásito.

Finalmente, se realizó un estudio comparativo en ovejas primoinfectadas y reinfectadas, comparando los niveles de citoquinas proinflamatorias (IL-1β, IFNγ, TNF-α) y reguladoras (IL-10, TGF-β, IL-4), así como los niveles del factor de transcripción Foxp3, tanto en ganglios linfáticos hepáticos como en hígado, en la fase temprana de la infección (4, 8 y 16 días p.i.) (Ruiz-Campillo *et al*., 2023). Tal y como se esperaba, en el hígado de ovejas primoinfectadas se detectó una regulación a la baja del IFNγ en etapas muy tempranas de la infección, seguida de una respuesta Th1/Th2/Treg. Por el contrario, en ovejas reinfectadas se describió una fuerte respuesta mixta Th1/Th2/Treg ya en etapas tempranas de la infección, mientras que en etapas tardías se encontró principalmente una respuesta inmune Th2/Treg. En ganglios, la primoinfección resultó en una respuesta Th1/Th2/Treg casi desde el inicio de la infección, seguida de una respuesta tardía tipo Th2, mientras que la reinfección resultó en una respuesta Th2 temprana liderada por altos niveles de IL-4, para pasar a una respuesta Th1/Th2/Treg en etapas crónicas. Este estudio mostró que la regulación de la respuesta inmune en animales reinfectados hacia una respuesta Th2 es más rápida que en aquellos que son infectados por primera vez. En resumen, se evidencia que, en fases muy tempranas de la infección en ovinos, *F. hepatica* produce una disminución de respuestas proinflamatorias y de linfocitos T CD4$^+$ colaboradores, y un aumento de las respuestas reguladoras, acompañada de apoptosis de leucocitos y producción de óxido nítrico, remarcando la enorme capacidad del parásito de influir en la respuesta inmune de su hospedador ya desde el primer día de infección.

Por otra parte, los modelos experimentales utilizando mamíferos de pequeño tamaño (principalmente ratones) han mostrado que, en este caso, los macrófagos son la

principal población observada en el peritoneo (Miller *et al*., 2009), con un fenotipo similar al observado en rumiantes (Stempin *et al*., 2016). Los eosinófilos apenas tienen presencia en el peritoneo, y solo se detectan de forma notoria durante la fase hepática (Walsh *et al*., 2009).

En cuanto a la información disponible sobre moléculas presentes en los NEJ que puedan tener un papel en la evasión y modulación de la respuesta inmune del hospedador, esta es también relativamente escasa. Los NEJ son capaces de expresar en su superficie un análogo del TGF-β, que inhibe la ADCC mediada por macrófagos (Sulaiman *et al*., 2016). En la misma línea, los oligosacáricos de la superficie del verme han sido caracterizados, mostrando capacidad inmunomoduladora (García-Campos *et al*., 2016b; Ravidà *et al*., 2016). Las proteasas secretadas por los vermes inmaduros también desempeñan un papel crucial en la evasión de la respuesta inmune, ya que permiten a los parásitos degradar los anticuerpos adheridos a su superficie, inhibiendo así el anclaje de células inmunes efectoras y la activación del sistema del complemento (Carmona *et al*., 1993). Además, la actividad de las proteasas se ve potenciada por el proceso de desprendimiento continuo al que se ve sometido el glicocálix (Hanna *et al*., 1980b). Las vesículas extracelulares constituyen otra de las grandes estrategias de adaptación del parásito. No obstante, para los NEJ la información es muy limitada y solo se refiere a estudios proteómicos de vesículas extracelulares de NEJ mantenidos durante 28 días *in vitro*, analizando las vesículas producidas durante este periodo como un conjunto, sin que se identificasen moléculas con un papel inmunomodulador específico en dichas vesículas (Trelis *et al*., 2022).

En definitiva, se constata que la caracterización de la respuesta defensiva concreta frente a los NEJ en el intestino del hospedador es todavía un campo poco estudiado. De forma similar, los mecanismos de evasión y modulación inmune en las fases tempranas de la fasciolosis son más conocidos para los vermes juveniles residentes en el peritoneo y el hígado, que en aquellos parásitos que atraviesan la pared intestinal del hospedador, en los que dichos mecanismos son poco o nada conocidos.

Hipótesis de trabajo y objetivos

El paso de los vermes juveniles de *Fasciola hepatica* a través de la pared intestinal de su hospedador definitivo constituye un punto crítico para el éxito de la infección, al suponer un factor limitante tanto para la viabilidad de los parásitos en su ruta migratoria hacia el hígado, como en el control terapéutico de la enfermedad. Sin embargo, y a pesar de su importancia, los mecanismos moleculares que rigen la relación parásito-hospedador durante este primer contacto entre ambos organismos han sido poco explorados. En el contexto actual de la fasciolosis como enfermedad zoonótica emergente, con un impacto tanto sanitario como económico considerable, y reportes crecientes de resistencias frente al fármaco de elección, es urgente considerar otras herramientas de control. En este sentido, la vacunación constituiría una estrategia más racional para el control de la fasciolosis, pues induciría una protección duradera y no dejaría residuos en los productos de origen animal, minimizando el riesgo de aumento de resistencias. Por ello, y para mejorar los resultados obtenidos en los experimentos de vacunación llevados a cabo hasta el momento, resulta imprescindible aumentar el conocimiento de los citados mecanismos parásito-hospedador en las fases tempranas de la fasciolosis, con el fin último de perseguir la eliminación precoz de la infección, antes de que los vermes adultos se establezcan en su localización definitiva.

Partiendo de esta idea subyacente, la hipótesis de trabajo de la presente Tesis Doctoral es que la interacción de los juveniles recién excistados de *F. hepatica* con la pared intestinal de su hospedador definitivo vertebrado desencadena cambios a nivel molecular en ambos organismos. Su conocimiento permitirá no solo mejorar la comprensión de la interacción parásito-hospedador en la fase temprana de la enfermedad, aportando información sobre los procesos biológicos esenciales para la supervivencia y patogenicidad del parásito y sobre los mecanismos defensivos del hospedador, sino también postular una serie de candidatos moleculares que permitan la formulación de una futurible vacuna frente al parásito. Para demostrar dicha hipótesis se formularon los siguientes objetivos:

1) Diseñar y desarrollar modelos experimentales que permitan replicar en condiciones de laboratorio la interacción con el epitelio intestinal y la penetración del intestino del hospedador llevada a cabo por los vermes juveniles recién excistados de *Fasciola hepatica*, así como estudiar la interacción molecular entre ambos organismos.

2) Identificar y caracterizar mediante aproximaciones proteómicas y transcriptómicas los cambios en el perfil de expresión, tanto de parásito como de hospedador, después de su interacción en los modelos experimentales puestos a punto.

Para abordar estos objetivos, se realizarán excistaciones *in vitro* de metacercarias de *F. hepatica* con el fin de obtener el material parasitario de partida, los juveniles recién excistados o NEJ, los cuales se pondrán en contacto con cultivos en placa de células de epitelio intestinal de ratón o con fragmentos de intestino obtenidos del hospedador murino, durante un tiempo de estimulación comprendido entre las 3 y las 24 horas.

A continuación, distintas fracciones de parásito y hospedador serán recuperadas de los modelos de interacción y, posteriormente, analizadas mediante las tecnologías iTRAQ y SWATH-MS, así como por RNA-Seq, determinando los cambios en el perfil de expresión proteómico y transcriptómico, respectivamente, en comparación con parásitos y células intestinales no estimulados. De manera secundaria, también se llevará a cabo la caracterización inmuno-histológica de los fragmentos de intestino del hospedador invadidos por los vermes juveniles de *F. hepatica*.

Capítulo 1:

Set up of an *in vitro* model to study early host-parasite interactions between newly excysted juveniles of *Fasciola hepatica* and host intestinal cells using a quantitative proteomics approach

Resumen de la publicación

La fasciolosis, causada por el trematodo *Fasciola hepatica*, es una enfermedad de carácter zoonótico responsable de pérdidas millonarias en la industria ganadera, así como un importante problema de salud humana en regiones endémicas. Al igual que otros endoparásitos, *F. hepatica* realiza un complejo ciclo migratorio en el organismo de su hospedador definitivo, que afecta al intestino, el peritoneo y el hígado, y termina con los vermes adultos en la red biliar. Aunque los vermes juveniles son responsables de la patología aguda de la enfermedad, el conocimiento relativo a las etapas larvarias es escaso, y la mayoría de estudios sobre este parásito se centran en su fase adulta.

Con el objetivo de facilitar el estudio de la fase juvenil del parásito, se ha puesto a punto un modelo *in vitro* de interacción parásito-hospedador que busca reproducir el primer contacto entre ambos organismos, el cual tiene lugar a nivel del intestino delgado entre los vermes juveniles recién excistados (*Newly Excysted Juveniles*; NEJ) de *F. hepatica* y la mucosa intestinal del hospedador. Para ello, se realizó la excistación *in vitro* de metacercarias del parásito, y los juveniles obtenidos se pusieron en contacto con un cultivo primario de células de epitelio intestinal de ratón (*Mouse Primary Small Intestinal Epithelial Cells*; MPSIEC) durante 24 horas.

Pasado este tiempo, ambos organismos se separaron y fraccionaron, y su perfil de expresión proteico se caracterizó utilizando la metodología iTRAQ (*Isobaric Tag for Relative and Absolute Quantitation*), detectándose un total de 191 y 62 proteínas sobreexpresadas, respectivamente, en el tegumento y la fracción somática de los NEJ de *F. hepatica*, junto con 112 y 57 proteínas subexpresadas, implicadas en procesos biológicos como la regulación de la actividad proteolítica, el metabolismo energético y los procesos de señalización. Paralelamente, se detectaron 87 proteínas sobreexpresadas en la fracción soluble del epitelio intestinal, así como 73 proteínas subexpresadas, relacionadas con procesos de transporte de vesículas y regulación de procesos metabólicos, entre otros.

Research paper

Set up of an in vitro model to study early host-parasite interactions between newly excysted juveniles of *Fasciola hepatica* and host intestinal cells using a quantitative proteomics approach

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ABSTRACT

Fasciola hepatica is the causative agent of fasciolosis, a parasitic zoonosis of global distribution causing significant economic losses in animal production and a human public health problem in low-income countries. Hosts are infected by ingestion of aquatic plants carrying metacercariae. Once ingested, the juvenile parasites excyst in the small intestine and, after crossing it, they follow a complex migratory route that lead the parasites to their definitive location in the bile ducts. Despite being a critical event in the progression of the infection, the available data on the cross-talk relationships between the parasite and the host at an early stage of the infection are scarce. The objective of the present work is to characterize the proteomic changes occurring in both the parasite and the host, through the development of a novel in vitro model, to shed light on the molecular pathways of communication between the newly excysted juveniles (NEJ) from *F. hepatica* and the host's intestinal epithelium. For this, in vitro excystation of *F. hepatica* metacercariae was carried out and NEJ were obtained. Additionally, optimal conditions of growth and expansion of mouse primary small intestinal epithelial cells (MPSIEC) in culture were fine-tuned. Tegumentary and somatic parasite antigens (NEJ-Teg and NEJ-Som), as well as host cell protein lysate (MPSIEC-Lys) were obtained before and after 24 h co-culture of NEJ with MPSIEC. We used an isobaric tags for relative and absolute quantitation (iTRAQ)-based strategy to detect 191 and 62 up-regulated, and 112 and 57 down-regulated proteins in the NEJ-Teg and NEJ-Som extracts, respectively. Similarly, 87 up-regulated and 73 down-regulated proteins in the MPSIEC-Lys extract were identified. Taking into account the biological processes in which these proteins were involved, interesting mechanisms related to parasite development, invasion and evasion, as well as manipulation of the host intestinal epithelial cell adhesion, immunity and apoptosis pathways, among others, could be inferred, taking place at the host-parasite interface. The further understanding of these processes could constitute promising therapeutic targets in the future against fasciolosis.

1. Introduction

Host invasion processes in fasciolosis start with the excystment of the juvenile forms of the parasite *Fasciola hepatica* in the small intestine. This process occurs after the ingestion of aquatic plants carrying infectious metacercariae by suitable hosts, including ruminants and humans. Following excystment, the newly excysted juveniles (NEJ) cross the intestinal wall in order to follow a complex migratory route leading the parasites to their definitive location in the bile ducts where they become sexually mature (Mas-Coma et al., 2014; Moazeni and Ahmadi, 2016). After that, the chronicity of the infection, as well as the acute form of the disease, typically associated with the liver migratory stages, determine a hepatic pathological process, with an impact on the production of meat, milk and wool, and decreased fertility (Schweizer et al., 2005) in a disease affecting millions of ruminants around the world with annual economic losses of more than 3 billion USD (Toet

et al., 2014). In addition, fasciolosis is an emerging zoonosis in many areas of Latin America, Africa and Asia, and its epidemiology in humans is linked to a high prevalence in livestock, being children a particularly vulnerable population (Nguyen et al., 2011). Despite its worldwide relevance, the classical vaccination approaches carried out so far, mostly based on the use of parasitic molecules from the adult phase, have not given the expected results (Dominguez et al., 2018); thus, studying the host-parasite relationships at an earlier phase of infection could provide important information to develop new control measures against this liver fluke.

NEJ are capable of crossing the intestinal wall by about 2 h after metacercariae ingestion (Moazeni and Ahmadi, 2016), representing a key process for the parasite life cycle as a "point of no return" in fasciolosis. Importantly, the first contact of the parasite with the host occurs between the cells of the intestinal epithelium and the NEJ, being this a determining phase for the subsequent development of the infection. The intestinal epithelial cells constitute a physical barrier but also interact with the pathogen and with the underlying immune cells, producing immunoeffector molecules involved in the first line of innate immune defense against the parasite (Goto and Ivanov, 2013). The deep characterization of the changes induced in the host cells and in the NEJ upon their interaction is of great importance to define the first events driving the host-parasite relationship inside the vertebrate host. Existing models on the study of these mechanisms, including ex vivo models using NEJ injection into a rat gut segment (van Milligen et al., 1998), as well as in vitro models placing NEJ into compartments containing rat distal jejunal sheets (Garcia-Campos et al., 2016a), are useful in the study of parasitic migration, but do not allow the study of the changes arising both in the parasite and in the host cells before and after their interaction. In vitro models based on cell cultures approaches could represent a better alternative to those already described, since they allow the co-culture of parasites and host cells directly involved in these mechanisms in order to obtain more robust conclusions. These types of in vitro models have been widely used for the study of host-parasite interactions in helminths with intestinal stages by co-culturing intestinal epithelial cells and infective third-stage larvae of *Trichostrongylus colubriformis*, *Ancylostoma ceylanicum* or *Ascaris suum* (Corvan et al., 2015; Feather et al., 2017; Ebner et al., 2018).

The main objective of this work is to develop an in vitro model to shed light on the molecular pathways of communication between *F. hepatica* NEJ and the host's intestinal epithelium. This new model is used to reveal cross-talk interactions between the parasite and its host in the early phase of infection. For this purpose, a high-throughput quantitative proteomics approach using isobaric tags for relative and absolute quantitation (iTRAQ) was performed to detect changes in the proteins expressed by NEJ and by the host intestinal epithelial cells after their in vitro interaction.

2. Material and methods

2.1. In vitro excystation of F. hepatica metacercariae

NEJ were obtained as follows: *F. hepatica* metacercariae (Italian strain) were purchased from Ridgeway Research Ltd. (UK). One thousand metacercariae were excysted in vitro as described previously (Hernández-González et al., 2010). In brief, pure CO_2 was bubbled for 30 s in 10 ml of cold distilled water. Sodium dithionite to a final concentration of 0.02 M was added to the water, and the tube closed and incubated at 37 °C until a fine cloudy precipitate was produced. Metacercariae were then added to the tube and further incubated at 37 °C for 1 h. After incubation, parasites were washed twice by sedimentation with warm distilled water, and 5 ml of Hank's balanced salt solution (Sigma, USA) plus 10 % (v/v) rabbit bile and 30 mM HEPES (Sigma) pH 7.4 were added to the dry metacercariae. Parasites were then incubated for 4 h at 37 °C. Emerging active parasites were collected with a 20 µl pipette, washed twice in cold PBS and immediately subjected to protein

extraction (see Section 2.3) or used for the in vitro interaction with host cells (see Section 2.2).

2.2. Growth and expansion of a mouse intestinal epithelial cell line in culture, and cell culture stimulation with NEJ

Mouse small intestinal epithelial cells (C57BL/6 MPSIEC) from Cell Biologics (ref. C57-6051) were grown in epithelial cell growth medium (Innoprot). MPSIEC plates were precoated with an attachment factor solution (0.2 % gelatin). Cells were cultured at 37 °C in a humidified atmosphere in the presence of 5 % CO_2 and 95 % air. Medium was changed every 3 days. Expansion was done by trypsinizing the cells (Trypsin/EDTA, Innoprot) and replating them when the proliferating cells reached a sufficient density. Passaging was done at ratios of 1:3. Cell counts were performed using the equipment Countess® Automated Cell Counter (Invitrogen) following the manufacturer's instructions. After cultures were fine-tuned, the optimum stimulation conditions were selected, which resulted in the use of 60 mm plates in passage five with a confluence of 100 %. Confluent cells were treated with 200 NEJ per plate for 24 h in triplicate. Parasite and cell culture viability were monitored by taking images under an inverted microscope. Cytotoxicity of co-culture was ruled out after calculation of the amount of lactate dehydrogenase (LDH) released to the cell media as measured with the Pierce™ LDH Cytotoxicity Assay Kit (Thermo Scientific) following the manufacturer's protocol. Then, NEJ were collected with a 20 µl pipette and cells were separately collected by detachment with a cell scraper, and centrifuged at 15,000 g for 10 min at 4 °C, both washed twice in cold PBS and immediately subjected to protein extraction (see Section 2.3). Untreated cells, as well as NEJ before cell co-culture were used as controls (Fig. 1).

2.3. Protein extraction

Both NEJ before and after cell co-culture were used for tegument protein isolation as previously described (Garcia-Campos et al., 2016b). In brief, parasites were washed three times in PBS followed by incubation for 30 min at room temperature with 1 ml of 1 % Nonidet P40 (NP40) in PBS. Then, NEJ were centrifuged at 300g for 5 min and the supernatant collected. NP40 was removed from the supernatant with 0.3 g of Bio beads (Bio-Rad) according to the manufacturer's recommendations. The pellet obtained after tegument removal, containing denuded NEJ, was suspended in RIPA buffer (Sigma-Aldrich) and used for somatic proteins isolation. After incubation, both samples were centrifuged at 1000 g for 5 min and the supernatants resulting from this process, named NEJ-Teg and NEJ-Som, respectively, were collected. On the other hand, treated and non-treated MPSIEC were lysed in ice-cold lysis buffer (20 mM Tris–HCl, pH 7.5), 140 mM NaCl, 10 mM ethylendiaminetetraacetic acid, 10 % glycerol, 1 % Igepal CA-630, aprotinin, pepstanin, pepstatin, and leupeptin at 1 µg/ml each, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate). The supernatant including soluble proteins was collected and the resulting extract was named MPSIEC-Lys. All biological triplicate extracts were mixed and labelled with the number 1, if they corresponded with controls, or with the number 2, if they had been obtained after cell culture stimulation with NEJ (Fig. 1). A cocktail of protease inhibitors was added to all samples following the methodology described by Maizels et al. (1991) and protein contents was determined by DC protein assay commercial kit (Bio-Rad). Finally, all samples were aliquoted and stored at −80 °C until use.

2.4. In-gel digestion and iTRAQ labelling

Protein extracts (10 µg for *F. hepatica* extracts; 40 µg for mouse cell extracts), were suspended up to 50 µl in sample buffer, and then applied onto 1.2-cm wide wells of a conventional SDS-PAGE gel (1 mm-thick, 4 % stacking, and 10 % resolving). The run was stopped as soon as the
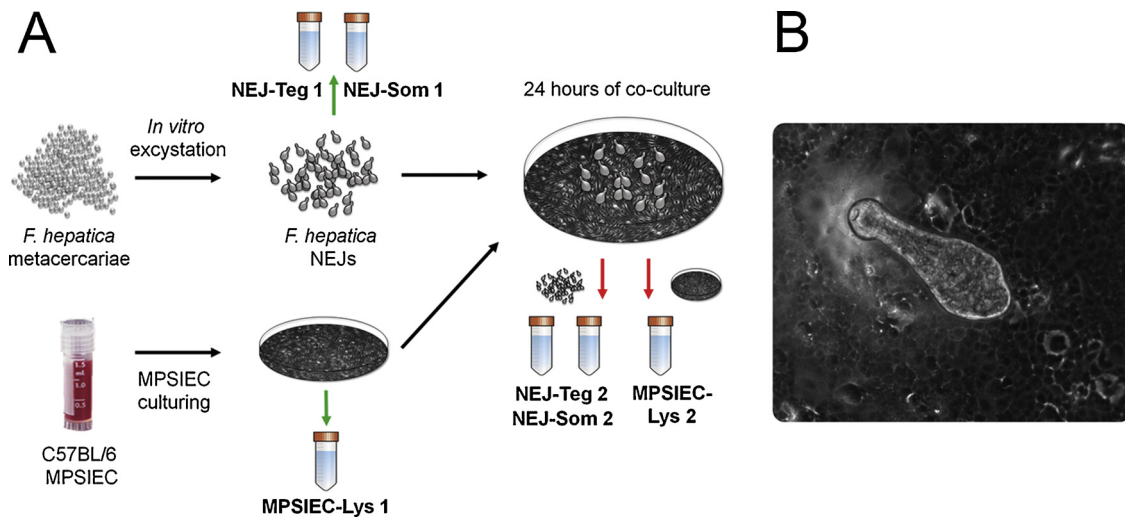
Fig. 1. Experimental design and in vitro model. *F. hepatica* juveniles after in vitro excystation and mouse intestinal epithelial cells were co-cultured for 24 h. Parasitic tegument and somatic extracts, as well as host cell lysates were obtained before and after co-culturing (A). Representative image of MPSIEC stimulated with the NEJ (100x) (B). Tegument extract from newly excysted juveniles, NEJ-Teg; somatic extract from newly excysted juveniles, NEJ-Som; mouse primary small intestine epithelial cell lysate; MPSIEC-Lys. In all extracts number 1 represents before co-culturing and number 2 represents after co-culturing.

front entered 3 mm into the resolving gel, so that the whole proteome became concentrated in the stacking/resolving gel interface. The unseparated protein bands were visualized by Coomassie blue staining, excised, cut into cubes (2 × 2 mm), and placed into 0.5 ml microcentrifuge tubes. The gel pieces were destained in acetonitrile:water (ACN:H_2O, 1:1), reduced with 10 mM DTT for 1 h at 56 °C and alkylated with 50 mM iodoacetamide for 1 h at room temperature in darkness. Samples were finally digested with sequencing grade trypsin (Promega, Madison, WI) as described by Shevchenko et al. (2001), supernatants dried down, desalted using OMIX Pipette tips C18 (Agilent Technologies) and dried down in a SpeedVac. The resultant peptide mixture was labeled using the iTRAQ reagent 8plex Multi-plex kit (Applied Biosystems) as follows: reagent 113 for NEJ-Teg 1, 114 for NEJ-Teg 2, 115 for NEJ-Som 1, 116 for NEJ-Som 2, 119 for MPSIEC-Lys 1 and 121 for MPSIEC-Lys 2 (Köcher et al., 2009). Briefly, peptides were dissolved in 0.5 M triethylammonium bicarbonate (TEAB), adjusted to pH 8. For labeling, each iTRAQ reagent was dissolved in 50 μL of isopropanol, added to the respective peptide mixture and incubated at room temperature for two hours. Labelling was stopped by the addition of 0.1 % formic acid. Whole supernatants were dried down and the six samples (4 samples from *F. hepatica* and 2 samples from mouse cells) were respectively mixed to obtain two labeled mixtures. Mixtures were desalted and fractionated using a Pierce High pH Reversed-Phase Peptide Fractionation Kit.

2.5. Reverse phase-liquid chromatography RP-LC–MS/MS analysis

Each labeled mixture were dried, resuspended in 10 μl of 0.1 % formic acid and analyzed by RP-LC–MS/MS in an Easy-nLC II system coupled to an ion trap LTQ-Orbitrap-Velos-Pro hybrid mass spectrometer (Thermo Scientific). Peptides were concentrated (on-line) by reverse phase chromatography using a 0.1 mm × 20 mm C18 RP precolumn (Proxeon), and then separated using a 0.075 mm x 250 mm C18 RP column (Proxeon) operating at 0.3 μl/min. Peptides were eluted using a 240-min dual gradient from 5 to 25 % solvent B in 180 min followed by gradient from 25 to 40 % solvent B over 240 min (Solvent A: 0.1 % formic acid in water, solvent B: 0.1 % formic acid, 80 % acetonitrile in water). ESI ionization was performed using a Nano-bore emitters Stainless Steel ID 30 μm (Proxeon) interface (Alonso et al., 2015). The instrument method consisted of a data-dependent top-20 experiment with an Orbitrap MS1 scan at a resolution ($m/\Delta m$) of 30,000 followed by either twenty high energy collision dissociation

(HCD) MS/MS mass-analyzed in the Orbitrap at 7500 ($\Delta m/m$) resolution. MS2 experiments were performed using HCD to generate high resolution and high mass accuracy MS2 spectra. The minimum MS signal for triggering MS/MS was set to 500. The lock mass option was enabled for both MS and MS/MS mode and the polydimethylcyclosiloxane ions (protonated (Si(CH3)2O))6; m/z 445.120025) were used for internal recalibration of the mass spectra. Peptides were detected in survey scans from 400 to 1600 amu (1 μscan) using an isolation width of 2 u (in mass-to-charge ratio units), normalized collision energy of 40 % for HCD fragmentation, and dynamic exclusion applied during 30 s periods. Precursors of unknown or +1 charge state were rejected.

2.6. Database searching and bioinformatic analysis

All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.5.1) and validated with X! Tandem (The GPM, thegpm.org; version CYCLONE 2010.12.01.1) against a database comprising the *F. hepatica* predicted proteome [WBPS13 database, corresponding to Bioproject PRJEB25283 (https://parasite.wormbase.org)] (Cwiklinski et al., 2015) appended to the *Mus musculus* proteome (Uniprot) and the cRAP database (https://www.thegpm.org/crap/). Parameters for Mascot and X! Tandem searches included a fragment ion mass tolerance of 0.30 Da and a parent ion tolerance of 50 ppm and fixed modifications of O^{18+} of pyrrolysine and iTRAQ8plex labelling of lysine and the n-terminus. In X! Tandem searches variable modifications specified were Glu- > pyro-Glu of the n-terminus, ammonia-loss of the n-terminus, Gln- > pyro-Glu of the n-terminus, oxidation of methionine, methylthio of cysteine and iTRAQ8plex labelling of tyrosine. In Mascot searches variable modifications specified were oxidation of methionine, and iTRAQ8plex labelling of tyrosine. Scaffold Q + (version Scaffold_4.2.1) was used to validate MS/MS based peptide and protein identifications. Peptide and protein identifications were accepted if they could be established at greater than 95 % and 99 % probability, respectively, as specified by the Peptide Prophet algorithm (Keller et al., 2002), and contained at least two identified peptides (Nesvizhskii et al., 2003). Proteins containing similar peptides that could not be differentiated based on MS/MS analysis were grouped to satisfy the principles of parsimony. A false discovery rate (FDR) of < 0.1 % was calculated using protein identifications validated by the Scaffold Q + program. Scaffold Q + was used to quantify the isobaric tag peptide and protein identifications. Channels were corrected in all

samples according to the algorithm described in i-Tracker (Shadforth et al., 2005) and acquired intensities in the experiment were globally normalized across all acquisition runs. Individual quantitative samples were normalized within each acquisition run, and intensities for each peptide identification were normalized within the assigned proteins. The reference channels were normalized to produce a 1:1 fold change. All normalization calculations were performed using medians to multiplicatively normalize data. Blast2GO (Conesa et al., 2005) was used to classify proteins according to gene ontology (GO) categories.

3. Results

3.1. Protein identification and quantification

An MS/MS approach using isobaric labelling was followed to identify and quantify the proteins present in the samples. A total of 67,966 and 33,764 spectra were obtained in both 4-plex labelled experiments (*F. hepatica*, 113–116, and *M. musculus*, 119–121, respectively), representing 3299 and 2822 unique proteins containing two or more unique peptides identified using an FDR < 0.1 % (Supplementary Files 1–2). Mouse proteins were also identified in NEJ-Teg 2 and NEJ-Som 2 samples and, similarly, *F. hepatica* proteins were identified in the MPSIEC-Lys 2 sample (Supplementary Files 1–2). The quantification analysis was performed using Scaffold Q+, and only proteins with a $\log_2$ fold-change > 0.6 or < −0.6 (for up-regulated and down-regulated proteins, respectively) were taken into consideration for further analysis. The expression level of 303 proteins in the tegument of *F. hepatica* NEJ was differentially regulated by the incubation of the parasites with mouse intestinal epithelial cells (191 up-regulated and 112 down-regulated; Fig. 2A; Supplementary File 3). Furthermore, the levels of 119 proteins were regulated in the somatic extract of *F. hepatica* NEJ after incubation with mouse small intestinal epithelial cells (62 up-regulated and 57 down-regulated; Fig. 2A; Supplementary File 4). Similarly, the expression levels of 87 proteins were up-regulated and 73 were proteins down-regulated in the protein lysate of mouse intestinal epithelial cells after incubation with *F. hepatica* NEJ (Fig. 2B; Supplementary File 5).

3.2. Incubation with mouse epithelial cells induces a protein composition shift in F. hepatica NEJ

Interestingly, up-regulated proteins from the tegument of *F. hepatica*

Fig. 3. Bar graph representing the top 10 biological processes from up- and down-regulated proteins in the tegument from *F. hepatica* NEJ after incubation with mouse intestinal epithelial cells. Y-axis represents the nodescore from Blast2GO.

NEJ after incubation with mouse cells were implicated in different biological processes such as phosphorylation, transport and proteolysis (Fig. 3). Among these proteins we positively identified several kinases (tyrosine kinase, PEPCK and serine/threonine kinase among others), ubiquitin-like proteins, legumain-like proteins and several cathepsin L enzymes (CL1 and CL1D). Conversely, up-regulated proteins from the somatic extracts of the parasite were implicated in several metabolic processes such as primary metabolic process, nitrogen compound metabolic process and cellular nitrogen compound metabolic process, as well as in other biological processes such as protein phosphorylation (i.e. tyrosine-protein kinase PR2) and cytoskeleton organization (i.e.
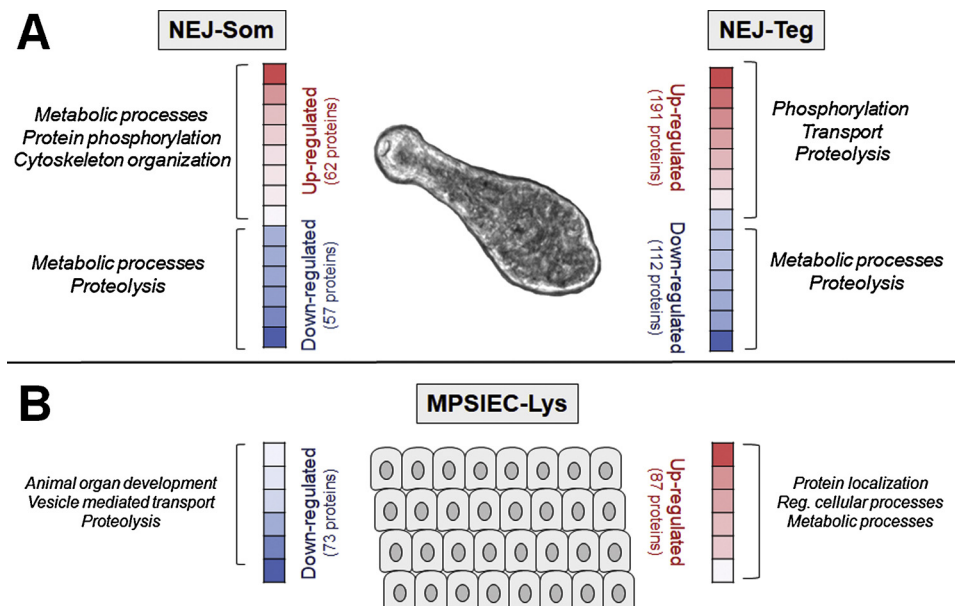

Fig. 2. Diagram showing the number of up- and down-regulated proteins and main associated biological processes from the somatic and tegumental extracts of *F. hepatica* NEJ (A) and mouse intestinal epithelial cells lysate extract (B) after experimental incubation. Gene ontology biological processes and the corresponding gene ontology identifiers are given in Supplementary File 6.
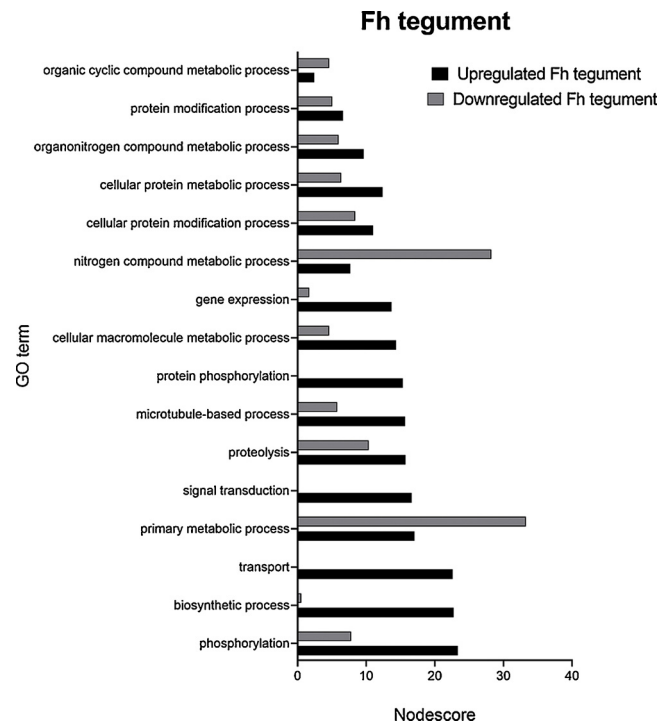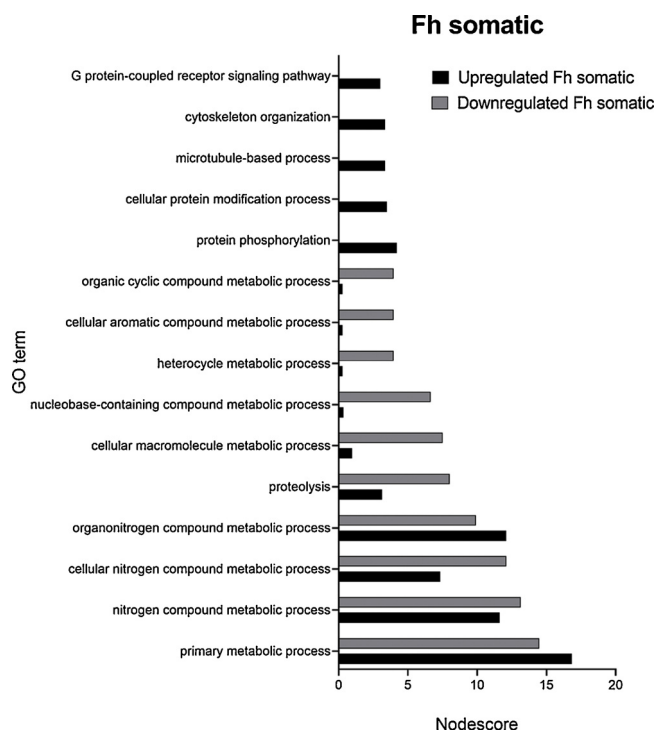
Fig. 4. Bar graph representing the top 10 biological processes from up- and down-regulated proteins in the somatic extracts from *F. hepatica* NEJ after incubation with mouse intestinal epithelial cells. Y-axis represents the nodescore from Blast2GO.

tubulin alpha chain and serine/threonine-protein kinase MARK2) among others (Fig. 4). Down-regulated proteins from both *F. hepatica* extracts (tegumental and somatic proteins) are heavily implicated in several metabolic processes (i.e. nitrogen compound metabolic processes and primary metabolic process) and other biological processes such as proteolysis (Figs. 3 and 4). Interestingly, among these proteins we found several cathepsin L (CL1D, pro-CL3, CL3 and CL4), as well as cathepsin B (CB) and other proteolytic enzymes such as a papain family cystein protease in the tegument and a serine protease (mastin precursor) in the somatic extract.

3.3. Mouse epithelial cells show specific proteomic adaptation after incubation with F. hepatica NEJ

Changes in the proteomic composition of mouse epithelial cells were detected after incubation with *F. hepatica* NEJ. The most important changes in up-regulated proteins from MPSIEC-Lys 2 at the biological level (based on the frequency and the nodescore value provided by Blast2GO) were related to protein localization, regulation of cellular processes and organic cyclic compound metabolic processes and nucleobase-containing compound metabolic processes (Fig. 5A). Among the proteins implicated in these processes, we found plastin-1, clathrin light chain and syntaxin-6 (protein localization) as well as inositol-1-monophosphatase, phosphoinositide phospholipase C and phosphodiesterase (regulation of cellular processes). Down-regulated proteins from MPSIEC-Lys 2 were mainly implicated in tissue development and regeneration such as animal organ development (i.e. tropomyosin alpha-3 chain and myosin 3), cell adhesion (i.e. polycistin-1 and stabilin 1), and other processes (i.e. annexin 5, nucleoside diphosphate kinase A and superoxide dismutase) as well as in protein metabolism such as proteolysis and vesicle mediated transport (Fig. 5B).

4. Discussion

It is suggested that the lack of knowledge regarding the underlying biological, biochemical and immunological components from the host-parasite interface represents one of the main reasons that could explain that currently not many targets have been successfully developed into vaccines against parasites (Stutzer et al., 2018). Therefore, understanding how parasites infect and migrate within their host tissues is of paramount importance to discover new interventions that strategically block host-parasite interactions (Cwiklinski et al., 2019). In the case of the study of fasciolosis caused by *F. hepatica*, these strategies should be ideally focused on the deep characterization of the first hours of cross-talk between the parasite and its host, e.g., in the changes induced in the NEJ and in the host intestinal epithelial cells upon their interaction, since this step is critical to define the first events driving the host-parasite relationship inside the vertebrate host. The aim of the present work is to characterize, starting from developing a novel in vitro model, the proteomic changes taking place during the early phase of fasciolosis between the NEJ and the host intestinal epithelial cells.

Regarding the parasite, we decided to work separately with tegument and somatic extracts, although it is expected to be difficult to obtain a complete separation of both antigenic compartments. Nevertheless, we obtained a tegumental enriched fraction as it has been postulated by other authors working with similar methodology (Garcia-Campos et al., 2016b). This extract also comprised excretory-secretory proteins, presumably regurgitated from the fluke gut, so it could be considered that it is a mixture of excretory/secretory and membrane-associated proteins (Morphew et al., 2013; Toet et al., 2014). The tegument of *Fasciola*, like that of other parasitic trematodes, is a biologically active and metabolically complex matrix continuously exposed to host tissues (Ravidà et al., 2016). It is a very dynamic structure, since changes in both its morphology and its composition from its juvenile to its adult form have been described, revealing the response of the migrating parasite to the changing demands of the host environment (Bennett and Threadgold, 1975). In our study, we found a large reorganization of the NEJ-Teg extract after 24 h of contact with the MPSIEC, which resulted in 303 differentially regulated proteins. In this regard, tegument turnover has been postulated as a successful strategy that ensures survival of platyhelminth worms helping them in reducing antigenicity of their outer surface (Skelly and Alan Wilson, 2006; Mulvenna et al., 2010). Observing the biological processes in which these proteins might be involved, we found 14 and 10 up-regulated proteins related to transport and signal transduction functions, respectively, in the NEJTeg 2 extract. This would be in agreement with the sophisticated communication mechanisms required by parasites with the aim of enhancing their own survival and transmission when facing a physical and molecular barrier from the host (Coakley et al., 2016; Roditi, 2016). These mechanisms should include not only the down-regulation of the host immune responses at the intestinal level, but also the expression of mediators and transporters allowing parasites to adapt and survive in a hostile environment, as can be inferred from the up-regulated proteins identified in this study. These included, among others, proteins related to innate immunity (leucine-rich repeat-containing protein), vesicle exocytosis (calcium-dependent secretion activator), pH homeostasis (voltage-gated hydrogen channel) and receptors for host molecules (cholecystokinin receptor). Interestingly, cholecystokinin is a hormone with important functions in pancreatic secretion, gallbladder function, intestinal motility and intestinal inflammation (Chandra and Liddle, 2007). Its synthesis and secretion is carried out by enteroendocrine cells in the duodenum and jejunum (Brubaker, 2012), the portion of the intestine in which NEJ should cross the intestinal barrier (Mas-Coma et al., 2014).

Analysis of the somatic proteome of the NEJ allowed us to identify 119 differentially regulated parasitic proteins after incubation with MPSIEC. In this case, many of the modified biological processes identified resulted in altered structural functions, such as cytoskeleton
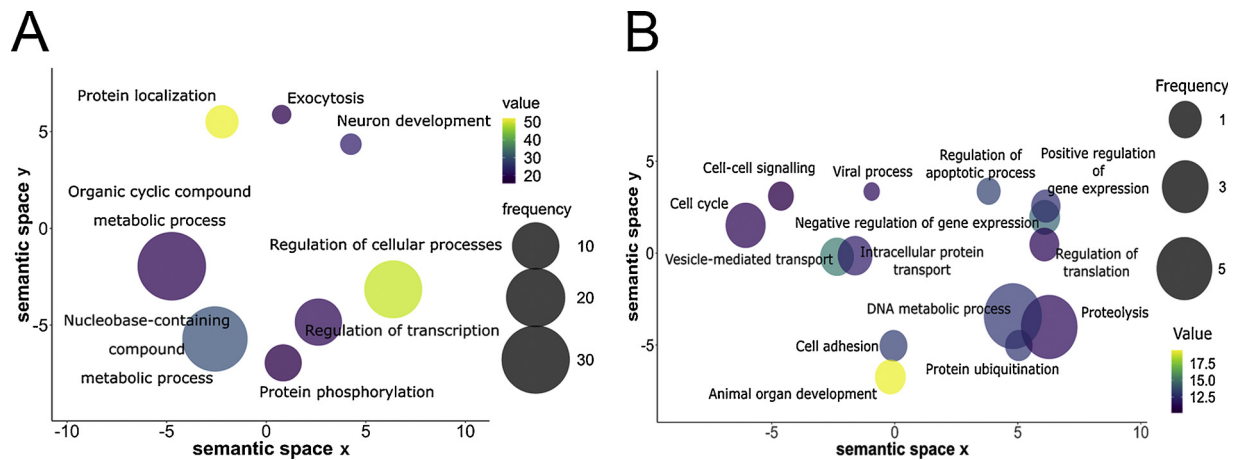
Fig. 5. Biological processes of up-regulated (A) and down-regulated (B) mouse primary small intestinal epithelial cells lysate (MPSIEC-Lys) proteins ranked by nodescore (Blast2GO) and plotted using REViGO. Semantically similar GO terms plot close together, increasing heatmap score signifies increasing nodescore from Blast2GO, while circle size denotes the frequency of the GO term from the underlying database.

organization or microtubule-based processes. In this sense, large-scale transcriptional changes have been described within the tubulin family as *F. hepatica* developed and migrated from the gut, across the peritoneum (Ryan et al., 2008; Cwiklinski et al., 2015). Tubulins are known targets of triclabendazole (TCBZ), a derivative of benzimidazoles, which are nitrogen containing heterocyclic compounds (Asif, 2017). Although the nature of the interaction of *F. hepatica* with TCBZ remains undefined and resistance has been described increasingly since the mid-1990s, it is considered the drug of choice to control fasciolosis (Kelley et al., 2016). Despite the fact that the actual biochemical mechanism of TCBZ resistance remains unclear, changes in the target molecule or the ability of parasites to metabolize the drug have been pointed out in this phenomenon (Brennan et al., 2007; Kelley et al., 2016). Intriguingly, we have found a down-regulation activity of nitrogen compound metabolic processes in both antigenic compartments (NEJTeg 2 and NEJSom 2). This might be related to the parasitic secretion of molecules with capacity to metabolize benzimidazoles, as well as with the ability to evade the action of the reactive nitrogen species. The production of these nitrogen intermediates by immune cells has been identified as a mechanism of cell-mediated cytotoxicity to NEJ (Piedrafita et al., 2001). Accordingly, NEJ would require metabolize host nitrogen compounds as a detoxification mechanisms in the early phase of the infection as have been previously studied in bacteria (Poole, 2005).

Finally, we could identify a representative number of differentially regulated proteins belonging to two important functional groups in both extracts NEJSom and NEJTeg: phosphorylation and proteolysis. Protein phosphorylation regulates many cellular processes by the action of protein kinases, in a mechanism that implies the transfer of phosphate groups from ATP to specific substrates (Manning et al., 2002). Their prominent roles in controlling parasite development and differentiation have been widely demonstrated in the study of schistosomes (Grevelding et al., 2018). In this species of trematodes, translocation of different groups of protein kinases from cytosol to membrane fractions has been related to transformation processes, as well as with motility and survival mechanisms of juvenile schistosomula (Wiest et al., 1992; Ressurreição et al., 2016; Grevelding et al., 2018). In the latter, the participation of host dopamine activating protein kinases in the early-stage schistosomules was demonstrated (Hirst et al., 2016). Interestingly, the up-regulated expression of a dopamine D2 receptor in the NEJTeg2 extract was shown in our study. In this sense, a dopamine D2 receptor with several potential phosphorylation sites for protein kinase has been related to parasite motility in *Schistosoma mansoni* (Taman and Ribeiro, 2009). We found in our study 13 and 3 up-regulated proteins related to phosphorylation processes, mainly kinases, in the tegument and somatic extracts from NEJ after contact with MPSIEC, respectively.

Phosphorylation over-expression has not been described in the study carried out by Cwiklinski et al. (2018), in which the authors analysed, among other aspects, the proteomic changes in the NEJ after 24 h of incubation in axenic culture medium (RPMI 1640 medium). This suggests that the up-regulated expression of protein kinases found in our study, which could be of importance in the development and differentiation of NEJ once they cross the intestine, would be induced only after the contact with the intestinal barrier of the host.

Proteolysis, as a result of parasitic secretion of molecules with protease activity, is considered as a key mechanism in order to ensure the invasion and survival of trematodes. Their pivotal role in a large number of host-parasite interactions supposes that some of these proteases have been considered promising targets for the development of novel chemotherapeutic drugs and vaccines against a number of trematodiases, including fasciolosis (Kasný et al., 2009). Within this type of molecules the cathepsin-like cysteine peptidases represent not only the most numerous group (> 80 % of the total protein secreted by adult flukes), but also the most studied for its important functions in virulence, infection, tissue migration and modulation of host innate and adaptive immune responses (Cwiklinski et al., 2019). Immunolocalisation studies of *F. hepatica* NEJ carried out by Cwiklinski et al. (2018) showed that cathepsin cysteine peptidases are localised within the somatic extracts of the parasite, specifically to the gastrodermis of the bifurcated gut, which would act as a reservoir of the proteolytic capacity of the parasite. Our results show a down-regulation of this type of proteins in both, somatic and tegumental parasitic extracts after the contact with MPSIEC. Therefore, we can hypothesize that interaction with host intestinal epithelial cells could cause the secretion of some parasitic cathepsins.

In addition, the expression associated with *Fasciola* development of these proteases has been correlated with the passage of the parasite through host tissues (Stack et al., 2011). Consequently, when the immature flukes enter the liver and feed on/migrate through the tissue, the secretion of FhCL3 and FhCB drops, while the percentage of other cathepsin L clade members (mainly FhCL1) within the secretome of the parasite become increases (Robinson et al., 2008). Interestingly, the results obtained in our study showed this shift in the regulation of cathepsin expression after the contact of the NEJ with MPSIEC (See Fig. 6). We observed a predominance of the cathepsin isotypes B, L3 and L4 in the NEJ-Teg 1 extract, which is substituted by an up-regulation of cathepsins L1 and L1D after co-culturing with MPSIEC [FhCL1_1 and FhCL_4 according to the terminology described by Cwiklinski et al. (2019)]. These results could suggest the importance of FhCB, FhCL3 and FhCL4 in the early stages of the parasite, playing important roles in excystment or gut penetration, as well as of the CL1
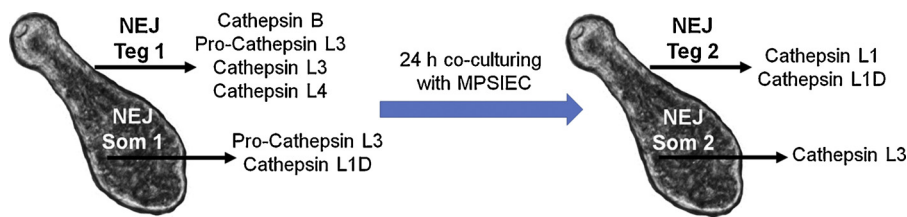
Fig. 6. Protein profile of the up-regulated cathepsin cysteine peptidases in the tegument and somatic extracts of the NEJ before and after co-culturing with the MPSIEC. Tegument extract from newly excysted juveniles, NEJ-Teg; somatic extract from newly excysted juveniles, NEJ-Som. In all extracts number 1 represents before co-culturing and number 2 represents after co-culturing.

clade in later stages (e.g. immature migratory flukes), as it has been reviewed by Cwiklinski et al. (2019). In their proteomic studies carried out with NEJ without the involvement of the host intestinal epithelial cells, Cwiklinski et al. (2018) found that FhCL3 proteases comprise the major components of the NEJ somatic proteins (including tegumental fraction), after 24 h of incubation in culture medium. This suggests that the interaction between the NEJ and the MPSIEC, and not only time after excystment, could be responsible for the triggering of the expression of a different protease profile in the parasite. This new cathepsin repertoire, including components typically associated with the adult stage of the parasite could be part of the secretome that the parasite requires for its proteolytic activity during its developmental/migratory phase, as well as to evade the host's defensive mechanisms in an early stage of the infection by the replacement of its cathepsin profile.

Secondly, we obtained MPSIEC lysates before and after stimulation with NEJ in order to identify the proteomic changes induced in the host intestinal epithelial cells upon contact with the parasite. We found 160 differentially regulated proteins from the MPSIEC cultures, representing approximately half of proteins whose expression had changed in the parasite's tegument extract. This is in line with a similar study carried out with the same cell type stimulated with larvae L3 of *Ascaris suum* in which authors are surprised to find such a low magnitude of response (indicated by fold-change values) and the lack of conclusive activation signatures (Ebner et al., 2018).

Among other processes, we found 43 and 21 up-regulated proteins in stimulated MPSIEC related to regulation of cellular processes and protein localization, respectively. Interestingly, we identified seven proteins belonging to the family of Rab proteins, within both groups. Rab family of proteins comprise Ras-like small GTPases with important roles in vesicle trafficking processes (Barr and Lambright, 2010). Furthermore, recently, their ability to regulate some processes related to innate immunity and inflammation by controlling the formation, transport and fusion of intracellular organelles has been described (Prashar et al., 2017). Similarly, we found an up-regulated ficolin-1 in the MPSIEC-Lys 2. Ficolins are innate pattern recognition receptors playing important roles within the innate immune response to numerous pathogens, including some parasites, via the recognition of pathogen-associated molecular patterns (PAMPs) (Cestari et al., 2013; Ren et al., 2014; Bidula et al., 2019). Taking into account that intestinal epithelial cells are considered as a first line of defense connecting pathogens and underlying immune cells in order to maintain intestinal homeostasis and promote host defense (Allaire et al., 2018), these up-regulated proteins suggest possible signaling mechanisms, which could be used by the host to trigger activation of the innate immune system through activity of the Rab pathway or ficolins, among others, as a response to parasite stimuli.

On the other hand, and interestingly, we found a decrease in the expression of the positive regulation of host immune system in the MPSIEC-Lys 2, including down-regulation of three proteins related to ubiquitination. Since surfaces of the mammalian intestine interact directly with the external environment, intestinal epithelial cells have evolved a number of strategies for controlling the invasion of infectious agents. In this regard, innate immunity is a highly effective first line of defence against pathogens (Hooper, 2015). The ubiquitin system have increasingly received attention for its important role in responding to

pathogen infection as a part of the host innate immune system, as well as its modulation by pathogenic antigens has recently pointed out in some bacteria responsible for intestinal diseases, such as *Escherichia coli*, *Salmonella* and *Shigella* (Bhoj and Chen, 2009; Zhou and Zhu, 2015; Li et al., 2016). Regarding *Fasciola* juveniles, it has been described that as soon as NEJ arrive at the intestinal wall, they secrete immunomodulatory molecules that influence the host innate response (Dalton et al., 2013). Consequently, the observed down-regulation of protein ubiquitination might be an immune evasion strategy carried out by NEJ in the early stage of infection.

Finally, parasite effects on the host intestinal barrier morphology could be related to 7 and 8 down-regulated proteins identified in the MPSIEC after contact with NEJ linked with cell adhesion and the regulation of the apoptotic processes, respectively. Cell-cell interactions, as part of cell adhesion mechanisms in intestinal epithelial cells are critical to maintain gastrointestinal homeostasis, especially during pathological conditions (Efstathiou and Pignatelli, 1998). Furthermore, disruption or reduced expression of cell adhesion proteins and loss of epithelial barrier function represents one of the main strategies used by intestinal parasites to invade host tissues (Di Genova and Tonelli, 2016). Our data showed a down-regulated expression of some cell adhesion proteins, such as polycystin 1, stabilin 1 and protein S100-A10 in MPSIEC-Lys 2. Among them, polycystin 1 has found to be part of a complex with the essential epithelial cell adhesion molecules E-cadherin and catenins (Huan and van Adelsberg, 1999). Intriguingly, parasite E-cadherin and catenin, as well as mouse polycystin 1 were up-regulated in NEJ-Teg extract after the contact with MPSIEC, suggesting a potential parasitic adhesion mechanism to the host intestinal barrier prior invasion. Regarding apoptotic processes, we found, among others, a down-regulated expression of host superoxide dismutase, a potent antioxidant that protects against oxidation-induced apoptosis (Briehl et al., 1997). Similarly, the induction of intestinal epithelial cell apoptosis by intestinal parasites such as *Giardia lamblia*, *Cryptosporidium sp.*, and *Entamoeba histolytica* have been showed as a mechanism contributing to tissue invasion (Di Genova and Tonelli, 2016).

In conclusion, "omics" technologies are helping advance our understanding of the host-parasite relationships in the study of fasciolosis (Cwiklinski and Dalton, 2018). Our data are in accordance with these studies showing the adaptation of NEJ to the host environment and the capacity for rapid evolution in terms of the number of differentially regulated proteins identified in the parasite extracts after the contact with the host epithelial barrier. The analysis of the biological processes in which these proteins are involved show different strategies allowing the parasite to develop and differentiate, some of them only triggered after communicating with its host, for invading its tissues or evading its defence mechanisms. In addition, the inclusion of the MPSIEC in our in vitro model allow us to know how the parasite stimuli could influence on the host intestinal epithelium functions, that are important for parasite invasion and survival, such as the modulation of the innate immune system, the disruption of cell-cell interactions or the regulation of apoptosis. Future studies aimed at unravelling these cross-talk relationships between the NEJ of *F. hepatica* and the host intestinal barrier could define new pharmacological or vaccine targets for the early elimination of fasciolosis, before the adult worms are established in its definitive location.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetpar.2020.109028.

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Capítulo 2:

Proteomics coupled with *in vitro* model to study the early crosstalk occurring between newly excysted juveniles of *Fasciola hepatica* and host intestinal cells

Resumen de la publicación

La fasciolosis es una enfermedad parasitaria originada por el trematodo *Fasciola hepatica*, que constituye una amenaza de primer nivel para la sanidad animal en todo el mundo, así como un problema de salud humana, especialmente en países en desarrollo. Aunque desde hace décadas se han caracterizado tanto el ciclo biológico del parásito como las respuestas de su hospedador definitivo a la infección, el conocimiento de la relación entre ambos organismos se limita principalmente a la fase crónica de la enfermedad, una vez que el verme se ha establecido en un entorno protegido. Por otra parte, la interacción parásito-hospedador temprana, incluyendo la respuesta del hospedador durante la migración que caracteriza la etapa inicial de la infección, y los mecanismos de adaptación del parásito, son poco conocidos.

En este sentido, las tecnologías -ómicas abren una puerta al estudio de fenómenos anteriormente inaccesibles, ya que proporcionan una gran cantidad de información al analizar el conjunto de biomoléculas de un tipo presentes en un contexto biológico determinado. En este trabajo, se empleó el modelo *in vitro* de interacción parásito-hospedador desarrollado en el primer capítulo de la presente Tesis Doctoral para realizar una estimulación corta (de 3 horas, similar al tiempo requerido por los vermes juveniles de *F. hepatica* para atravesar la pared intestinal) de ambos organismos, caracterizando sus respuestas a nivel molecular mediante la aproximación proteómica SWATH-MS (*Sequential Window Acquisition of All Theoretical Mass Spectra*).

Esta metodología permitió identificar un total de 38 proteínas sobreexpresadas y 172 subexpresadas por el parásito tras el contacto con el hospedador, involucradas principalmente en procesos de proteolisis, modulación de la actividad del citoesqueleto y respuesta al estrés. Por su parte, en las células de ratón se detectaron 36 proteínas sobreexpresadas y 97 subexpresadas tras dicho contacto, relacionadas con procesos de secreción, respuesta al estrés oxidativo o control de la expresión génica, y entre las que destaca un amplio grupo de proteínas ribosomales que vieron reducida su expresión. Estos resultados demuestran que una estimulación de tan solo 3 horas es capaz de desencadenar cambios de expresión relevantes para el resultado de la infección.
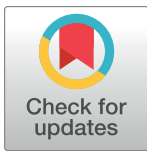
RESEARCH ARTICLE

Proteomics coupled with *in vitro* model to study the early crosstalk occurring between newly excysted juveniles of *Fasciola hepatica* and host intestinal cells

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Abstract

Fasciolosis caused by the trematode *Fasciola hepatica* is a zoonotic neglected disease affecting animals and humans worldwide. Infection occurs upon ingestion of aquatic plants or water contaminated with metacercariae. These release the newly excysted juveniles (FhNEJ) in the host duodenum, where they establish contact with the epithelium and cross the intestinal barrier to reach the peritoneum within 2–3 h after infection. Juveniles crawl up the peritoneum towards the liver, and migrate through the hepatic tissue before reaching their definitive location inside the major biliary ducts, where they mature into adult worms. Fasciolosis is treated with triclabendazole, although resistant isolates of the parasite are increasingly being reported. This, together with the limited efficacy of the assayed vaccines against this infection, poses fasciolosis as a veterinary and human health problem of growing concern. In this context, the study of early host-parasite interactions is of paramount importance for the definition of new targets for the treatment and prevention of fasciolosis. Here, we develop a new *in vitro* model that replicates the first interaction between FhNEJ and mouse primary small intestinal epithelial cells (MPSIEC). FhNEJ and MPSIEC were co-incubated for 3 h and protein extracts (tegument and soma of FhNEJ and membrane and cytosol of MPSIEC) were subjected to quantitative SWATH-MS proteomics and compared to respective controls (MPSIEC and FhNEJ left alone for 3h in culture medium) to evaluate protein expression changes in both the parasite and the host. Results show that the interaction between FhNEJ and MPSIEC triggers a rapid protein expression change of FhNEJ in response to the host epithelial barrier, including cathepsins L3 and L4 and several immunoregulatory proteins. Regarding MPSIEC, stimulation with FhNEJ results in alterations in the protein profile related to immunomodulation and cell-cell interactions, together with a drastic reduction in the expression of proteins linked with ribosome function. The molecules identified in this model of early host-parasite interactions could help define new tools against fasciolosis.

Author summary

Fasciolosis caused by *Fasciola hepatica* poses a serious concern for animal and human health worldwide. Treatment and prevention of this disease is challenging due to the appearance of parasites that are resistant to the current treatment and the suboptimal efficacy of assayed vaccines. In this context, the importance of early interactions with the host for parasite migration and infection progression prompted us to develop and study new models of interaction between *F. hepatica* juveniles and their host. Here, an *in vitro* model coupled with a quantitative proteomics approach was developed to evaluate the changes in *F. hepatica* newly excysted juveniles right after their contact with intestinal epithelial cells, which represent the first line of host cells that the parasite encounters upon host infection. Additionally, this model has been used to decipher potential changes elicited by the parasite in the host intestine. A proteomic study of parasites and host cells upon co-incubation revealed specific changes in the repertoire of proteins from both host and parasite following their interaction. These proteins could be of importance for parasite migration and survival inside the host, and give new important insights to understand the host-parasite molecular crosstalk in early fasciolosis.

Introduction

Early host-parasite interactions are of paramount importance in the context of helminth infections as these mechanisms occur during the first contact between both organisms and the resulting molecular crosstalk determines the overall success of the parasite invasion process [1]. From a clinical point of view, knowledge of this initial infection stage is essential for diagnosis and treatment, as an early detection of infection would allow for the disease to be managed more effectively and before the parasite is able to trigger immune evasion mechanisms that favour its establishment in a niche that is poorly accessible to the host immune response. *In vitro* models to dissect the early infection stages of parasitic infections constitute a valuable tool that has been successfully implemented for the study of different endoparasites of medical and veterinary interest [2,3]. Moreover, the usefulness of these models has been substantially enhanced by the introduction of proteomic and transcriptomic tools [4], which facilitate the analysis of the host-parasite molecular repertoire and offer a powerful resource for the identification of therapeutic targets.

Fasciola hepatica is the causative agent of fasciolosis, a foodborne parasitic disease responsible for enormous economic losses in the livestock industry (mainly affecting large-size herbivorous species) of around $3 billion per year [5], as well as a major human health problem in several endemic regions around the planet [6,7]. The first contact with the definitive host takes place in the small intestine after ingestion of food or water contaminated with metacercariae, which excyst in the duodenum and release the newly excysted juveniles (FhNEJ). These are able to cross the intestinal wall and reach the peritoneum in approximately 3 h, and from this point continue their migratory cycle towards the liver [8,9]. Although the migratory phases that appear after crossing of the intestinal wall and which are responsible for the acute phase of the disease have attracted significant research interest over the past decades, parasite interaction with the intestinal epithelium itself has received little attention and, in fact, the body of knowledge about the molecular responses triggered by the interaction between FhNEJ and the intestinal epithelium is scarce [10]. In this context, molecular characterisation of host-parasite

interactions at the intestinal level could provide new biomarkers for early diagnosis and treatment of fasciolosis as well as potential vaccine candidates against this disease [11].

Several experimental models have been developed over the past decades to study the passage through the intestinal wall of FhNEJ based on reproducing this migration step by using sections of the rat jejunum under *ex vivo* [12] and *in vitro* conditions [13]. Although these studies provided valuable information on the path followed by *F. hepatica* juveniles during migration, they did not emphasise the molecular events that occur in both the parasite and the host in response to their first interaction. Previously, our group developed an *in vitro* host-parasite interaction model aimed at reproducing the contact of FhNEJ with the host's intestinal epithelium by co-culturing these parasites with mouse primary small intestinal epithelial cells (MPSIEC). In this model, proteomic changes induced by host-parasite interactions within the first 24 h of contact were assessed using Isobaric Tag for Relative and Absolute Quantitation (iTRAQ) [14].

In the present study, we employed this *in vitro* system to address protein expression changes in both FhNEJ and MPSIEC after a short and biologically-relevant interaction time of 3 h, followed by proteomic analyses of all samples using a new era quantitative proteomic approach known as Sequential Window Acquisition of All Theoretical Mass Spectra (SWATH-MS). This approach comprises a label-free identification and quantification methodology, which allows for improved accuracy and reproducibility [15] by computing mass spectra in multiple windows of a few *m/z* units. The ultimate goal is to be able to identify the proteins playing a key role in the first host-parasite contact in fasciolosis, as well as exploring the potential molecular interactions and relevant biological processes involved in the early phase of the infection.

Materials and methods

Primary intestinal epithelial cell culture

Mouse small intestinal epithelial cells (C57BL/6 MPSIEC) from Cell Biologics (ref. C57-6051) were grown and expanded as previously described [14]. Briefly, cells were plated in petri dishes precoated with 0.2% porcine gelatine, and cultured in epithelial cell growth medium (Innoprot) at 37°C in a humidified atmosphere in the presence of 5% CO_2. Medium was replaced every 24–48 h and when confluence was reached (approximately every 48 h), cells were split in a 1:3 ratio.

F. hepatica metacercariae *in vitro* excystment

F. hepatica metacercariae (Italian strain) were purchased from Ridgeway Research LTD (UK) and *in vitro* excystment was performed as previously described [16]. In brief, CO_2 was bubbled for 30 sec in a tube containing 10 ml of distilled water, and sodium hydrosulphite (Sigma) was added to a final concentration of 0.02 M. This solution was added to metacercariae and incubated for 1 h at 37°C. Then, the metacercariae were washed twice with warm distilled water, resuspended in 5 ml of Hank's balanced salt solution (Sigma) supplemented with 0.03 M HEPES (Sigma) and 10% lamb bile (from a local abattoir) and incubated at 37°C for 4–5 h. FhNEJ were manually collected with a micropipette, washed twice with sterile PBS and immediately incubated with host cells.

In vitro interaction model

The *in vitro* interaction model used in this study was previously described by González-Miguel *et al.* [14], with some modifications. Briefly, MPSIEC on passage 5 were cultured in 60 mm

gelatin-coated plates (n = 3) until cell confluence was reached, and they were stimulated with 400 FhNEJ per plate. The co-culture was incubated for 3 h at 37°C in a 5% CO_2 atmosphere, after which MPSIEC and FhNEJ were separated by successive washes with sterile PBS. Additionally, 3 batches of 400 FhNEJ and 3 confluent plates of passage 5 MPSIEC were incubated under the same conditions without stimulation, consisting of MPSIEC and FhNEJ left alone for 3 h in the same experimental conditions that co-cultured cells and FhNEJ, and were used as negative controls. Number of cells (3.2 x 10^6 intestinal epithelial cells per plate) and number of FhNEJ for *in vitro* stimulation experiments was settled to maximize the chances to have the majority of cells in contact with the FhNEJ and thus properly stimulated and to obtain enough stimulated host and parasite material for downstream analyses, although this specific host cells/parasite ratio could not reflect the *in vivo* situation.

Protein extraction

All FhNEJ samples were treated as described by García-Campos *et al.* [17] for isolation of the tegument and somatic fractions. This protocol is based on the procedure described earlier in [18], with minor modifications, in which the tegumental nature of the antigens extracted from *F. hepatica* intact worms was tested by the peroxidase-antiperoxidase immunocytochemical technique at the light microscope level [18]. Thus, and although a complete separation of tegument and somatic FhNEJ extracts is expected to be difficult to achieve due to technical constraints, enriched protein fractions of the tegument can be obtained as described by other authors [17, 18]. FhNEJ were washed twice with sterile PBS, resuspended in an appropriate volume of PBS + 1% Nonidet P-40 (Sigma) and incubated at room temperature with gentle stirring for 30 min in order to extract the tegument fraction. After incubation, the samples were centrifuged (300 *g*, 5 min) and the supernatant of the detergent-soluble extract containing the enriched protein fraction of the FhNEJ tegument was transferred to clean tubes. The pellet containing naked FhNEJ was resuspended in RIPA buffer (Sigma) and disrupted by ultrasound (5 cycles of 30 s). Finally, samples were centrifuged (1,000 *g*, 5 min) and the clarified supernatant containing somatic proteins was transferred to clean tubes. All *F. hepatica* samples were treated with protease inhibitors and stored at -80°C until use.

Cell culture plates containing MPSIEC were washed twice with sterile PBS and incubated on ice for 10 min to weaken cell adhesion. Cells were carefully lifted from the plate using a cell scraper, collected with a pipette tip and deposited into 1.5 ml tubes. Cytosolic and membrane proteins were separately isolated using the Mem-PER Plus Membrane Protein Extraction kit (Thermo Fisher) following the manufacturer's instructions. Protease inhibitors were added at the appropriate ratio and the samples were stored at -80°C until use.

Proteomic analysis

Quantitation of all protein samples was performed using a detergent-compatible kit (Protein Quantification Assay; Machery-Nagel) following the manufacturer's instructions. In-gel digestion of proteins was carried out in accordance to the described protocols [19] with the following modifications: 20 μg of each sample were dissolved in 20 μl of Laemmli Sample Buffer (Bio-Rad) supplemented with β-mercaptoethanol and heated for 5 min at 95°C, after which they were loaded onto an Any kD precast 1D PAGE gel (Bio-Rad) and run at 200 V for 5 min. After separation, samples were fixed with 40% ethanol/10% acetic acid and stained with colloidal Coomasie.

Gel bands were cut into pieces and reduction of peptides was performed using 10 mM dithiothreitol (DTT) in 50 mM of ammonium bicarbonate (20 min at 60°C, 850 rpm), and for alkylation 55 mM iodoacetamide in 50 mM ammonium bicarbonate (30 min at room

temperature in the dark, 850 rpm) were used. Trypsin digestion was performed using a 1:25 ratio (ng enzyme/μg protein) in 50 mM ammonium bicarbonate (overnight at 37˚C with no stirring), and subsequent extraction of peptides was carried out using pure acetonitrile, which was dried in a rotary evaporator at 37˚C. Finally, peptides were resuspended in 2% ACN, 0.1% TFA (5 min at room temperature, 850 rpm). For library construction, all *F. hepatica* samples were pooled together and loaded onto an analytical column (LC Column, 3 μ C18-CL, Nikkyo) equilibrated in 5% ACN/0.1% formic acid. Elution was performed over 120 min in a linear gradient of 5–35% solvent B (A: 0.1% FA; B: ACN, 0.1% FA) at a flow rate of 300 nl/min and analysed in a mass spectrometer nanoESI qQTOF (5600 TripleTOF, ABSCIEX).

Analysis was carried out in a data-dependent mode (DDA). Survey MS1 scans were acquired from 350–1250 *m/z* for 250 ms, whereas the quadrupole resolution was set to 'UNIT' for MS2 experiments, which were acquired 100–1500 *m/z* for 150 ms in high sensitivity mode. *Mus musculus* samples were treated using the same workflow in order to generate a mouse-specific peptide library. Individual samples were acquired with the tripleTOF operating in SWATH mode (DIA), in which a 0.050-s TOF MS scan from 350–1250 *m/z* was performed, followed by 0.080-s product ion scans from 350–1250 *m/z* on the 32 defined windows (3.05 sec/cycle).

Top 100 were used in DDA mode. In DIA mode, the quadrupole was operated using 'Unit' resolution, using 15 Da windows with 1 Da overlap between windows. Collision energy was determined using the following equation: CE = (Slope) * x * (*m/z*) + intercept. Slope = 0.625 and intercept = -3 coefficients were used, and all peptides were fragmented as z = 2. MS/MS scans were performed using a resolution of 15000, and no precursors were selected, as they all entered the collision chamber and were fragmented as z = 2 charge. No technical replicates were used. Instead of that, reproducibility was evaluated with a quality control of 3 injections of 500 ng K562.

The *.wiff* files corresponding to the peptide libraries were processed via Protein Pilot v5.0 (SCIEX) to generate a peak list used as reference for individual sample quantitation. The Paragon algorithm [20] was applied to reference databases with the following parameters: trypsin specificity, iodoacetamide cys-alkylation and taxonomy not restricted. Only proteins with at least 2 identified peptides and < 1% FDR were considered for subsequent analysis. The databases used in this study (downloaded the 25/02/2020) contained the predicted proteome of *F. hepatica* (PRJEB25283, https://parasite.wormbase.org/Fasciola_hepatica_prjeb25283/Info/ Index) or the proteome of *Mus musculus* (https://www.uniprot.org/proteomes/UP000000589), both appended to the cRAP contaminant database (https://www.thegpm.org/crap/).

The *.wiff* files obtained from SWATH individual acquisitions were analysed using PeakView 2.1 (SCIEX) and MarkerView 3.0 (SCIEX). Protein areas were calculated and normalized to the total sum of the areas of all the quantified proteins, and proteins identified within the contaminant database were removed from the dataset prior to the differential expression analyses.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [21] partner repository with the dataset identifier PXD033952.

The proportion of proteins showing transmembrane domains in the proteins identified in both the membrane and the cytosol fractions of MPSIEC was evaluated with the DeepTMHMM tool (https://dtu.biolib.com/DeepTMHMM). Additionally, the presence/ absence of the membrane marker COX-IV protein was assessed in both extracts.

Statistical and computational analysis

Log2 of each detected protein was determined prior to quantitative analysis. Statistical treatment was performed using GraphPad Prism 9.0.1, and evaluation of differences between

stimulated and control samples was performed using *Student's t-test* coupled with Benjamini, Krieger and Yekutieli *post-hoc* corrections. Only proteins with a q-value <0.05 were considered as differentially expressed. Principal Component Analysis (PCA) of all samples was performed with the online tool ClustVis [22], and the Volcano plots showing the distribution of up- and down-regulated proteins were obtained using the *ggplot2* package of R software.

Functional annotation of the differentially expressed proteins was performed with Blast2GO 5.2 using the blastp and InterProScan algorithms to obtain protein descriptions against the NCBI and EBI databases, respectively. Gene Ontology (GO) terms in the Biological Process (BP), Molecular Function (MF) and Cellular Component (CC) categories were computed, and GOs obtained by both algorithms were merged.

GO terms within the BP and MF categories were parent removed and represented using the ReViGO tool (http://revigo.irb.hr/), showing the size of each term in proportion to its Node-score. Differentially expressed proteins from MPSIEC samples were represented in interaction networks using STRING (https://string-db.org/).

Results and discussion

Prior to invading host tissues, FhNEJ must interact with cells of the intestinal epithelium, which comprise the first physical barrier and the first line of innate immune defence encountered by the parasite [23]. Consequently, in this study we aimed to reproduce the physical and biological interactions between the juvenile worms of *F. hepatica* and the cells of the intestinal epithelium in an *in vitro* model, and study the proteomic changes induced during the first 3 h of interaction to shed light on host-parasite relationships occurring before the 'point of no return' in fasciolosis is reached.

The experimental design that we proposed is based on co-culturing FhNEJ with MPSIEC for 3 h at 37°C, a time frame that is similar to that required by FhNEJ to migrate through the host intestinal wall in a physiological setting (Fig 1). After establishing contact with the mouse intestinal cell monolayer, FhNEJ actively displayed their typical locomotion process, which is based on a smooth sequence of whole-body waves coordinated with alternate attachment and release of the oral and ventral suckers and a vermiform peristalsis [24,25] (Fig 1, S1 Video). After 3 h of co-culture, both FhNEJ and epithelial cells were separated and subjected to protein extraction from two compartments: detergent-soluble extract enriched with tegument and soma in the case of FhNEJ, and cytosol and membrane for mouse cells. Thereby, we aimed to isolate the proteins comprising the host-parasite interface. It is worth mentioning that the tegument of *F. hepatica* includes a biologically active and metabolically complex matrix that is continuously exposed to host tissues, whose dynamic composition is directly related to the changing environment that the juvenile parasite encounters during its migration process [26–28]. For that reason, the FhNEJ tegument has been proposed as a potential source of therapeutic targets against fasciolosis [10]. Nevertheless, it should be mentioned that our detergent-soluble extract could contain components of the FhNEJ tegument, together with components of other areas of FhNEJ that could be exposed to the extraction protocol.

Protein identification and quantification

After establishing the *in vitro* interaction model, we applied a SWATH-MS-based proteomic approach [29] in order to identify protein expression changes in both the parasite and host cells upon their interaction. Despite its robustness in comparing protein abundance of different samples, this technique has been infrequently used in parasitology research. In our study, the application of SWATH-MS analysis resulted in a total of 6,169 and 24,965 spectra identified in FhNEJ and MPSIEC samples, respectively, corresponding to 541 and 2,012 proteins
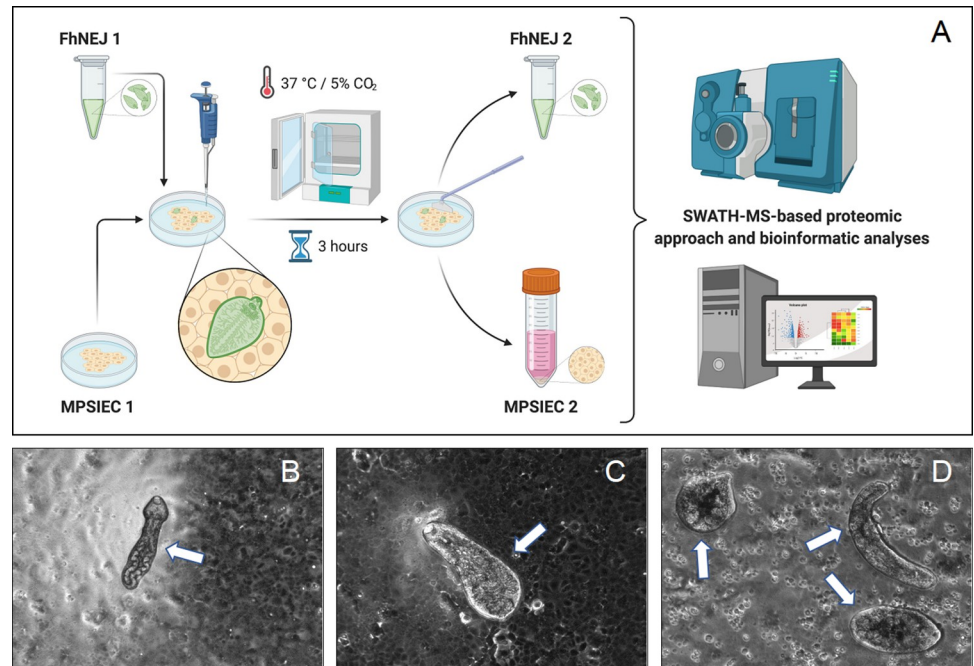
Fig 1. Experimental design of the *in vitro* interaction model between *F. hepatica* juveniles (FhNEJ) and mouse intestinal epithelial cells (MPSIEC). (A) Parasitic extracts and host cell lysates were obtained before (FhNEJ 1, MPSIEC 1) and after co-culturing for 3 h (FhNEJ 2, MPSIEC 2) and their proteins analyzed by SWATH-MS. (B, C, D) Representative images of MPSIEC (at the background of each image) stimulated with the FhNEJ (arrows) are shown (optical microscope, 10x). Panel A created with BioRender.com.

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detected with at least 2 peptides using an FDR below 1%. From the identified proteins, 505 and 427 were present in all replicates from the detergent-soluble extract enriched with tegument and soma extract of FhNEJ, respectively, and 1,497 and 1,508 proteins were found in all replicates from the cytosol and membrane extracts of MPSIEC, and could therefore be quantified. Common contaminant proteins represented 31 and 33 of the proteins identified in the tegument and soma of FhNEJ, respectively, and 134 and 177 contaminant proteins, respectively, were detected in the cytosol and membrane of MPSIEC and were excluded from further statistical analysis. After contaminant removal, a total of 474 and 394 common proteins were identified in all FhNEJ tegument and soma samples, respectively. This represents a 4.87% and 4.05% of the predicted *F. hepatica* proteome (9732 proteins). As for mouse, 1363 were identified in all cytosol samples whereas 1331 proteins were listed in the membrane fraction, which represents a 2.46% and a 2.41% of the *Mus musculus* theoretical proteome (55342 proteins).

The identified proteins were defined as variables in PCA analyses, in which FhNEJ stimulated samples formed compact clusters, whereas the rest of the samples displayed a less optimal clustering (Fig 2). However, separation among stimulated and control samples was clear in all cases, especially along the horizontal axis represented by the first principal component which constitutes the main source of variability, depicting that the stimulation of only 3 h is enough to trigger changes at the proteomic level in all studied compartments. This analysis suggests that FhNEJ undergo an orchestrated pattern of protein expression with a low degree of variability after establishing contact with MPSIEC. Such response would be important in the tegument of *F. hepatica* provided its dynamic composition and continuous adaptation to the changing demands of the juvenile parasite during its migration and developmental process [28]. On the other hand, changes in MPSIEC showed a higher intragroup variation compared
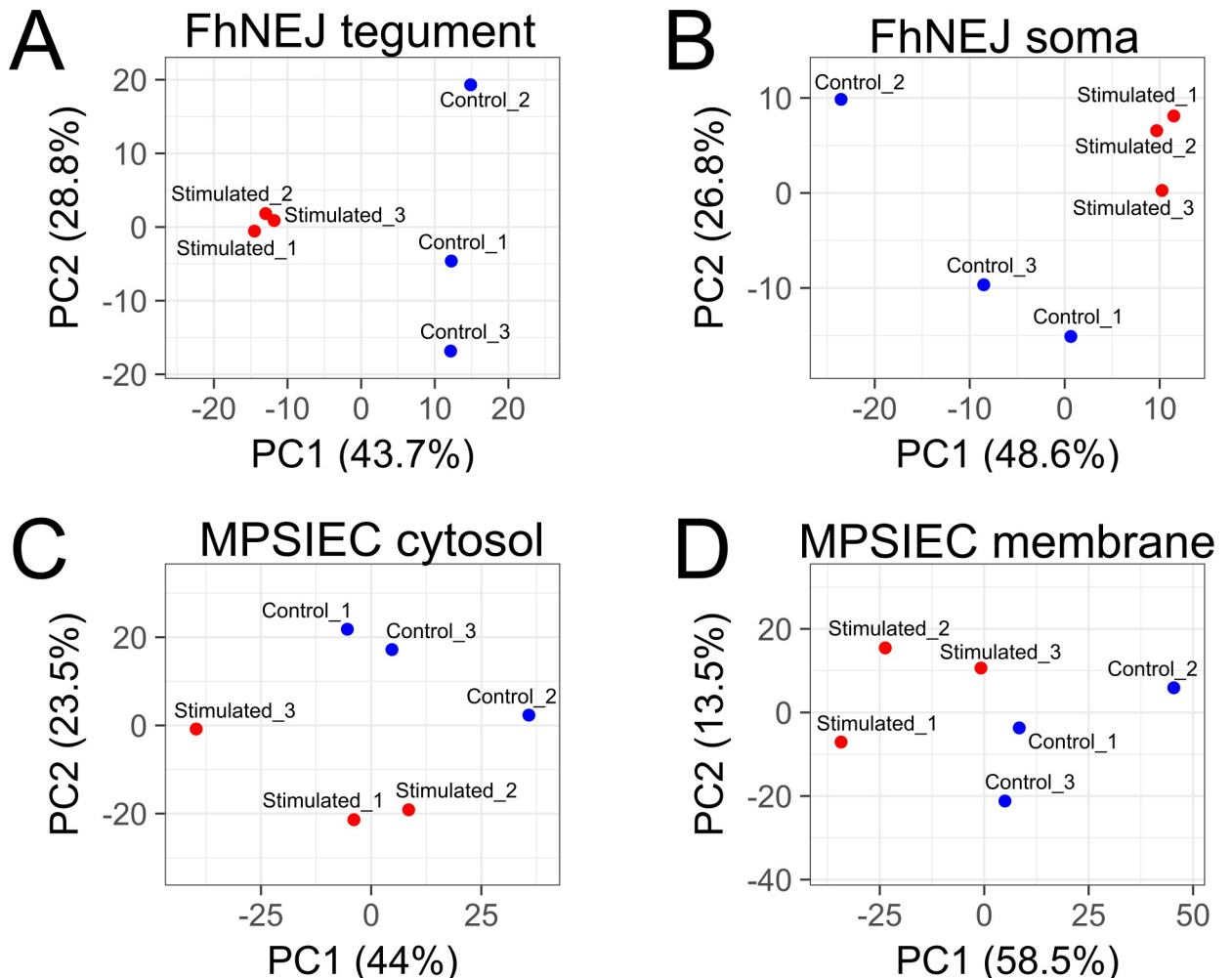
Fig 2. Principal Component Analysis (PCA) of each group of samples analysed in the study. PCA analyses of FhNEJ detergent-soluble extract enriched with tegument (FhNEJ tegument; A), FhNEJ somatic (B), MPSIEC cytosol (C) and MPSIEC membrane (D) protein extracts. Blue dots represent the three control replicates (incubated alone for 3h; control) whereas red dots represent the three replicates co-incubated for 3h (stimulated). The percentage of variance explained by each principal component (PC) is indicated on its corresponding axis.

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with the compartments analyzed in FhNEJ, similar to what has been described in comparable studies [30].

The presence of transmembrane (TM) domains in proteins identified in the two fractions (membrane and cytosol) of MPSIEC was assessed in order to determine the effectiveness of the enrichment procedure of the two obtained fractions. Seventy-four cytosolic proteins were predicted to contain at least one TM domain in their structure, which represents a 5.43% of the proteins identified in this fraction, while 170 proteins within the membrane fraction were predicted to contain TM domains, representing a 12.77% of the identified proteins, a proportion similar to other studies in which membrane-enriched fractions of mammalian cells extracts were obtained (e.g., [31]). Additionally, the protein COX-IV (Uniprot ID P19783), used to prove the enrichment of membrane proteins (e.g., [32]) was detected only in the membrane fraction and not in the cytosol extract. This indicates the success of the enrichment procedure.

Differentially expressed proteins (DEPs) were assessed through Student's t-test, and only DEPs with a q-value <0.05 were used for further analysis. Since fold change cutoffs are
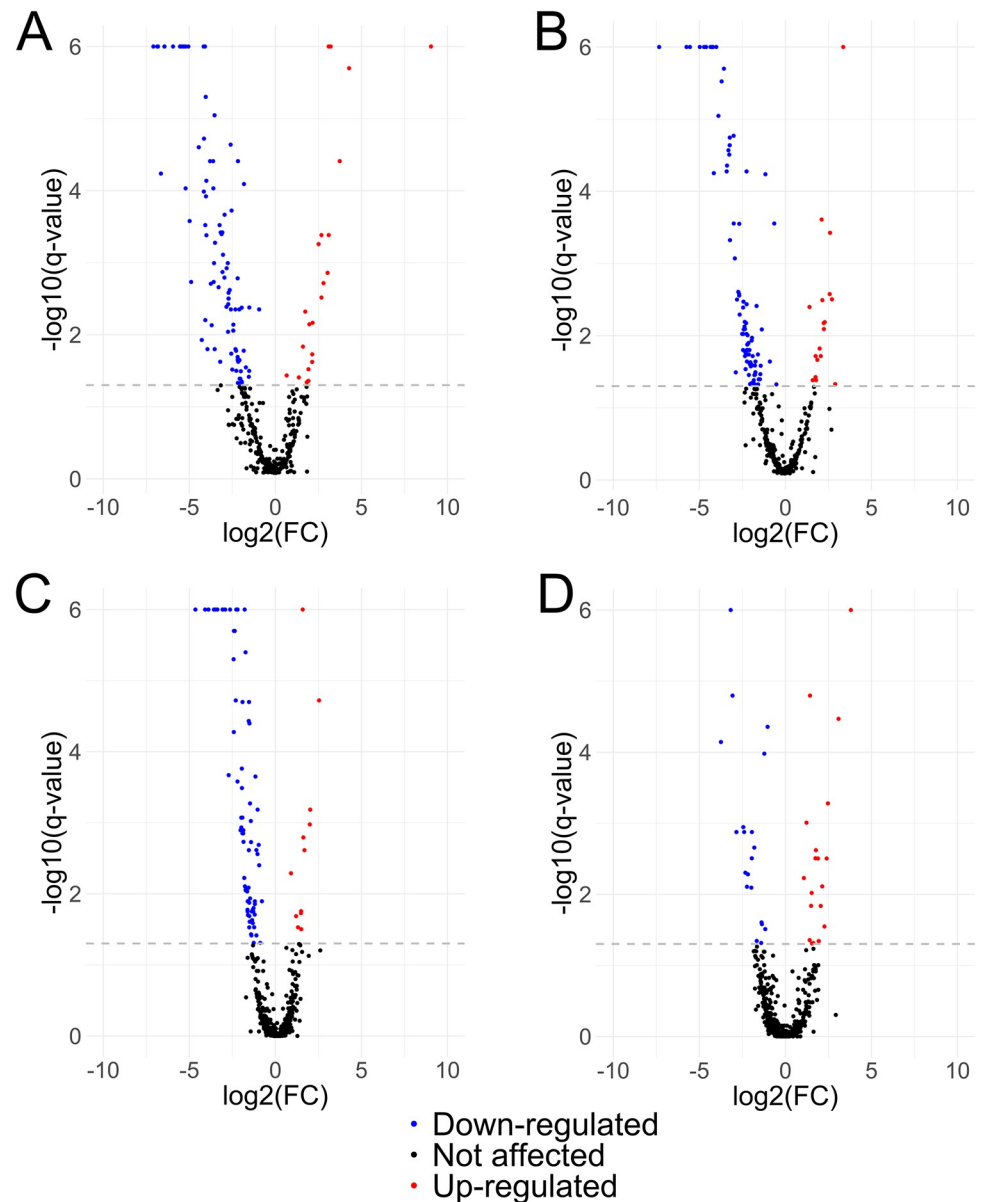
Fig 3. Volcano plots representing the changes in the proteomic profile of each group of samples analysed in the study. Volcano plots corresponding to FhNEJ detergent-soluble extract enriched with tegument (FhNEJ tegument; A), FhNEJ somatic (B), MPSIEC cytosol (C) and MPSIEC membrane (D) protein extracts. Red dots represent up-regulated proteins after stimulation challenge, and blue dots represent down-regulated proteins. Only DEPs with q-value <0.05 were considered as differentially expressed, and no fold change threshold was considered.

arbitrary and depend largely on the dynamic range of each technique, significance of differentially abundant proteins was selected based only on q-value. A total of 109 DEPs meeting the above-mentioned criteria were found in the detergent-soluble extract enriched with tegument of FhNEJ (21 up- and 88 down-regulated) (Fig 3A), while 101 DEPs (17 up- and 84 down-regulated) were identified in the somatic extract (Fig 3B). Concerning MPSIEC extracts, a total of 93 DEPs (16 up- and 77 down-regulated) were identified among cytosolic proteins (Fig 3C), and 40 DEPs (20 up- and 20 down-regulated) were found in the membrane (Fig 3D). In sum, the number of DEPs from MPSIEC is 1.5 times lower than from FhNEJ, providing a similar

FhNEJ Tegument extract

109 DEP
21 Up-regulated
88 Down-regulated

Description	Log2FC
Cathepsin L	9.04
Histone H4	4.28
cathepsin L3 peptidase	3.74
UDP-glucuronic acid decarboxylase	3.21
Histone deacetylase	3.1
glutathione S-transferase	3.09
histone H2B	3.03
Peptidase D	2.8
Mu-class gst glutathione S-transferase	2.68
Tegumental antigen	2.68
hypothetical protein D915_003049	2.51
Tegumental calcium-binding EF-hand protein 4	2.16
Phosphoglycerate mutase	2.14
serpin 2	2.14
Cytochrome c oxidase subunit IV	1.97
actin, putative	-5.06
Cytosolic large ribosomal subunit protein L30	-5.21
hypothetical protein D915_005536	-5.22
ubiquinol-cytochrome c reductase cytochrome c1 subunit	-5.3
hypothetical protein D915_004677	-5.37
peptidyl-prolyl cis-trans isomerase FKBP1B	-5.45
Chaperonin-containing T-complex protein 1 eta subunit	-5.51
Heterogeneous nuclear ribonucleoprotein A2	-5.54
thioredoxin-glutathione reductase	-5.95
Programmed cell death protein 6	-6.44
hypothetical protein D915_010660	-6.45
putative smirv1 protein	-6.65
putative serine/threonine-protein kinase STE20	-6.82
Troponin i	-6.87
CD59 molecule complement regulatory protein	-7.09

FhNEJ Somatic extract

101 DEP
17 Up-regulated
84 Down-regulated

Description	Log2FC
putative ATP-dependent 26s proteasome regulatory subunit	3.36
Delta-1-pyrroline-5-carboxylate synthetase	2.89
hypothetical protein PHET_02717	2.59
cathepsin L3 peptidase	2.57
T-complex protein 1 subunit gamma	2.29
Dipeptidyl peptidase	2.23
Mitogen-activated protein kinase	2.22
probable dynein light chain	2.15
Tetraspanin	2.1
Collagen alpha-1(IV) chain	2.05
Phosphoglycerate mutase	1.99
serpin 4	1.86
hypothetical protein D915_008568	1.78
Ras-related protein Rap-1A	1.75
Mu-class gst glutathione S-transferase	1.75
Fatty acid-binding protein type 2	-3.26
Mitochondrial-associated endoribonuclease MAR1	-3.31
hypothetical protein D915_000531	-3.39
Cytosolic large ribosomal subunit protein L30	-3.41
40S ribosomal protein S8	-3.71
Annexin	-3.89
Dihydrolipoamide acetyltransferase	-4.02
Spliceosome rna helicase bat1	-4.16
Uracil-DNA glycosylase	-4.21
putative vacuolar protein sorting 26 vps26	-4.26
CD59 molecule complement regulatory protein	-4.61
Programmed cell death protein 6	-4.73
Tubulin alpha-1C chain	-4.98
Heterogeneous nuclear ribonucleoprotein A2	-5.75
Troponin i	-7.34

Fig 4. Differentially expressed proteins in FhNEJ after incubation with MPSIEC. The first 15 proteins with the highest (represented in red) or lowest (represented in blue) fold change within the FhNEJ detergent-soluble extract enriched with tegument (FhNEJ Tegument extract) and somatic extract (FhNEJ Somatic extract) are shown. Icons created with BioRender.com.

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proportion to that obtained in our previous *in vitro* interaction model after 24 h co-culture of FhNEJ with MPSIEC [14]. This ratio is also in line with a similar study in which dual-species RNA-Seq analysis of porcine intestinal epithelial cells upon co-culture with L3 larvae of *Ascaris suum* showed a low magnitude response by host cells after parasitic stimulation [3].

Our data also showed an imbalance towards down-regulated proteins, especially concerning FhNEJ extracts upon MPSIEC co-culture, which has been observed in similar studies (e.g., [33, 34]). This fact suggests that silencing of protein expression could be faster than its induction in FhNEJ after a short time of stimulation with host tissues, which could also be related to the turnover and/or shedding mechanisms that take place on the FhNEJ surface shortly after excystment as part of their immune evasion strategies [35, 36].

Up and down-regulated proteins in FhNEJ and MPSIEC were identified by database search and bioinformatic analysis (S1 Table for FhNEJ and S2 Table for MPSIEC). The top 15 regulated proteins with highest fold changes are shown in Figs 4 (FhNEJ) and 5 (MPSIEC).

Functional annotation

FhNEJ protein expression changes rapidly in response to interaction with the host intestinal epithelial barrier. In the detergent-soluble extract enriched with tegument of FhNEJ,

MPSIEC Cytosol extract

93 DEP
16 Up-regulated
77 Down-regulated

Description	Log2FC
Acyl-Coenzyme A dehydrogenase family, member 9	2.54
cadherin-3 isoform a preproprotein	2.02
interferon-induced transmembrane protein 3	2
signal recognition particle 9 kDa protein	1.69
1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase	1.62
actin-related protein 2/3 complex subunit 4	1.59
atlastin-2 isoform 2	1.5
Mitochondrial ribosomal protein L12	1.49
coatomer subunit gamma-2	1.49
solute carrier family 2, facilitated glucose transporter	1.45
catenin alpha-1 isoform X1	1.42
lanosterol synthase	1.37
CUGBP Elav-like family member 1 isoform X5	1.31
transferrin receptor protein 1	1.2
Hspa1b protein	0.9
ribonuclease inhibitor isoform X2	-2.36
protein cordon-bleu isoform 2	-2.41
Peroxiredoxin 1	-2.42
Ubiquitin-conjugating enzyme E2 variant 2	-2.43
dynein light chain 1, cytoplasmic	-2.64
tubulin alpha-1C chain	-2.71
40S ribosomal protein S6	-2.92
60S ribosomal protein L37a	-3.06
methionine aminopeptidase 2	-3.07
protein CDV3 homolog isoform X2	-3.37
NAD(P)H dehydrogenase [quinone] 1	-3.47
40S ribosomal protein S11	-3.57
prostaglandin E synthase 3 isoform X3	-3.89
RL36A protein	-4.09
elongin-C	-4.65

MPSIEC Membrane extract

40 DEP
20 Up-regulated
20 Down-regulated

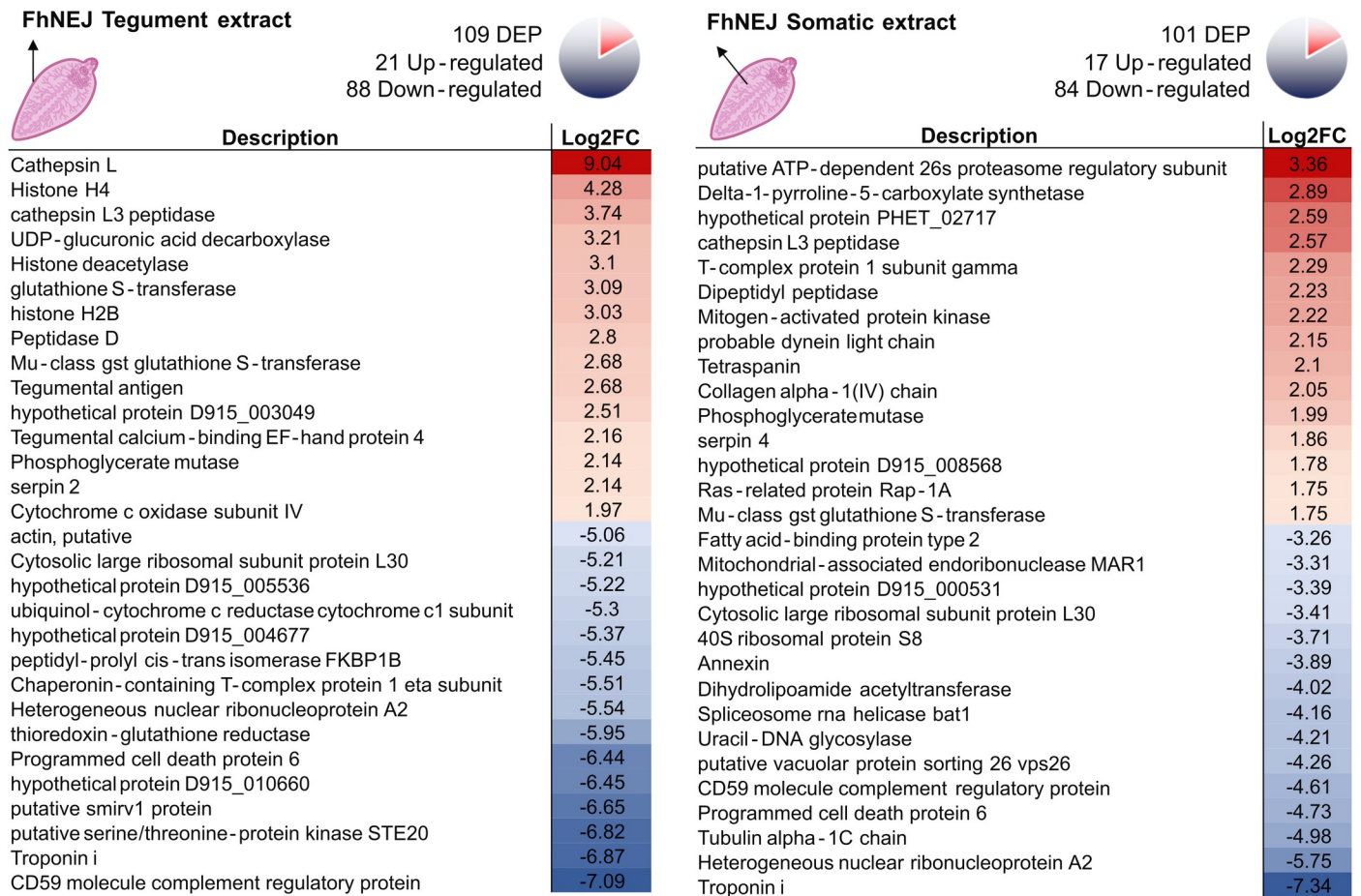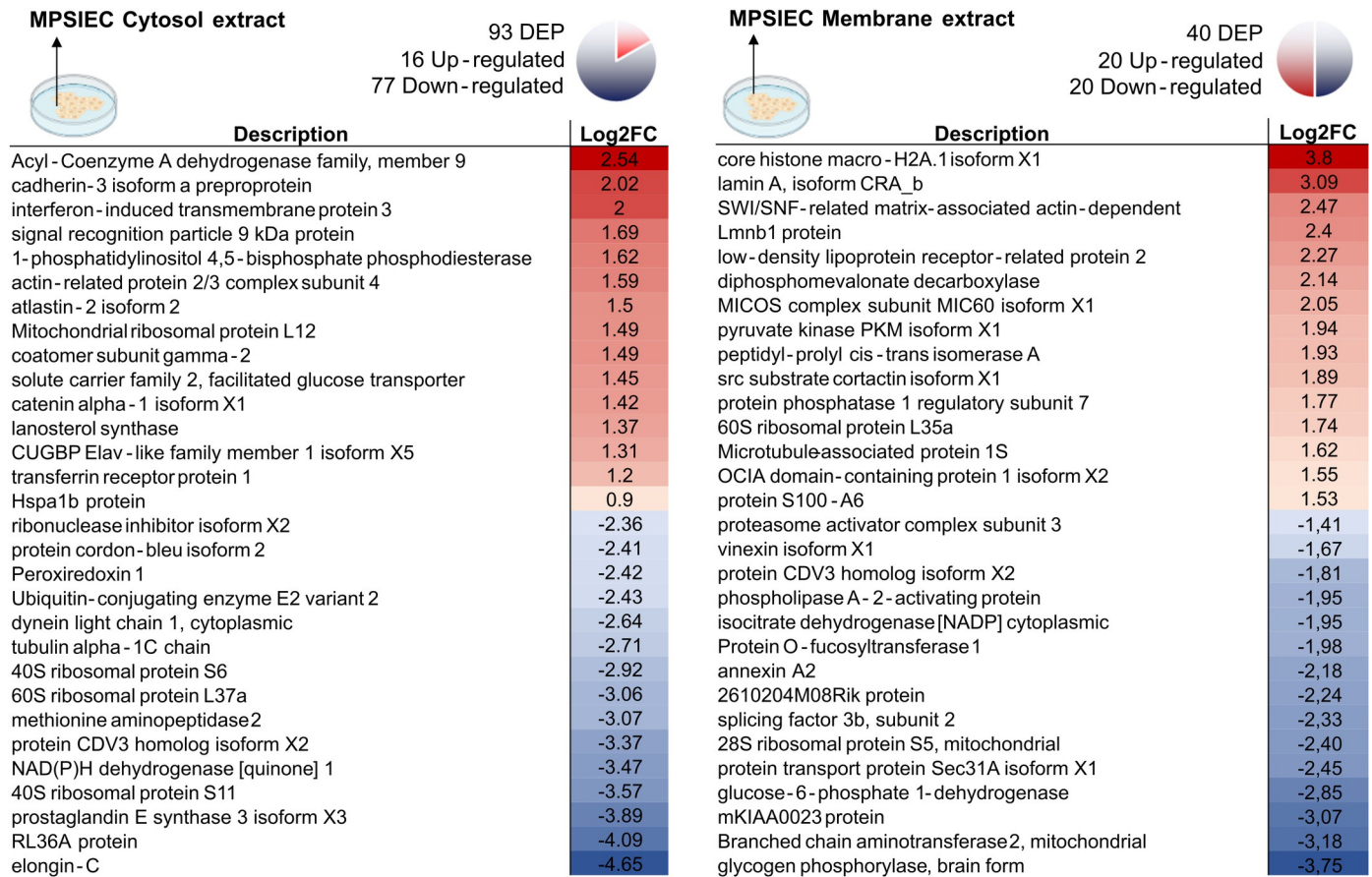| Description	Log2FC
core histone macro-H2A.1 isoform X1	3.8
lamin A, isoform CRA_b	3.09
SWI/SNF-related matrix-associated actin-dependent	2.47
Lmnb1 protein	2.4
low-density lipoprotein receptor-related protein 2	2.27
diphosphomevalonate decarboxylase	2.14
MICOS complex subunit MIC60 isoform X1	2.05
pyruvate kinase PKM isoform X1	1.94
peptidyl-prolyl cis-trans isomerase A	1.93
src substrate cortactin isoform X1	1.89
protein phosphatase 1 regulatory subunit 7	1.77
60S ribosomal protein L35a	1.74
Microtubule-associated protein 1S	1.62
OCIA domain-containing protein 1 isoform X2	1.55
protein S100-A6	1.53
proteasome activator complex subunit 3	-1,41
vinexin isoform X1	-1,67
protein CDV3 homolog isoform X2	-1,81
phospholipase A-2-activating protein	-1,95
isocitrate dehydrogenase [NADP] cytoplasmic	-1,95
Protein O-fucosyltransferase 1	-1,98
annexin A2	-2,18
2610204M08Rik protein	-2,24
splicing factor 3b, subunit 2	-2,33
28S ribosomal protein S5, mitochondrial	-2,40
protein transport protein Sec31A isoform X1	-2,45
glucose-6-phosphate 1-dehydrogenase	-2,85
mKIAA0023 protein	-3,07
Branched chain aminotransferase 2, mitochondrial	-3,18
glycogen phosphorylase, brain form	-3,75

Fig 5. Differentially expressed proteins in MPSIEC after incubation with FhNEJ. The top 15 proteins with highest (represented in red) or lowest (represented in blue) fold change within the MPSIEC membrane and cytosol extracts are shown. Icons created with BioRender.com.

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the top over-represented BPs were proteolysis, microtubule-based process and transport (Fig 6A), while the main MFs identified in this antigenic compartment were transferase activity, metal ion binding and oxidoreductase activity (S1 Fig).

Regarding proteolysis, a considerable number of proteomic studies have demonstrated the high level of protein complexity of the juvenile stages of *F. hepatica* in terms of proteolysis-related molecules [16,37,38]. This situation is not unexpected considering that this function is pivotal for the parasite invasion process. More specifically, trematode peptidases have been pointed out as key molecules in facilitating parasite migration, nutrition, immune evasion and other important host-parasite interactions [39]. Cathepsin peptidases are among the major proteolytic enzymes involved in FhNEJ virulence and tissue migration and they represent the most abundant proteases in the secretions of *F. hepatica* and a paradigmatic example of evolutionary divergence in response to selection pressure [40]. In fact, 23 and 11 different sequences of cathepsins belonging to the L and B classes have been identified in the *F. hepatica* genome [41], and their expression shows a stage-specific temporal shift that correlates with juvenile development and the organ invasion process [42,43].

The high abundance of proteases among the up-regulated proteins in the detergent-soluble extract enriched with tegument of FhNEJ upon incubation with MPSIEC is remarkable (S1 Table). These included cathepsin L (identified as cathepsin L4 (CL4) after comparison of its prosegment with those corresponding to *F. hepatica* consensus sequences of CL1 and CL5
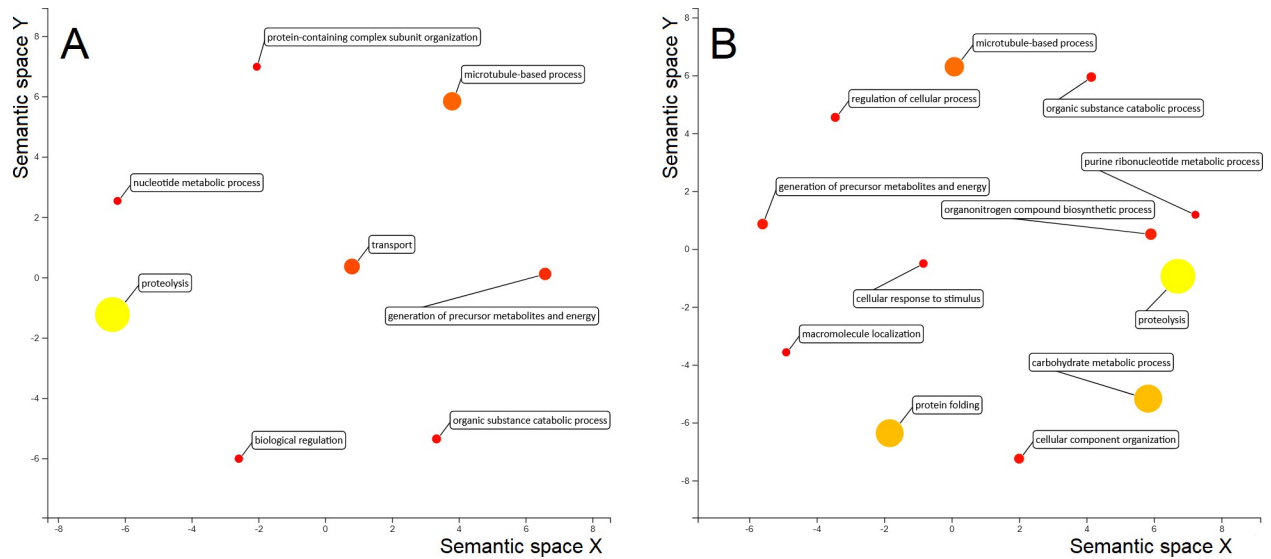
Fig 6. ReViGO plot showing the main GO terms within the Biological Process (BP) category in FhNEJ. The BP GO terms corresponding to up- and down-regulated proteins in the detergent-soluble extract enriched with tegument (A) and soma (B) extracts of FhNEJ after incubation with MPSIEC are shown. The size and colour of each circle represents the Nodescore of each GO term, and the spacing between circles refers to the similarity between the represented terms.

https://doi.org/10.1371/journal.pntd.0010811.g006

prosegments [44]), cathepsin L3 peptidase (CL3) and peptidase D. The first and third upregulated proteins in FhNEJ tegument upon contact with host cells (CL4 and CL3, respectively) have been found in the transcriptome of FhNEJ axenically maintained *in vitro* for 1, 3 and 24 h [37], but only CL3, and not CL4, has been found in protein extracts of FhNEJ tegument [17]. Our results suggest that CL4 would be overexpressed in the detergent-soluble extract enriched with tegument of FhNEJ only when subjected to interact with host cells. On the contrary, CL3 would be expressed in FhNEJ after *in vitro* excystment without requiring host stimulus. Thus, CL4 may play a role at the host-parasite interface upon contact of FhNEJ with the host. Importantly, CL3 is known for its collagenolytic activity, which is especially efficient in digesting type I and II collagens [45]. Provided that FhNEJ must overcome four layers in the small intestine wall (mucosa, submucosa, muscularis externa and serosa) that are highly rich in collagen [46] in order to reach the peritoneum, the over-expression of proteases with collagenolytic activity in the host-parasite interface could be crucial for parasite invasion. Moreover, this important function might be followed by the activity of other up-regulated proteases such as peptidase D (also known as prolidase), whose most studied substrates are dipeptides that are generated during collagen breakdown [47]. Together with these proteases, the protease inhibitor serpin 2 is also upregulated in this compartment of FhNEJ after incubation with MPSIEC. Serpin 2, characterized as a surface molecule in this parasite, can both protect the FhNEJ from host proteolysis and play a role in immune evasion [48].

Interestingly, some proteins traditionally associated with the nucleus such as histones H4 and H2B and histone deacetylase were also up-regulated in the FhNEJ detergent-soluble extract enriched with tegument after co-incubation with host intestinal epithelial cells. These proteins have important functions in chromosomic DNA organization [49] and they can also be released to the cytosol and the extracellular space to perform other functions, such as antimicrobial responses [50]. In fact, it has been demonstrated that histones (e.g. H4 or H2B) and their derivatives, which are cleaved from histones by cathepsins [51], directly eliminate a wide range of pathogens in a similar way to other antimicrobial peptides [50]. This mechanism, by which proteins exhibit 'unexpected' functions separate from their canonical activities, is

referred to as 'moonlighting'. Moonlighting is directly linked to unconventional protein secretion routes that allow for the translocation of cytosolic antigens to the host-parasite interface [52]. This is also the case of surface-located glycolytic enzymes, such as phosphoglycerate mutase, which is up-regulated in the tegument and the soma of FhNEJ in this study. This enzyme normally participates in energy metabolism, but when located extracellularly it also plays a role in parasite invasion and establishment within the host [53].

Potentially linked to immune regulation, we found glutathione-S-transferase (GST) and the calcium-binding EF-hand protein 4 (CABP4) to be overexpressed in this protein fraction of FhNEJ upon incubation with MPSIEC. *F. hepatica* GST (also found here overexpressed in the somatic extract of FhNEJ) has shown anti-inflammatory properties [54], and *F. gigantica* CABP4 stimulated IL-2 and IFN-γ Th1-type cytokines and reduced IL-10 (Th2) expression levels by activated goat monocytes [55]. As observed in other trematode species (e.g., schistosomes [56]), these proteins could play a role in the first steps of infection by protecting FhNEJ from inflammatory responses as well as eliciting a transient Th1-type immune response during early infection.

Down-regulated proteins in the detergent-soluble extract enriched with tegument of the parasite include components of the muscle contraction machinery (troponin, actin, dynein, titin, etc.) and proteins involved in metabolite transport (fatty acid binding protein, vesicle-associated membrane protein-associated protein A) along with other multi-purpose proteins (annexin, peptidyl-prolyl cis-trans isomerase). Noteworthy, some biological processes with up-regulated proteins (i.e. proteolysis or antioxidant response) also exhibit down-regulated representatives, and might therefore be finely regulated during the early phases of *F. hepatica* infection. This could be the case of the above-mentioned cathepsins. In fact, the results obtained in this study showed a down-regulation in the expression of two isoforms of cathepsin B after contact of FhNEJ with MPSIEC. FhCB peptidases appear to be highly expressed by FhNEJ and down-regulated in adult flukes [40, 57], although defined isoforms of CBs could have important roles in the excystment process but not in the subsequent migration of FhNEJ. Overall, these results suggest an important role of the interaction between FhNEJ and the host intestinal wall in the cathepsin expression shift observed in *F. hepatica* in correlation to parasite development and migration through host tissues.

Interestingly, the downregulated protein with the highest Log2FC in this FhNEJ extract, also corresponding to the fifth most downregulated protein in somatic extracts of FhNEJ upon MPSIEC incubation, is CD59, a complement regulatory protein that protects cells from the destructive action of the complement system. Homologue CD59 proteins have been previously described to be expressed and secreted by FhNEJ *in vitro* [37, 58], together with other parasite proteins that could impair the host's complement response, such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [37, 38, 58], which is similarly downregulated in the tegument extract of FhNEJ upon incubation with MPSIEC. Thus, it is possible that the down-regulation of CD59-like molecule and GAPDH herein identified could be an indication of depletion of these molecules in the tegument compartment as a result of increased secretion upon interaction with the host to counteract complement attack. However, it is also known that FhNEJ employ multiple mechanisms to inhibit complement attack during invasion [59], so alternative strategies to avoid this host defence mechanism could also be taking place.

In the somatic FhNEJ extract, the main GO terms in the BP category were proteolysis, carbohydrate metabolic process, protein folding and microtubule-based process (Fig 6B), and transferase activity, peptidase activity and metal ion binding in the MF category (S1 Fig). Up-regulated proteins in this compartment exhibited a profile similar to that described in the tegument, including proteins involved in peptide degradation (CL3, dipeptidyl peptidase), antioxidant response (GST) or metabolic enzymes (phosphoglycerate mutase), as well as proteins

involved in signalling cascades (Mitogen-activated protein kinase) or cell-cell adhesion (tetraspanin). Tetraspanins are transmembrane proteins involved in important functions linked with the maintenance of the integrity of the adult fluke tegument [60]. Noteworthy, tetraspanins from other helminth trematodes such as *Schistosoma mansoni* or *Opistorchis viverrini* have elicited promising results as vaccine candidates against schistosomiasis and opisthorchiasis, respectively [61, 62]. However, the biological role of the identified up-regulation of this protein in the internal tissues of the FhNEJ should be further investigated.

Down-regulated proteins in the soma of FhNEJ included proteins also down-regulated in their tegument (fatty acid binding protein, GAPDH, peptidyl-prolyl cis-trans isomerase). Additionally, legumain like and leucyl aminopeptidase, superoxide dismutase, transketolase and pyruvate dehydrogenase, representing functions similar to those down-regulated in the tegument of FhNEJ, were also found as down-regulated in the somatic extracts of FhNEJ.

Regarding antioxidant enzymes, our data revealed that the expression of four isoforms of one of their main representatives, GST, is up-regulated within the FhNEJ somatic and tegument extracts after co-culture with MPSIEC. It has been described that GSTs account for 4% of *F. hepatica* excretory/secretory products and that these enzymes have a key role in detoxifying both endogenous and exogenous toxins arising from the oxidative host defence response [63]. For that reason, this parasitic antigen has been proposed as a vaccine candidate against fasciolosis [64]. However, our data also revealed the presence of a GST isoform that is down-regulated within the FhNEJ somatic extract. This particular shift in the expression pattern of parasitic isoforms of the same protein upon interaction with host cells could be explained by finely regulated biochemical redundancy mechanisms, which would hamper the deleterious effects of the immune response against these specific antigens. This bidirectional regulation of molecules that are important for parasite survival and development could also explain the reported high variability in protection rates and the partial success of vaccination studies against fasciolosis that included these diversely-regulated antigens in their formulations [10,65].

The comparison of the results of the present analysis with that performed before by our group by using the iTRAQ methodology after 24 h co-incubation of FhNEJ and MPSIEC [14], shows that proteolysis seems to play a key role in FhNEJ after co-culture with MPSIEC, at both 3h and 24h post-incubation (pi), as this functional category is up-regulated at both times. However, the protease profile shows some differences between the two incubation times, as cathepsin L3 is the major isoform together with peptidase D after 3h pi, whereas at 24h pi cathepsin L1 and peptidase C1 are up-regulated and cathepsins L3, L4 and B, along with papain and legumain, are down-regulated. This suggests that at 24h pi the parasite is already expressing adult stage specific isoforms, while juvenile isoforms are already decaying. Regarding regulation of proteolysis, this seems to play a more important role in the earliest stages (3h pi), as serpin 2 is up-regulated at this time, while cystatin is found as down-regulated at 24h pi. Similarly, response to oxidative stress is represented by several GST isoforms that are up-regulated at 3h pi but not at 24h pi.

On the other hand, muscle contraction and cytoskeleton reorganization processes seem to be more represented at 24h pi, as at this time several related proteins are found to be up-regulated while they are not found at 3h pi, including dynein, cadherin, myophilin and catenin alpha. Several metabolic enzymes could be also differentially expressed at both times pi. The landscape at 3h pi is mainly aerobic (including several up-regulated respiratory enzymes such as cytochrome c oxidase and quinone oxidoreductase), while at 24h pi it seems to evolve to an anaerobic profile with a greater abundance of glycolytic enzymes (e.g., aldolase, GAPDH). Interestingly, histone isoforms are found as up-regulated proteins at both times pi, with histone H4 present at both times, while histone H2B is found at 3h and histone H2A at 24h. In

summary, comparison of the results obtained here at 3h pi obtained by SWATH and the previous results at 24 h pi obtained by iTRAQ, show that proteolysis and its regulation, muscle contraction and metabolism could be differentially regulated in FhNEJ upon MPSIEC co-incubation at different times pi. Nevertheless, this should be further explored in more comparable studies, since the methods used to check up- and down-regulated proteins in FhNEJ after co-culture with host MPSIEC between the two above-mentioned studies are different regarding the proteomic and statistical approaches.

Stimulation of MPSIEC with FhNEJ results in the modulation of protein expression in host cells. In vertebrate animals, the small intestine is the organ with the largest surface area in contact with the outside environment. Although its main function is the absorption of water and nutrients from the diet, it is also the entry point for a multitude of pathogens, including foodborne parasites [66]. As a matter of fact, intestinal epithelial cells are considered the first line of defence against pathogens, since they can detect them and trigger appropriate responses by underlying immune cells in order to maintain intestinal homeostasis [67,68]. However, the mechanisms by which the host intestinal epithelium detects parasitic invasion and stimulates appropriate immune responses is still a matter of debate [69].

Our proteomics approach showed a considerable number of altered processes that were identified in the extracts of MPSIEC upon interaction with FhNEJ. In particular, some of the main modified BPs in MPSIEC cytosolic extracts were regulation of catalytic activity, actin cytoskeleton organisation, response to inorganic substance and vesicle-mediated transport (Fig 7A), whereas the membrane fractions showed differential expression of proteins involved in intracellular protein transport, response to oxidative stress and negative regulation of transcription (Fig 7B). Within the MF category, the most represented GO terms were RNA binding, structural constituent of ribosome and protein-containing complex binding among cytosolic proteins, and protein-containing complex binding, RNA binding and actin binding in the membrane extracts (S2 Fig).

Over-expressed proteins in the cytosol of mouse cells were involved in functions such as vesicle transport (coatomer subunit gamma-2, atlastin-2) and lipid biosynthesis (acyl-
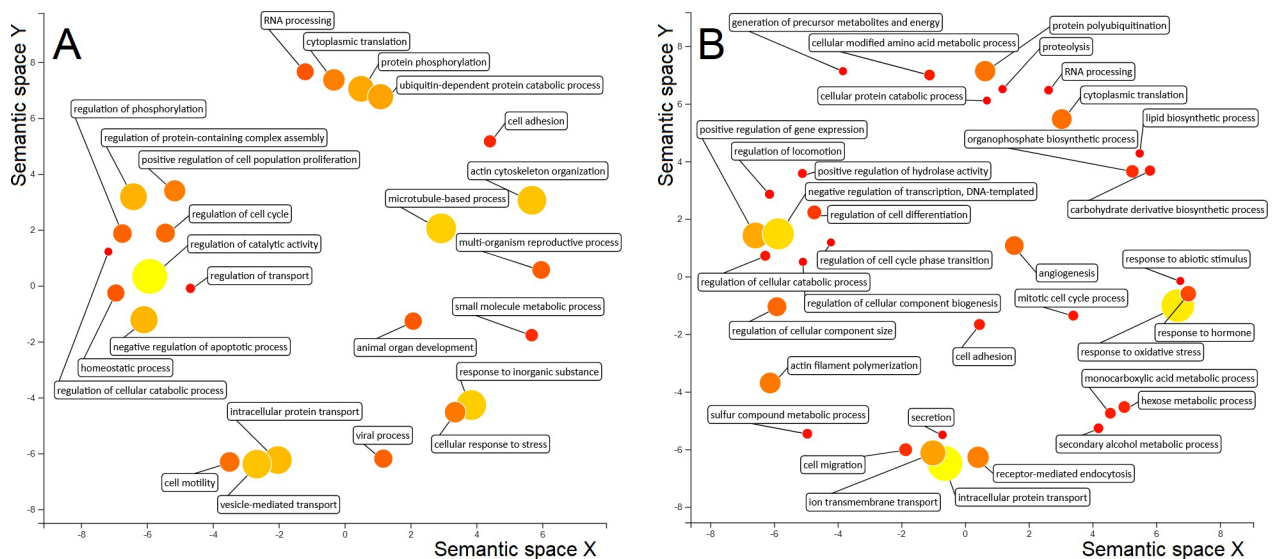

Fig 7. ReViGO plot showing the main GO terms within the Biological Process (BP) category in MPSIEC. The BP GO terms corresponding to up- and down-regulated proteins in the cytosol (A) and membrane (B) extracts of MPSIEC after incubation with FhNEJ are shown. The size and colour of each circle represents the Nodescore of each GO term, and the spacing between circles refers to the similarity between the represented terms.

Coenzyme A dehydrogenase family member, lanosterol synthase) along with proteins involved in the response to stress (Hspa1b protein) or viruses (interferon-induced transmembrane protein 3 (IFITM3)). Among down-regulated proteins, there was an abundance of ribosomal constituents together with cytoskeleton-related proteins. Moreover, our results revealed that the expression of pro-inflammatory mediators (prostaglandin E synthase 3 (PTGES3)) and peptides with antimicrobial activity (Lysophospholipase 1) was also decreased.

Up-regulated proteins within the MPSIEC membrane extracts included nuclear lamin components (lamin A, CRA_b isoform, Lmnb1 protein), chromatin-interacting proteins (SWI/SNF-related matrix-associated actin-dependent regulator of chromatin, chromobox protein homolog 1) and enzymes involved in energy metabolism (diphosphomevalonate decarboxylase, pyruvate kinase PKM). Down-regulation of proteins related to carbohydrate catabolism (glycogen phosphorylase, glucose-6-phosphate 1-dehydrogenase, cytoplasmic isocitrate dehydrogenase (NADP)) or response to pathogens (phospholipase A-2-activating protein (PLAA), annexin A2) was observed in this extract.

Taken together, these results suggest a potential modulation of MPSIEC immune responses by FhNEJ via the regulation of important host defence mediators. These mechanisms could have a crucial role not only in relation to the overall success of parasite invasion but also in the outcome of concomitant infections. Particularly, phospholipase A-2 plays a key role in the initiation, propagation and resolution of inflammation at the intestinal level. This enzyme is considered one of the main components of the innate immune system of the intestinal epithelial barrier against invading microbes due to its bacteriolytic properties [70]. Likewise, prostaglandin E2 is a principal mediator of inflammation and its role in mucosal defence is of paramount importance to maintain the integrity of the gastrointestinal tract [71]. Accordingly, down-regulation of host PTGES3 and PLAA in response to FhNEJ stimulation could potentially facilitate FhNEJ invasion as well as bacterial colonization of the intestinal epithelium. This interspecies interaction would be in line with an increased risk of concomitant bacterial infections that has been reported in fasciolosis [72].

The stimulation of MPISEC with FhNEJ also resulted in the upregulation of IFITM3, an interferon-induced protein that is also constitutively expressed by virtually all human and mouse cell types. IFITM3 restricts cellular entry of enveloped viruses, including SARS coronaviruses [73], by either accelerating endosomal acidification towards a degradative state or by altering the mechanical properties and composition of lipid bilayers, which disfavours the fusion between virus and target cell membranes [74]. This finding opens up a new perspective related to the potential of defined FhNEJ-derived proteins to modulate the susceptibility to viral infections [75].

Notwithstanding, and although a number of proteins with potential immune-related functions have been identified, further experiments aimed at testing the ability of MPSIEC exposed to FhNEJ to respond to specific Th1-inducing ligands should be performed in the future to clarify the immunomodulatory capacity of the FhNEJ in this model.

Protein association analysis reveals an orchestrated reduction in host ribosome function after co-culture with FhNEJ. The sum of up-regulated proteins in both compartments of MPSIEC was further analysed for interactions using STRING in order to establish possible protein-protein relationships that could be relevant to *F. hepatica* infection (Fig 8). This set presented a more diffuse interaction network with three clusters of association. One of them is characterized by the up-regulation of membrane proteins, specifically of components of the nuclear lamina (lamin A isoform CRA_b and Lmnb1 protein). Lamin A, B and C, also known as the nuclear intermediate-filament proteins, provide a scaffold for the nuclear envelope and protect the chromatin from physical damage [76]. The second association cluster of MPSIEC up-regulated proteins upon co-culture with FhNEJ is formed by an isoform of cadherin-3, catenin alpha-1 isoform X1, src substrate cortactin isoform X1, protein flightless-1 homolog
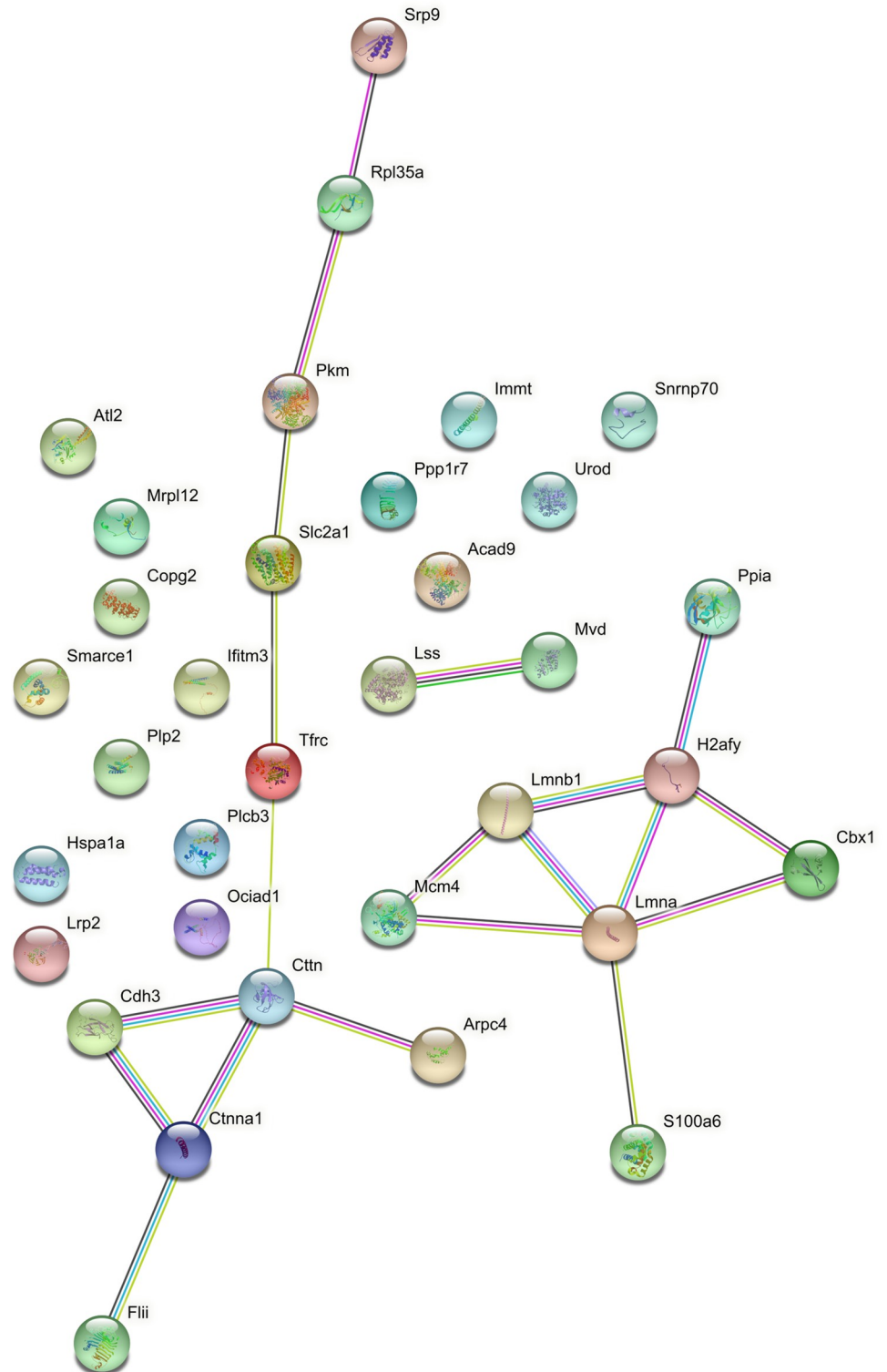
Fig 8. Interaction plot (STRING) showing protein-protein relations within the set of up-regulated MPSIEC proteins. Edges between network nodes represent protein-protein associations, which are classified as "known interaction" (light blue: from curated databases; purple: experimentally determined), "predicted interactions" (green: gene neighbourhood; red: gene fusions; blue: gene co-occurrence) or "others" (yellow: textmining; black: co-expression; pale blue: protein homology) (https://version-11-5.string-db.org/cgi/network?networkId= blUqyaKyKA9R).

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isoform X1 and an actin-related protein. The association between cadherins and catenins creates a complex that is linked to the underlying actin cytoskeleton and involved in the expansion and completion of cell-cell adhesion processes, among others [77]. The up-regulation of these proteins could be part of a host defence mechanism aimed at increasing the adhesion properties of the epithelial cell barrier and play a role in cadherin-mediated host-parasite interactions, which have been described to be involved in tissue invasion by different pathogens [78]. The third cluster of host up-regulated proteins is represented by proteins involved in cholesterol biosynthesis (diphosphomevalonate decarboxylase and lanosterol synthase). Parasites are capable of exploiting cholesterol metabolism to impair the host immune response and also make use of this pathway for invasion purposes, since they employ cholesterol rich domains as an assembly platform for invasion [79].

The same analysis was performed for MPSIEC down-regulated proteins upon interaction with FhNEJ (Fig 9), and we detected a prominent cluster of ribosomal proteins as the most representative feature. Moreover, we found associations of these proteins with other important
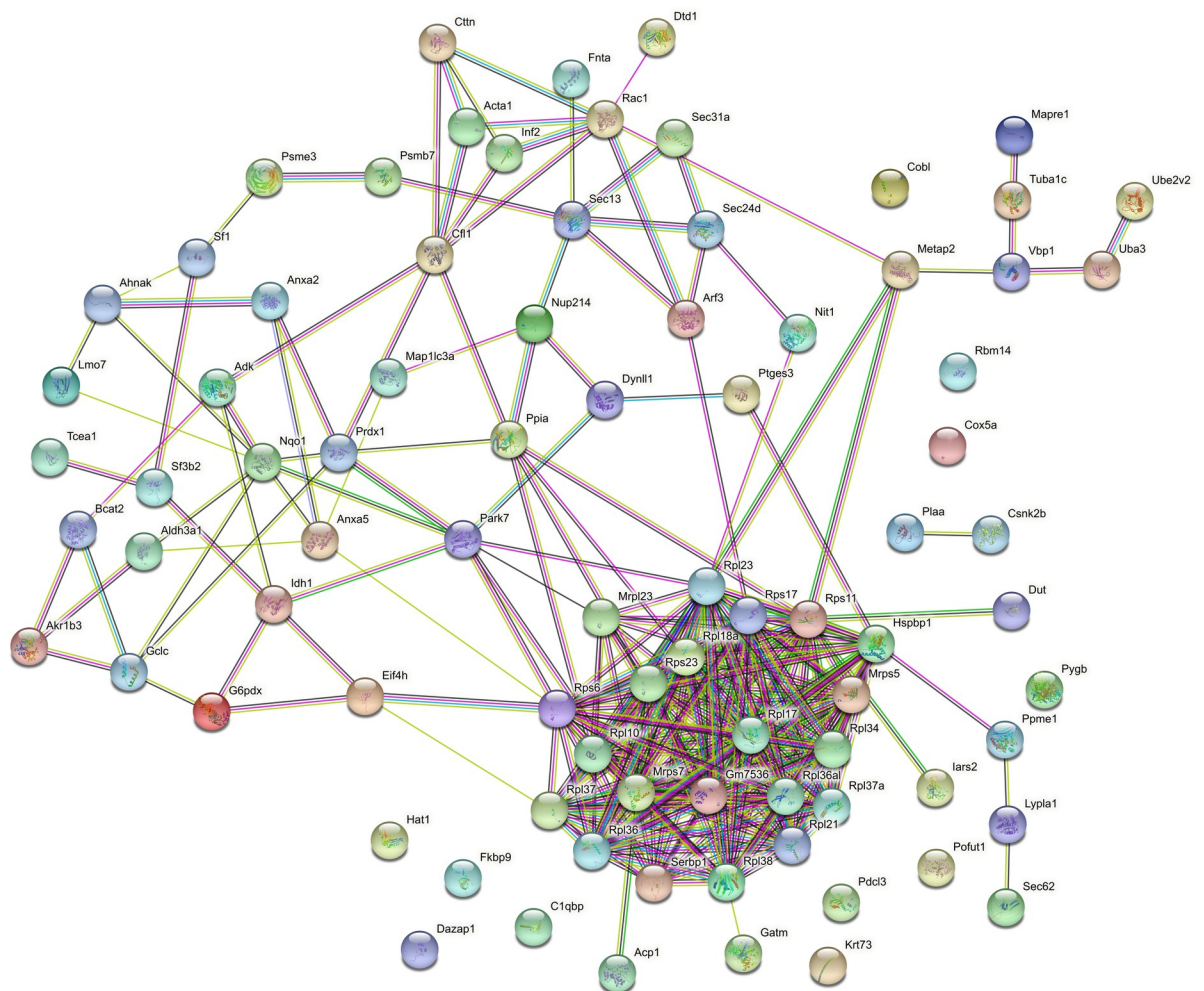

Fig 9. Interaction plot (STRING) showing protein-protein relationships within the set of down-regulated MPSIEC proteins. Edges between network nodes represent protein-protein associations, which are classified as "known interaction" (light blue: from curated databases; purple: experimentally determined), "predicted interactions" (green: gene neighbourhood; red: gene fusions; blue: gene co-occurrence) or "others" (yellow: textmining; black: co-expression; pale blue: protein homology) (https://version-11-5.string-db.org/cgi/network?networkId=bEnbwSoK41Pk).

https://doi.org/10.1371/journal.pntd.0010811.g009

representatives, such as Hsp70-binding protein 1, plasminogen activator inhibitor 1 RNA-binding protein isoform X2 and protein/nucleic acid deglycase DJ-1. In addition to these interactions, additional proteins were detected as interactors in smaller networks, such as cofilin-1, peroxiredoxin-1, or protein SEC13 homolog. A downstream analysis of MPSIEC down-regulated proteins using the Cytoscape MCODE package also detected the abovementioned cluster of ribosomal proteins, and annotation with DAVID revealed that the functions involving this protein family include translation, ribosomal small subunit biogenesis and ribosomal assembly. Intriguingly, a similar reduced ribosome function in intestinal epithelial cells was observed in an analogous *in vitro* model carried out to study host-parasite interactions in porcine ascariosis [3]. Ribosomes are not only essential constituents of the protein synthesis machinery but can also be involved in the so-called extra-ribosomal functions, which include regulation of cell growth and proliferation, differentiation, apoptosis, and DNA repair [80]. Additionally, down-regulation of ribosome function has been reported in response to various types of stress, such as cell cycle arrest and apoptosis [81], so the down-regulation of ribosomal function herein described could be representative of a general loss of homeostasis in the host upon infection with *F. hepatica*.

Conclusions

After ingestion of infective metacercariae, FhNEJ are released in the host duodenum and start invading host tissues by crossing the intestinal epithelial barrier in a finely regulated process that takes place in 2–3 h and represents the first direct contact between *F. hepatica* and its host. In this work, we have set up an *in vitro* model that replicates this important stage of the infection process by co-culturing FhNEJ and MPSIEC. The use of SWATH-MS to analyze and quantify the dynamic changes in the protein repertoire of both the parasite and the host after co-stimulation has allowed us to identify a set of proteins and processes that shed light into the intricate molecular crosstalk that occurs between both organisms. The obtained results show rapid changes in the protein expression pattern of FhNEJ in response to interaction with the host intestinal epithelium, including proteolytic, antioxidant and immunoregulatory differentially expressed proteins. MPSIEC also responded to parasite stimulus by showing alterations in the expression of proteins related to immunomodulation and cell-cell interactions, as well as a remarkable decrease in ribosome function. Future studies aimed at confirming the functional consequences of host-parasite interactions at this stage of infection could help us define new targetable candidates for an efficient elimination of *F. hepatica* during early fasciolosis.

Supporting information

S1 Fig. ReViGO plot showing the main GO terms within the Molecular Function (MF) category in FhNEJ. The MF GO terms corresponding to up- and down-regulated proteins in the detergent-soluble extract enriched with tegument (A) and soma (B) extracts of FhNEJ are shown. The size and colour of each circle represents the Nodescore of each GO term, and the spacing between circles refers to the similarity between the terms represented.
(JPG)

S2 Fig. ReViGO plot showing the main GO terms within the Molecular Function (MF) category in MPSIEC. The MF GO terms corresponding to up- and down-regulated proteins in the cytosol (A) and membrane (B) extracts of MPSIEC are shown. The size and colour of each circle represents the Nodescore of each GO term, and the spacing between circles refers to the similarity between the terms represented.
(JPG)

S1 Table. Quantitative and annotation data of the differentially expressed proteins within the FhNEJ fractions. Uniprot accession codes were assigned as the match with the highest score in a BLAST search against the *F. hepatica* sequences available in this database (downloaded on 25 February 2020), whereas the remaining data were obtained during the Blast2GO annotation pipeline described previously.
(XLSX)

S2 Table. Quantitative and annotation data of the differentially expressed proteins within the MPSIEC fractions. Data were obtained during the Blast2GO annotation pipeline described previously.
(XLSX)

S1 Video. Footage showing the *in vitro* host-parasite interaction model developed in this study. In the video, a single FhNEJ can be observed crawling over the MPSIEC monolayer with a characteristic piston movement.
(MOV)

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Methodology: Judit Serrat, Marta López-García, Javier González-Miguel, Mar Siles-Lucas.

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Validation: Javier González-Miguel, Mar Siles-Lucas.

Visualization: David Becerro-Recio, Judit Serrat, Marta López-García, Javier González-Miguel, Mar Siles-Lucas.

Writing – original draft: David Becerro-Recio, Javier González-Miguel, Mar Siles-Lucas.

Writing – review & editing: Judit Serrat, Marta López-García, Javier Sotillo, Fernando Simón, Javier González-Miguel, Mar Siles-Lucas.

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Capítulo 3:

Study of the migration of *Fasciola hepatica* juveniles across the intestinal barrier of the host by quantitative proteomics in an *ex vivo* model

Resumen de la publicación

Fasciola hepatica es uno de los parásitos más ampliamente distribuidos del planeta, con casos reportados en los cinco continentes tanto en humanos como en animales, presentando además elevadas tasas de morbimortalidad en el ganado rumiante. Pese a los esfuerzos por desarrollar estrategias de protección eficaces, en la actualidad las medidas de control disponibles se reducen a un puñado de fármacos, efectivos, en su mayor parte, contra la fase adulta del parásito. Esto, unido a la creciente aparición de resistencias frente a los fármacos disponibles, hacen deseable el desarrollo de estrategias que permitan el diagnóstico y tratamiento temprano de la enfermedad.

El proceso infectivo comienza tras la ingesta de las metacercarias del parásito, que liberan los juveniles recién excistados (*Newly Excysted Juveniles*; NEJ) en el duodeno del hospedador, y estos atraviesan la pared intestinal para iniciar un complejo ciclo migratorio por los tejidos. Este momento es considerado el punto de no retorno en la infección, por lo que se hace necesario disponer de modelos experimentales que permitan conocer en detalle este fenómeno. Para ello, en este estudio se desarrolló un modelo *ex vivo* en el que se extrajeron fragmentos de intestino delgado de ratón, en cuyo interior se introdujeron NEJ previamente excistados, y se incubaron durante 2,5 horas.

Pasado este tiempo, los NEJ que atravesaron la pared del intestino, que supusieron alrededor de un 22 % del total, fueron fraccionados y sometidos a un análisis proteómico mediante SWATH-MS (*Sequential Window Acquisition of All Theoretical Mass Spectra*). De este modo, se detectaron cambios en el perfil de expresión relacionados con procesos de proteolisis, toma de nutrientes, detoxificación de radicales libres y respuesta a estrés, entre otros. Por su parte, los fragmentos de intestino se sometieron a un estudio histológico que permitió evaluar las lesiones causadas por la migración de los vermes, observando alteraciones en la integridad de las capas del intestino que no se encontraron en muestras sin contacto con vermes, además de una mayor presencia de caspasa-3, un marcador de apoptosis temprana.
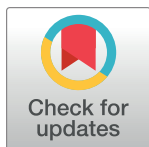
Study of the migration of *Fasciola hepatica* juveniles across the intestinal barrier of the host by quantitative proteomics in an *ex vivo* model

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Abstract

Fasciola hepatica is a trematode parasite that infects animals and humans causing fasciolosis, a worldwide-distributed disease responsible for important economic losses and health problems. This disease is of growing public health concern since parasite isolates resistant to the current treatment (triclabendazole) have increasingly been described. *F. hepatica* infects its vertebrate host after ingestion of the encysted parasite (metacercariae), which are found in the water or attached to plants. Upon ingestion, newly excysted juveniles of *F. hepatica* (FhNEJ) emerge in the intestinal lumen and cross the intestinal barrier, reach the peritoneum and migrate to the biliary ducts, where adult worms fully develop. Despite the efforts made to develop new therapeutic and preventive tools, to date, protection against *F. hepatica* obtained in different animal models is far from optimal. Early events of host-FhNEJ interactions are of paramount importance for the infection progress in fasciolosis, especially those occurring at the host-parasite interface. Nevertheless, studies of FhNEJ responses to the changing host environment encountered during migration across host tissues are still scarce. Here, we set-up an *ex vivo* model coupled with quantitative SWATH-MS proteomics to study early host-parasite interaction events in fasciolosis. After comparing tegument and somatic fractions from control parasites and FhNEJ that managed to cross a mouse intestinal section *ex vivo*, a set of parasite proteins whose expression was statistically different were found. These included upregulation of cathepsins L3 and L4, proteolytic inhibitor Fh serpin 2, and a number of molecules linked with nutrient uptake and metabolism, including histone H4, H2A and H2B, low density lipoprotein receptor, tetraspanin, fatty acid binding protein a and glutathione-S-transferase. Downregulated proteins in FhNEJ after gut passage were more numerous than the upregulated ones, and

included the heath shock proteins HSP90 and alpha crystallin, amongst others. This study brings new insights into early host-parasite interactions in fasciolosis and sheds light on the proteomic changes in FhNEJ triggered upon excystment and intestinal wall crossing, which could serve to define new targets for the prevention and treatment of this widespread parasitic disease.

Author summary

Fasciolosis caused by the helminth parasite *Fasciola hepatica* is a serious health and economic problem worldwide. Treatment and prevention of this disease pose several drawbacks that have so far not been solved. The definition of suitable parasite molecular targets to overcome such drawbacks should be based on thoroughly deciphering host-parasite interactions, and in this regard most studies have focused on the adult stages of *F. hepatica*. Nevertheless, in this context, the study of the transient juvenile stages of this parasite could be of higher utility due to the importance of early interactions with the host for parasite migration and the successful establishment of infection. In this work, we set-up an *ex vivo* model and performed a quantitative proteomics approach to study the changes in *F. hepatica* juveniles upon gut passage. We found that the parasite tegument and somatic compartments experienced deep changes in their composition and showed that the host triggers the expression of specific molecules that are important for parasite migration and survival at this stage. The molecules described here could serve to better understand host-parasite interactions and to define new targets against fasciolosis.

Introduction

Fasciola hepatica is responsible for most cases of fasciolosis, which is considered as the food-borne parasitic disease with the widest geographical distribution [1] as a result of the ability of *F. hepatica* to persist in different ecosystems and to develop in a wide variety of intermediate and definitive hosts [2]. Economic losses derived from this disease are estimated to exceed $3 billion per year [3] and mainly affect ruminant livestock, arising from reduced production of animal by-products as well as an increased susceptibility to other diseases. Fasciolosis is also considered a major food-borne zoonotic disease as human cases have been reported in up to 51 countries [4]. Current estimations suggest that around 2.6 million people could be affected by this disease, and up to 90 million may be at risk of infection [5].

The life cycle of *F. hepatica* in the vertebrate host starts upon ingestion of raw aquatic plants or water contaminated with metacercariae, which rapidly excyst in the host duodenum and release the newly excysted juvenile flukes (FhNEJ). The first contact between the parasite and host tissues occurs at the intestinal level, from where FhNEJ start migrating by crossing the host's intestinal wall in around 2–3 hours after excystment [6]. This mechanism could be considered as the "point of no return" in fasciolosis in terms of disease progression, since it represents the first step of an intricate migratory route followed by FhNEJ that eventually drives them towards the major intra-hepatic biliary ducts, a location that is poorly accessible to effectors of the host immune response [7].

Despite the efforts to obtain a better understanding of host-parasite relationships in fasciolosis carried out over the past decades, relatively little is known about the molecular cross-talk between *F. hepatica* and its hosts during the early stages of infection. In order to fill this

knowledge gap, different models have been developed to study the molecular milieu at the FhNEJ-host interface by using *ex vivo* [8] or *in vitro* [9] experimental rat models that mimic the passage through the intestine by FhNEJ. In this line, our group recently developed an *in vitro* model aimed at replicating the early contact between FhNEJ and the intestinal epithelium of the host [10]. In this model, *F. hepatica* metacercariae were excysted *in vitro* and placed over a primary cell culture representing the mouse intestinal epithelium. After 24 hours of co-incubation, both FhNEJ and cells were subjected to proteomic analysis using Isobaric Tag for Relative and Absolute Quantitation (iTRAQ) to determine which proteins were differentially expressed in both species during their interaction. This strategy resulted in the identification of 191 and 62 up-regulated, and 112 and 57 down-regulated proteins in the FhNEJ tegument and somatic extracts, respectively. Similarly, 87 up-regulated and 73 down-regulated proteins in the extract of host cells were identified. Regulated proteins were related to parasite development, invasion and evasion, as well as manipulation of the host intestinal epithelial cell adhesion, immunity and apoptosis pathways, amongst others. -Omics approaches can be useful not only to investigate the changes arisen in the host-parasite interface but also to find new and effective therapeutic targets against fasciolosis [11]. In this context, the development of innovative and more accurate proteomic approaches such as Sequential Window Acquisition of All Theoretical Mass Spectra (SWATH-MS) could provide an improvement in terms of reproducibility and sensitivity [12–15].

In this work, we set-up an *ex vivo* model to replicate the passage of FhNEJ through the intestinal wall of the host using a murine model combined with SWATH-MS analysis to understand how the expression profile of FhNEJ is modified during this process, which reveals a list of candidate proteins that might be relevant during the intestinal stage of *F. hepatica* infection.

Materials and methods

Ethics statement

All animals received humane care in conformity with the Directive for the protection of animals utilized for scientific purposes (Directive 2010/63/UE, Decision 2020/569/UE and RD 1386/2018). All methods have been authorized by the Ethical Animal Experimentation Committee of the University of Córdoba and by the Junta de Andalucía (project no. 2021PI/22).

F. hepatica in vitro excystment

A total of 10,000 metacercariae from the *F. hepatica* Italian strain were purchased from Ridgeway Research LTD (UK), and *in vitro* excystment was performed according to previous reports [16]. Briefly, CO_2 was bubbled into a sterile tube containing 10 ml of distilled (d) H_2O for 30 s, and sodium dithionite was added to a final concentration of 0.02 M. The tube was incubated at 37˚C for 5 min, the mix was added to metacercariae and incubated at 37˚C for 1 hour. After incubation, metacercariae were washed twice with warm dH_2O and resuspended in 10 ml of excystment medium consisting of 0.03 M HEPES, 10% rabbit bile (from a local abattoir) and Hank's balanced salt solution. Metacercariae were distributed in a 6-well plate, incubated at 37˚C for 4 h and the excystment process was monitored every hour under a microscope. While FhNEJ emerged, empty metacercariae cysts and unexcysted metacercariae were removed using a micropipette. Around 1,500 FhNEJ were collected to be used as negative controls (see *Ex vivo* model section), while the rest were centrifuged (5 min, 300 x *g*) and left in 200 μl of supernatant (containing the FhNEJ), which was reserved for further use.
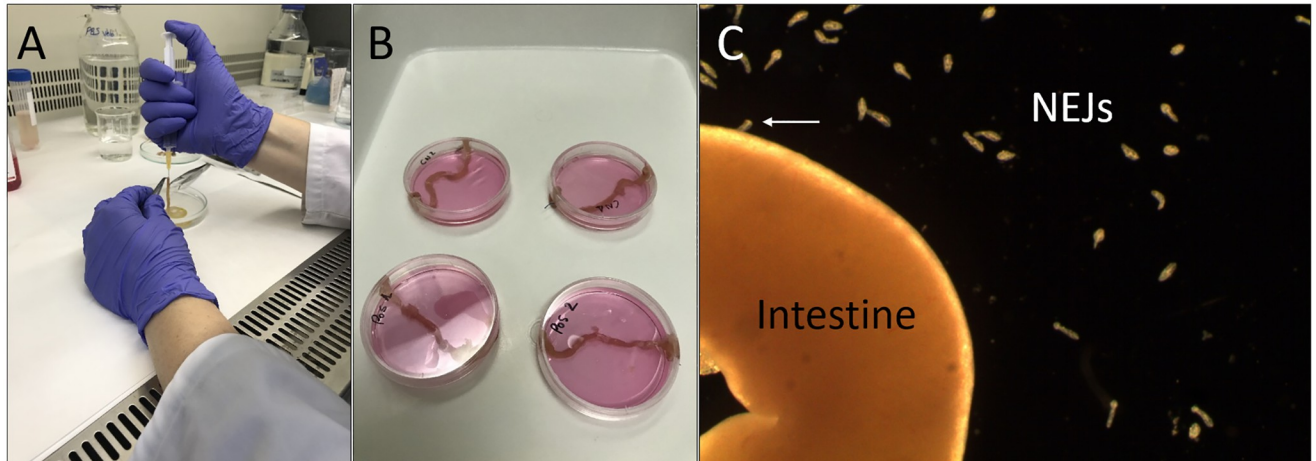
Fig 1. *Ex vivo* model for the migration of FhNEJ through mouse intestine. A) Preparation of the intestines before FhNEJ infection; B) Incubation of ligated intestines in RPMI medium at 39˚C 5%CO$_2$ for 2.5 h; C) Image obtained in a stereomicroscope showing the FhNEJ after intestinal passage (white arrow).

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Ex vivo model

A total of nine 12 week old male C57Bl/6 mice were housed by the Central Service of Experimental Animals at the University of Córdoba and divided in 3 replicates. The experimental procedure of the *ex vivo* model began with a 24 h period of fasting prior to necropsy. The day of the experiment, mice were euthanized by CO$_2$ overdose and cervical dislocation, and the stomach and the subsequent 15 cm of the small intestine were collected to perform the *ex vivo* assay. Intestines were cut close to the pylorus to remove the stomach and only the duodenum and jejunum portions were kept. Intestinal contents were flushing out under a laminar flow hood by two gentle washes with a 5 mL syringe coupled to 21G needles containing sterile phosphate-buffered saline (PBS) (Fig 1A). One end of the intestines was firmly ligated and a solution of 200 μl of excystment medium, containing an average of 2,500 FhNEJ per intestine, was pipetted inside the intestinal lumen through the caudal section of the intestine, which was immediately ligated after FhNEJ addition. Intestines injected with excystment medium devoid of FhNEJ were used as negative controls. Both experiment (injected with FhNEJ) and control intestines were placed in 60 mm petri dishes containing RPMI medium (leaving the ligated ends outside the plate), which were incubated for 2.5 hours at 39˚C and 5% CO$_2$ (Fig 1B). Additionally, untreated intestines were placed in fixative immediately after collection and washed as described in the section "Histopathological and immunohistochemical study". The plates were examined under a stereomicroscope before and after incubation to visualize the appearance of FhNEJ outside the intestines. The *ex vivo* assay was performed in triplicate. FhNEJ used as negative controls were incubated alone after excystment in petri dishes (each one with 500 FhNEJ) containing RPMI medium for 2.5 hours at 39˚C. After incubation, FhNEJ that crossed the intestinal walls, and those used as negative controls, were manually collected with a micropipette and washed twice with sterile PBS, centrifuged at 300 x *g* for 5 min and immediately subjected to protein extraction.

Protein extraction

In order to study *F. hepatica* tegument and somatic proteins separately, tegument extraction of FhNEJ samples was performed as previously described [9]. Briefly, FhNEJ were resuspended in 500 μl sterile PBS containing 1% Nonidet P-40 and incubated at room temperature with soft

stirring (60 rpm using a rotatory mixer) for 30 min. FhNEJ were then centrifuged (300 x *g* for 5 min) and the supernatant containing the tegument protein fraction was stored at -80˚C until use. The resulting pellet was resuspended in 500 μl RIPA lysis buffer and disrupted by ultrasonic pulsing (5 cycles of 30 s, leaving the samples on ice between cycles to avoid overheating) to release the somatic fraction. Finally, protein samples were centrifuged (1,000 x *g* for 5 min) and the supernatant containing the somatic proteins was stored at -80˚C. All samples were treated with the Protease Inhibitor Cocktail (Sigma) at 1x to avoid protein degradation.

Mass spectrometry analysis

Protein samples were quantified using a detergent compatible kit (Protein Quantification Assay; Machery-Nagel) following the manufacturer's instructions. Protein samples were in-gel digested as previously described [17] with slight modifications: 20 μg of each sample was resuspended in 20 μl of Laemmli Sample Buffer (Bio-Rad) and denatured at 95˚C for 5 min, after which they were loaded onto an Any kD precast 1D PAGE gel (Bio-Rad) and run at 200 V for 5 min. After separation, proteins were fixed with 40% ethanol/10% acetic acid. Each lane of the gel was cut into pieces and treated with reducing and alkylating agents (dithiothreitol and iodoacetamide, respectively), after which the proteins were overnight digested with sequencing grade trypsin (Promega). The digestion was stopped with 1% trifluoroacetic acid (TFA) and the resulting peptides were extracted with acetonitrile (ACN). Finally, each sample was dried on a rotary evaporator and resuspended in 20 μl of 2% ACN; 0.1% TFA.

For library construction, all samples were pooled and loaded onto an analytical column (LC Column, 3 μ C18-CL, Nikkyo) equilibrated in 5% ACN 0.1% formic acid. Peptides were eluted in a linear gradient of 5–35% solvent B (A: 0.1% FA; B: ACN, 0.1% FA) over 120 min at 300 nl/min flow rate and analyzed in a mass spectrometer nanoESI qQTOF (5600 TripleTOF, ABSCIEX). Analysis was carried out in a data-dependent mode (DDA). Survey MS1 scans were acquired from 350–1250 m/z for 250 ms, whereas the quadrupole resolution was set to "UNIT" for MS2 experiments, which were acquired 100–1500 m/z for 150 ms in high sensitivity mode.

For individual sample acquisition, the tripleTOF was operated in SWATH mode (DIA), in which a 0.050-s TOF MS scan from 350–1250 m/z was performed, followed by 0.080-s product ion scans from 350–1250 m/z on the 32 defined windows (3.05 sec/cycle).

Database search and bioinformatics analysis

Protein Pilot v5.0 (SCIEX) was used to generate a peak list directly from 5600 TripleTof.*wiff* files corresponding to the peptide library. The database used contained the predicted proteome of *F. hepatica* (PRJEB25283, https://parasite.wormbase.org/Fasciola_hepatica_prjeb25283/ Info/Index), appended to the cRAP contaminant database (https://www.thegpm.org/crap/). The Paragon algorithm [18] was applied to the database with the following parameters: trypsin specificity, IAM cys-alkylation, taxonomy no restricted. Only proteins with at least 2 identified peptides and < 1% FDR were considered for subsequent analysis.

The.*wiff* files obtained from the SWATH experiment were analyzed using PeakView 2.1 (SCIEX) and MarkerView 3.0 (SCIEX). Protein areas were normalized by the total sum of the areas of all the quantified proteins, and proteins matching the contaminant database were removed from the dataset prior to differential expression analysis.

Principal Component Analysis (PCA) of all samples was conducted with the online tool ClustVis [19] and statistical evaluation was performed using GraphPad Prism 9.0. Data was Log2 transformed prior to differential expression analysis, and differences between control

and incubated samples were determined by Student's *t*-Test. *P* values were adjusted using the Benjamini, Krieger and Yekutieli post-hoc corrections. Differentially expressed proteins were identified by q value < 0.05.

Changes in the expression profile within the two analyzed compartments (tegument and soma) were represented in Volcano plots using the ggplot2 package of R software. Differentially expressed proteins were analyzed using Blast2GO 5.2 in order to obtain the associated Gene Ontology (GO) terms in the Biological Process, Molecular Function and Cellular Component categories, and the most enriched GO terms were visualized using the WeGO 2.0 tool (http://wego.genomics.cn/) [20] and the REVIGO tool (http://revigo.irb.hr/) [21]. Unidentified proteins were manually identified using the NCBI blastp algorithm.

Immunoblotting

Protein samples used in mass spectrometry analysis were first concentrated using centrifugal filters with a nominal molecular weight limit of 3 kDa (Merck Millipore) by centrifuging 30 min at 5,000 x *g* and 4°C and protein concentrations were measured using the Pierce BCA Protein Assay (Thermo Scientific). Equal amounts of protein were separated by SDS-PAGE in 12% gels and blotted onto nitrocellulose membranes. After transfer, total protein staining was performed with SYPRO Ruby (Thermo Fisher) according to manufacturer's instructions and membranes were blocked in PBS-0.05% Tween containing 2% BSA. The primary antibody anti-*Fasciola hepatica* serpin 2 (FhSrp2) produced in rabbit against the recombinant protein rFhSrp2 of *F. hepatica* [22] was diluted 1:500 in blocking buffer and added to the blot overnight at 4°C. After incubation with anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (diluted 1:2,000 in blocking buffer; Sigma), the bands were detected using enhanced chemiluminiscence (Clarity Western ECL Substrate, Bio-Rad) on a ChemiDoc MP Imaging System (Bio-Rad).

Histopathological and immunohistochemical study

For the histopathological study, a solution of 10% buffered formaldehyde was injected into the intestinal lumen and the intestines were immersed in the same fixative solution for 24 h. Next, intestines were sequentially trimmed in sections of 0.3 cm and embedded in paraffin wax, sectioned (4 μm thick) and stained with haematoxylin/eosin. For immunohistochemistry, sections (3 μm thick) were dewaxed, rehydrated and endogenous peroxidase activity was exhausted by incubation with 0.3% hydrogen peroxide in methanol for 30 min at room temperature. Antigen retrieval was performed by heating samples for 10 min in 0.01 M sodium citrate buffer (pH 6). Sections were washed in PBS (pH 7.2) and incubated with 20% normal goat serum (Vector Laboratories, Burlingame, California, USA) for 30 min at room temperature. Rabbit anti-mouse Caspase-3 (Biorbyt) primary antibody was diluted 1:400 in PBS containing 10% normal goat serum and incubated overnight at 4°C. Following washing in PBS, sections were incubated with goat anti-rabbit biotinylated secondary antibody (Dako) diluted 1:200 in PBS containing 10% normal goat serum for 30 min at room temperature. After washing in PBS, all sections were incubated with the ABC complex (Vectastain ABC Elite Kit) for 1 h at room temperature in darkness, washed in 0.05 M Tris buffered saline (pH 7.6) and then incubated with the chromogen solution (Vector NovaRED Peroxidase Substrate Kit). Finally, all sections were counterstained with Harris' hematoxylin and mounted with Eukitt (Freiburg, Germany). As a negative control, the specific primary antibody was substituted with non-immune isotype-matched sera.

Results

Ex vivo model

The *ex vivo* experimental system herein described allowed us to successfully replicate the passage of FhNEJ through the intestinal wall of the vertebrate host using a murine model. First, FhNEJ were excysted *in vitro* and collected as they emerged from the metacercariae, resulting in an excystment rate of 80% after 3 hours of *in vitro* incubation. Viability of excysted parasites was checked under a microscope, and only actively moving juveniles were used for the *ex vivo* experiment. By periodically monitoring the passage of parasites under the microscope, we observed that most of FhNEJ crossed the intestinal wall within the first 30 minutes after injection into the intestinal lumen. By the end of incubation (2.5 hours later), approximately 22% of the total FhNEJ injected had managed to break through the intestine (Fig 1C and S1 Video).

Protein identification and quantification

Proteins extracted from the tegument and soma of control parasites and FhNEJ that crossed the intestinal wall were subjected to SWATH-MS. A data-dependent acquisition approach was performed to identify the proteins contained in the library constructed from the pool of all samples. A total of 6,169 mass spectra were obtained and data deposited to the proteomeXchange Consortium via the PRIDE [23] partner repository with the dataset identifier PXD033945. The mass spectra corresponded to 3,384 unique peptides using a local FDR < 1%. These peptides represented 541 different proteins containing two or more unique peptides. Of these, 53 were found in the contaminant database and were not considered for further analyses.

Principal Component Analysis (PCA) of the FhNEJ tegument and somatic extracts was performed for the three replicates of each experimental condition. PC1 was the highest contributor to the variance of both tegument and somatic extracts (Fig 2A and 2B). For tegument extracts, control samples showed a close clustering, and samples from FhNEJ after gut passage showed some variation in the PC1 component, although they were clearly separated from control samples by either PC1 or PC2 variation (Fig 2A). Similar results were obtained for somatic extracts, although samples for each condition showed less optimal clustering as compared to their tegument counterparts (Fig 2B).

After SWATH-MS analysis, a total of 475 proteins were detected in all tegument samples and could therefore be quantified, while 416 proteins were identified in all somatic samples. 404 of those proteins (83% of total quantified proteins) were common to both compartments, while 71 and 12 proteins, representing 14.6% and 2.5% of their total quantified proteins, were uniquely detected in tegument and soma, respectively (Fig 2C). Statistical analysis of the SWATH-MS data from FhNEJ recovered after crossing the mouse intestine revealed 18 upregulated and 54 downregulated proteins in the tegument fraction, whereas 7 upregulated and 6 downregulated proteins were found in the soma, in comparison with control FhNEJ (Fig 3A and 3B, respectively).

Functional annotation

Gene Ontology annotation analysis (in the categories of Biological Process–BP–and Molecular Function–MF–) was performed on the differentially expressed proteins, and the results were plotted using the WEGO 2.0 tool (Fig 4A and 4B, respectively). In the tegument of FhNEJ, the main GO terms identified included terms mainly related to metabolic and biosynthetic processes in the BP category, and mainly related to binding functions in the MF category (Fig 4). REVIGO analysis of these extracts showed that some of the most representative terms in the
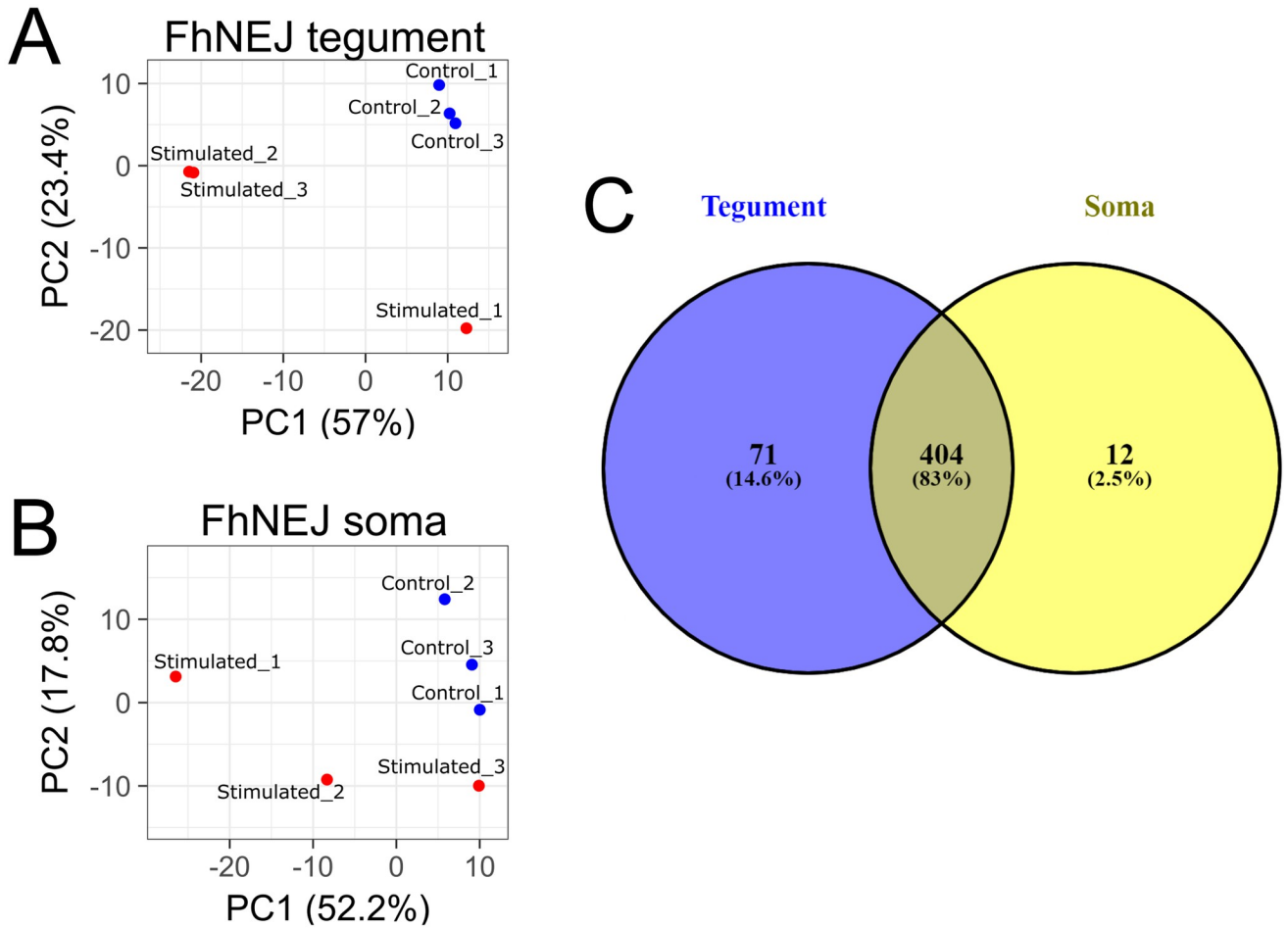
Fig 2. Principal Component Analysis (PCA) of FhNEJ (A) tegument and (B) somatic extracts. Blue dots represent the control replicates (Control_1 to _3) whereas red dots represent the replicates of FhNEJ after migration across the gut wall (Stimulated_1 to _3). The percentage of variance showed by each principal component (PC1 and 2) is indicated in its corresponding axis. (C) Venn's diagram showing the distribution of quantified proteins.

BP category were linked with proteolysis, transcription and glutathione metabolic process within upregulated proteins, and within the tricarboxylic acid cycle and mechanisms of cellular oxidant detoxification within down-regulated proteins (S1 Fig).

Details on annotation and analysis of upregulated and downregulated proteins in both tegument and somatic extracts are shown in Fig 5 and S1 Table. The topmost upregulated protein in the tegument was the protease cathepsin L (Log2FC 7.21), followed by histones H4 and H2B and the protease inhibitor Fh serpin 2. For the histones identified in *F. hepatica* extracts, the mass spectra were compared with the corresponding proteins from both *F. hepatica* and *Mus musculus*, and the identifiers assigned as preferred were those of *F. hepatica*, so despite being very conserved molecules, we could unequivocally assign those identifications to the parasite. Serpin protease inhibitors were overexpressed in the tegument extract with three different UniProt identifiers (see S1 Table) corresponding to three different proteins, and the sum of their respective Log2FC values place serpins in the first position of overexpressed proteins in the tegument of FhNEJ after gut passage. Overexpressed proteins in the tegument of FhNEJ upon gut passage also included a tegument antigen, the hypothetical protein D915_002431 containing a sperm-protein, enterokinase and agrin domain [24], and a low-density
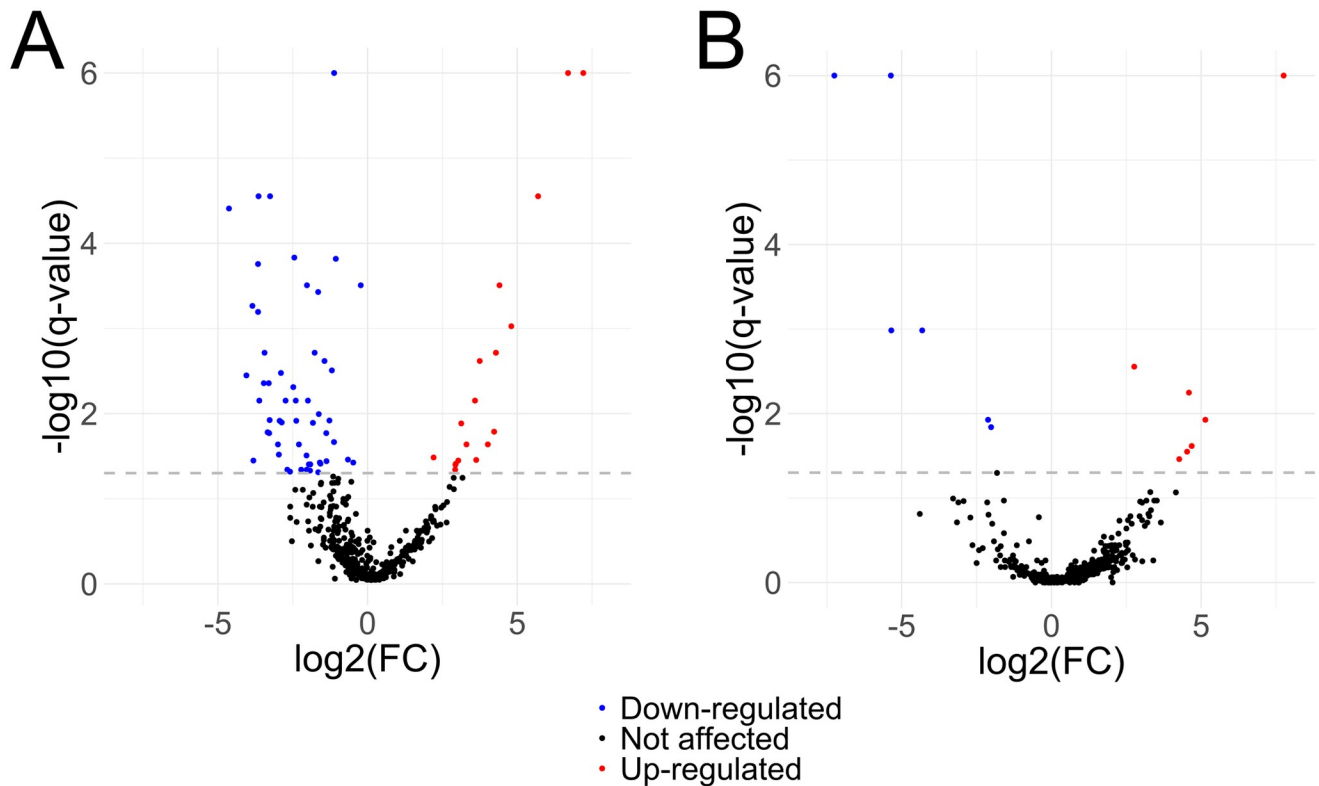
Fig 3. Volcano plots of the significantly differentially expressed proteins in tegument (A) and somatic extract (B) of *F. hepatica* juveniles after passing through mouse intestinal wall. The grey dashed line represents the threshold delimiting the differentially expressed proteins. Red dots represent upregulated proteins, while blue dots represent downregulated proteins.

lipoprotein (LDL) receptor, followed by two components of the electron transport chain, tetraspanin, two fatty acid binding proteins and the glutathione S-transferase Mu-class, among others (Fig 5).

Among downregulated tegument proteins, we identified numerous proteins related to peptide synthesis and degradation together with proteins with antioxidant activity and metabolic enzymes (Fig 5). The top ten downregulated proteins in terms of Log2FC included alpha crystallin, ribosomal protein 55a, retinol dehydrogenase 12 and endophilin B1, among others (S1 Table).

In the somatic fraction of FhNEJ that crossed the intestinal wall, the most represented GO terms were also related to metabolic processes in the BP category, and with binding and catalytic activity in the MF category (Fig 4). REVIGO analysis showed that BP terms related to glycolysis and gluconeogenesis were enriched in upregulated proteins, while downregulated proteins were mostly related to ribosome biogenesis, mRNA processing and protein folding (S1 Fig).

Accordingly, upregulated somatic proteins (Fig 5 and S1 Table) included enzymes involved in proteasomal degradation and protein secretion, together with vesicle trafficking related proteins, while downregulated proteins were mainly involved in protein synthesis and folding, as well as in mRNA processing. Noteworthy, five out of six proteins found downregulated in the somatic extract behaved likewise in the tegument extract.

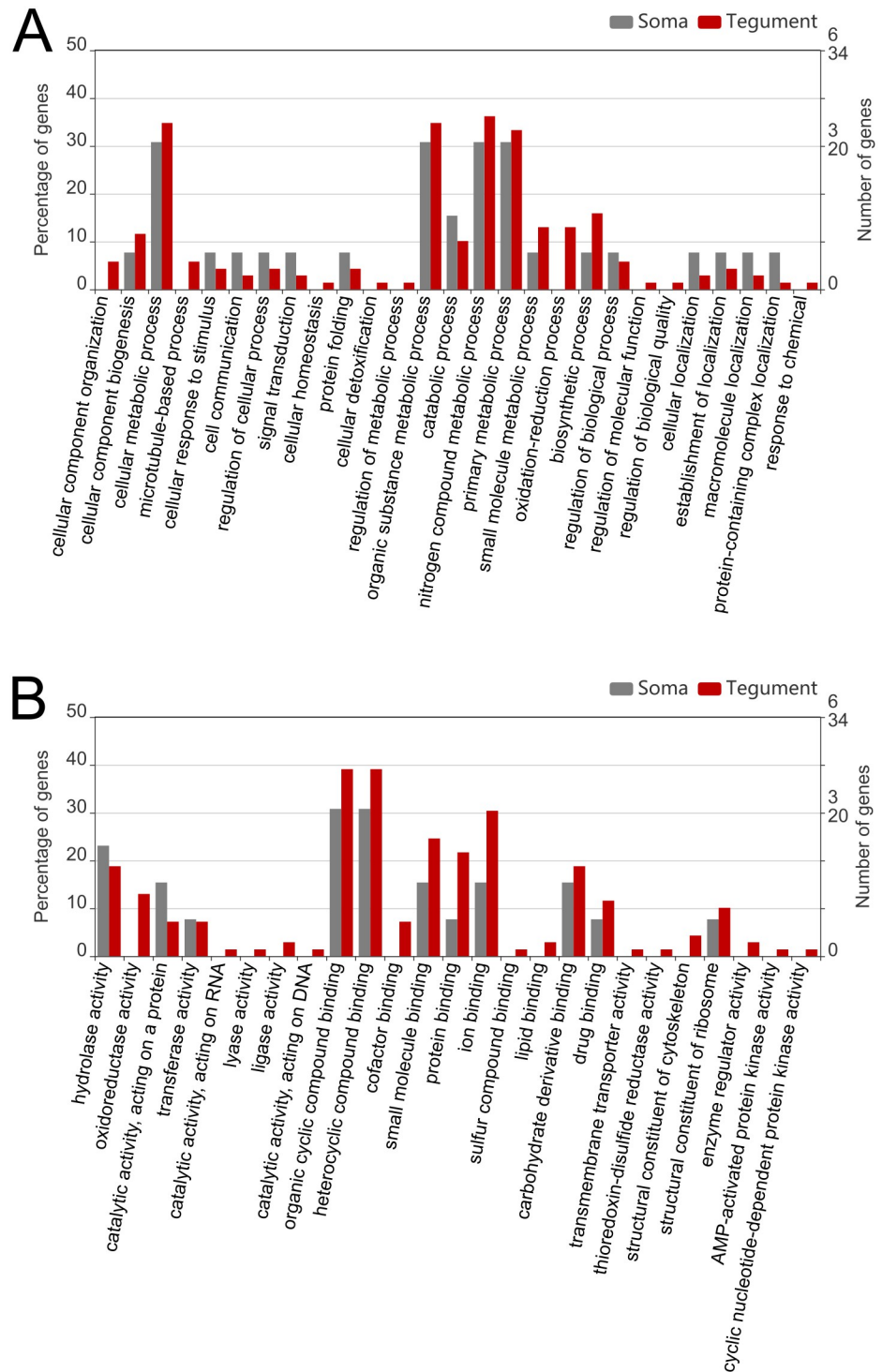**Fig 4. Bar graphs representing the results of the Gene Ontology analysis performed on the differentially expressed proteins, referred to Biological Process (A) and Molecular Function (B) categories.** Red bars represent the proportion of proteins found in the tegument samples, and grey bars represent the proportion of proteins found in the somatic samples.

https://doi.org/10.1371/journal.pntd.0010766.g004

Log2FC	Description
7,21	Cathepsin L
6,70	Histone H4
5,70	histone H2B
4,80	serpin 2
4,41	Tegumental antigen
4,29	serpin 2
4,23	hypothetical protein D915_002431
4,02	Low-density lipoprotein receptor (Ldl)
3,75	Succinate dehydrogenase complex subunit C
3,63	Serpin protein
3,59	Tetraspanin
3,30	Histone H2A
3,13	26S proteasome non ATPase regulatory subunit
3,03	NADH dehydrogenase (ubiquinone) Fe-S protein 2
2,94	Fatty acid binding protein a
2,94	glutathione S-transferase
2,93	Immunoglobulin domain-containing protein
2,21	fatty acid binding protein

Log2FC	Description
-0,23	Heterogeneous nuclear ribonucleoprotein A2
-0,48	Tubulin beta chain
-0,66	Guanine nucleotide-binding protein subunit beta
-1,06	Cytosolic large ribosomal subunit protein L30
-1,12	Glyceraldehyde-3-phosphate dehydrogenase testis-specific
-1,12	2-oxoglutarate dehydrogenase mitochondrial
-1,19	Mitochondrial-associated endoribonuclease MAR1
-1,27	Eukaryotic ribosomal protein L18
-1,37	Trans-2-enoyl-CoA reductase mitochondrial
-1,38	Small subunit ribosomal protein S2e
-1,44	thioredoxin-glutathione reductase
-1,57	Ubiquitinyl hydrolase 1
-1,59	Speckle-type POZ protein
-1,63	protein kinase A
-1,65	UDP-n-acetylglucosamine pyrophosphorylase
-1,65	Proteasome subunit beta type
-1,77	Eukaryotic-type carbonic anhydrase
-1,82	actin, putative
-1,91	glycine-rich cell wall structural protein isoform X3*
-1,92	RuvB helicase
-1,96	Chaperonin containing TCP1 subunit 2 beta
-1,99	L-lactate dehydrogenase A chain
-2,02	Major egg antigen
-2,03	Ubiquitinyl hydrolase 1
-2,04	Tubulin beta chain*
-2,22	Large subunit ribosomal protein L22e
-2,29	40S ribosomal protein S8
-2,38	hypothetical protein D915_010660
-2,40	Chaperonin-containing T-complex protein 1 eta subunit
-2,45	ubiquinol-cytochrome c reductase cytochrome c1 subunit
-2,48	Small COPII coat GTPase
-2,59	Stress-induced-phosphoprotein 1
-2,68	Long-chain-fatty-acid--CoA ligase
-2,74	N-myc downstream regulated
-2,87	Tubulin alpha-1C chain
-2,89	Small nuclear ribonucleoprotein D1 polypeptide
-2,94	Heat shock protein heat shock protein 90 alpha
-2,96	UMP-CMP kinase
-2,99	Cytosolic purine 5' nucleotidase
-3,26	Troponin i
-3,27	Ribosomal protein S4 Y-linked 1 Y isoform variant
-3,29	Alpha-adducin
-3,30	Developmentally-regulated GTP-binding protein 1
-3,34	Trifunctional enzyme subunit beta
-3,44	ATP-dependent RNA Helicase PI10
-3,47	Ubiquitin activating enzyme
-3,62	hypothetical protein D915_004677
-3,64	hypothetical protein D915_005536
-3,66	Endophilin-B1*
-3,66	ATP synthase subunit delta mitochondrial
-3,81	putative merlin/moesin/ezrin/radixin
-3,85	Retinol dehydrogenase 12
-4,05	Ribosomal protein S5a
-4,63	Alpha crystallin-containing small heat shock protein variant NtermFhHSP35a

72 dif. regulated proteins

TEG

SOM

13 dif. regulated proteins

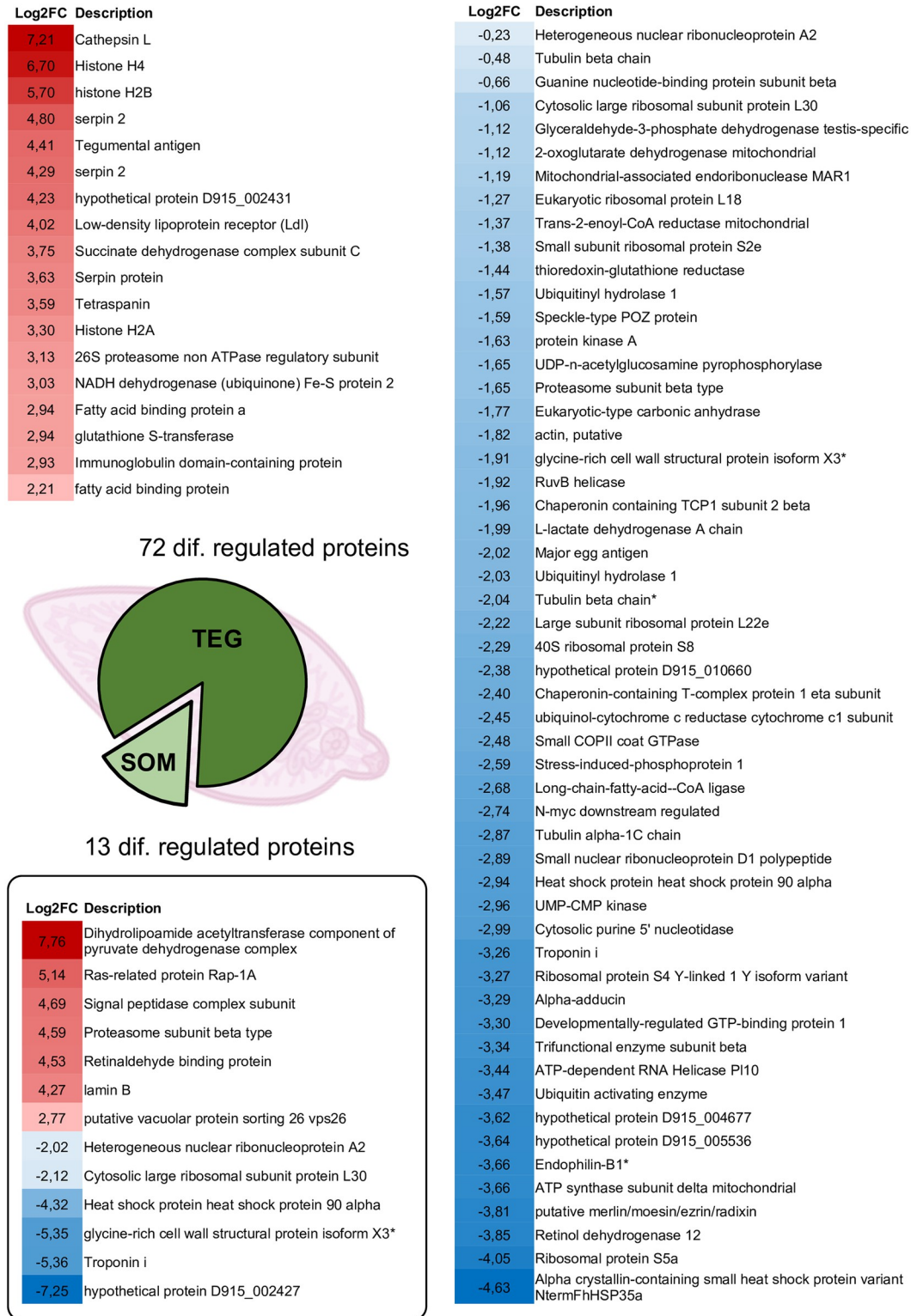| Log2FC	Description
7,76	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex
5,14	Ras-related protein Rap-1A
4,69	Signal peptidase complex subunit
4,59	Proteasome subunit beta type
4,53	Retinaldehyde binding protein
4,27	lamin B
2,77	putative vacuolar protein sorting 26 vps26
-2,02	Heterogeneous nuclear ribonucleoprotein A2
-2,12	Cytosolic large ribosomal subunit protein L30
-4,32	Heat shock protein heat shock protein 90 alpha
-5,35	glycine-rich cell wall structural protein isoform X3*
-5,36	Troponin i
-7,25	hypothetical protein D915_002427

Fig 5. Upregulated (in red) and downregulated (in blue) proteins in tegument (upper panels) and somatic (box lower panel) extracts of *F. hepatica* juveniles after intestinal passage, compared with control FhNEJ. Both protein description and the respective Log2FC values are shown. Colour scales represent the relative Log2FC value in each panel.
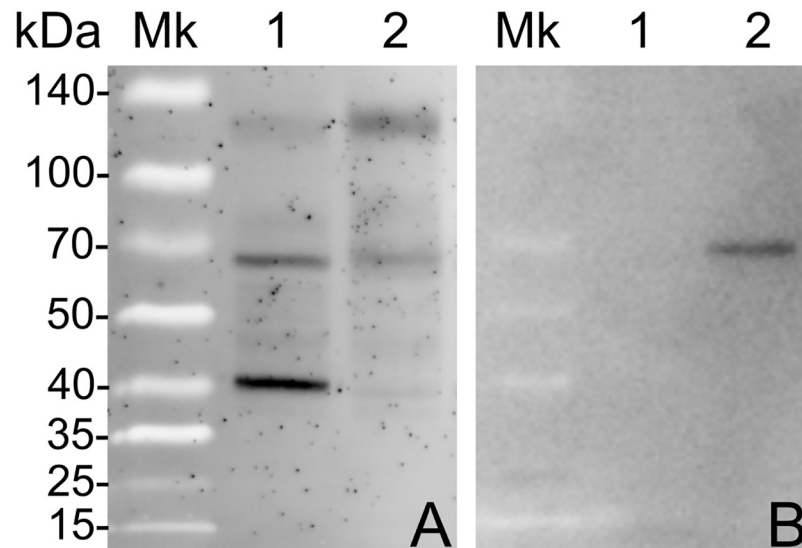
Fig 6. SDS-PAGE (A) and immunoblot (B) of tegument extracts from control FhNEJ (1) and FhNEJ after intestinal passage (2). (A) Sypro staining of total proteins; (B) anti-rFhSrp2 immunoblot. Molecular weights are shown in kDa.

Immunoblot

In order to validate our model, tegument extracts from control FhNEJ and FhNEJ that crossed the intestinal wall were subjected to SDS-PAGE and immunoblot using antibodies against recombinant *F. hepatica* serpin 2, rFhSrp2 [22]. Reactivity was detected at ~60 kDa in the tegument of FhNEJ after intestinal passage, while no reactivity was detected in tegument extracts of control FhNEJ (Fig 6).

Immunohistological analysis of the intestine

Histological examination of mouse intestine samples was performed by comparing hematoxylin-eosin staining of fresh intestine samples (Fig 7A) with those incubated for 2.5 hours at 39°C, including both FhNEJ-infected and uninfected samples. Although both control (uninfected control intestines kept at the same conditions that the infected intestines) and infected intestines showed loss of villi after incubation (Fig 7B and 7C, respectively), the infected intestines showed thinner walls together with separation of the muscular and serosal layers. Detailed analysis revealed the presence of FhNEJ in the apical region and deeper areas, reaching the crypts of Lieberkühn, although no inflammatory infiltrate around FhNEJ was observed (Fig 7C and 7D). Examination of these tissues also showed the presence of pycnotic and fragmented nuclei, suggesting cellular apoptosis. This was further assessed by immunohistochemical labelling with an anti-cleaved caspase 3 antibody, whose presence was more frequently detected in certain areas of FhNEJ-infected intestine samples, compared with fresh or noninfected intestines (Fig 8). Specifically, the percentage of caspase-3 positive cells or cell debris per field (x400) examined in 12 different fields was 80.5% and 76.1% lower in fresh and noninfected intestines, respectively, compared that of infected intestines.

Discussion

Migration through host tissues is a mechanism commonly used by the larval stages of helminth parasites. Despite the high energy and adaptive costs that this process implies, it confers
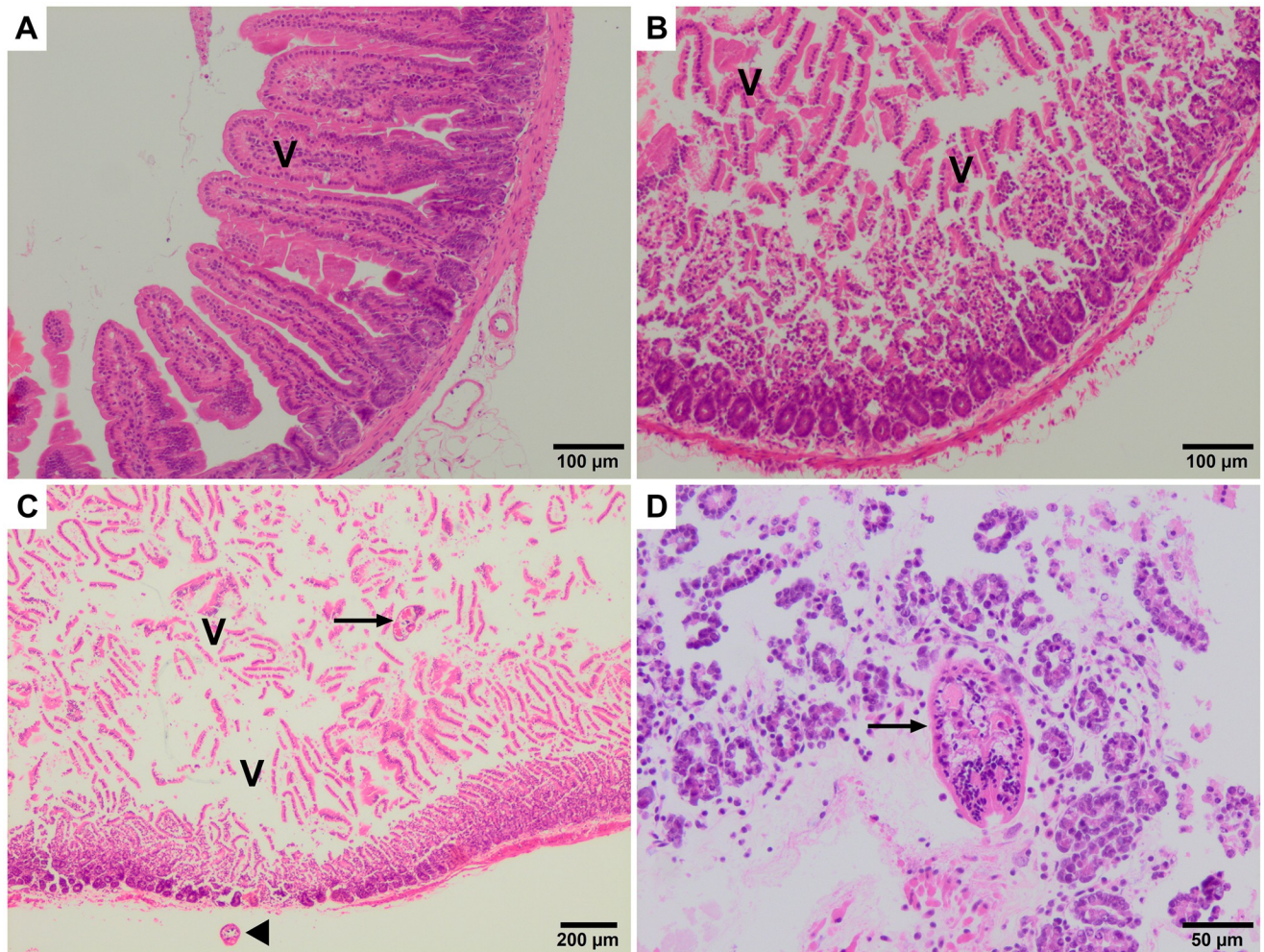
Fig 7. Histological features of the intestine of mice after hematoxylin-eosin staining. (A) Fresh intestine from a non-infected mouse showing normal villi (V). (B) Non-infected intestine after incubation for 2.5 hours at 39˚C and 5% of CO_2 showing detachment of villi (V). (C) Infected intestine after incubation and FhNEJ passage showing detached villi (V) and FhNEJ in the intestinal lumen (arrow) and in the intestinal serosa (arrowhead), both without inflammatory infiltrate. D) Detail of a FhNEJ (arrow) crossing the intestinal crypts. Note that there is no inflammatory infiltrate in the periphery of the FhNEJ.

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evolutionary advantages and constitutes an immune evasion strategy, allowing the parasite to grow in size and modify its antigenic profile [7, 25]. In this line, FhNEJ have a dynamic composition that undergoes deep changes within the first hours post excystment, as it has been demonstrated through the establishment of *in vitro* models that include FhNEJ culture in the presence or absence of host stimuli and downstream -omics analyses [10, 26, 27]. However, none of these approaches has replicated the passage of FhNEJ through the host intestinal barrier coupled with SWATH-MS, a highly sensitive quantitative proteomics technique, which has been barely employed in the field of parasitology [12, 13]. This technique comprises two steps: an identification phase in which a protein library is constructed from all the identified peptides using a traditional HPLC-MS methodology, followed by a quantification phase in which proteins from individual samples are acquired using multiple narrow m/z windows [14]. When compared to conventional proteomic methodologies, SWATH-MS combines the sensitivity of Data-Dependent Acquisition (DDA) approaches, such as Selected Reaction
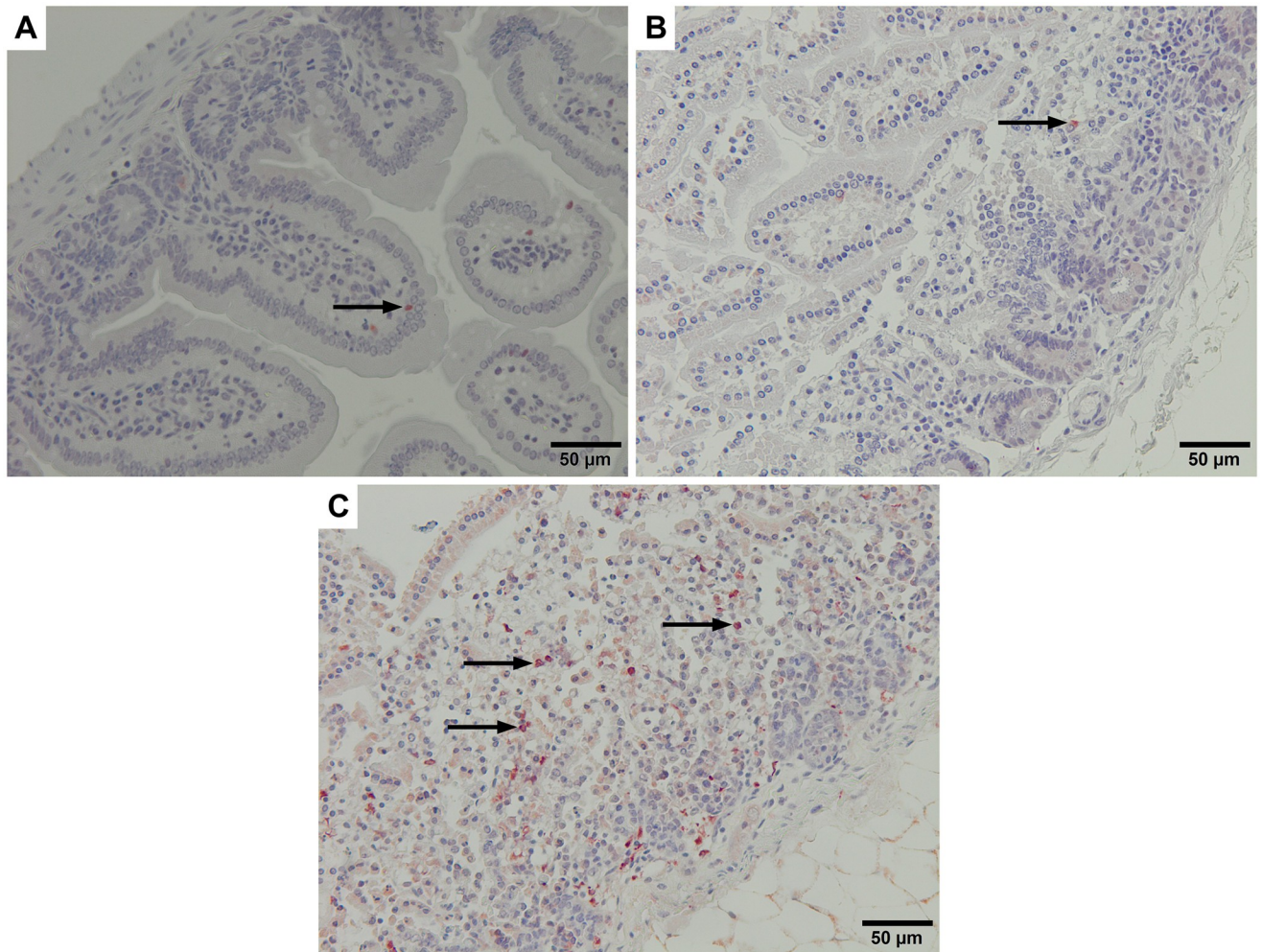
Fig 8. Mouse intestine subjected to the ABC method and hematoxylin counterstain. Cleaved caspase 3 in non-infected fresh intestine (A), non-infected incubated intestine (B) and infected and incubated intestine (C) all of them showing caspase 3+ cells (arrow). Specific areas of the infected and incubated intestine (C) showed a higher number of caspase 3+ cells in the lamina propria (arrows), compared with the fresh and the incubated and non-infected intestine.

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Monitoring (SRM), with the high-throughput analytical capacity of Data Independent Acquisition (DIA)- based shotgun proteomics [15]. Thus, the main objective of this study was to set up an *ex vivo* model of FhNEJ intestinal crossing coupled to a quantitative proteomic comparison of these parasites before and after gut passage, by using the recently developed SWATH-MS technology. The ultimate goal of this work is to unravel the main changes in protein composition of the tegument and somatic fractions of FhNEJ driven by gut passage.

In the *ex vivo* murine model used here, FhNEJ (Italian isolate) started appearing at the extra-luminal space in as little as 30 min. This observation, together with the recovery rate of FhNEJ is similar to those made by García-Campos *et al.* using an *in vitro* model [28], in which small pieces of rat intestine were used to evaluate FhNEJ passage using the Oregon isolate (20.96% passage after 2.5 hours), although these authors described a percentage of passage at 2.5 hours of 7.44% for the Italian isolate. An additional *ex vivo* model using intestinal loops of rats non separated from the whole body and injected with FhNEJ showed comparable results to ours with an in-house obtained *F. hepatica* isolate (35% passage at 2.5 hours of incubation

[8]). This suggests that different parasite isolates show a similar intestinal migratory behaviour, and that differences in percentage of migration could be attributed to differences in the viability of different parasite batches.

Our approach was planned to separately study a tegument-enriched fraction [10] and the somatic extract of FhNEJ after gut passage in comparison with control FhNEJ, to bring into focus the changes taking place at the host-interacting surface of the parasite, and to compare these changes with those occurring at internal tissues. Principal Component Analysis (PCA) discriminated between FhNEJ that were incubated alone and those that crossed an intestinal wall, although some intragroup variation was detected, similar to what has been described in comparable studies [29]. Annotation of proteins showed that both parasite compartments shared a high percentage of proteins, as expected. The percentage of proteins differentially regulated in each antigenic compartment was higher in the tegument than the somatic extract, indicating that the influence of the host on the regulation of protein expression mainly affects the parasite compartment that is directly exposed to host tissues.

Annotation of GO terms for each of the differentially regulated proteins revealed notable changes in metabolic and binding processes in both extracts, similar to the findings reported by Cwiklinski *et al.* [30] after GO annotation of the transcriptome of *F. hepatica* juveniles collected from mice 21 days post infection. The upregulated GO terms reported in our study (proteolysis, catalytic activity, transcription initiation and glutathione metabolic process) are also in agreement with those found by Cwiklinski *et al.* for FhNEJ [27], both in transcriptomic and excretory/secretory proteomic analyses of FhNEJ at several times post excystment. Conversely, we identified downregulation of TCA- and detoxification-related GO terms upon gut passage that were found to be well represented in previous proteomic and transcriptomic studies of early stages of *F. hepatica* (rev. in [11,30]). Nonetheless, comparison of our results with those reported by other authors could be hampered by the relative similarity between parasite stages and compartments used. Two important considerations could explain this discrepancy. First, none of the previous studies focused on profiling the proteome of *ex vivo* obtained FhNEJ after gut passage. Second, the performance of the SWATH-MS proteomic approach used in our study in terms of quantitative power is not comparable to that of semiquantitative proteomics approaches used until now. This said, our finding that TCA- related proteins are downregulated in the tegument of FhNEJ after *ex vivo* gut passage would be in line with the transition from aerobic to anaerobic metabolic states described both *in vitro* and *in vivo* in *F. hepatica* after FhNEJ excystment and during juvenile development (rev. in [7]). Nevertheless, it is important to mention that GO analysis could result in a broad description of the detected pathways. Notably, some reports show that the switch from aerobic to anaerobic metabolism of this parasite is a phenomenon that also occurs independently of host stimuli, given that it is detectable in the proteome of *in vitro* maintained FhNEJ 24 hours post excystment, compared with metacercariae and with FhNEJ 3 hours post excystment (e.g., [27]). Our results show that this metabolic switch seems to be enhanced by host stimulation, and that it first occurs at the host-parasite interface rather than deeper tissues of the parasite, since here TCA-related GO terms were found unaltered in the somatic extracts of *ex vivo* FhNEJ. Thus, and despite oxygen diffusion to the internal tissues of the parasite is expected to be more limited than that of its surface, we can still reason that the tegument could be the tissue ahead of the switch between aerobic and anaerobic metabolism in parasites facing the vertebrate host environment. Consistent with this assumption, REVIGO analysis of somatic extracts showed upregulation of GO terms related to gluconeogenesis and glycolysis, both linked to aerobic metabolism, similar to the observations made by Cwiklinski *et al.* [27] when comparing *in vitro* maintained FhNEJ during 3 hours and 24 hours. REVIGO analysis of both up- and downregulated GO terms in somatic extracts displayed a specific pattern different from that of the tegument. This

demonstrates that proteomic changes triggered by host stimulation results in differential patterns of parasite protein regulation at the host-parasite interface, compared with those occurring in inner compartments.

It has been extensively described that CL3 and CL4 isoforms are the CL proteases mainly expressed by NEJ, while CL1, CL2 and CL5 are more abundant in adult worms (rev. in [7]). Annotation of the tegument CL isoform identified in the present study (UniProt code A0A4E0RQP0) did not manage to assign it to a specific isoform. Previously, sequence analysis of *Fasciola* cathepsin L prosegments, which regulate enzyme activity by binding to the substrate cleft, allowed for the definition of 22 amino acid consensus sequences that are specific to every CL isoform prosegment [31]. This enabled grouping of CL isoforms from CL1 to CL5 in *Fasciola*, which facilitates annotation of these proteases. When we compared the prosegment of the CL identified in our set of proteins with the abovementioned consensus sequences, we found that the 22 amino acid consensus in the prosegment sequence herein identified was almost identical to the prosegment of CL4 (90.9% identity), and less similar to the prosegment of CL1A, CL1B, CL2, CL3 and CL5 (72.7%, 81.8%, 63.6%, 40.9% and 72.7% identity, respectively; S2 Fig). Transcriptomic analysis of metacercariae and FhNEJ maintained *in vitro* for 1, 3 and 24 hours revealed high gene expression of both CL3 and CL4, which declined in juveniles collected from mice 21 days post infection and adult worms [32]. CL4 is expected to play an intracellular housekeeping function, since it has not been identified in the excretory/secretory fraction and extracellular vesicles of FhNEJ by several authors [27,32–35], with the exception of Di Maggio *et al.* [36]. These last authors identified CL4 in both secretions and somatic extracts of FhNEJ maintained in culture for 48 hours, but its presence in the secretory fraction could be attributed to contamination with tegument proteins. Noteworthy, CL3 but not CL4 has been identified in tegument extracts of FhNEJ [9]. Consequently, our results suggest that CL4 is in fact most probably restricted to tegument expression, and that this specific isoform is overexpressed in FhNEJ upon gut passage. By contrast, CL3 seems to be expressed in FhNEJ without requiring host stimulus, as it is upregulated around 1 hour after excystment in FhNEJ maintained *in vitro* [11]. Furthermore, our results and those previously reported by others suggest that CL4 in not synthesized within the gastrodermal epithelial cells of the parasite and stored in "secretory" vesicles as FhCL3 is, and raises the possibility that CL4 may also play a role at the host-parasite interface during gut penetration of the parasite. The biological relevance of CL4 expression in the intestinal phase of *F. hepatica* infection may have been neglected in previous studies based on *in vitro* systems that did not include host cells and/or tissues during FhNEJ incubation.

The members of the serpin superfamily have been mainly related to the regulation of peptidase activity in FhNEJ (rev. in [7]) and described as highly expressed on their surface. Here, we found three serpins over-expressed in the tegument of FhNEJ after gut passage, corresponding to the WormBase identifiers maker-scaffold10x_794_pilon-snap-gene-0.129, maker-scaffold10x_113_pilon-augustus-gene-0.45 and maker-scaffold10x_293_pilon-augustus-gene-0.19, as detailed in S1 Table. These identifiers correspond to Fh serpin 4, Fh serpin 2 and Fh serpin 5, following the phylogenetic analysis performed by [22]. More specifically, Fh serpin 2 has been observed to be associated with the apical region of the spines within the parasite tegument [37]. Fh serpin 2, which is a potent inhibitor of the small intestine protease chymotrypsin, is more abundantly transcribed in juveniles 21 days post infection than in FhNEJ maintained *in vitro* at various times post excystment and adult worms [22]. However, our results show that this molecule is overexpressed early after gut invasion by FhNEJ, suggesting that host stimulation enhances the process of Fh serpin 2 production in FhNEJ. Fh serpin 2 has also been described as an inhibitor of inflammatory-related molecules, including chymase, neutrophil elastase and cathepsin G [22], indicating that Fh serpin 2 can both protect FhNEJ

during gut passage from host-derived proteolysis and play a role in immune evasion. In the present study, Fh serpin 2 overexpression was used to validate our proteomic results by immunoblot, which revealed a specific band at ~60 kDa in tegument extracts of FhNEJ that crossed the intestinal wall, but not in FhNEJ maintained *in vitro* for an equivalent time. The described molecular weight of *F. hepatica* native serpin 2 is ~40 kDa, although it has been shown that serpins can be found in native protein extracts complexed with other proteins or with themselves [22]. Specifically, Fh serpin 2 can form a covalent complex with chymotrypsin, resulting in the formation of a highly stable SDS complex of ~60 kDa, compatible with the band detected in our experiments. Our results confirm that Fh serpin 2 is one of the main proteins produced at the tegument of FhNEJ during gut passage, most likely to inhibit intestinal host proteases, although a role in the regulation of the activity of FhNEJ surface expressed proteases (e.g., CL4) cannot be ruled out given that Fh serpin 2 can also inhibit cathepsin L [22].

In relation to nutrition, both a low-density lipoprotein (LDL) receptor and several fatty acid binding proteins (FABPs) were found overexpressed in FhNEJ tegument after gut passage. *F. hepatica* is unable to synthesise lipids, and expresses several proteins for host lipid uptake [38]. This seems to be an especially active process in FhNEJ crossing the intestinal barrier, where we have found both LDL receptors and FABPs overexpressed, which could use host lipids as an essential energy source, transitioning from the use of their own endogenous energy sources to rely on host nutrients. In these very early stages, host nutrient intake still depends mainly on the tegument, since *F. hepatica* gastrodermal cells start their uptake activity later in the development of the parasite [39]. Additionally, both LDL receptors and FABPs have been also related to immune evasion mechanisms in *Schistosoma* and *Fasciola* [30,40].

Also linked to immune evasion due to its anti-inflammatory properties [41], and to antioxidant defence mechanisms of the parasite, glutathione-S-transferase (GST) has been extensively studied in adult worms as one of its excretory-secretory antigens, and has been identified in FhNEJ and juvenile secretions [30]. GST has been described in the cytoplasmic extensions of parenchymal cells in FhNEJ [42], which could account for its presence in the tegument of *ex vivo* obtained FhNEJ. Intriguingly, transcriptional analysis of several *F. hepatica* developmental stages showed that immature (21 days post-infection) flukes favour the thioredoxin-dependent antioxidant defence system instead of GST-based defence mechanisms, contrary to *F. gigantica* [30]. Whether GST could play a central anti-inflammatory role in the tegument of FhNEJ during gut passage, or just an accessory one (e.g., absorptive), is a matter of further investigation. In this context, immunohistological study of the intestinal fragments used in our experiments showed a lack of inflammatory infiltrate in the periphery of migrating FhNEJ, which could contribute to an overall anti-inflammatory environment in the intestine together with overexpressed Fh serpin 2 and GST in the tegument of FhNEJ. Additionally, immunostaining of intestinal fragments used in our *ex vivo* experiments showed a high number of caspase 3 positive leucocytes in the intestine after FhNEJ passage. Similar to our results, peritoneal leucocyte apoptosis driven by early stages of *F. hepatica* was described in sheep [43]. Thus, the lack of inflammation found in sections adjacent to FhNEJ during gut passage could also be explained by cell apoptosis.

The overexpression of the hypothetical protein D915_002431 in the tegument of *ex vivo* obtained FhNEJ is conspicuous. This protein contains the so called SEA domain, which is closely associated to regions receiving extensive O-glycosylation at the cell surface and adjacent to transmembrane proteins, including mucin-1 [24]. Glycans are rapidly shed from the tegument of FhNEJ and have important roles in invasion and tissue penetration, probably through binding to lectin receptors on host intestinal epithelial cells (rev. in [28]). Whether this specific hypothetical protein is involved in the pathogenicity and life cycle progression of *F. hepatica* deserves further investigation.

An additional number of proteins involved in trafficking, membrane dynamics and binding, transcriptional regulation/DNA replication and cell cycle progression were found overexpressed in the tegument of *ex vivo* obtained FhNEJ, among them several histones. The analysis of the transcriptome of *F. hepatica* showed that genes related to neoblasts such as histone 2A are constitutively expressed from NEJ to adult stages and have increased transcription in the juvenile parasites [27]. Additionally, regulation of histones had also been described for *Schistosoma mansoni* schistosomula maintained *in vitro*, in which expression of H2A, H2B and H4 was higher in the tegument of 5 days schistosomula, compared with 2 days and 3 hours schistosomula [44]. Similarly, a number of proteins related to cell cycle progression and cellular differentiation in early development were found overexpressed in the somatic extracts of *ex vivo* obtained FhNEJ. These proteins could be involved in the control of cell proliferation and differentiation during the intensified cell multiplication and transformation of FhNEJ towards adult worms. Overexpressed proteins in the somatic fraction of FhNEJ also included the putative vacuolar protein sorting vps26. This protein has been described as one of the main components of the retromer complex, which is involved in the trans-Golgi network that packages proteins into vesicles destined to lysosomes, secretory vesicles, or the cell surface. Notably, a similar protein was described in *Entamoeba histolytica* and was related to trafficking of cysteine proteases in this parasite [45], and it has similarly been linked to vesicular protein sorting and biogenesis of secretory organelles in *Plasmodium falciparum* and *Toxoplasma gondii* [46,47]. Vps26 could also be participating in vesicular trafficking in FhNEJ and could therefore be of crucial importance for the survival and development of the parasite at early stages of infection.

Regarding downregulated proteins, it has been described that HSP-90 and alpha crystallin are more highly expressed in metacercariae and FhNEJ maintained *in vitro* 1 hour after excystment compared to FhNEJ at 3 and 24 hours post excystment [27]. These proteins have been associated with the response to sudden environmental changes encountered by "dormant" stages of different parasites. Our results show that protection against reactive oxygen species of the host by HSPs could be crucial for FhNEJ found within the intestinal lumen, and may be put on the back burner once FhNEJ have passed through the intestinal wall.

In summary, we have shown that host stimulation (gut passage) triggers changes in the proteome of FhNEJ both at the tegument and somatic levels, which results in the expression of defined sets of proteins as well as an acceleration in the expression of additional proteins that would otherwise occur at a slower rate in FhNEJ axenically maintained *in vitro*. Our proteomic findings of upregulated and downregulated expressed proteins in the two fractions are similar to previous reports, as above-mentioned for each protein or group of proteins. Noteworthy, Hanna and Jura [48] described that significantly more *F. gigantica* flukes successfully established in mice via intraperitoneal injection of NEJ than oral infection with metacercariae. The authors attributed these differences to inappropriate physicochemical conditions for excystment in the gut of the mouse, although the mouse model has been considered suitable for studies on migration and establishment of *F. gigantica* by several authors (e.g., [49]). Whether these differences are also true for *F. hepatica* is a matter of future investigations and could contribute to decipher the importance of intestinal passage in the physiology and establishment of *F. hepatica* in the vertebrate host.

Supporting information

S1 Fig. REVIGO analysis of regulated proteins. The analysis shows the enriched GO terms in the Biological Process (BP) category, for upregulated (green) and downregulated (pink) proteins annotated in somatic and tegument extracts of FhNEJ after gut passage, compared with

control FhNEJ. Size of each circle represents the relative abundance of each BP term. Performed at http://revigo.irb.hr/.
(TIF)

S2 Fig. Comparison of the consensus amino acid sequence of the prosegment of cathepisns L1 to L5 with CL_A0A4E0RQP0. Alignment of the consensus sequences of the non-conserved *Fasciola* cathepsin L (CL1A, CL1B, CL2, CL3, CL4 and CL5) protease prosegment C-terminal regions, as described in [31], and the cathepsin L sequence identified as over-expressed in the tegument of FhNEJ after gut passage (CL_A0A4E0RQP0) in shown. As shown in the figure, the cathepsin L found in our study shows the highest percentage of identity in this region (90.9%) with the consensus sequence of CL4. Gaps in the alignment are represented by a point.
(TIF)

S1 Video. Intestinal passage of FhNEJ. The video shows FhNEJ crossing the intestine in the *ex vivo* model used in our experiments.
(AVI)

S1 Table. Up- and down-regulated proteins in FhNEJ upon intestinal passage. Details on annotation and analysis of upregulated and downregulated proteins in both tegument and somatic extracts are shown, including Protein ID, Uniprot Accession number, Description, Log2FC and GO IDs for Cellular component (CC), Molecular function (MF) and Biological process (BP). Teg up: proteins upregulated in FhNEJ tegument; Teg down: proteins downregulated in FhNEJ tegument; Som up: proteins upregulated in FhNEJ somatic extract; Som down: proteins downregulated in FhNEJ somatic extract.
(XLSX)

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Capítulo 4:

Study of the cross-talk between *Fasciola hepatica* juveniles and the intestinal epithelial cells of the host by transcriptomics in an *in vitro* model

Resumen de la publicación

La fasciolosis es una parasitosis de gran importancia veterinaria y clínica, responsable de pérdidas millonarias en la industria ganadera, así como un importante problema de salud en regiones endémicas. La capacidad de adaptación del verme, así como la aparición de cepas resistentes a los principales antihelmínticos conocidos, han convertido a la fasciolosis en una preocupación de primer orden, y recalcan la necesidad de encontrar alternativas terapéuticas que permitan el tratamiento eficaz de la enfermedad en sus etapas tempranas, idealmente mediante la utilización de vacunas. No obstante, aunque la etapa inicial de la enfermedad es determinante para el desarrollo de la infección, y responsable de la mayor parte de la mortalidad asociada, el conocimiento de la misma es relativamente escaso.

En este estudio, se utilizó la tecnología de secuenciación de ARN (RNA-Seq) acoplada al modelo *in vitro* de interacción parásito-hospedador desarrollado en el segundo capítulo de la presente Tesis Doctoral, con el fin de caracterizar las respuestas desplegadas por ambos organismos tras su primer contacto a nivel de la mucosa intestinal. El RNA-Seq constituye una herramienta ideal para el estudio de las etapas iniciales de la infección por su capacidad para analizar el transcriptoma completo de ambos organismos, así como por la pequeña cantidad de ARN requerida para su uso, que permite el estudio de los vermes juveniles.

El análisis transcriptómico detectó cambios significativos en la expresión de 342 genes en los vermes juveniles de *Fasciola hepatica*, involucrados en procesos como la degradación proteolítica y la incorporación de nutrientes, entre otros, mientras que en las células de ratón se identificaron 140 transcritos diferencialmente expresados tras el contacto con el parásito, relacionados con procesos de respuesta inmune, transporte intracelular y señalización, así como una importante cantidad de pseudogenes. En conjunto, estos datos proporcionan nuevo conocimiento acerca de las etapas iniciales de la fasciolosis, además de nuevas dianas moleculares para la formulación de futuras estrategias terapéuticas.

Study of the cross-talk between *Fasciola hepatica* juveniles and the intestinal epithelial cells of the host by transcriptomics in an *in vitro* model

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ABSTRACT

Fasciolosis is a globally widespread trematodiasis with a major economic and veterinary impact. Therefore, this disease is responsible for millions of dollars in losses to the livestock industry, and also constitutes an emerging human health problem in endemic areas. The ubiquitous nature of *Fasciola hepatica*, the main causative agent, is one of the key factors for the success of fasciolosis. Accordingly, this parasite is able to subsist in a wide variety of ecosystems and hosts, thanks to the development of a plethora of strategies for adaption and immune evasion. Fasciolosis comprises a growing concern due to its high prevalence rates, together with the emergence of strains of the parasite resistant to the treatment of choice (triclabendazole). These facts highlight the importance of developing novel control measures which allow for an effective protection against the disease before *F. hepatica* settles in a niche inaccessible to the immune system. However, knowledge about the initial phases of the infection, including the migration mechanisms of the parasite and the early innate host response, is still scarce. Recently, our group developed an *in vitro* host-parasite interaction model that allowed the early events to be unveiled after the first contact between the both actors. This occurs shortly upon ingestion of *F. hepatica* metacercariae and the emergence of the newly excysted juveniles (FhNEJ) in the host duodenum. Here, we present a transcriptomic analysis of such model using an approach based on RNA sequencing (RNA-Seq), which reveals changes in gene expression related to proteolysis and uptake of metabolites in FhNEJ. Additionally, contact with the parasite triggered changes in host intestinal cells related to pseudogenes expression and host defence mechanisms, including immune response, among others. In sum, these results provide a better understanding of the early stages of fasciolosis at molecular level, and a pool of targets that could be used in future therapeutic strategies against the disease.

1. Introduction

Foodborne trematodiasis comprise a heterogeneous group of diseases caused by helminth parasites delivered to their definitive host through contaminated food or water. These are responsible for high morbidity in both humans and animals and constitute an emerging health issue, especially in low-income countries (Robinson and Sotillo, 2022). Despite their importance, which includes *ca.* 56 million people affected and substantial economic losses to livestock worldwide, these diseases remain poorly documented (Fürst et al. 2012). Generally, foodborne trematodes undergo a migratory cycle while renewing their antigenic repertoire that involves several host organs before settling into a definitive niche. This strategy confers these parasites with evolutionary advantages in terms of ability to avoid immune destruction by the vertebrate host (Ryan et al., 2020). Migration route of the fluke *Fasciola hepatica* begins with the ingestion of metacercariae. These excyst in the duodenum releasing the newly excysted juveniles (FhNEJ) that are able to cross the gut wall within 2–3 h after infection. Then, immature parasites crawl up the peritoneum towards the liver, where the migratory process continue until they find their definitive location, the biliary ducts, where *F. hepatica* reaches maturity (Siles-Lucas et al. 2021). The passage of the FhNEJ through the intestine is regarded as the

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'point of no return' in fasciolosis, as the success of this process appears to be the major determinant for the overall outcome of the infection (González-Miguel et al. 2021). Therefore, a thorough understanding of the events governing early host-parasite contact is of paramount importance for the effective treatment of the disease.

Over the last decades, different experimental models have been proposed aiming to reproduce in the laboratory the early stages of the contact between FhNEJ and their definitive host, using *ex vivo* (Van Milligen et al., 1998; Becerro-Recio et al. 2022a) or *in vitro* (Garcia-Campos et al., 2016; González-Miguel et al. 2020; Becerro-Recio et al. 2022b) approaches. Such models provide direct evidence of the passage of FhNEJ through the intestinal wall. Either alone or in combination with these models, -omic technologies have revealed themselves as a valuable tool for the study of *F. hepatica* (Cwiklinski et al. 2018) and other foodborne trematodes (Pakharukova et al. 2022). Thereby, these methodologies facilitate the molecular dissection of the host-parasite interface by examining the behaviour of hundreds or even thousands of biological features (*e.g.*, genes, proteins, metabolites) in a single experiment. This allows to identify key mechanisms for parasite migration and survival and to determine changes in their expression along time or in response to certain stimuli. Additionally, host responses can be similarly characterised in order to determine the processes triggered by the first contact with the parasite, as well as the incipient defensive mechanisms that occur early in the course of infection.

Recently, our group developed an *in vitro* proteomic approach on a co-culture consisting on mouse primary small intestinal epithelial cells (MPSIEC) together with FhNEJ. In this way, we performed analyses by sequential window acquisition of all theoretical mass spectra (SWATH-MS) to identify changes in protein expression as a consequence of parasite contact with the intestinal lumen (Becerro-Recio et al. 2022b). After stimulation, parasite and host cells showed a clear deviation towards down-regulation, with a total of 210 and 133 differentially expressed proteins in *F. hepatica* and mouse cells, respectively. These were involved in processes such as proteolysis and its regulation, antioxidant response or metabolic adaptation in FhNEJ, or ribosomal function, cytoskeletal regulation and cell adhesion processes in MPSIEC, among others. The present study shows a complementary approach at the transcriptomic level, aiming to identify changes in mRNA expression.

2. Materials and methods

2.1. MPSIEC cell culture

MPSIEC obtained from C57BL/6 mice (Cell Biologics) (ref. C57–6051) consisting on mouse primary small intestinal epithelial cells, were cultured in 60 mm diameter Petri dishes previously coated with 0.2% porcine gelatine, and incubated in epithelial cell medium (Innoprot) supplemented with 2% fetal bovine serum (FBS), 1% penicillin/ streptomycin and 1% epithelial cell growth factor. Cells were cultured at 37 ºC in a humidified atmosphere containing 5% CO_2, and culture medium was replaced every 24–48 h. Whenever cells reached confluence, they were split in a 1:3 ratio. All the described experiments were performed using passage 1–5 cultures (Becerro-Recio et al. 2022b).

2.2. F. hepatica metacercariae in vitro excystment

F. hepatica Italian strain metacercariae, acquired from Ridgeway Research LTD (UK), were excysted *in vitro* using a methodology previously described in the literature (Hernández-González et al. 2010). Briefly, a sterile tube containing 10 ml of distilled water was supplemented with gaseous CO_2 and sodium dithionite (Sigma-Aldrich) to a final concentration of 0.02 M, whereupon the tube was incubated at 37 ºC for 5 min. At this point, the metacercariae were added to the mixture and incubated at 37 ºC for 1 h. After this, the metacercariae were washed thrice with distilled water and resuspended in an excystment medium containing Hank's balanced salt solution (Sigma-Aldrich), 0.03 M HEPES (Sigma-Aldrich) and 10% lamb bile (obtained from a local abattoir). Metacercariae were disposed into a 6-well plate and incubated at 37 ºC for 3–5 h, and during this time the excystment process was monitored under the microscope, collecting the FhNEJ with a micropipette as they were released from the metacercariae. Finally, the FhNEJ were washed twice with sterile PBS and immediately put into contact with the MPSIEC.

2.3. Co-culture model

The methodology used to replicate the host-parasite interaction was previously developed by our group (González-Miguel et al., 2020; Becerro-Recio et al. 2022b). In brief, passage 5 MPSIEC were cultured in 60 mm gelatin-coated plates (n = 3) and grown in epithelial cell growth medium (Innoprot) until cell confluence was reached. Then, freshly excysted FhNEJ were placed on MPSIEC culture plates at a rate of 500 parasites per plate. The co-culture was incubated for 3 h at 37 °C in a 5% CO_2 atmosphere, after which MPSIEC and FhNEJ were separated by successive washes with sterile PBS. Negative non-stimulated controls consisted of 3 batches of 500 FhNEJ and 3 confluent plates of passage 5 MPSIEC separately incubated under the same conditions (growth medium and incubation time, temperature and atmosphere) than the co-culture. All cultures were performed in triplicate (n = 3). Number of intestinal epithelial cells per plate and number of FhNEJ for *in vitro* stimulation experiments were settled to maximize the chances to have the majority of cells in contact with the FhNEJ and thus properly stimulated and to obtain enough stimulated host and parasite material for downstream analyses, although this specific host cells/parasite ratio could not reflect the *in vivo* situation.

2.4. Total RNA extraction and high-throughput RNA-Seq

Total RNA extraction from MPSIEC was performed using the RNeasy Mini kit (Qiagen), whereas for FhNEJ the miRNeasy Mini kit (Qiagen) was used together with the QIAshredder (Qiagen) cell homogenizer columns. In both cases, extraction was performed according to manufacturer's instructions including a DNaseI treatment step, and total RNA was stored at − 80 ºC until use. Quality of every RNA sample was assessed *via* the Thermo Scientific NanoDrop ND-1000 spectrophotometer so as to determine its concentration along with the A260/A280 and A260/A230 ratios, and further *via* the Agilent 2100 Bioanalyzer system in order to determine the RNA Integrity Number (RIN) of each sample. Only samples with a RIN > 7 were subjected to Illumina TruSeq RNA library preparation. Due to the particular nature of *F. hepatica* ribosomal RNA (Haçariz and Sayers, 2013), it was not possible to determine such parameter in some cases, where samples were sequenced without quality check.

RNA-Seq library preparation and sequencing were performed by Macrogen, Inc. (South Korea) facilities. MPSIEC samples were treated with the TruSeq Stranded mRNA kit (Illumina), while FhNEJ samples were processed with the TruSeq Stranded Total RNA with Ribo-Zero kit (Illumina). The purified RNA was then randomly fragmented, and the fragmented RNA was retrotranscribed and adaptor ligated at both ends. Finally, the fragments were spread in an appropriate flow cell, amplified by PCR and sequenced from both ends (paired-end sequencing). All samples were sequenced on an Illumina NovaSeq 6000 equipment. In MPSIEC, 2 × 150 bp reads; 30 M reads/sample were acquired, while in FhNEJ 2 × 150 bp reads; 40 M reads/sample were acquired. In the generation of fastq files with the resulting reads, the quality values of each nucleotide were expressed on the Phred+ 33 scale. The raw RNA-Seq read data were deposited and can be found in the European Nucleotide Archive (ENA) repository, through the accession number PRJEB57383.

2.5. Processing of RNA-Seq data

An overview of the computational analysis workflow is available at Supplementary Figure 1. A preliminary quality control of the sequencing process was performed on all samples using the FastQC v0.11.9 software (Wingett and Andrews, 2018), a quality control tool for high throughput sequence data which allowed to determine multiple general parameters from the reads so as to guide further analysis. Illumina sequencing adapters were then removed with the Trimmomatic v0.39 tool (Bolger et al. 2014), that was also used to trim the first 10 nucleotides of each read as they showed higher sequencing instability. Additionally, rRNA of *F. hepatica* samples was removed using SortMeRNA 4.3.2 (Kopylova et al. 2012), filtering against the consensus 18 S and 28 S rRNA sequences provided by the software. A summary of the quality control process was performed with the MultiQC v1.13 software (Ewels et al. 2016).

Once the processing of the reads was completed, these were mapped over the *Mus musculus* (GRCm39; https://www.ensembl.org/Mus_musculus/Info/Index) and *Fasciola hepatica* (PRJEB25283; https://parasite.wormbase.org/Fasciola_hepatica_prjeb25283/Info/Index) reference genomes (genome data), providing the corresponding GTF annotation files to enhance the performance of the process. The mapping was conducted using STAR 2.7.9a (Dobin et al. 2013), and the read count for each gene was computed with the HTSeq-Count software embedded into STAR.

2.6. Differential gene expression analysis and biological annotation

An Exploratory Data Analysis (EDA) was then performed by Sparse Partial Least Squares Discriminant Analysis (sPLS-DA) using the *MixOmics* package from the Bioconductor suite (Rohart et al. 2017) to determine the overall degree of similarity among samples prior to a more specific analysis. Differential expression analysis was performed with the Bioconductor package *DESeq2* (Love et al. 2014), that normalized and transformed the read counts, as well as determining the up- and down-regulated genes after interaction between MPSIEC and FhNEJ. Only genes with p-value < 0.05 and present in at least one replicate for each experimental condition were considered as differentially expressed and were further analysed in subsequent steps. The statistical requirement was considered to be sufficiently robust, and therefore no minimum fold change threshold was considered. Graphical representation of this analysis was performed with R software, using the *ggplot2* package.

Once the list of differentially expressed genes (DEGs) in both MPSIEC and FhNEJ was obtained, the amino acid sequences encoded by the FhNEJ DEGs were obtained using the BioMart tool from WormBase ParaSite and annotated using Blast2GO 6.0 (Conesa et al. 2005) with the blastp algorithm against the NCBI protein database restricted to the *Platyhelminthes* phylum. In MPSIEC, the Bioconductor package *BiomaRt* was used to obtain the gene descriptions and associated Gene Ontology (GO) terms of all DEGs, and the corresponding sequences were translated and annotated using Blast2GO against a database containing the mouse proteome available at Uniprot (https://www.uniprot.org/proteomes/UP000000589). Graphical overview of the annotation results was performed using the ReViGO tool (Supek et al. 2011) for the GO categories Biological Process (BP) and Molecular Function (MF), using the Nodescore provided by Blast2GO as quantitative criteria.

3. Results

3.1. Quantitative analysis

An RNA-Seq strategy was used to determine the expression changes triggered by the host-parasite interaction between *F. hepatica* juveniles and intestinal epithelial cells in co-culture. The performance of the mapping process was similar in both FhNEJ and MPSIEC samples, with around 45% of the total reads mapping to a single locus. As for the read

quality control, the Phred logarithmic scale gave values of 35–36 after trimming, even in the last positions, indicating that the sequencing quality is optimal (error between 0.1% and 0.01%; accuracy between 99.9% and 99.99%). After this, Sparse Partial Least Squares Discriminant Analysis (sPLS-DA) was performed for all replicates of FhNEJ and MPSIEC samples (Fig. 1). These charts showed that the control group of FhNEJ samples displayed a clear separation from stimulated samples. Additionally, the distance among replicates of the same condition was smaller especially along the horizontal axis, which gathered the highest amount of variability among samples. In MPSIEC samples, all control replicates were clustered together and clearly separated from stimulated samples, whereas one replicate from this group displayed some distance from the others on both horizontal and vertical axes.

As mentioned, no minimum fold change was considered for DEGs, in order to include all transcripts that were differentially expressed and could have biological relevance independently from their fold change magnitude. Although the total reads mapping was low, our analysis allowed the identification of a high number of DEGs in both samples, most of them (around 86%) corresponding to coding genes. A total of 342 DEGs were detected in FhNEJ samples (113 up-regulated and 229 down-regulated) (Fig. 2A) after stimulation, whereas 140 DEGs were found in MPSIEC samples (103 up-regulated and 37 down-regulated) (Fig. 2B).

3.2. Functional annotation

Functional annotation of the differentially expressed genes was performed to obtain their description, as well as their associated GO terms in the BP and MF categories, in order to determine the most relevant events triggered by the *in vitro* interaction.

In the case of FhNEJ, 296 of the detected DEGs corresponded to coding genes (111 up- and 185 down-regulated), whose nucleotide sequence was translated into amino acids and identified using the Blast2GO software. In MPSIEC, descriptions of DEGs were assigned using the *BiomaRt* R package, followed by an analysis analogous to that performed in FhNEJ, where the proteins encoded by DEGs were obtained, resulting in a total of 240 proteins (184 up- and 56 down-regulated). Full descriptions of the differentially expressed genes within the FhNEJ and MPSIEC samples are available in Supplementary Table 1 and 2, respectively. In addition, in order to facilitate the visualization of the results, the top 15 annotations with the highest or lowest fold change for both sample types, as an arbitrary amount of the more representative transcripts, are shown in Fig. 3.

For FhNEJ, 3 of the 15 top up-regulated transcripts were hypothetical proteins. Additionally, among the top 15 up-regulated transcripts in FhNEJ upon MPSIEC incubation, 4 are related to the regulation of gene expression. These include cilia- and flagella-associated protein 20, tetratricopeptide repeat protein, lipopolysaccharide-induced tumor necrosis factor-alpha factor and ubiquitin-conjugating enzyme E2. Transcripts potentially linked with tegument turnover included a putative glycosyltransferase 14 family member and a tegument antigen. The predicted protein sequence of the tegument antigen shows the motifs commonly found in the so-called Calcium Binding Proteins (CABP) of *F. hepatica* (Thomas and Timson, 2016). These include an EF-hand, a dynein complex and a calcium-binding motif. Nevertheless, the similarity between its predicted sequence and those CABP described in *F. hepatica* is low (data not shown). Other transcripts represented in the top 15 up-regulated molecules in FhNEJ included the neutral sphingomyelinase, the transmembrane protease serine 3, the epididymal secretory protein E1, a putative drug transporter potentially related to multidrug resistance (Catalano et al., 2022), the brain-specific serine protease 4 that could enable serine-protease activity, and the dynein light chain LC8-type, linked with the muscle contraction machinery (Becerro-Recio et al., 2022b). The top 15 down-regulated proteins in FhNEJ included 8 unidentified and hypothetical proteins, and the cell differentiation protein rcd1, which may play a role in FhNEJ cell
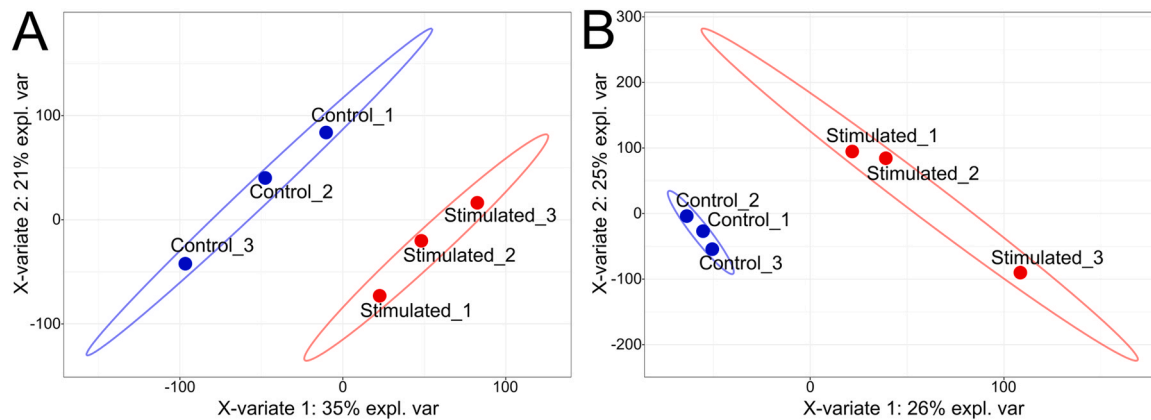
Fig. 1. Sparse Partial Least Squares Discriminant Analysis (sPLS-DA) of FhNEJ (A) and MPSIEC (B) samples. Blue dots represent non-stimulated control samples, whereas red dots represent samples stimulated by co-culture for 3 h. The percentage of variability explained by each component is indicated in the corresponding axis, and the ellipse around each group of samples represents a 95% confidence interval for the modelled distribution.
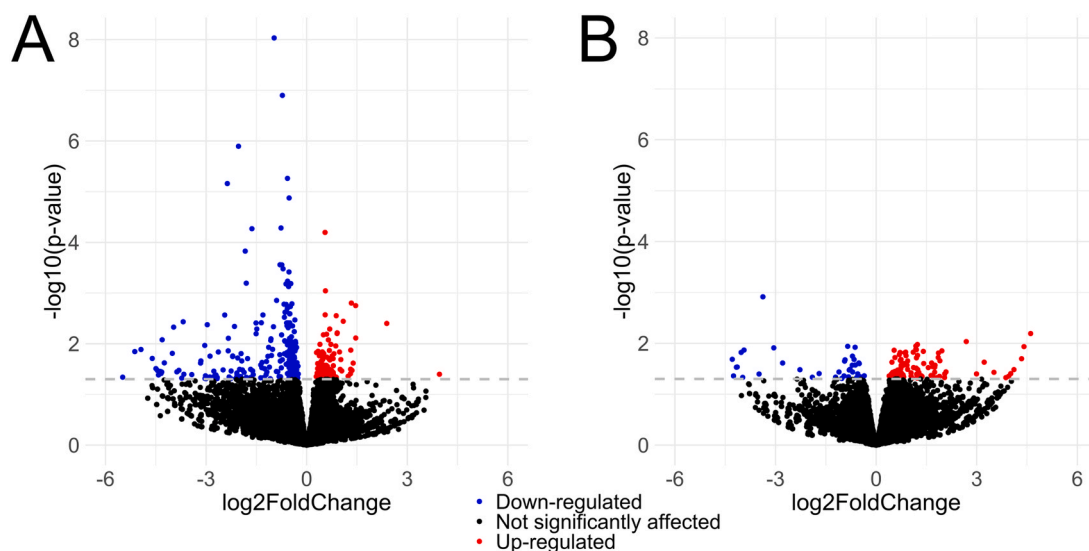

Fig. 2. Volcano plots showing the differential expression pattern of FhNEJ (A) and MPSIEC (B). Blue dots represent significantly down-regulated genes after stimulation and red dots represent up-regulated genes. The grey dotted line delimitates the statistical significance cut-off (p-value <0.05).

differentiation, the cell wall integrity and stress response component 1, a putative beta-arrestin, the arginine methyltransferase, the calcium-binding mitochondrial carrier protein SCaMG1, and the purine nucleoside phosphorylase.

For MPSIEC, 8 out of top 15 up-regulated and 9 out of top 15 down-regulated transcripts represent pseudogenes (Fig. 3). Additionally, the junction adhesion molecule 2, a pore-forming protein-like, the zinc finger protein 953, a hedgehog acyltransferase-like, the antigen identified by monoclonal antibody Ki 67, the shugoshin 1 and the FAM3 metabolism regulated signalling molecule 3 were found in the top 15 up-regulated proteins in MPSIEC. Among down-regulated proteins, the MFNG O-fucosylpeptidase 3-beta-N-acetylglucosaminyltransferase, the apical junction component 1, the EP300 interacting inhibitor of differentiation 3, the protein tyrosine phosphatase non-receptor type 7, the histidine ammonia lyase and the keratin 36 were found in MPSIEC.

Additionally, tables comparing the relative quantitation (fold change) and the absolute quantitation (read counts) for all the identified transcripts in both samples were generated (Supplementary Table 3). In this regard, absolute transcription rate of the most overexpressed genes in MPSIEC was not particularly high. In FhNEJ a high abundance of DEGs dynein light chain, tegumental antigen and epididymal secretory protein E1 was observed. Additionally, two highly expressed legumain

isoforms were found, despite their relative change in expression fall outside the top 15 DEGs.

Secondly, the functional annotation was plotted using the ReViGO software, which displayed the most representative GO terms among DEGs found in the analysed samples. In FhNEJ, these GO terms in the BP category included macromolecule biosynthesis, signal transduction, proteolysis and cytoskeleton organisation (Fig. 4). Furthermore, the MF category within the DEGs in FhNEJ samples after MPSIEC stimulation was majorly represented by binding to different types of biomolecules, as well as peptidase and kinase activity (Supplementary Figure 2A).

In the case of MPSIEC, visualisation of the annotation using ReViGO showed several clusters of closely related BP. These included response to endogenous and abiotic stimuli, vesicle secretion and transport, and regulation of gene expression, as well as specific processes such as innate immune response, epithelial cell differentiation, or regulation of morphogenesis and cytoskeletal function (Fig. 5). Within the MF category, the most remarkable feature was a group of GO terms related to binding to different biomolecules, including receptor, signalling and cytoskeletal proteins, as well as catalytic activity (Supplementary Figure 2B). A detailed GO annotation of the BP categories found as up- and down-regulated in FhNEJ and MPSIEC upon interaction can be found in Supplementary Tables 4 and 5, respectively.
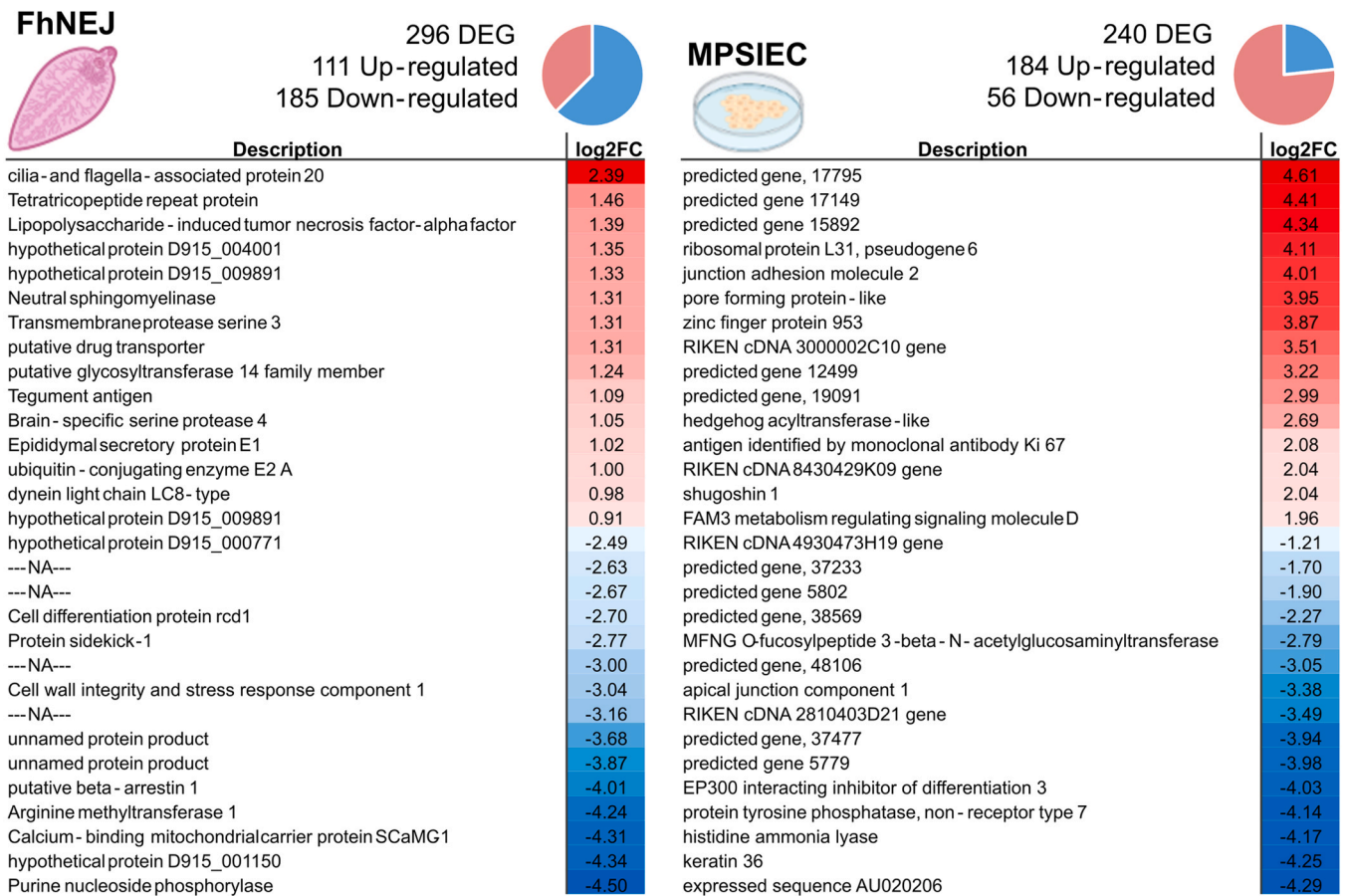
Fig. 3. Differentially expressed genes after *in vitro* stimulation. The top 15 annotations with the highest (red) or lowest (blue) fold change are shown for both FhNEJ and MPSIEC.
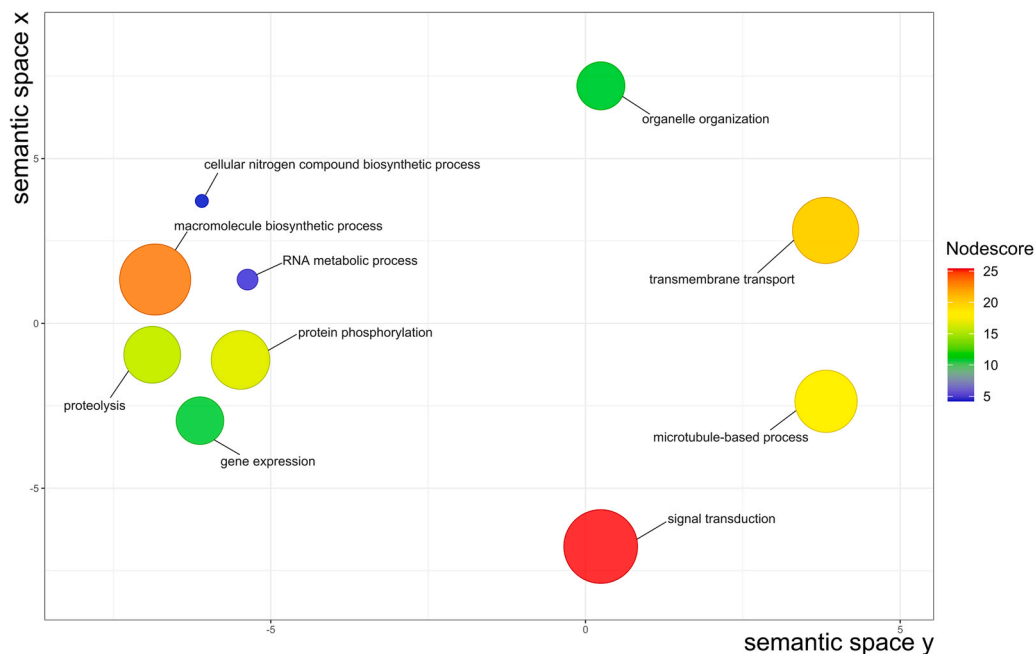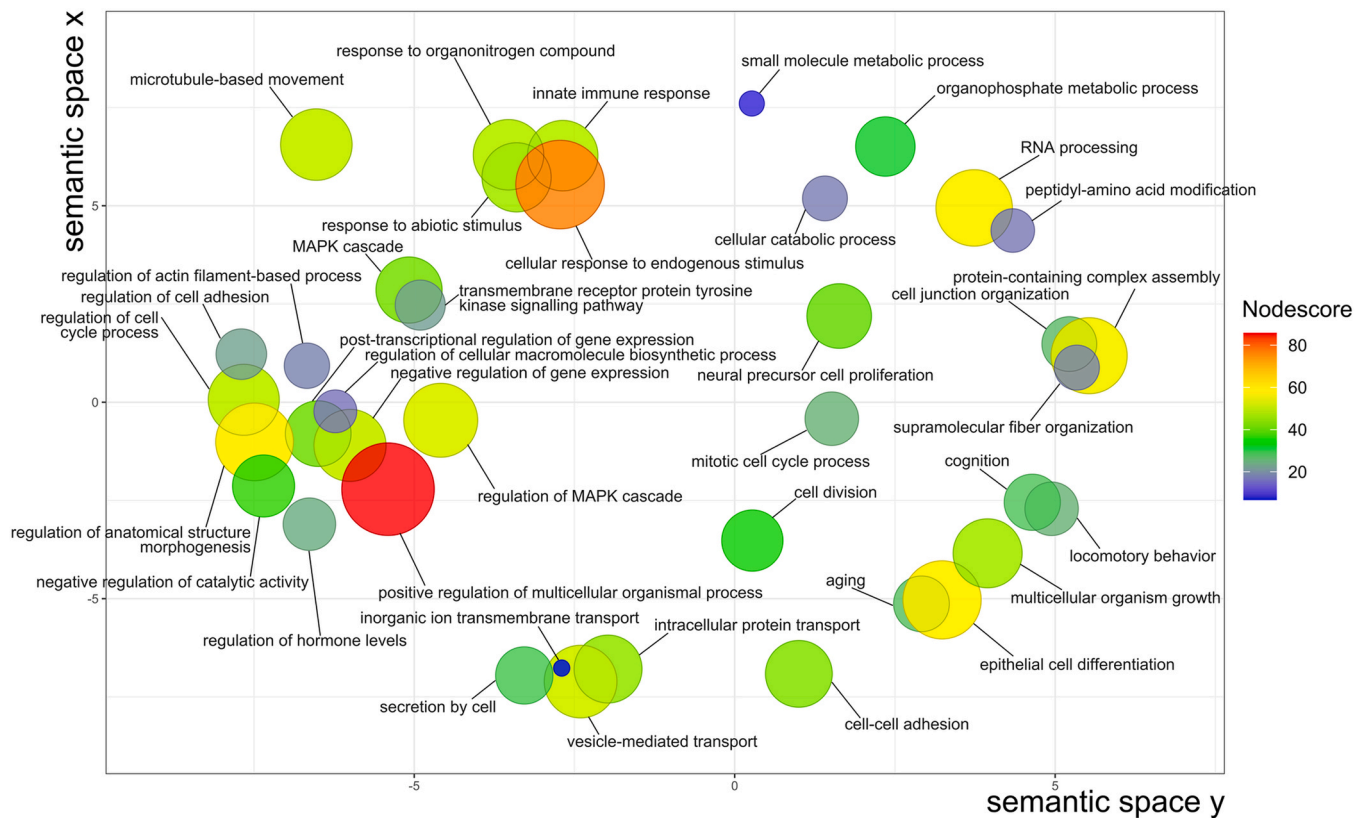

Fig. 4. ReViGO plots showing the most representative GO terms among DEGs found in FhNEJ samples, in the Biological Process category. The size and colour of each circle represents the Nodescore of each GO term, while the proximity between circles is proportional to the degree of similarity of the represented terms.

Fig. 5. ReViGO plots showing the most representative GO terms among DEGs found in MPSIEC samples, in the Biological Process category. The size and colour of each circle represents the Nodescore of each GO term, while the proximity between circles is proportional to the degree of similarity of the represented terms.

4. Discussion

The host-parasite relationships during the early phases of infection by *F. hepatica* in the vertebrate host have been scarcely studied at molecular level. Recently, we described the proteomic changes in an *in vitro* model that allows the interaction between FhNEJ and MPSIEC (Becerro-Recio et al., 2022b). These are the first host cells that the parasite meets upon excystment in the small intestine. Changes were studied by SWATH-MS, a proteomic approach that facilitates the quantitation of differentially expressed proteins among different samples. This methodology showed rapid changes in the protein expression pattern of FhNEJ in response to interaction with the host intestinal epithelial cells. MPSIEC also responded to parasite stimulus by showing alterations in the expression of proteins related to crucial biological processes. Here, we extend this analysis, evaluating the changes triggered by the host-parasite interaction in the transcriptome of MPSIEC and FhNEJ.

Identification of differentially expressed transcripts and their corresponding proteins was performed with the Blast2GO software. GO analysis of up-regulated transcripts in FhNEJ after MPSIEC stimulation showed that one of the dominant BP categories is proteolysis. The expression of proteases along the development of *F. hepatica* inside its definitive host has been found to be tightly regulated, due to their implications in a variety of processes crucial for parasite development, including parasite migration and feeding. Proteases are also specifically related to pathogenic and virulence phenomena (Cwiklinski and Dalton, 2022). Among the up-regulated molecules in this GO category, we found the transmembrane protease serine 3, described in other organisms as related to the initiation of proteolytic cascades and belonging to the S1 family of the SA clan of serine type peptidases, for which the prototype is chymotrypsin. Unfortunately, *F. hepatica* serine proteases are largely understudied in comparison with other groups of proteases, thus the biological and biochemical functions of these proteases are poorly

understood. In other trematodes, this class of proteases seems to play a role in host-parasite interactions and parasite survival (Dvorak and Horn, 2018). Also linked with proteolysis, although not in the top 15 of over-expressed transcripts in FhNEJ after MPSIEC co-incubation, we identified the transcript of the peptidase legumain, an asparaginyl endopeptidase cysteine protease. Legumains have been found in the secretions of FhNEJ (Tkalcevic et al. 1995; Robinson et al. 2009). Later proteomic studies in *F. hepatica* metacercariae and juveniles also identified a range of legumains, suggesting that these peptidases are activated once the parasite reached the intestine (Cwiklinski et al. 2018). This assumption is compatible with the results obtained in our *in vitro* model showing a high abundance of legumain transcripts in FhNEJ after their interaction with the intestinal epithelial cells of the host. Importantly, legumains are involved in the activation of cathepsin L proteases by cleavage of their pro-peptides (Cwiklinski et al., 2022), allowing their proteolytic activity. Our previous proteomic results in this *in vitro* model showed that the interaction between FhNEJ and MPSIEC triggers a rapid protein expression change of FhNEJ in response to the host epithelial barrier, including cathepsins L3 and L4 (Becerro-Recio et al. 2022b). Due to the tightly regulated expression of legumains during the *F. hepatica* development and their role in activating cathepsins linked with collagenolytic and endopeptidase activities, the over-expression of peptidases detected here could be related to host invasion. In line with this, cathepsin B is also overrepresented in transcripts of FhNEJ after MPSIEC incubation.

As mentioned, four of the top 15 up-regulated transcripts in FhNEJ upon MPSIEC incubation are linked with the regulation of gene expression. Increased expression of these genes is consistent with increased cell proliferation in FhNEJ, thus most likely having a role in the rapid growth and transformation of the parasite inside its vertebrate host. During FhNEJ migration and development, the turnover of the tegument and the expression of different glycoconjugates on their

surface have been found of paramount importance for parasite survival and pathogenesis (McVeigh et al. 2018; González-Miguel et al. 2021). The tegument antigen and the glycosyltransferase found here in the top 15 transcripts of MPSIEC-stimulated FhNEJ could be associated with these processes. Importantly, the predicted protein of the tegument antigen transcript shows high similarity with the peptide sequences of the tegument antigens previously found by us as over-expressed in FhNEJ tegumental extracts both after interaction with MPSIEC *in vitro* (UniProt identifier A0A4E0RZ43) (Becerro-Recio et al. 2022b) and gut passage *ex vivo* (UniProt identifier A0A4E0RZ43) (Becerro-Recio et al. 2022a). Thus, this family of proteins seems to be localized to the FhNEJ tegument. Although the function of these molecules remains to be determined, it is worth mentioning that they are unique to trematodes. Thus, they are of interest in understanding their function in the biogenesis and rapid change of the T bodies that support the turnover of the tegument of *F. hepatica* inside the mammalian host (González-Miguel et al. 2021). Regarding the identified glycosyltransferase, this could be related to the activity required for protein glycosylation, a pattern that changes during the intra-mammalian parasite development (Cwiklinski et al. 2021). Carbohydrate-containing glycoconjugates are expressed on the surface of *F. hepatica* developing juveniles, and are released in their secretions, playing an important role during infection due to their immunomodulatory properties (Rodríguez et al., 2015).

Parasite trematodes do not synthesize lipids, including cholesterol. Trematodes need these molecules as energy source and they acquire them from the host (Cwiklinski et al., 2021). In this regard, among the 15 top overexpressed transcripts in FhNEJ, we found sphingomyelin, which attaches to cholesterol and helps to form other lipids and mobilizes the cell surface cholesterol to the interior of cells (Chatterjee, 1993). Additionally, we found the transmembrane protease serine 3, which shows an LDL receptor-like domain, mediating the endocytosis of cholesterol-rich low-density lipoprotein (Südhof et al., 1985). We also found the epididymal secretory protein E1, known as NPC2 (Niemann-Pick intracellular cholesterol transporter 2), already described in extracellular vesicles of *F. hepatica* (Cwiklinski et al., 2015). In this context, these proteins could assist FhNEJ in the uptake of metabolic substrates from the host, suggesting that the lipid energy source taken from the host is of great importance for this developmental stage of the parasite.

Our *in vitro* model also permitted us to examine transcriptional changes in the MPSIEC cells. This revealed fast and profound changes in MPSIEC gene expression after their interaction with FhNEJ. Notably, 8 out of top 15 up-regulated and 9 out of top 15 down-regulated transcripts represent pseudogenes. Interestingly, these genes did not tend to have high read counts in terms of absolute quantitation (Supplementary Table 3). Pseudogenes are regions of the genome that are similar to functional genes but are thought to be non-functional. However, some recent studies have suggested that pseudogenes have the potential to act in complex transcriptional and post-transcriptional modulation processes (Muro et al., 2011). Thus, pseudogenes are emerging as crucial regulators of development and disease in which are commonly regulated. However, systematic functional characterization and evolution of pseudogenes remain largely unexplored in mammals (Qian et al., 2022). Notwithstanding, there is growing evidence of the importance of the regulation of the transcription pseudogenes in pathological processes, including non-infectious and infectious diseases, that affect mammals (Cristiano, 2022).

Besides pseudogenes, up-regulated transcripts in MPSIEC upon incubation with FhNEJ included molecules potentially linked with host defence, as the Junction adhesion molecule 2 (JAM2), the Pore forming protein-like (PFP-L) and the FAM3 metabolism regulating signalling molecule D. JAM2 promotes lymphocyte trans-endothelial migration (Johnson-Léger et al. 2002), a process triggered by antigens of numerous infectious agents associated with the inflammatory response. Several mechanisms play a critical role in trans-endothelial migration, including disruption or loosening of adherens junctions (Muller, 2011).

Noteworthy, the Apical junction component 1, predicted to be located in adherens junctions and involved in cell-cell junction organization, is one of the top 15 down-regulated transcripts in MPSIEC after incubation with FhNEJ. Thus, the cross-talk between the host intestinal epithelial cells and the FhNEJ results in the activation of signals related to trans-endothelial immune cells migration at intestinal level, that are crucial to fight against the parasite during the very early steps of infection. The triggering of these molecules in reference to inflammation by FhNEJ in MPSIEC could be related to pathogen-associated molecular patterns (PAMPs) released by the parasite. PAMPs have been described in *F. hepatica*, *e.g.* CL1 and GST (Dowling et al., 2010), but which specific molecules derived from FhNEJ could act as PAMPs in our settings should be further investigated.

Together with the triggering of this inflammatory mechanism, the up-regulation of the PFP-L in the intestinal epithelial cells could also result in the damage of FhNEJ in the gut lumen, since PFP are immune effectors that can target pathogens directly, resulting in formation of pores in cells and cell death. The PFP-L detected here is most similar in its amino acid sequence to the macrophage-expressed gene 1 protein-like or perforin-2, as indicated by alignment of its sequence with those available in GenBank through BLASTP (data not shown). Inside this family of proteins, perforin-2 could exert a similar and alternative function to the complement membrane attack complex (Krawczyk et al. 2020).

Potentially related to host defenses, the over-expression of FAM3D in mouse gastrointestinal tract is broadly linked with intestinal homeostasis, and more specifically with anti-microbial peptide production (Liang et al. 2020). Antimicrobial peptides (AMPs) are a class of small peptides that widely exist in nature and they are an important part of the innate immune system, showing a wide range of inhibitory effects against different pathogens, including parasites (Huan et al. 2020). Triggering of this molecule by FhNEJ in MPSIEC could result in the activation of additional defense mechanisms of MPSIEC against FhNEJ mediated by AMPs.

Other proteins found to be in the top 15 of over-expressed molecules in MPSIEC included proteins potentially related to DNA repair and chromatin organization (EP300, shugoshin, antigen identified by monoclonal antibody Ki 67), regulation of transcription (zinc finger protein 953) and post-translational modifications (hedgehog acyltransferase). Among the top 15 down-regulated proteins in MPSIEC, we also found proteins that could be linked with immune responses. The EP300 interacting inhibitor of differentiation 3 has been pointed out as important for the regulation of the function of Foxp3 Treg cells (Liu et al., 2013). Intriguingly, *F. hepatica* induces Foxp3 T cell over-expression in liver from infected sheep during early stages of infection (Pacheco et al., 2018). Additionally, the protein tyrosine phosphatase non-receptor type 7 plays a role in the negative regulation of T cell antigen receptor signal transduction (Saxena et al., 1998), and its down-regulation in MPSIEC cells upon FhNEJ interaction could have a connection with the enhancement of antigen presentation by T cells.

Comparison of the relative quantitation (fold change) and the absolute quantitation (read counts) for all the identified transcripts in both samples showed that absolute transcription rate of the DEGs transcripts in MPSIEC was not particularly high. Conversely, a high abundance of DEGs such as dynein light chain, tegumental antigen and epididymal secretory protein E1 was observed in FhNEJ. Additionally, we found two highly expressed legumain isoforms, despite their relative change in expression fall outside the top 15. This correlates with the high expression of these proteases described during the early stages of infection (Robinson et al., 2009). This comparison shows that DEGs do not necessarily represent the most abundant transcripts in this setting.

Finally, and despite the obvious difficulties in integrating these transcriptomic data with the previously published proteomic analysis using the same *in vitro* model by us (Becerro-Recio et al., 2022b), it is possible to establish some potential connections. Thus, there is a correlation in the differential expression of molecules belonging to some

protein families such as proteases (including legumain, cathepsins B, CL2 and 4, but not CL3) or epigenetic regulation proteins (histone acetyltransferase). On the contrary, no changes in the levels of metabolic enzymes, antioxidant proteins or protease inhibitors (observed in the proteomic approach) were found in the transcriptomic analysis. With regard to MPSIEC, both proteomic and transcriptomic approaches display expression alterations in common processes such as intracellular transport, cell adhesion and cell cycle regulation, as well as phosphorylation-mediated signalling. Notably, the decrease in ribosomal function in the cytosol, as the most prominent phenomenon in the proteomic study, is also present at the transcript level, represented by several ribosomal protein-coding genes with reduced expression after contact with the parasite. On the other hand, while at the protein level hardly any changes indicating the triggering of immune reactions were noticed, some transcripts related to this process were now found, most of which up-regulated. These include an interferon regulatory factor, suggesting that induction of a Th1-type response may be occurring during the initial stage of infection.

In summary, a high-throughput transcriptomic analysis coupled with an *in vitro* model of co-culture by FhNEJ and MPSIEC has been carried out for the first time to explore the host-parasite relationships in fasciolosis. This model allows the depiction of the interaction between the early stages of *F. hepatica* with a specific set of host cells facilitating the evaluation of the changes triggered by both organisms, among which we have found regulation of numerous transcripts specifically related to essential processes of the host-parasite relationship. The implementation of this model for other transcriptomic studies in fasciolosis will also allow more accurate comparisons with data sets produced by other authors, which currently entail complexity due to the different methodological approaches that are employed.

CRediT authorship contribution statement

David Becerro-Recio: Methodology, Investigation, Formal analysis, Data curation, Writing - original draft, Writing - review & editing. **Judit Serrat:** Methodology, Writing - review & editing. **Marta López-García:** Methodology, Writing - review & editing. **María Torres-Valle:** Project administration, Methodology, Writing - review & editing. **Francisco Colina:** Formal analysis, Data curation, Writing - review & editing. **Iván M. Fernández:** Formal analysis, Data curation, Writing - review & editing. **Javier González-Miguel:** Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization, Writing - original draft, Writing - review & editing. **Mar Siles-Lucas:** Supervision, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetpar.2023.109981.

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Conclusiones

1) Se ha desarrollado un novedoso modelo experimental *in vitro* que replica el primer contacto entre *Fasciola hepatica* y su hospedador vertebrado a nivel del intestino delgado, permitiendo su estudio y caracterización mediante técnicas -ómicas. Este se basa en el cocultivo experimental de los juveniles recién excistados del parásito con células epiteliales intestinales de ratón.

2) Se ha puesto a punto un innovador modelo experimental *ex vivo* que permite el estudio, la caracterización histológica y -ómica, así como la monitorización del atravesamiento de la pared intestinal llevado a cabo por *F. hepatica* durante la primera etapa de su migración dentro del hospedador vertebrado. Dicho modelo se fundamenta en la introducción y posterior cocultivo experimental de juveniles recién excistados del parásito en segmentos de duodeno de ratón.

3) A nivel proteómico y transcriptómico, se revela la capacidad de adaptación de los vermes juveniles de *F. hepatica* en los modelos desarrollados, los cuales llevan a cabo una rápida renovación antigénica en relación con el gran número de moléculas que diferencialmente se expresan, especialmente en su superficie tegumental, tras el contacto o invasión de la barrera intestinal del hospedador. Estos cambios incluyeron, principalmente, procesos de desarrollo y diferenciación, de invasión de tejidos, así como de evasión de las respuestas del hospedador, representados por enzimas y transcritos metabólicos, proteolíticos, antioxidantes e inmunorreguladores.

4) El estímulo parasitario influye también en las funciones del epitelio intestinal del hospedador tras la interacción entre ambos organismos en los modelos puestos a punto. Este hecho se fundamenta, principalmente, en alteraciones en la expresión de proteínas y transcritos relacionados con la modulación de la respuesta inmune innata, las interacciones célula-célula y la presencia de pseudogenes, así como con una disminución notable en la función ribosomal, lo que ilustra una pérdida generalizada de la homeostasis tras la infección por *F. hepatica*.

5) Los resultados obtenidos en la presente Tesis Doctoral contribuyen a la caracterización molecular de la interacción parásito-hospedador en una fase crítica de la fasciolosis, como lo es el contacto e invasión parasitaria de la barrera intestinal del hospedador. Por ello, las moléculas identificadas en este estudio pueden ayudar a definir nuevas dianas farmacológicas o vacunales para la eliminación temprana de la fasciolosis, antes de que los vermes adultos se establezcan en su localización definitiva.

Bibliografía

Se recoge, a continuación, la lista de referencias citadas en la Revisión Bibliográfica de la presente Tesis Doctoral. Las citas utilizadas en los Capítulos 1 a 4 se encuentran listadas dentro la correspondiente publicación de cada capítulo.

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