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Advances in *Fasciola hepatica* research using –omics technologies

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Abstract

The liver fluke *Fasciola hepatica* is an economically important pathogen of livestock worldwide, as well as being an important neglected zoonosis. Parasite control is reliant on the use of drugs, particularly triclabendazole (TCBZ), which is effective against multiple parasite stages. However, the spread of parasites resistant to TCBZ has intensified the pursuit for novel control strategies. Emerging –omic technologies are helping advance our understanding of liver fluke biology, specifically the molecules that act at the host-parasite interface and are central to infection, virulence and long-term survival within the definitive host. This review discusses the sequencing technological advances that have facilitated the unbiased analysis of liver fluke biology, resulting in an extensive range of -omics datasets. In addition, we highlight the –omics studies of host responses to *F. hepatica* infection, that, when combined with the parasite datasets, provide the opportunity for integrated analyses of host-parasite interactions. These extensive datasets will form the foundation for future in-depth analysis of *F. hepatica* biology and development and the search for new drug or vaccine interventions.

1. Introduction

DNA sequencing technologies have rapidly evolved over the past few decades, stemming from the traditional Sanger methodology used to map the first human genome (Lander et al., 2001; Venter et al., 2001) to the recent high-throughput sequencing technologies such as Roche 454 and Illumina (Reuter et al., 2015) that we use today. More recently, single cell sequencing has emerged, pioneered by Pacific Biosciences and Oxford Nanopore Technologies, through the PacBio and MinION platforms, respectively (Reuter et al., 2015). As the technology for sequencing DNA has progressed, so too have the routine protocols for the extraction of nucleic acids and library preparation (Price et al., 2009); this has allowed sequencing projects to be carried out on even the most challenging species to propagate in the laboratory and those for which it was previously difficult to obtain adequate quantities of nucleic acids. Consequently, the number of sequencing projects undertaken has exploded, including recent ambitious proposals to sequence 10000 vertebrate genomes (Genome 10K project; Koepfli et al., 2015), 5000

arthropods (i5K project; Poelchau et al., 2015) and all 10500 species of birds (B10K project; Jarvis, 2016), to name but a few.

In the area of parasitology, a similar large-scale collaboration was initiated with the aim of sequencing 50 helminth genomes from human and veterinary parasites of global importance (50 Helminth Genomes Project, 50HGP; http://www.sanger.ac.uk/science/collaboration/50hgp). The advances in sequencing technologies enabled the number of genomes sequenced under this directive to be exceeded. Now in its ninth release, the database housing these genomes, WormBase ParaSite, comprises 134 genomes, representing 114 species (Howe et al., 2017). In addition to acting as a central repository and publically-accessible database for the wider research community, WormBase ParaSite integrates all available genomic and transcriptomic data to provide functional annotation and expression information for each species and thus facilitate comparative genomics analysis.

How we profile the repertoire of transcripts expressed by an organism, at a particular time-point or in response to external cues, has also evolved with advances in sequencing technology. Studies first focused on analysing partial sequences, known as expressed sequence tags (ESTs) derived from libraries of cDNA clones (Parkinson and Blaxter, 2009). In conjunction, serial analysis of gene expression (SAGE) methodology facilitated differential or temporal gene expression studies, as well as the detection and analysis of low abundant transcripts (Sun et al., 2004). However, it was the development of gene expression microarrays that initially instigated high throughput transcriptome analyses that are still used today (Schena et al., 1995; Malone and Oliver, 2011). Since microarrays only detect known gene transcripts immobilised on microchips they are less useful for gene discovery. By contrast, the emergence of RNA sequencing (RNAseq) allowed the analysis of all gene transcripts present within a given sample and now, advanced through the development of next-generation sequencing (NGS) technologies, has largely replaced microarrays for gene transcription analysis.

This emerging array of transcriptome profiling tools has been applied extensively to helminth parasites. Approximately 508,000 ESTs have been generated from Platyhelminth parasites and are housed in the NCBI database

dbEST (dbEST release 130101; https://www.ncbi.nlm.nih.gov/dbEST/). SAGE methodology has also been employed for the analysis of gene expression across different lifecycle stages (Knox and Skuce, 2005; Williams et al., 2007; Taft et al., 2009). More recently, large scale RNAseq analyses have been completed for a range of Platyhelminth parasites, several of which have been disseminated through the site Helminth.net (Martin et al., 2015). These freely-accessible datasets have complemented ongoing genome projects.

In parallel with techniques to analyse nucleic acids, advances in modern proteomic technologies have allowed the high throughput identification and characterization of complex proteins preparations (Yarmush and Jayaraman, 2002; Brewis and Brennan, 2010). Progress has also been made in developing extraction protocols for soluble and membrane-bound proteins, as well increasing the sensitivity of proteomic technologies, including gel-free protocols that can be carried out on very small amounts of proteins (micrograms) (Scherp et al., 2011; Nature Method of the year 2012. 2013). By integrating proteomic data with genomic/transcriptomic data functional annotation is more precise and can provide qualitative and quantitative information regarding the expression of genes and their products, as well as data such as the existence of splice variants or the nature of post-translational modifications.

Parasite-host interaction is a complex phenomenon involving molecules produced by both partners. The ability of helminth parasites to invade, migrate and survive within their hosts is expedited by the range of proteins they secrete/excrete. The roles these released proteins play during infection have been investigated in many studies using proteomic tools and have provided a rich source of immunomodulators, diagnostic reagents and vaccine candidates that can be cherry-picked at will to bring forward into commercialisable biotherapeutics. The available genomic/transcriptomic data, including those present in WormBase ParaSite, complement these proteomic studies, providing publically-available databases that can be used during the identification/annotation process to further our understanding of helminth parasites and their interaction with their hosts.

In this review, we focus on the datasets available for the liver fluke parasite, *Fasciola hepatica*, and in particular how they are currently analysed and interrogated

to enhance our knowledge of liver fluke biology with a particular emphasis towards elucidating how these parasites invade and survive within their hosts. The lifecycle of this digenean trematode includes a snail intermediate host, within which the parasite undergoes a clonal expansion, and a mammalian definitive host, where the parasite develops into sexually mature adults, releasing 20000–24000 eggs per fluke per day (Boray, 1969). Infection of the mammalian host occurs following the ingestion of the infective encysted stage, the metacercariae. Within the intestine, the parasite excysts, as newly excysted juveniles (NEJ) that migrate across the intestinal wall, across the peritoneal cavity to the liver and bile ducts. *F. hepatica* is known to infect a broad range of mammalian hosts, including rodents, ruminants, ungulates, kangaroos and primates (Robinson and Dalton, 2009), implying the parasite has evolved a universal process(s) of infection. As a hermaphroditic parasite, *F. hepatica* has the ability to self- and cross-fertilise. In addition, studies have shown that hybridisation with the sister species, *Fasciola gigantica* can occur, resulting in intermediate or hybrid forms as determined by analysis of mitochondrial genes and intergenic genome sequences (Le et al., 2008; Itagaki et al., 2011; Ichikawa-Seki et al., 2017).

The extensive collection of -omics datasets now available for *F. hepatica* includes the draft genome, stage-specific transcriptomes, and proteomic datasets for the somatic proteome, secretome, extracellular vesicles and glycoproteome of the outer tegumental surface. These datasets can now be used to investigate the complex features of the *Fasciola* lifecycle, particularly their effects on life history traits that directly impact on gene flow within liver fluke populations, influencing the spread of drug resistance and virulence/pathogenicity traits.

2. Genomics

2.1 The *F. hepatica* mitochondrial genome

The characterisation and differentiation of various *Fasciola* species using morphological features is often unreliable and can only be used for the differentiation of adult parasites found within the bile ducts. Molecular identification based on nuclear ribosomal and mitochondrial genes is a more robust method of species

143 classification. These molecular tools also provide markers for population genetic
144 studies and epidemiological analysis of *Fasciola* spp. The complete *F. hepatica*
145 mitochondrial (mt) genome was the first to be sequenced from a trematode species
146 (Le et al., 2001) and has since been used for several population genetics studies of
147 *F. hepatica* (Walker et al., 2007; Walker et al., 2011; Walker et al., 2012; Bargues et
148 al., 2017). Similarly, the complete mt genome from *F. gigantica* has been reported
149 (Liu et al., 2014), which now provides species-specific references that can be used in
150 species characterization studies. For example, Liu and colleagues (2014)
151 sequenced the complete mt genome from an intermediate form of *F. hepatica* and *F.*
152 *gigantica* found in the Heilongjiang province, China (Peng et al., 2009). Based on
153 intergenic spacer regions (ITS-1 & ITS-2) this isolate is indeed inferred to be a hybrid
154 between *F. hepatica* and *F. gigantica*, although comparative analysis between
155 *Fasciola* spp. mt genomes revealed that the intermediate form was more closely
156 related to *F. gigantica* than to *F. hepatica.* This study shows that hybridisation is not
157 uniform across the genome and that sequence variations at different sites can occur,
158 in this case within the nuclear ribosomal genes and the maternally inherited
159 mitochondrial genes. Thus, the study also highlighted the complexity incurred during
160 hybridization of *Fasciola* species and challenges that their subsequent
161 characterization presents.

162

163 **2.2 Nuclear genome**

164 To date 33 Platyhelminthes genomes are publically available within
165 WormBase ParaSite, comprising species from the Trematoda, Cestoda, Monogenea
166 and Rhabditophora Classes. Analysis of the genome assembly sizes shows that
167 although individual species vary in respect to their genome size, trends can be
168 observed. In general, the cestode tapeworms have considerably smaller genomes
169 compared the other members of the Phylum Platyhelminthes. The major exception
170 to this statement is *Spirometra erinaceieuropaei*, which has one of the largest
171 Platyhelminth genomes (1.3 Gb; Bennett et al., 2014). Concerning the Class
172 Trematoda, the blood flukes of the species *Schistosoma* have smaller genomes
173 compared with other members of the Class.

Surprisingly, *F. hepatica* has the largest trematode genome sequenced to date (1.3 Gb; Cwiklinski et al., 2015a). For a parasite such as *Fasciola* that ensures its own species survival through the daily generation of large numbers of eggs, the evolution of a large genome appears counter-intuitive as it potentially imposes a cost on egg production. The reason for the large genome size has yet to be determined, but our studies indicate that it has not arisen through genome duplication or an increase in the percentage of the genome that is comprised of repeat regions. Although an equivalent number of genes have been identified across the trematode genomes, comparative analysis reveals that increases in genome size are reflective of increases in average exon and intron length, though this alone does not fully explain the increased genome size of the *F. hepatica* genome. Further analysis of the non-coding regions is required to determine their function and, in particular, their importance in gene regulation (ENCODE Project Consortium 2012).

The recent genome sequencing of *F. hepatica* isolates from the Americas by McNulty and colleagues, confirmed that the large genome size is comparable between fluke isolates (McNulty et al., 2017). Interestingly, the analysis of these American isolates revealed the presence of a *Neorickettsia* endobacterium within the parasite, which was further demonstrated by immunolocalisation studies that found the bacterium within the eggs, reproductive system and the oral suckers of adult fluke. Consistent with other studies of trematode-*Neorickettsia* interactions, *Neorickettsia* could also be detected in the *Fasciola* eggs by PCR methods. To date no other liver fluke isolates from other geographical locations have reported the presence of any *Neorickettsia* endobacteria, indicating that the acquisition of this endobacteria may have occurred since the introduction of *F. hepatica* to the Americas. The study by McNulty and colleagues (2017) highlights the potential interaction between *Fasciola* and endosymbionts/endobacteria and warrants further investigation.

Single nucleotide polymorphism (SNP) analysis of UK *F. hepatica* isolates, including isolates resistant to the frontline anthelminthic, triclabendazole (Hodgkinson et al., 2013) has revealed high levels of sequence polymorphism in the *F. hepatica* genome (Cwiklinski et al., 2015a). In particular, a marked over-representation of genes with high levels of non-synonymous polymorphism was associated with axonogenesis and chemotaxis, reflecting the changing environments

the parasite encounters during its migration in the host. This data has recently been complemented by microsatellite analysis that revealed high levels of genetic diversity and gene flow within field isolates in the UK (Beesley et al., 2017). High levels of genetic diversity and gene flow may be important to counter the decline of allele diversity as a result of self-fertilisation (Noel et al., 2017).

The current *F. hepatica* genome assembly (PRJEB6687; Cwiklinski et al., 2015a) is comprised of a large number of scaffolds and contigs (20,158 scaffolds and 195,709 contigs, with a scaffold N50 of 204kb), mainly due to the size of the genome and the high percentage of repeat regions, which has hindered the assembly. In the future, utilising sequencing platforms that generate longer reads as well as technologies such as optical mapping should resolve this problem. The sequencing reads can then be mapped to the ten *F. hepatica* chromosomes (Sanderson, 1953), allowing analysis of genome structure and genomic comparison of Platyhelminth genome organisation.

3. Transcriptomics

The development of novel control strategies, vaccine and diagnostics aimed at specific *F. hepatica* lifecycle stages, requires an understanding of the genes that are transcribed at each time-point in development as well as their specific transcriptional abundance. Initial studies of gene identification and analysis were based on a limited number of unannotated expressed sequence tags (ESTs; 6819 sequences) generated from adult *F. hepatica* parasites by the Wellcome Trust Sanger Institute (ftp://ftp.sanger.ac.uk/pub/pathogens/Fasciola/hepatica/ESTs/). This EST database was also an essential resource for blasting peptide sequences for *F. hepatica* proteomic studies (Chemale et al., 2006; Robinson et al., 2009; Chemale et al., 2010; Hacariz et al., 2014; Morphew et al., 2014).

The formative analysis of these EST sequences identified several key molecules of interest for further characterisation, including glutathione transferases (GSTs; Chemale et al., 2006), calcium binding proteins (Banford et al., 2013), mucin-like proteins (Cancela et al., 2015) and the helminth defence molecule (Robinson et al., 2011; Martinez-Sernandez et al., 2014). Enhancing our understanding of the *F.*

hepatica lifecycle, Robinson and colleagues (2009) utilised an integrated transcriptomic and proteomic approach based on these adult-specific *Fasciola* ESTs, to profile the expression of proteins secreted by *Fasciola* parasites as they migrate through the host. However, this analysis was based on the premise that similarities could be drawn between the proteins expressed by the adult parasites residing in the bile ducts and those expressed by the migrating NEJ parasites. Utilising an adult-specific database, especially one with a limited number of sequences, likely resulted in NEJ-specific proteins being overlooked.

In 2010, Cancela and colleagues (2010) reported the generation of 1684 ESTs from the excysted NEJ. The limited number of ESTs is reflective of the amount of total RNA that could be extracted from 1200 NEJ and subsequently used for cDNA synthesis (200ng). Nevertheless, analysis of these sequences identified several sequences that had not been previously reported within the adult ESTs, implying that they were NEJ-specific. Specifically, several cathepsin cysteine proteases and antioxidant enzymes were characterised and showed that *F. hepatica* has adapted stage-specific proteases and enzymes to utilise throughout its lifecycle. The identification of novel stage-specific genes within this study highlighted the need for more extensive lifecycle stage-specific transcriptomes to further *Fasciola* research.

Led by the developments in sequencing technologies, Young and colleagues (2010) reported the first extensive adult *F. hepatica* transcriptome sequenced using 454 sequencing technology. In comparison to the 6819-unannotated adult-specific EST sequences available, this study generated a total of 590, 927 high quality reads that were clustered into approximately 48,000 sequences, of which 15,423 supercontigs of 745 bp (± 517bp) were enriched for open reading frames (ORF). These sequences were subjected to extensive homology searches and protein prediction, using tools such as InterProScan, gene ontology (GO) and KOBAS (KEGG Orthology-Based Annotation System) to annotate the predicted proteins. Based on the publically available datasets at the time, approximately 44% of the sequences were classified, identifying proteins representative of the adult stage parasite. In keeping with the fact that *F. hepatica* expresses a range of cathepsin cysteine proteases, several cysteine peptidase family members were identified within the adult transcriptome. The predicted protein sequences were also screened for

signal peptide and transmembrane domains to profile those proteins secreted by classical pathways within the ES proteins by the adult parasites; this analysis identified all the 160 ES proteins reported by Robinson et al. (2009). Importantly, comparing the Robinson et al. (2009) proteomic dataset with this more extensive adult *F. hepatica* database resulted in the annotation of previously unclassified proteins, including a group of fatty acid binding proteins and redox antioxidant enzymes. A further 18,347 contigs have been generated using 454 sequencing of adult fluke cDNA by Wilson et al. (2011) during their interrogation of the adult tegument. This more extensive dataset for adult *F. hepatica* has been interrogated by various research groups and has led to the identification of a range of proteins including, SCP/TAPS proteins (Cantacessi et al., 2012), glutathione transferases (Morphew et al., 2012) and cathepsin cysteine proteases (Morphew et al., 2011).

The available transcriptome data and subsequent analysis for *Fasciola* spp. has since been further improved with the development of short read Illumina sequencing that has increased sequence depth and coverage (Reuter et al., 2015). Investigation of the similarities between *F. hepatica* and *F. gigantica*, particularly those molecules important at the host-parasite interface, has been carried out following the first characterisation of the *F. gigantica* adult transcriptome (Young et al., 2011). Similarly, in depth Illumina sequencing has been applied to the study of virulence and immunomodulation-related genes of adult *F. hepatica*, identifying 62 previously uncharacterised virulence-related genes. *In silico* characterisation, subsequently implied that these genes have immunomodulatory properties since they were comparable to various immune related molecules, including cytokines and immune receptors (Hacariz et al., 2015).

In particular, the development of Illumina sequencing technology has advanced our knowledge of other *F. hepatica* lifecycle stages that have been previously difficult to analyse. We have reported the sequencing of several early lifecycle stages, namely the infective metacercariae stage, the NEJ parasites 1hr, 3hr and 24hr post-excystment, as well as juvenile parasites at 21-day post infection and adult parasites which has provided a transcriptional profile of *F. hepatica* during infection (Cwiklinski et al., 2015a). Differential gene transcription analysis showed that the parasite regulates the transcription of many of its genes with progressively

more genes being highly transcribed as the parasite rapidly grows and develops in preparation for migration through the host liver (>8000; Cwiklinski et al., 2015a).

The integration of transcriptome data with the *F. hepatica* genome has also revealed that gene family expansion is a key feature of *F. hepatica* adaptation and survival; we have shown that *F. hepatica* transcribes different members of these gene families during different stages of the lifecycle (Cwiklinski et al., 2015a). Key examples of such expanded gene families are the cathepsin cysteine proteases and the microtubule-related alpha and beta tubulins genes. Biochemical analysis of the family of cathepsin proteases has shown that the different clades have evolved distinctive peptidolytic activity specific to the requirements of different lifecycle stages (Robinson et al., 2008). Similarly, the transcription of the diverse range of beta tubulin isotypes that are temporally regulated could explain the stage-specific efficacy of benzimidazole anthelminthics (Sanabria et al., 2013).

Coupled with comprehensive proteomic analyses, a current focus of our work is to investigate the infective and invasive lifecycle stages, namely the metacercariae and NEJ to elucidate how the parasite prepares for infection and undergoes alterations to ensure its own survival (Cwiklinski and Dalton, unpublished). In depth interrogation of the transcriptome data available for these lifecycle stages has shown that the infective stage, metacercariae, is metabolically active and that early juvenile stages regulate the transcription of metabolic pathways, particularly those related to aerobic energy metabolism (Cwiklinski and Dalton, unpublished).

McNulty et al. (2017) reported a transcriptome analysis of *F. hepatica* eggs, metacercariae and adult stages, as part of their genome characterisation of American *F. hepatica* isolates, and identified several gene sets that were overexpressed by specific lifecycle stages. In particular, consistent with our analysis (Cwiklinski et al., 2015a), the cathepsin proteases were found to be highly regulated; different clade isotypes were over-expressed by the metacercariae compared with the adult parasite. The most significantly over-expressed gene in eggs was found to be the rate-limiting enzyme of the pentose phosphate pathway, glucose-6-phosphate dehydrogenase.

Recently, there has been an interest in extracellular vesicles within parasite secretomes and the role they play at the host-parasite interface (Marcilla et al., 2014;

Coakley et al., 2015). Extracellular vesicles (EVs) enable cell-to-cell communication by transferring proteins, lipids and microRNA (El Andaloussi et al., 2013; Record et al., 2014; Huang-Doran et al., 2017). At least two sub-populations of EVs with different protein content have been shown by centrifugation methods to be secreted by *F. hepatica*, including large EVs released from the parasite gut (15K EVs) and smaller exosome-like vesicle released from the tegumental surface (120K EVs) (Marcilla et al., 2012; Cwiklinski et al., 2015b). Transcriptomic analysis of the genes involved in the EV biogenesis pathway suggests that the synthesis of these two sub-populations of EVs occur via separate pathways, namely ESCRT and lipid-related/ESCRT-independent pathways, respectively (Cwiklinski et al., 2015b; de la Torre-Escudero et al., 2016).

Further analyses of *Fasciola* microRNAs (miRNAs) has been carried following the generation of three small RNAseq libraries from adult parasites (Xu et al., 2012; Fromm et al., 2015), the NEJ (Fontenla et al., 2015) and extracellular vesicles isolated from adult parasite secretion (Fromm et al., 2015). These studies have identified 52 non-coding microRNAs (miRNAs) corresponding to 32 metazoan-conserved miRNA families (Fromm et al., 2017). In addition, five *F. hepatica*-specific sequences were identified. Comparative analysis with *F. gigantica* indicates that these five sequences are specific to *F. hepatica* and are not shared across the *Fasciola* genus. Correspondingly, eight miRNAs have been identified as *F. gigantica*-specific (Xu et al., 2012). Whether or not these *Fasciola*-specific miRNAs are important for infection of the mammalian host has yet to be determined. Throughout the lifecycle the abundance of the miRNAs expressed by *F. hepatica* varies, indicating stage-specific roles (Fromm et al., 2017), with those miRNAs present within the extracellular vesicles most likely important for host-parasite interactions. In particular, the predicted targets of five immuno-regulatory miRNAs found to be enriched within the EV warrant further investigation (Fromm et al., 2015; Fromm et al., 2017).

4. Proteomics

Molecules that are secreted/excreted from liver flukes, also known as the ES proteins, are considered necessary for their migration through the tissues of the host

and evasion from immune responses. While the early migrating stages of *F. hepatica* are mainly tissue feeders, adult parasites residing in the bile ducts are obligate blood feeders. The adult flukes are readily recovered from the bile ducts of infected livestock and ES proteins are released in abundance when the adult parasites are maintained in culture medium (even microgram quantities can obtained from 10 adult parasites *in vitro*). Thus, the ES proteins of adult parasites have been extensively studied using proteomic tools. Early studies of *F. hepatica* proteins used radio-metabolic labelling to differentiate between the proteins of the various lifecycle stages (Irving and Howell, 1982; Dalton et al., 1985). Isoelectric focusing and densitometry were also carried out to characterise the ES proteins secreted by flukes maintained in different mammalian systems, namely llamas, rats, mice and cattle, which showed a different banding pattern (Lee et al., 1992a; Lee et al., 1992b). Jefferies and colleagues (2000; 2001) improved this analysis using two-dimensional gel electrophoresis and subsequent characterisation and annotation of protein spots to identify a range of cathepsin L proteases, superoxide dismutase, peroxiredoxin, glutathione S-transferases and fatty acid binding proteins. This study formed the basis for further in-depth analyses of these protein families using modern proteomic techniques and phylogenetic tools to elucidate how these protein families have diverged and adapted (Chemale et al., 2006; Robinson et al., 2008; Marcilla et al., 2008; Morphew et al., 2011; Morphew et al., 2012; Morphew et al., 2013; Cwiklinski et al., 2015b; Morphew et al., 2016; Di Maggio et al., 2016). Furthermore, proteomic analysis of the proteins within the extracellular vesicles released within the secreted/excreted proteins has revealed that the 15K and 120K sub-populations of EVs released by *F. hepatica* vary in their protein cargo composition (Cwiklinski et al., 2015b).

Analysis of the adult liver fluke secretome has been used to assess the mode of action of the anthelminthic drug triclabendazole (TCBZ), and suggest that TCBZ broadly effects liver fluke metabolism (Chemale et al., 2010). In particular, protein signatures of liver fluke parasites susceptible and putatively-resistant to TCBZ can be discerned based on the parasite's response to the TCBZ metabolite triclabendazole sulphoxide (TCBZ-SO) (Morphew et al., 2014). Parasite susceptibility to TCBZ characterised by lethal activity was indicated by the presence of actin, gelsolin, DJ-1 and triose phosphate isomerise, whereas putative resistance

400 characterised by sub-lethal activity was indicated by the presence of calreticulin,
401 cathepsin L proteases and enolase. These highly-specific protein profiles provide
402 potential markers that can be used for future TCBZ efficacy studies.

403 In contrast to the large amount of protein secreted by the adult parasites,
404 analysis of the early developmental and migratory stages of *F. hepatica* is more
405 challenging given their small size and difficulty in locating them in hosts tissues.
406 Accordingly, fewer proteomic studies have been reported for these stages. The
407 molecular investigation of egg embryonation, however, characterised 28 proteins
408 within the somatic proteome from 200,000 eggs (Moxon et al., 2010), and revealed
409 that protein complexity increases as eggs mature, consistent with the development
410 of the miracidia stage. This study also demonstrated that eggs have a substantially
411 different protein profile to the other developmental stages of *F. hepatica* (Moxon et
412 al., 2010). Similarly, a study of the secretome of an intra-molluscan stage, *in vitro*
413 transformed mother sporocysts, required 388,000 parasites to generate sufficient
414 protein for analysis (Gourbal et al., 2008). Seventeen of the most abundant proteins
415 were analysed, in particular two antioxidant enzymes, Cu/Zn superoxide dismutase
416 and thioredoxin (Gourbal et al., 2008). These enzymes were previously reported
417 within the adult secretome (Jefferies et al., 2001; Robinson et al., 2009), suggesting
418 a uniform process of detoxification of reactive oxygen species.

419 The development of proteomic tools and the accessibility of *F. hepatica*
420 parasites have facilitated the expansion of the available proteome datasets for the
421 NEJ migratory stages. Profiling the proteins secreted by the early infective stages,
422 namely the NEJ 24hr post-excystment and juvenile fluke 21 days post-infection with
423 those of adult parasites has allowed stage-specific proteins to be determined
424 (Robinson et al., 2009). A greater level of protein complexity was observed within
425 the juvenile secretome (45 proteins), compared with the NEJ 24hr (29 proteins) and
426 adult secretome (22 proteins), with a wider range of cathepsin isotypes and
427 antioxidant enzymes being secreted. This is consistent with the migratory and
428 feeding traits of this stage, and the upregulation of gene transcription observed
429 during this stage (Andrews, 1999; Cwiklinski et al., 2015a). The NEJ 24hr
430 secretome profile also confirmed the initial characterisation by N-terminal
431 sequencing of the NEJ secreted proteins carried out in the Meeusen laboratory

(Tkalcevic et al., 1995), which described an abundance of cathepsin L and asparaginyl endopeptidase cysteine proteases.

Facilitated by the sequencing of the *F. hepatica* genome, Di Maggio and colleagues (2016) recently reported a comprehensive analysis of the secreted proteins of adult liver fluke and NEJ 48hr post-excystment and compared these with the somatic proteome of the NEJ 48hr. Using gel free proteomic techniques, this study identified 202 proteins within the adult secretome, 90 proteins within the NEJ 48hr secretome and 575 proteins in the somatic proteome of the NEJ 48hr. Consistent with other secretome analyses, a range of proteases and protease inhibitors were detected by both developmental stages, representing >70% and <10% of the total protein secreted, respectively. Furthermore, previously unreported proteins were identified within the NEJ somatic proteome, including structural proteins and proteins related to metabolism, expanding our knowledge of this lifecycle stage.

Untangling the complexities of host-parasite interactions is key to furthering our understanding of how this helminth evades the host immune system. The adult liver fluke parasites reside within the bile ducts immersed in bile composed of bile acids, phospholipids, cholesterol, bilirubin and inorganic salts (Farina et al., 2009). Safe from the host's immune response (Andrews, 1999; Correia et al., 2001), proteomic analysis has shown that the adult secretome in the bile of sheep infected with *F. hepatica* is dominated by cathepsin L proteases (Morphew et al., 2007) similar to that shown *in vitro* (Robinson et al., 2009; Morphew et al., 2011).

Key to the survival within the mammalian host is the parasite tegument that can be rapidly turned over to prevent attachment of immune effector cells. Proteomic characterisation of the adult tegument was found to be enriched in structural proteins, transporters, proteins involved in secretory pathways and antioxidant enzymes (Wilson et al., 2011; Hacariz et al., 2012). A similar range of proteins were also identified within the somatic proteome of the outer tegumental surface of NEJ (Hernandez-Gonzalez et al., 2010). Furthermore, recent analysis has been carried out on the tegumental immunoprecipitate formed following the incubation of live adult *F. hepatica* flukes in purified IgG from *F. gigantica*-infected Thin Tailed sheep (Cameron et al., 2017). This study identified several molecules

consistent with previous analyses of the tegument (Wilson et al., 2011; Hacariz et al., 2012), as well as a range of proteins associated with *F. hepatica* exosomes (15K EVs; Cwiklinski et al., 2015b). In addition, it highlighted the cross-reactivity between antibodies elicited against *F. gigantica* during infection of Thin Tailed sheep and *F. hepatica* tegumental proteins, and raised the interesting question of whether different proteins and EV components are secreted/released under different 'environmental' situations.

5. Glycomics

To date *F. hepatica* glycomic analyses have focussed on the outer surface of the parasite, the glycocalyx, that is rich in glycoproteins and glycolipids (Threadgold, 1976). Analysis has shown that the tegumental surface is highly glycosylated, with an abundance of mannose-rich N-linked glycoproteins present on the surface, spines and suckers (Garcia-Campos et al., 2016; Ravida et al., 2016a; Ravida et al., 2016b). The exact role these N-glycoproteins play at the host-parasite interface is currently unknown, though parasite glyco-conjugates that been implicated in evading the host immune response (van Die and Cummings, 2010). Moreover, blocking of the N-glycans on the surface on the NEJ using lectins has been shown to inhibit their migration across the intestinal wall (Garcia-Campos et al., 2017). Studies of the *F. hepatica* glycans have shown that they have immune modulatory properties modulating toll-like receptor-induced maturation of dendritic cells through carbohydrate specific receptors (CLR) (Rodriguez et al., 2015; Ravida et al., 2016a; Rodriguez et al., 2017). In contrast to the analysis of the *F. hepatica* N-glycans, the composition of the O-glycans has yet to be determined, though potential sites have been identified within the glycoproteins of the tegument (Ravida et al., 2016b).

The glycocalyx is also rich in glycolipids that are highly antigenic (Wuhrer et al., 2003) and share terminal Galα1-4Gal and Galβ1-6Gal motifs with cestodes that result in serological cross-reactivity (Wuhrer et al., 2004). As well as being cross-reactive with other members of the Phylum Platyhelminthes, these glycolipids also mimic mammalian-type glycolipids (Wuhrer et al., 2001; Wuhrer et al., 2004), facilitating parasite survival.

6. Proteomic and transcriptomic analysis of host responses to *F. hepatica*

Several recent studies have utilised -omics approaches to investigate the responses of the host during infection with *F. hepatica* with the aim to elucidate host responses that mirror the stage of infection and the developmental changes that occur within the advancing parasite. These large-scale investigations of the host responses give an unbiased global view of the effects of fasciolosis on host immune tissues, and have revealed novel aspects of pathogenesis associated with infection. In addition, these approaches are being used to evaluate potential vaccine candidates, identifying the genes involved in conferring protection (Wesołowska et al., 2013; Rojas-Caraballo et al., 2017).

Transcriptomic responses within macroscopic lesions of *F. hepatica*-infected liver at 8 weeks post infection (wpi) in sheep revealed that gene expression is highly regulated (Alvarez Rojas et al., 2015), consistent with comparable microarray studies of mice (Rojas-Caraballo et al., 2015). Several processes characteristic of acute fasciolosis and upregulated in response to the damage caused by the parasite were identified. Genes corresponding to fibrosis and tissue repair were found to be upregulated, consistent with the subsequent tissue regeneration required following the invasive migration of the parasite. In keeping with observations that helminth infections typically skew host immune responses towards a Th2 type, genes associated with Th2 differentiation and B cell activation were found to be upregulated, while Th1 type responses were down-regulated. Interestingly, this study also reported that an increased abundance of circulating reticulocytes is associated with the blood feeding activity of *F. hepatica*, which can cause anaemia. Increased transcription of haemoglobin-related genes and four genes putatively associated with Fanconi anaemia were also observed.

In other investigations of the host responses to fasciolosis, two recent studies analysed the transcriptomic responses of ovine peripheral blood mononuclear cells (PBMC) at stages throughout infection (Alvarez Rojas et al., 2016; Fu et al., 2016). Despite different protocols being used for sample preparation, RNA extraction and subsequent analysis of the RNAseq data, both studies reveal that gene transcription is highly regulated during *F. hepatica* infection of sheep, particularly during acute

infection (1-2 wpi). Both studies also observed the upregulation of genes associated with TGF beta signalling, including the genes TGF beta, collagen type 1 and the downstream SMAD signalling genes. These genes play a major role in fibrosis, which were also observed in the transcriptome analysis of infected liver described above (Alvarez Rojas et al., 2015). Upregulation of genes associated with the complement and coagulation cascades, chemokine signalling pathway and cytokine-cytokine receptor interaction pathway were also reported by Alvarez Rojas et al. (2016).

Consistent with the polarization of immune responses towards a Th2 type, the gene encoding inducible nitric oxide synthase (iNOS) was shown to be down-regulated in response to infection with *F. hepatica* at both acute and chronic stages in the study by Fu et al. (2016). Transcription of Th17 related genes were also down–regulated, suggesting that *F. hepatica* is able to inhibit the differentiation and stability of Th17 cells. In contrast with the study by Fu and colleagues (2016), however, genes encoding interleukins, particularly those related to Th2 type responses, such as IL-4, were not reported by Alvarez Rojas et al. (2016) to be differentially expressed at statistical levels. This difference may reflect the different protocols used to process the PBMCs for RNA extraction, namely processing fresh cells versus storage in RNAlater, which may have had an effect on the stability of gene expression profiles (Debey-Pascher et al., 2011; Eikmans et al., 2013). Equally, the type of strategy used for sequence analysis may contribute to the differences observed.

As infection by *F. hepatica* progresses, the amount and composition of immune cells present both within the peritoneal cavity and circulating in the peripheral blood shifts to an abundance of eosinophils, which is associated with a polarisation to Th2 type immune responses. Differential eosinophil cell counts were only reported in the Alvarez-Rojas et al. study (2016), and showed the infected animals had substantially more eosinophils than the control non-infected animals. At 4 wpi, the eosinophil count in the infected group ranged from 12-39% compared with the control group counts of 1-5%. Therefore, the changes of transcriptomic response are reflective of both a change in transcription during infection as well as a change in the number and type of cells within the PBMC fraction and must be interpreted accordingly.

Compared with inbred mouse strains, large animal mammalian hosts of helminth parasites, in particular sheep and cattle are genetically more variable, at both individual animal level and between breeds. This can have a significant effect on how an animal or animal breed responds to infection with *F. hepatica* (Ardia et al., 2011). In the case of the two studies analysing transcriptomic responses in PBMC, each used different sheep breeds sorted into groups of four animals. To address the possible between-animal variation Alvarez Rojas et al. (2016) employed two strategies to analyse their data; (1) assessment of each animal as an independent experiment and (2) treating each group (control non-infected and infected) as biological replicates. Differentially expressed genes identified by both strategies were then used for further analysis, resulting in the identification of 183 and 76 genes differentially expressed at 2 wpi and 8 wpi, respectively. In comparison, the study by Fu et al. (2016), which compared animals as biological replicates, identified 6490 differentially expressed genes at 1 wpi, indicating that many genes of interest were overlooked by the stringent process employed by Alvarez-Rojas et al. (2016). However, the sheep breed-specific responses may also be a factor in the differences observed between these trials. Therefore, it is important for studies of host-parasite interactions in ruminants that sufficient numbers of animals are used and that the type of analysis utilised is appropriately considered and validated.

The host responses to the migrating parasites within the peritoneal compartment during early infection and in the bile ducts consistent with chronic infection have also been investigated using proteomic tools. Early fasciolosis is characterised by the migration of *F. hepatica* through the intestinal wall to the liver via the peritoneal cavity. Proteomic analysis of the peritoneal fluid from sheep infected by *F. hepatica* at 18 days post infection (dpi) identified an abundance of proteins associated with the complement system and proteins associated with the liver extracellular matrix (ECM) (Ruiz-Campillo et al., 2017). The presence of proteins associated with the liver ECM, including collagen VI, fibronectin and fibrocystin, is likely the result of the damage caused by the parasite as it invades and migrates through the liver. Intriguingly, this study also detected two ECM-related molecules, periostin and vascular cell adhesion protein 1 (VCAM-1), that mediate leukocyte infiltration and are associated with marked eosinophilia, which warrant further investigation as biomarkers of infection.

Systemic responses have been analysed using proteomic tools to identify biomarkers of infection within the serum. Rioux et al. (2008) showed that there were significant changes within the sera beginning within 3 weeks of infection, consistent with the transcriptomic analysis of PBMCs that highlighted a greater level of differential gene expression during acute infection (Fu et al., 2016; Alvarez Rojas et al., 2016). These striking changes also coincide with marked expression of >8000 genes that accompany the rapid growth and development of *F. hepatica* in the first three weeks of infection (Cwiklinski et al., 2015a). Two markers of particular interest, namely transferrin and apolipoprotein A-IV (Apo A-IV), were upregulated during this early period (Rioux et al., 2008). Transferrin is associated with the anaemia caused by the blood feeding parasites, whereas Apo A-IV is associated with regulation of appetite within the intestine of mammals (although studies in rats and mice insinuate a possible role within the liver; VerHague et al., 2013). In comparison, levels of transferrin detected within the bile by Morphew et al. (2007) were reduced compared with levels in the serum, further highlighting that the data can vary significantly depending of the sample type (serum, bile, peritoneal fluid etc) and time of infection.

7. Concluding remarks

Over the last few decades major advances that have been made through –omics technologies have provided the liver fluke community with an extensive array of datasets that can be interrogated to further our understanding of liver fluke biology (Fig. 1). The number of genes encoded within the *F. hepatica* genome has been clarified. In particular, this information has been crucial in elucidating gene family organisations, which in the past have been complicated by the large number of gene sequences of similar classification present within the NCBI database. In addition, the genes transcribed by *F. hepatica* have been found to be highly regulated throughout the lifecycle within the mammalian host. This knowledge is vital for our continuing efforts to develop control strategies that, in particular, target the early stage parasites. Proteomic analysis of the secreted/excreted proteins has highlighted key molecules that play an important role at the host-parasite interface. Biochemical characterization of these key molecules has also revealed stage-specific adaptations, including the activity of cathepsin L proteases that includes

collagenolytic activity specific to the juvenile parasites and haemolytic activity restricted to the adult parasites (Robinson et al., 2008). It has also revealed some unexpected adaptations such as the kunitz-type serpin inhibitors that have inhibitory activity against cathepsin L cysteine proteases and not serine proteases (Smith et al., 2016).

However, the function of a large proportion of *F. hepatica* genes and the proteins they encode still remain unknown. In general, these genes only share homology with uncharacterised genes from other Platyhelminthes, indicating that they are Phylum-specific. Further investigation is therefore required to decipher the function these genes play and specifically their importance for host-parasite interactions. This can be achieved utilising post-genomic tools such as RNA interference (RNAi) and CRISPR, as well as protein structural analysis to increase our knowledge of these uncharacterised genes, facilitating the annotation of Platyhelminthes datasets. Furthermore, the addition of this information into the various software packages available for omics analyses, such as STRING (Szklarczyk et al., 2015) and PANTHER (Mi et al., 2017) where there is a current lack of data relating to the Phylum Platyhelminthes, is essential to expand our knowledge of parasite protein-protein interaction networks.

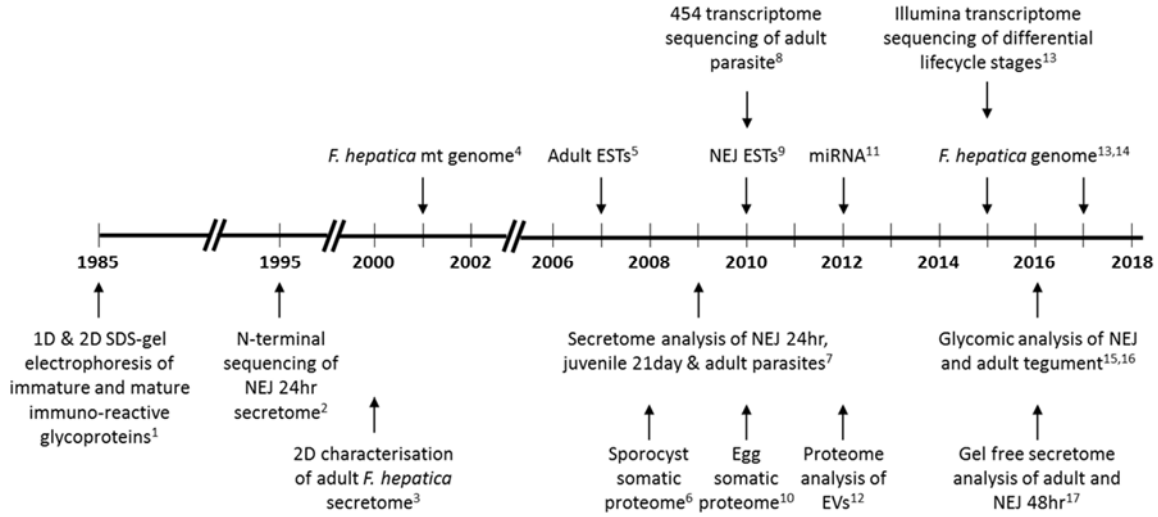
How parasites regulate their genes, specifically in response to their environment, particularly the host immune response, is becoming an area of intense interest. In particular, this analysis has encompassed understanding the epigenetic process of gene regulation, through DNA methylation, histone modification and non-coding RNA associated with gene silencing (Egger et al., 2004). The role these epigenetic processes play to facilitate *Fasciola* invasion and survival has yet to be investigated. However, studies of closely related Platyhelminthes have indicated that it warrants further investigation. In particular, the study of DNA methylation across the Phylum Platyhelminthes has shown that cytosine methylation is a functionally conserved epigenetic feature (Geyer et al., 2013). Furthermore, recent analysis of the epigenome of *Schistosoma mansoni* cercariae has revealed that histone modifications play an important role in regulating the transcription of genes, with the cercariae being transcriptional inactive (Roquis et al., 2015). In-depth of analysis of the *Fasciola* genome has already revealed an array of non-coding small RNAs that may play a part in the post-transcriptional regulation of *Fasciola* genes

and/or be important for the regulation and manipulation of the mammalian host. Similar analysis of the epigenome of the different lifecycle stages will show if there are any lifecycle stage-regulatory factors associated with liver fluke gene regulation.

For the future development of control strategies, a greater understanding of host helminth interactions is paramount. This review has discussed the large-scale datasets available for the study of liver fluke infection, from both the parasite and the mammalian host. Going forward the analyses of these data should be integrated to elucidate the delicate interplay that occurs during infection and to determine if the pathogenicity/virulence of liver fluke isolates within field populations plays a role in this interaction.

Figure Legend

Fig. 1. Schematic of the major *Fasciola* –omics advances detailed over time. The principal references are denoted by numbers as follows: 1) Dalton et al., 1985; 2) Tkalcevic et al., 1995; 3) Jefferies et al., 2000; 4) Le et al., 2001; 5) ftp://ftp.sanger.ac.uk/pub/pathogens/Fasciola/hepatica/ESTs/; 6) Gourbal et al., 2008; 7) Robinson et al., 2009; 8) Young et al., 2010; 9) Cancela et al., 2010; 10) Moxon et al., 2010; 11) Xu et al., 2012; 12) Marcilla et al., 2012; 13) Cwiklinski et al., 2015a; 14) McNulty et al., 2017; 15) Garcia-Campos et al., 2016; 16) Ravida et al., 2016b; 17) Di Maggio et al., 2016.



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