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Advances in Fasciola hepatica research using -omics technologies

Cwiklinski, K., & Dalton, J. (2018). Advances in Fasciola hepatica research using -omics technologies. International Journal for Parasitology. <https://doi.org/10.1016/j.ijpara.2017.12.001>

Published in:

International Journal for Parasitology

Document Version:

Peer reviewed version

Queen's University Belfast - Research Portal:

[Link to publication record in Queen's University Belfast Research Portal](#)

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1 **Advances in *Fasciola hepatica* research using –omics technologies**

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14 Keywords: *Fasciola hepatica*, helminth, trematode, genome, transcriptome,
15 proteome.

16 **Abstract**

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

32

33 **1. Introduction**

34 DNA sequencing technologies have rapidly evolved over the past few
35 decades, stemming from the traditional Sanger methodology used to map the first
36 human genome (Lander et al., 2001; Venter et al., 2001) to the recent high-
37 throughput sequencing technologies such as Roche 454 and Illumina (Reuter et al.,
38 2015) that we use today. More recently, single cell sequencing has emerged,
39 pioneered by Pacific Biosciences and Oxford Nanopore Technologies, through the
40 PacBio and MinION platforms, respectively (Reuter et al., 2015). As the technology
41 for sequencing DNA has progressed, so too have the routine protocols for the
42 extraction of nucleic acids and library preparation (Price et al., 2009); this has
43 allowed sequencing projects to be carried out on even the most challenging species
44 to propagate in the laboratory and those for which it was previously difficult to obtain
45 adequate quantities of nucleic acids. Consequently, the number of sequencing
46 projects undertaken has exploded, including recent ambitious proposals to sequence
47 10000 vertebrate genomes (Genome 10K project; Koepfli et al., 2015), 5000

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

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

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

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

80 dbEST (dbEST release 130101; <https://www.ncbi.nlm.nih.gov/dbEST/>). SAGE
81 methodology has also been employed for the analysis of gene expression across
82 different lifecycle stages (Knox and Skuce, 2005; Williams et al., 2007; Taft et al.,
83 2009). More recently, large scale RNAseq analyses have been completed for a
84 range of Platyhelminth parasites, several of which have been disseminated through
85 the site Helminth.net (Martin et al., 2015). These freely-accessible datasets have
86 complemented ongoing genome projects.

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

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

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

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

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

136

137 **2. Genomics**

138 **2.1 The *F. hepatica* mitochondrial genome**

139 The characterisation and differentiation of various *Fasciola* species using
140 morphological features is often unreliable and can only be used for the differentiation
141 of adult parasites found within the bile ducts. Molecular identification based on
142 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.

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

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

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

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

212 The current *F. hepatica* genome assembly (PRJEB6687; Cwiklinski et al.,
213 2015a) is comprised of a large number of scaffolds and contigs (20,158 scaffolds
214 and 195,709 contigs, with a scaffold N50 of 204kb), mainly due to the size of the
215 genome and the high percentage of repeat regions, which has hindered the
216 assembly. In the future, utilising sequencing platforms that generate longer reads as
217 well as technologies such as optical mapping should resolve this problem. The
218 sequencing reads can then be mapped to the ten *F. hepatica* chromosomes
219 (Sanderson, 1953), allowing analysis of genome structure and genomic comparison
220 of Platyhelminth genome organisation.

221

222 **3. Transcriptomics**

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

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

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

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

257 Led by the developments in sequencing technologies, Young and colleagues
258 (2010) reported the first extensive adult *F. hepatica* transcriptome sequenced using
259 454 sequencing technology. In comparison to the 6819-unannotated adult-specific
260 EST sequences available, this study generated a total of 590, 927 high quality reads
261 that were clustered into approximately 48,000 sequences, of which 15,423
262 supercontigs of 745 bp (\pm 517bp) were enriched for open reading frames (ORF).
263 These sequences were subjected to extensive homology searches and protein
264 prediction, using tools such as InterProScan, gene ontology (GO) and KOBAS
265 (KEGG Orthology-Based Annotation System) to annotate the predicted proteins.
266 Based on the publically available datasets at the time, approximately 44% of the
267 sequences were classified, identifying proteins representative of the adult stage
268 parasite. In keeping with the fact that *F. hepatica* expresses a range of cathepsin
269 cysteine proteases, several cysteine peptidase family members were identified within
270 the adult transcriptome. The predicted protein sequences were also screened for

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

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

295 In particular, the development of Illumina sequencing technology has
296 advanced our knowledge of other *F. hepatica* lifecycle stages that have been
297 previously difficult to analyse. We have reported the sequencing of several early
298 lifecycle stages, namely the infective metacercariae stage, the NEJ parasites 1hr,
299 3hr and 24hr post-excystment, as well as juvenile parasites at 21-day post infection
300 and adult parasites which has provided a transcriptional profile of *F. hepatica* during
301 infection (Cwiklinski et al., 2015a). Differential gene transcription analysis showed
302 that the parasite regulates the transcription of many of its genes with progressively

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

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

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

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

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

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

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

363

364 **4. Proteomics**

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

367 and evasion from immune responses. While the early migrating stages of *F.*
368 *hepatica* are mainly tissue feeders, adult parasites residing in the bile ducts are
369 obligate blood feeders. The adult flukes are readily recovered from the bile ducts of
370 infected livestock and ES proteins are released in abundance when the adult
371 parasites are maintained in culture medium (even microgram quantities can be obtained
372 from 10 adult parasites *in vitro*). Thus, the ES proteins of adult parasites have been
373 extensively studied using proteomic tools. Early studies of *F. hepatica* proteins used
374 radio-metabolic labelling to differentiate between the proteins of the various lifecycle
375 stages (Irving and Howell, 1982; Dalton et al., 1985). Isoelectric focusing and
376 densitometry were also carried out to characterise the ES proteins secreted by flukes
377 maintained in different mammalian systems, namely llamas, rats, mice and cattle,
378 which showed a different banding pattern (Lee et al., 1992a; Lee et al., 1992b).
379 Jefferies and colleagues (2000; 2001) improved this analysis using two-dimensional
380 gel electrophoresis and subsequent characterisation and annotation of protein spots
381 to identify a range of cathepsin L proteases, superoxide dismutase, peroxiredoxin,
382 glutathione S-transferases and fatty acid binding proteins. This study formed the
383 basis for further in-depth analyses of these protein families using modern proteomic
384 techniques and phylogenetic tools to elucidate how these protein families have
385 diverged and adapted (Chemale et al., 2006; Robinson et al., 2008; Marcilla et al.,
386 2008; Morphew et al., 2011; Morphew et al., 2012; Morphew et al., 2013; Cwiklinski
387 et al., 2015b; Morphew et al., 2016; Di Maggio et al., 2016). Furthermore, proteomic
388 analysis of the proteins within the extracellular vesicles released within the
389 secreted/excreted proteins has revealed that the 15K and 120K sub-populations of
390 EVs released by *F. hepatica* vary in their protein cargo composition (Cwiklinski et al.,
391 2015b).

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

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

434 Facilitated by the sequencing of the *F. hepatica* genome, Di Maggio and
435 colleagues (2016) recently reported a comprehensive analysis of the secreted
436 proteins of adult liver fluke and NEJ 48hr post-excystment and compared these with
437 the somatic proteome of the NEJ 48hr. Using gel free proteomic techniques, this
438 study identified 202 proteins within the adult secretome, 90 proteins within the NEJ
439 48hr secretome and 575 proteins in the somatic proteome of the NEJ 48hr.
440 Consistent with other secretome analyses, a range of proteases and protease
441 inhibitors were detected by both developmental stages, representing >70% and
442 <10% of the total protein secreted, respectively. Furthermore, previously unreported
443 proteins were identified within the NEJ somatic proteome, including structural
444 proteins and proteins related to metabolism, expanding our knowledge of this
445 lifecycle stage.

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

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

464 consistent with previous analyses of the tegument (Wilson et al., 2011; Hacariz et al.,
465 2012), as well as a range of proteins associated with *F. hepatica* exosomes (15K
466 EVs; Cwiklinski et al., 2015b). In addition, it highlighted the cross-reactivity between
467 antibodies elicited against *F. gigantica* during infection of Thin Tailed sheep and *F.*
468 *hepatica* tegumental proteins, and raised the interesting question of whether different
469 proteins and EV components are secreted/released under different 'environmental'
470 situations.

471

472 **5. Glycomics**

473 To date *F. hepatica* glycomic analyses have focussed on the outer surface of
474 the parasite, the glycocalyx, that is rich in glycoproteins and glycolipids (Threadgold,
475 1976). Analysis has shown that the tegumental surface is highly glycosylated, with
476 an abundance of mannose-rich N-linked glycoproteins present on the surface, spines
477 and suckers (Garcia-Campos et al., 2016; Ravida et al., 2016a; Ravida et al.,
478 2016b). The exact role these N-glycoproteins play at the host-parasite interface is
479 currently unknown, though parasite glyco-conjugates that been implicated in evading
480 the host immune response (van Die and Cummings, 2010). Moreover, blocking of
481 the N-glycans on the surface on the NEJ using lectins has been shown to inhibit their
482 migration across the intestinal wall (Garcia-Campos et al., 2017). Studies of the *F.*
483 *hepatica* glycans have shown that they have immune modulatory properties
484 modulating toll-like receptor-induced maturation of dendritic cells through
485 carbohydrate specific receptors (CLR) (Rodriguez et al., 2015; Ravida et al., 2016a;
486 Rodriguez et al., 2017). In contrast to the analysis of the *F. hepatica* N-glycans, the
487 composition of the O-glycans has yet to be determined, though potential sites have
488 been identified within the glycoproteins of the tegument (Ravida et al., 2016b).

489 The glycocalyx is also rich in glycolipids that are highly antigenic (Wuhrer et
490 al., 2003) and share terminal Gal α 1-4Gal and Gal β 1-6Gal motifs with cestodes that
491 result in serological cross-reactivity (Wuhrer et al., 2004). As well as being cross-
492 reactive with other members of the Phylum Platyhelminthes, these glycolipids also
493 mimic mammalian-type glycolipids (Wuhrer et al., 2001; Wuhrer et al., 2004),
494 facilitating parasite survival.

495

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

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

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

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

527 infection (1-2 wpi). Both studies also observed the upregulation of genes associated
528 with TGF beta signalling, including the genes TGF beta, collagen type 1 and the
529 downstream SMAD signalling genes. These genes play a major role in fibrosis,
530 which were also observed in the transcriptome analysis of infected liver described
531 above (Alvarez Rojas et al., 2015). Upregulation of genes associated with the
532 complement and coagulation cascades, chemokine signalling pathway and cytokine-
533 cytokine receptor interaction pathway were also reported by Alvarez Rojas et al.
534 (2016).

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

549 As infection by *F. hepatica* progresses, the amount and composition of
550 immune cells present both within the peritoneal cavity and circulating in the
551 peripheral blood shifts to an abundance of eosinophils, which is associated with a
552 polarisation to Th2 type immune responses. Differential eosinophil cell counts were
553 only reported in the Alvarez-Rojas et al. study (2016), and showed the infected
554 animals had substantially more eosinophils than the control non-infected animals. At
555 4 wpi, the eosinophil count in the infected group ranged from 12-39% compared with
556 the control group counts of 1-5%. Therefore, the changes of transcriptomic
557 response are reflective of both a change in transcription during infection as well as a
558 change in the number and type of cells within the PBMC fraction and must be
559 interpreted accordingly.

560 Compared with inbred mouse strains, large animal mammalian hosts of
561 helminth parasites, in particular sheep and cattle are genetically more variable, at
562 both individual animal level and between breeds. This can have a significant effect
563 on how an animal or animal breed responds to infection with *F. hepatica* (Ardia et al.,
564 2011). In the case of the two studies analysing transcriptomic responses in PBMC,
565 each used different sheep breeds sorted into groups of four animals. To address the
566 possible between-animal variation Alvarez Rojas et al. (2016) employed two
567 strategies to analyse their data; (1) assessment of each animal as an independent
568 experiment and (2) treating each group (control non-infected and infected) as
569 biological replicates. Differentially expressed genes identified by both strategies
570 were then used for further analysis, resulting in the identification of 183 and 76
571 genes differentially expressed at 2 wpi and 8 wpi, respectively. In comparison, the
572 study by Fu et al. (2016), which compared animals as biological replicates, identified
573 6490 differentially expressed genes at 1 wpi, indicating that many genes of interest
574 were overlooked by the stringent process employed by Alvarez-Rojas et al. (2016).
575 However, the sheep breed-specific responses may also be a factor in the differences
576 observed between these trials. Therefore, it is important for studies of host-parasite
577 interactions in ruminants that sufficient numbers of animals are used and that the
578 type of analysis utilised is appropriately considered and validated.

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

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

609

610 **7. Concluding remarks**

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

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

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

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

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

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

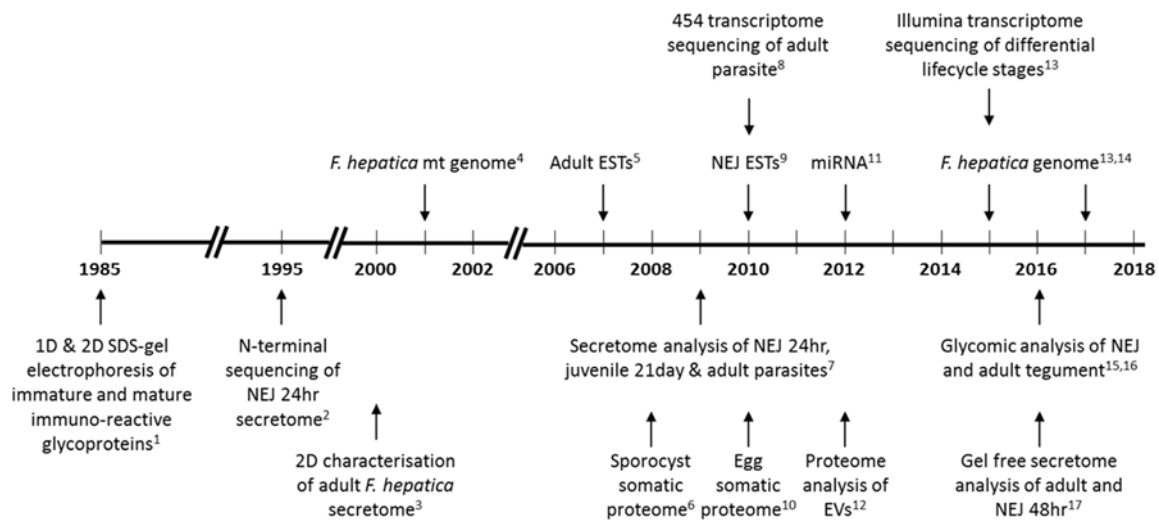
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669

670 **Figure Legend**

671 Fig. 1. Schematic of the major *Fasciola* –omics advances detailed over time. The
 672 principal references are denoted by numbers as follows: 1) Dalton et al., 1985; 2)
 673 Tkalcevic et al., 1995; 3) Jefferies et al., 2000; 4) Le et al., 2001; 5)
 674 ftp://ftp.sanger.ac.uk/pub/pathogens/Fasciola/hepatica/ESTs/; 6) Gourbal et al.,
 675 2008; 7) Robinson et al., 2009; 8) Young et al., 2010; 9) Cancela et al., 2010; 10)
 676 Moxon et al., 2010; 11) Xu et al., 2012; 12) Marcilla et al., 2012; 13) Cwiklinski et al.,
 677 2015a; 14) McNulty et al., 2017; 15) Garcia-Campos et al., 2016; 16) Ravida et al.,
 678 2016b; 17) Di Maggio et al., 2016.

679



680

681 **Acknowledgements**

682 KC and JPD are funded by a European Research Council Advanced Grant
683 (HELIVAC, 322725) awarded to JPD and are members of the Horizon 2020-funded
684 Consortium PARAGONE.

685

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