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1 **Revisiting the *Schistosoma japonicum* life cycle transcriptome for new** 2 **insights into lung schistosomula development**

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6

7 **Abstract**

8 Schistosome parasites are complex trematode blood flukes responsible for the disease
9 schistosomiasis; a global health concern prevalent in many tropical and sub-tropical countries.
10 While established transcriptomic databases are accessed *ad hoc* to facilitate studies characterising
11 specific genes or gene families, a more comprehensive systematic updating of gene annotation and
12 survey of the literature to aid in annotation and context is rarely addressed. We have reanalysed an
13 online transcriptomic dataset originally published in 2009, where seven life cycle stages of
14 *Schistosoma japonicum* were examined. Using the online pathway analysis tool Reactome, we have
15 revisited key data from the original study. A key focus of this study was to improve the interpretation
16 of the gene expression profile of the developmental lung-stage schistosomula, since it is one of the
17 principle targets for worm elimination. Highly enriched transcripts, associated with lung
18 schistosomula, were related to a number of important biological pathways including host immune
19 evasion, energy metabolism and parasitic development. Revisiting large transcriptomic databases
20 should be considered in the context of substantial new literature. This approach could aid in the
21 improved understanding of the molecular basis of parasite biology. This may lead to the
22 identification of new targets for diagnosis and therapies for schistosomes, and other helminths.

23

24 **Keywords**

25 Schistosomiasis; Transcriptomics; Schistosomula; Pathway Analysis; Helminths.

1. Introduction

Schistosomiasis, originally referred to as Bilharzia, is a parasitic disease caused by several species of trematode of the genus *Schistosoma*. The blood fluke is a zoonotic parasitic helminth, with some species infecting humans and agriculturally important animals (Gordon, et al., 2016). Schistosomiasis affects more than 230 million people worldwide, however there is risk of future infection in an additional 500-779 million individuals (Crompton, 1999, Gryseels, et al., 2006, McManus, et al., 2020). The World Health Organisation (WHO) identifies schistosomiasis as a Neglected Tropical Disease (NTD) which is endemic in 78 tropical and sub-tropical countries (Chitsulo, et al., 2000, Colley and Secor, 2014). Three different strains are predominately responsible for human schistosomiasis; *Schistosoma japonicum*, *S. mansoni*, and *S. haematobium*, however *S. japonicum* is the main causative agent in Southeast Asia (Gordon, et al., 2015a, Gordon, et al., 2015b).

2. The Importance of Lung-Schistosomula during the Schistosome Life Cycle

Parasitic organisms often demonstrate complex life cycles including the requirement of several hosts. Trematodes exploit specific species of freshwater snails, with *Oncomelania spp* acting as an intermediate host for *S. japonicum*. Snails release hundreds of free swimming cercariae, which infiltrate the skin of the definitive mammalian host. In the course of host penetration the cercariae activate their development into schistosomula, larvae which will further mature and develop in the host blood stream. The extensive forebody transformation to schistosomula corresponds with intense biochemical and physiological changes to establish their further maturation within the definitive mammalian host (Gobert, et al., 2007, Gobert, et al., 2010). Schistosomula transverse into the blood or lymphatic vessels of the pulmonary circulation, where they will travel to infect the lung. Schistosomula exit the lungs and re-enter the systemic venous system, facilitating migration to the hepatic portal system, where the pre-adult can sexually mature and reside (Gobert, et al., 2007).

The biochemical alterations of the lung schistosomula inherently provide the host immune system with an array of antigens (Wilson, 2009). However, while in the lungs migrating schistosomula are resistant to both adaptive and innate host immune responses (Riengrojpitak, et al., 1998). This successful host-parasite relationship demonstrates the ability of the schistosome to adapt and thrive within the hostile conditions of the host venous system. Published literature highlights possible immune evasion mechanisms (Gobert, et al., 2007, Wilson, 2009), including the rapid

turnover of the heptalaminate tegument membrane (Gobert, et al., 2003), the secretion of schistosome derived immune modulators (Wilson, 2012), and epitope concealment and antigen masking (Sepulveda, et al., 2010), all of which will contribute to schistosome survival and the down regulation of the host immune response. The lung-stage of the schistosome is the principle phase for potential worm elimination, by host immunological response. Schistosomula could provide insights for possible vaccine targets, due to their endurance regarding the host immune attack (Wilson, 2009).

3. Previously reported Schistosomula Transcriptomics

The original study carried out high throughput microarray analysis of the blood fluke *S. japonicum*, in order to profile genes which were enriched throughout seven differential stages of the schistosome life cycle (Gobert, et al., 2009). At the time, Gene Ontology analysis was carried out enabling the identification of genes involved in developmental changes, and stage specific functions and processes within the parasite; host immune evasion, the acquisition of nutrients, energy production, calcium signalling, sphingolipid metabolism, egg production and tegument function. However, comprehensive pathway analysis was not presented. Due to their biological importance, we now focus on the enriched or upregulated genes in the Day 3 *in vivo* derived schistosomula as identified in the original study.

Data from the original findings published in 2009 had additional annotation provided and was then mapped to pathways for re-mining, in order to review and update (Gobert, et al., 2009). This included the use of supplementary tables in the publication and GEO Gene Expression Omnibus, <http://www.ncbi.nlm.nih.gov/geo/> accession numbers GPL7160, GSE12704. All data was filtered for all of the 19,222 contigs previously present on the schistosome microarray. Signals that were flagged “present” during the feature extraction process were included, as well as p-values (ANOVA with multiple testing correction: Benjamini and Hochberg False Discovery Rate) ≤ 0.05 . Finally an arbitrary ≥ 2 fold up-regulation relative to the mean fold intensity of combined life cycle stages, was used to identify lung schistosomula enriched genes.

3.1 Updated annotation and Reactome pathway analysis online database

All genes were subjected to DIAMOND BLASTX, a heuristic open source algorithm able to translate contig nucleotide sequences into viable and accurate protein description, using a protein database. BLASTX, often referred to as the gold standard tool for offering such high sensitivity, is much too

86 slow for application of such a large nucleotide sequence dataset. Appropriate for multiple
87 sequences, long read analysis and capable of translation speeds 20,000 times faster than BLASTX
88 alone, the DIAMOND sequence aligner was selected to provide high sensitivity of large data input
89 within a much shorter time period (Buchfink, et al., 2015). The closest human homologue was
90 identified, with accession numbers used for subsequent Reactome analysis.

91 Reactome is an online (<https://reactome.org/>) curated database, representative of human
92 biological pathways and reactions (Fabregat, et al., 2018). It provides an in-built bioinformatics tool
93 for the interpretation of pathway analysis through visual representation. Along with protein
94 description and gene ontology (GO), BLASTX analysis also provided accession numbers, required by
95 Reactome. It is not a specialised bioinformatics tool for parasites. As a result, contig nucleotide
96 sequences must be converted to the closest human homologue. Analysis was performed against
97 Reactome version 68. Human homologue accession numbers were then compared to genes
98 upregulated within human pathways to identify relevant biological pathways. Pathways were
99 organised in order of significance as determined by *p-value* (Fabregat, et al., 2017) and the pathway
100 hierarchy was visualised through Reactome, which allowed for the final identification of genes
101 expressed in relation to key aspects of lung-stage schistosomula biology.

102 The Reactome database incorporates a species comparison tool, enabling comparative analysis
103 between human pathways and those predicted in the model organism *Caenorhabditis elegans* (Haw
104 and Stein, 2012, Fabregat, et al., 2018). Widely used for research in developmental biology, there is
105 extensive knowledge of its biology and genome, *C. elegans* is very well characterised (Duguet, et al.,
106 2020). Therefore, a comparison between the free-living nematode; *C. elegans*, and the parasitic
107 platyhelminth *S. japonicum* can be used to reveal the molecular basis of parasitism. *C. elegans* and *S.*
108 *japonicum* through releasing extracellular vehicles (EVs) may play a vital role in both organisms and
109 aspects of their behaviour (de la Torre-Escudero, et al., 2019, Duguet, et al., 2020). In this context,
110 using the model organism as a comparator was beneficial in terms of developmental biology and
111 host-parasite interactions. Comparison between *C. elegans* and *S. japonicum* to reveal the basis of
112 parasitism may be problematic. Any significant transcriptional differences between the two
113 organism may not be related to parasitism. Since one is a nematode and the other a platyhelminth,
114 these represent species relatively diverged in evolution. Therefore, differences may be associated
115 with physiological characteristics instead of parasitism. Comparison with a free-living platyhelminth
116 (Martin-Duran and Egger, 2012) may provide stronger indicators of parasitism.

117 Re-annotation and re-analysis of the gene expression data was extremely advantageous, in the
118 context of the substantial new literature published following 2009 (Gobert, et al., 2009). When
119 compared to GO and KEGG pathway analysis, Reactome can provide a better way to interpret and
120 visualise data. Reactome is however not a specialised bioinformatics tool for helminths. Nucleotide
121 sequences must be matched to human homologues, as such analysis may be influenced by this
122 reliance on human databases. For example topics such as the immune system, may be over
123 represented.

124 **3.2 Data subset for reanalysis and reinterpretation**

125 A gene list containing 1,311 contigs upregulated in the lung schistosomula was generated, including
126 the removal of any technical duplicates (see **Supplementary Table 1**). The 10 most up-regulated
127 transcripts by relative fold change, are presented in **Table 1**. Examples of enriched lung
128 schistosomula genes included cytochrome c oxidase subunit iii (Contig4489 2,805.95 fold) and
129 eukaryotic translation elongation factor 1 alpha (Contig560 533.95 fold).

130 **4. Pathway Analysis**

131 Reactome analysis of enriched genes present in the lung schistosomula, mapped pathways for 728
132 of the 1,311 identifiers (genes) in the data. The transcriptome wide pathway overview (**Fig. 1**)
133 enabled the hierarchical visualisation of generated pathways, biologically important to the lung-
134 stage schistosomula (Fabregat, et al., 2017). Reactome analysis was able to depict the ten most
135 significant pathways (**Table 2**), determined by *p-value*. The most significant pathway identified was
136 the *Endosomal/Vacuolar pathway*, with a *p-value* = 1.11E-16. The 5 most significant pathways are
137 all related to functions within the immune system.

138

139 **Table 1. The ten most highly enriched transcripts of lung schistosomula as originally identified by Gobert**
140 **et al., 2009.**

Contig/Probe	Nucleotide Description/Protein Homology	<i>p</i> -value ¹	Fold Change ²
TC17941	ferritin heavy chain	1.10E-04	12231.52
TC14376	Zinc phosphodiesterase ELAC protein 2	5.70E-04	5701.36
Contig4489	cytochrome c oxidase subunit iii	1.42E-04	2805.95
TC19335	Tetratricopeptide repeat protein 25	1.44E-03	1273.01
TC11085	T cell receptor beta variable 5-8	5.92E-04	1240.04
Contig6759	Immunoglobulin superfamily member 10 (IgSF10) CMF608	1.07E-03	1206.84
TC12767	N-acetylglucosamine-1-phosphotransferase subunits GNPTAB	1.87E-03	982.79
TC15743	Alpha-2,8-sialyltransferase 8B	1.91E-03	971.16
Contig560	eukaryotic elongation factor 1 alpha	9.83E-04	533.95
TC7263	Cystic fibrosis transmembrane conductance regulator (CFTR)	1.35E-03	512.88

141 ¹ ANOVA with multiple testing correction.

142 ² Fold Change is relative to the average expression of all seven *S. japonicum* life cycles stages examined.

143

144

145 **Table 2. The ten most significant Reactome pathways enriched in lung schistosomula determined by *p*-**
146 ***value*.**

Pathway Name	Entities found	Ratio	<i>P</i> -value	FDR*
<i>Endosomal/Vacuolar pathway</i>	36/82	0.004	1.11E-16	2.05E-13
<i>Antigen Presentation Folding, assembly and peptide loading of class I MHC</i>	44/217	0.011	9.20E-07	7.00E-04
<i>ER-Phagosome pathway</i>	49/298	0.015	1.30E-06	7.00E-04
<i>Antigen processing-Cross presentation</i>	51/320	0.016	1.52E-06	7.00E-04
<i>Neutrophil degranulation</i>	56/480	0.024	4.82E-05	0.018
<i>SRP-dependent cotranslational protein targeting to membrane</i>	20/119	0.006	1.92E-04	0.059
<i>Formation of a pool of free 40S subunits</i>	18/110	0.005	5.30E-04	0.139
<i>Class I MHC mediated antigen processing & presentation</i>	76/670	0.033	0.003	0.606
<i>RHO GTPases activate IQGAPs</i>	9/45	0.002	0.004	0.646
<i>Eukaryotic Translation Termination</i>	16/111	0.006	0.004	0.646

147 * False Discovery Rate.

148 Due to the large quantity of data demonstrating function within the immune system, pathways were
149 further analysed using a hierarchy overview. Results reveal a pathway hierarchy, each step away
150 from the centre will represent a level lower in the hierarchy. The highest level pathway is *Class I*
151 *MHC mediated antigen processing & presentation*, projected outwards from the central node of the
152 immune system (**Fig. 2A**). Lower level pathways were also identified as shown in **Fig. 2b**. Reactome

analysis reports identified individual accession numbers responsible for pathway generation. The data reveals numerous genes which contribute to several different pathways related to immune function. For example, the 26S proteasome non-ATPase subunit RPN5 (Contig7591) was detected in four different pathways; *ER-Phagosome pathway*, *Antigen processing-Cross presentation*, *Antigen processing: ubiquitination & proteasome degradation* and *Cross presentation of soluble exogenous antigens (Endosomes)* (Supplementary Table 2).

159
160

Supplementary Table 2. Examples of genes contributing to immune pathways.

Pathway	Genes	Contig	p value ¹	Fold Change ²
<i>Endosomal/Vacuolar pathway</i>	HLA class I antigen Cw-7 α -chain	TC14680	1.10E-02	15.87
	HLA class I antigen A-3 α -chain	Contig8280	2.77E-02	5.09
	Cathepsin L	Contig1966	3.83E-02	2.78
<i>Antigen Presentation: Folding, assemble and peptide loading of class I MHC</i>	HLA class I antigen, Cw-7 α -chain	TC14680	1.10E-02	15.87
	HLA class I antigen, A-3 α -chain	Contig8280	2.77E-02	5.09
<i>ER-Phagosome pathway</i>	Proteasome subunit α - 8	TC13609	4.01E-03	16.97
	26S proteasome non-ATPase subunit RPN5	Contig7591	1.04E-02	11.26
	26S proteasome non-ATPase subunit RPN8	TC10760	2.90E-03	10.59
	26S proteasome non-ATPase subunit RPN5	Contig7514	2.63E-02	7.58
	26S proteasome non-ATPase subunit RPN1	Contig780	1.03E-02	5.31
	Proteasome subunit β -5	TC10632	1.67E-02	5.16
<i>Antigen processing-Cross presentation</i>	Proteasome subunit α -8	TC13609	4.01E-03	16.97
	26S proteasome non-ATPase subunits RPN5,8,1		See Above	
	Proteasome subunit β -5	TC10632	1.67E-02	5.16
	Cathepsin L	Contig1966	3.83E-02	2.78
<i>Antigen processing: ubiquitination & proteasome degradation AND Cross presentation of soluble exogenous antigens (Endosomes)</i>	Proteasome subunit α -8	TC13609	4.01E-03	16.97
	26S proteasome non-ATPase subunits RPN5,8,1		See Above	
	Proteasome subunit β -5	TC10632	1.67E-02	5.16
<i>Cross presentation of particulate exogenous antigens (Phagosomes)</i>	Integrin β -5	Contig2213	1.44E-02	25.13

¹ ANOVA with multiple testing correction.

² Fold Change is relative to the average expression of all seven *S. japonicum* life cycles stages examined.

Fig. 1. Overview of Reactome pathway analysis of lung schistosomula enriched genes. The pathways are arranged hierarchically, with the centre of each circular “burst” representing the root of a top level pathway, such as “Immune System”. Each stride away from the centre signifies a lower level in the hierarchy of pathways. The circled areas indicates a pathway(s) that is over-represented in the dataset. Grey denotes pathways which are not significantly over-represented during pathway analysis.

169

170 **Fig. 2. Hierarchy overview of high level pathways observed in the immune system. A.** *Class 1 MHC*
 171 *mediated antigen processing & presentation* was identified as the highest level pathway, followed
 172 by *Antigen processing ubiquitination & proteasome degradation*, *Antigen processing-cross*
 173 *presentation* and *Antigen presentation: Folding, assemble and peptide loading of class I MHC*. **B.** *The*
 174 *endosomal/vascular pathway*, *ER-Phagosome pathway*, *Cross presentation of soluble exogenous*
 175 *antigens (Endosomes)* and *Cross presentation of particulate exogenous antigens (Phagosomes)*,
 176 represent the lowest level pathways within the immune system. The highlighted regions are
 177 representative of innate and adaptive immunity, green (highlighted central to down in B.) signifies

178 a unit of the innate immune pathway including neutrophil degranulation. Orange (all highlights in
179 A. and central to left highlights in B) is representative of adaptive immune pathways, involving
180 antigen processing and presentation.

183 *Neutrophil degranulation* (**Fig. 2B**) was found to be a significant pathway within the lung-stage
184 schistosomula (**Table 2**), with 64 genes contributing to the pathway. Novel genes of interest were
185 identified (**Supplementary Table 3**), including Elongation factor 1-alpha (Contig560) with a large fold
186 change of 533.95. Elongation factor 1-alpha is also one of the top 10 most upregulated transcripts
187 in the data set, shown above in **Table 1**. An overview of the immune system demonstrates different
188 mechanisms of relevance, such as those involved in Innate and Adaptive Immunity (**Fig. 2B**).

189
190 **Supplementary Table 3. Selected lung schistosomula enriched genes associated within the *neutrophil***
191 ***degranulation* pathway.**

Gene	Contig	<i>p</i> value ¹	Fold Change ²
Eukaryotic elongation factor 1 alpha	Contig560	9.83E-04	533.95
Secretory carrier-associated membrane protein	TC8138	1.19E-02	85.29
Tetraspanin	TC18168	1.39E-02	72.23
Endoplasmic reticulum resident protein	Contig3800	2.72E-02	38.07
Vesicle-associated membrane protein	TC13962	5.03E-03	18.79
Cathepsin D	Contig6337	5.86E-03	14.99
26S proteasome non-ATPase subunit RPN5	Contig7591	1.04E-02	11.26
26S proteasome non-ATPase subunit RPN8	TC10760	2.90E-03	10.59
Tetraspanin cd53 antigen	Contig5336	5.54E-03	9.09
26S proteasome non-ATPase subunit RPN5	Contig7514	2.63E-02	7.58
26S proteasome non-ATPase subunit RPN1	Contig780	1.03E-02	5.31
annexin a2a	Contig7025	9.94E-03	4.57
Cathepsin A	Contig7606	5.18E-03	4.57
Heat shock cognate 71 protein (cDNA FLJ77848)	Contig7768	3.02E-02	3.81
TGF beta receptor associated protein -1	TC19148	4.67E-02	3.64
Cathepsin B	Contig8688	7.76E-03	3.44
Cathepsin L	Contig1966	3.83E-02	2.78

192 ¹ ANOVA with multiple testing correction.

193 ² Fold Change is relative to the average expression of all seven *S. japonicum* life cycles stages examined.

194 Lists of highly expressed genes that are involved in Energy metabolism, Immune evasion and
195 Development are presented in **Supplementary Table 4**. Several genes are shown to contribute to
196 different functions, such as Proteasome subunit alpha-type 8 (TC13609), a subunit of the *Ubiquitin*

197 *Proteasome* Pathway; with a fold change of 16.97, it has been shown to function in several pathways
 198 relating to both immune evasion and development. Nutritional transporters are shown to be highly
 199 expressed in the lung-stage schistosomula, such as the ATP-binding cassette (ABC) type transporter
 200 AbcG6 (TC12977), upregulated at a fold change of 103.86.

201

202 **Supplementary Table 4. Selected novel highly enriched genes, associated with *Nutritional metabolism*,**
 203 ***Proteasome degradation/Immune evasion* and *Development* within the lung-stage schistosomula.**

Function	Gene	Contig	<i>p</i> value ¹	Fold Change ²
Energy metabolism	ABC transporter AbcG6	TC12977	2.61E-02	103.86
	Glucose transporter protein (SGTP4)	Contig4074	4.78E-02	2.45
	Pepsin A-3	TC13201	1.63E-02	25.51
	Cathepsins A, B, D, L		See Table 4	
Immune evasion	Proteasome subunit α -8	TC13609	4.01E-03	16.97
	26S proteasome non-ATPase subunits RPN1,5,8		See Table 4	
	Proteasome subunit β -5	TC10632	1.67E-02	5.16
	HLA class I antigen Cw-7 α -chain	TC14680	1.10E-02	15.87
	HLA class I antigen A-3 α -chain	Contig8280	2.77E-02	5.09
	Eukaryotic elongation factor 1-alpha	Contig560	9.83E-04	533.95
	Tetraspanin	TC18168	1.39E-02	72.23
	Tetraspanin cd53 antigen	Contig5336	5.54E-03	9.09
	Endoplasmic reticulum resident protein	Contig3800	2.72E-02	38.07
Development	Annexin	TC12719	1.00E-02	17.33
	Annexin a2a	Contig7025	1.00E-02	4.57
	Paramyosin	Contig7928	1.61E-02	18.60
	Proteasome subunit α -8	TC13609	4.01E-03	16.97
	26S proteasome non-ATPase subunits RPN1,5,8		See Table 4	
	Proteasome subunit β -5	TC10632	1.67E-02	5.16

204 ¹ ANOVA with multiple testing correction.

205 ² Fold Change is relative to the average expression of all seven *S. japonicum* life cycles stages examined.

206

207 Out of the 1,311 enriched contig nucleotide sequences subject to Reactome analysis, only 728
 208 identifiers (genes) were mapped to appropriate pathways. Owing to many regions of the *S.*
 209 *japonicum* genome remaining uncharacterised with unknown functions, a large proportion of the
 210 *Schistosoma* transcriptional picture is incomplete. Consequently, more research in functional
 211 genomics, including gene knock-out, would be extremely beneficial to improve this situation.

212 5. New interpretations

213 The new annotations for genes enriched during the lung schistosomula stage were updated and
214 placed in broad biological relevance, using both pathway analysis and literature primarily published
215 since the original study (Gobert, et al., 2009), when the transcriptomics was originally generated,
216 analysed, interpreted and discussed. In the original paper, 1,782 genes were analysed using the
217 bioinformatics platform Blast2Go, providing functional annotation in gene ontology (GO) and KEGG
218 pathway analysis. During the schistosomula lung-stage, gene function was catalogued into two of
219 three main categories modulated during development; *biological process* and *molecular function*.
220 Genes enriched within the GO category of *biological processes* were found to be heavily involved in
221 *metabolism* (Gobert, et al., 2009). In the present study, we were able to confirm a correlation
222 between the microarray data and prominent GO; *metabolic process*. Protein metabolism pathways;
223 SRP-dependent co-translational protein targeting to membrane, formation of a pool of free 40S
224 subunits and Eukaryotic Translational Termination, were all significant Reactome pathways enriched
225 within the lung schistosomulum (Table 2).

226 Reactome analysis has identified new pathways containing multiple genes involved in the immune
227 system. Originally, the GO term *immune system* was not found to be enriched amongst genes
228 upregulated in the lung-stage, compared to all other life cycle stages. However, results did highlight
229 the GO *protein binding* within the *molecular function* category and during the present re-annotation,
230 eukaryotic elongation factor-1-alpha (eEF1 α) was identified as a highly enriched transcript (Table
231 1).

232 **5.1 eEF1 α - a potential regulator for apoptosis and host immunomodulation**

233 Eukaryotic elongation factor 1-alpha (eEf1 α) (Contig560) was revealed to be one of the most highly
234 upregulated gene transcripts involved in the developmental lung-stage of the schistosome.
235 Reactome pathway analysis also placed this upregulated gene within the *neutrophil degranulation*
236 pathway. Conserved in many organisms, eEf1 α plays a central role in eukaryotic protein synthesis
237 (Billaut-Mulot, et al., 1994). Functionally, eEF1 α is an enzyme responsible for the catalysis of the
238 GTP-dependent binding of aminoacylated-tRNAs to the A site of the ribosome (Timm, et al., 2017,
239 Wang, et al., 2017). However, eEf1 α also plays a key role in functions other than translation,
240 including apoptosis (Alves, et al., 2015).

241 The schistosome is a multicellular parasite known to induce apoptosis in host cells (DosReis and
242 Barcinski, 2001, Chen, et al., 2002). Genomic studies have revealed that there are similarities in the

243 apoptotic processes present between schistosomes and higher organisms (*Schistosoma japonicum*
244 Genome Sequencing Functional Analysis Consortium, 2009, Peng, et al., 2010, Peng, et al., 2011,
245 Han, et al., 2013). Features of apoptosis, such as morphological change and condensed chromatin,
246 were characteristic of schistosomula extracted from the resistant mammalian host *Microtus fortis*
247 (Peng, et al., 2011). The expansion of research concerning the schistosome genome has recognised
248 apoptosis as a vital process, for their growth and host-parasite interactions in each developmental
249 stage of the life cycle (Chen, et al., 2002, *Schistosoma japonicum* Genome Sequencing Functional
250 Analysis Consortium, 2009, Peng, et al., 2011).

251 Our reanalysis reveals that eEF1 α was highly expressed in lung schistosomula. The function of eEF1 α
252 as a selective apoptotic regulator has been noted in the unicellular parasitic organism *Trypanosoma*
253 *cruzi*, however, there is limited information concerning the contribution of schistosome eEF1 α with
254 regards to apoptotic mechanisms (Billaut-Mulot, et al., 1994, Alves, et al., 2015). eEF1 α proteins can
255 be found in secreted exosome vesicles of *S. japonicum* (Zhu, et al., 2016) and *T. cruzi* (Bayer-Santos,
256 et al., 2013). Extracellular vesicles (EV) are known to play an important role in parasite-host
257 interactions (de la Torre-Escudero, et al., 2019). eEF1 α is a pleiotropic protein also involved in non-
258 canonical function, such as apoptotic modulation (both pro- and anti-) through the presence of
259 eEF1 α 1 and eEF1 α 2 isoforms (Abbas, et al., 2015). Parasite survival through the release of an anti-
260 apoptotic signal, which blocks apoptosis induced by host cells would be advantageous, in this case
261 to the lung-stage schistosomula. This may promote immune evasion by the schistosomula and
262 immunomodulation of the host response, during a time of increased schistosome development.

263 Parasitic pathogens have developed strategies to regulate the host immune response to enable
264 immune evasion (Maizels, et al., 2018). The membranes of the schistosome tegument are enriched
265 in abundant phospholipid classes such as phosphatidylcholine (PC) (Retra, et al., 2015), which has
266 also been identified in the *S. mansoni* lipidome (Giera, et al., 2018). *Leishmania infantum* are able
267 to form PC-epitopes through the active modification of the eEF1 α protein (Timm, et al., 2017). *L.*
268 *infantum* eEF1 α was further confirmed to be significant with regards to immunosuppression and
269 priming of the host cells in order to facilitate *Leishmania* attack (Babu and Nutman, 2019). Our
270 reanalysis and the supported literature has identified eEF1 α protein as a potential virulence factor
271 within the lung-schistosomula. It is also possible that exosome vesicles present on the developing
272 tegument of *S. japonicum* contain eEF1 α modified with PC. PC modified antigen molecules may have
273 an important role in the degradation of host protective cells, through the introduction of eEF1 α and

274 its ability to regulate apoptosis; this would disrupt attempts by the host to exterminate the worm
275 or potentially cultivate and acquire a protective response (Jenkins, et al., 2005).

276 The modification of biomolecules, such as eEF1 α , may contribute to the immunomodulatory
277 capacity of the schistosomula. PC-bearing antigens have been found to intervene with host immune
278 cells, potentially interfering with crucial signalling pathways of both B and T cells, dendritic cell
279 maturation and mast cell degranulation. This would enable the schistosome to accomplish
280 immunological resistance to the host and ensure schistosomula survival during the lung-stage of
281 their life cycle. Our hypothesis supports initial claims that lung schistosomula protect themselves
282 through inducing changes in their membrane surface together with the confinement of antigen
283 molecules in lipid-rich sites (El Ridi, et al., 2003, Fonseca, et al., 2012). The presence of eEF1 α as an
284 apoptotic regulator would provide an immunological barrier in addition to a physical obstruction
285 provided by membrane alterations, such as PC modification. Through the use of Reactome, we could
286 better appreciate and visualise eEF1 α as a complex and pleiotropic moonlighting protein subunit
287 (Jakobsson, et al., 2018) implicated in actin-binding (Demma, et al., 1990, Yang, et al., 1990),
288 apoptotic regulation (Abbas, et al., 2015), host immunomodulation and host immune evasion, in
289 addition to its role in protein synthesis.

290 **5.2 Nutrient Transporters-Schistosome Glucose Transporter Proteins**

291 Schistosomes within the blood vessels of the mammalian host can transport glucose and other
292 nutrients across their outer tegument membrane (Skelly, et al., 1998, Gobert, et al., 2003). This
293 involves the presence of schistosome glucose transport proteins (SGTP) which are host-exposed and
294 distinctively localised to the tegument of the adult schistosome (Krautz-Peterson, et al., 2010). Our
295 reanalysis revealed the upregulated expression of several genes involved in energy metabolism. The
296 upregulated expression of the gene for SGTP4 was expected due to the presence of the
297 schistosomula heptalaminate tegument, commonly associated with SGTP4. SGTP1 is also expressed
298 in schistosomula, however the gene was not present within our data set as it was < 2 fold enriched.

299 RNAi studies by Krautz-Peterson et al. (2010) proposed that glucose demands are higher for *in vivo*
300 parasites, since SGTP-knockdown parasites displayed less viability *in vivo* following the infection of
301 mice. It was argued that in order to migrate through host tissues, and in occupying the vasculature
302 and fighting the host immune response, it is vital for the parasite to generate more energy (Krautz-
303 Peterson, et al., 2010). Our reanalysis supports this theory, as does the upregulated gene expression
304 of SGTP4 in *in vivo* lung schistosomula (Gobert, et al., 2009).

305 5.3 Nutrient Transporters-Efflux Transporters

306 The ATP-binding cassette (ABC) type transporter AbcG6 (TC12977), an efflux transporter that
307 translocates compounds across the tegumental membrane (Greenberg, 2014), was shown in our
308 reanalysis to be highly enriched within lung schistosomula. The presence of transporter molecules
309 is essential for parasitic survival (Fonseca, et al., 2012), and lung schistosomula may potentially have
310 a greater requirement for membrane transporters compared to mature adults (Gobert, et al., 2007).
311 The distinction in tegument thickness, an apparent lack of gut function and the absence of a large
312 absorptive surface may suggest that the schistosomula are not fully functional when equated to the
313 adult parasite regarding nutrition. There are opposing views concerning the lung-stage
314 schistosomula and their ability to ingest host blood; some studies claim that a functional gut is
315 absent within the lung schistosomula, however other research suggests that it is present and could
316 be partially functional (Bennett and Caulfield, 1991, El Ridi, et al., 2003). The upregulated expression
317 of transporter genes could enable schistosomula to thrive without a functional gut, through the
318 direct importation of sugar and fatty acids across the tegument (Krautz-Peterson, et al., 2007,
319 Krautz-Peterson, et al., 2010, Da'dara, et al., 2012).

320 5.4 Proteases and schistosomula nutrition

321 Proteases, including cysteine and aspartic proteases, are a group of enzymes capable of the catalytic
322 hydrolysis and digestion of peptide bonds. Multiple functions within the schistosome have been
323 proposed including haemoglobin digestion and host invasion (Skelly, et al., 2014, Tan-ariya, et al.,
324 2014). Our reanalysis reveals the upregulated expression of several classes of proteolytic
325 endopeptidase enzymes; cathepsins D (Contig6337), B (Contig8688) and L (Contig1966) in lung-
326 stage schistosomula.

327 Schistosome cathepsin B, cathepsin L and papain-superfamily lysosomal cysteine endopeptidases
328 were upregulated within lung-stage schistosomula, potentially supporting parasite nutrition by the
329 digestion of host haemoglobin released from ingested host erythrocytes (El Ridi, et al., 2014).
330 Cysteine proteases are regulated proteolytic enzymes, essential for schistosome survival and growth
331 within its developmental intra-mammalian stages (Zerda, et al., 1988), due to their association with
332 the invasion of host tissues. The current reanalysis correlates with further transcriptomic studies by
333 Gobert et al. (2010). In the presence of red blood cells, *in vitro* schistosomula genes encoding
334 cathepsin B and cathepsin L were upregulated greatly in *S. mansoni* (Gobert, et al., 2010). Gene

335 silencing studies have exhibited the inability of schistosomes to feed on host haemoglobin *in vitro*
336 and failure to survive *in vivo* without the presence of the proteolytic enzymes cathepsin B and
337 cathepsin L (Correnti, et al., 2005).

338 Cathepsin D (Contig6337) is an aspartic proteinase also shown to be upregulated within the
339 reanalysed data. The proteolytic enzyme is expressed in the gut of the adult parasite similar to
340 cysteine proteinases and has been shown to be critical in haemoglobin digestion and therefore the
341 survival of the schistosome (Figueiredo, et al., 2015). *S. mansoni* schistosomula, when subjected to
342 RNAi against cathepsin D (Morales, et al., 2008), exhibited reduced growth *in vitro* and a retardation
343 of gut function. Furthermore, Cathepsin D knockdown schistosomes were not able to survive
344 following injection into a mouse host (Morales, et al., 2008). *In vitro* the cathepsin D⁻ schistosomula
345 gut appeared red in colour; this is in distinct contrast with the black coloured gut of the controls
346 which contain hematin, the final product of haemoglobin proteolysis (Toh, et al., 2010, Toh, et al.,
347 2015). It was hypothesised that the red colour was due to human haemoglobin, which had not been
348 digested as a result of cathepsin D gene silencing (Morales, et al., 2008).

349 Our reanalysed data and previous studies both suggest that proteinases are essential for functions
350 in the overall maintenance and survival of schistosomes, particularly schistosomula (Morales, et al.,
351 2008, Gobert, et al., 2010). The upregulated expression of cathepsins within our reanalysis provides
352 further support for cathepsins important role in nutrition, indicating that lung schistosomula have a
353 functional gut and therefore the ability to feed from the blood stream of the host.

354 **6. Future prospects**

355 Traditionally, DNA microarrays have been used to answer a wide range of questions as the standard
356 application for gene expression profiling in *Schistosoma* genomics and transcriptomics. Microarrays
357 are a reliable and well-established technology that can still provide new insights into biological
358 processes under investigation. Nevertheless, it is an older technology and its limitations still remain
359 omitting RNA-editing events and exon-exon splice junctions for novel expressed regions, and only
360 profiling predefined genes; available from the *S. japonicum* genome and transcriptome, through
361 hybridization (Malone and Oliver, 2011).

362 The advent of high-throughput sequencing technology in recent years and general advancements
363 in the field of transcriptomics have enabled Wangwiwatsin et al. to investigate, and describe the
364 developmental time-course of *S. mansoni*, with particular emphasis on the lung-stage

365 (Wangwiwatsin, et al., 2020). The study includes the first description of RNA-sequencing (RNA-seq)
366 transcriptome analysis on day-6 *in vivo* derived lung schistosomula, when 864 genes were found to
367 be upregulated in comparison to day-13 juveniles (Wangwiwatsin, et al., 2020). In contrast, our
368 hybridization-based method was able to generate a gene list of 1,311 identifiers upregulated during
369 the lung-stage. Compared to the original microarray study (Gobert, et al., 2009), this level of analysis
370 is based on a more mature and streamlined genomic structure, enabling a highly sensitive
371 investigation of gene expression. RNA-seq reads can be directly mapped to the *S. mansoni* genome,
372 which has been collectively re-annotated more than the *S. japonicum* microarray data (Gobert, et
373 al., 2009).

374 Wangwiwatsin and colleagues also expand on gene ontology enrichment (GO), highlighting the GO
375 term; *iron homeostasis*, enriched amongst those transcripts; ferritin-2, up-regulated in the lung-
376 stage of development (Wangwiwatsin, et al., 2020). Ferritin, the highly conserved iron storage
377 protein, can store extensive levels of iron within its isoforms; Ferritin-2, characteristic of somatic
378 tissues, and Ferritin-1 (Schussler, et al., 1995, Glanfield, et al., 2007). Compared to day-6
379 schistosomula, Ferritin-2 genes were down regulated in day-13 parasites. Although downregulated,
380 ferritin-2 was still expressed during a time when iron should be necessary for schistosome growth
381 and development (Wangwiwatsin, et al., 2020). In the present study, the ferritin heavy chain
382 (TC17941) was the single most upregulated transcript in the schistosomula, within the re-annotated
383 data set, with a fold change of 12,231.51 (Table 1). Consequently, both studies support evidence
384 that iron is used by the schistosome for growth, development and maintenance. This research could
385 also advocate claims that lung schistosomula are able to absorb and scavenge iron, before their gut
386 is differentiated. It is evident that RNA-seq holds the promise of the future for bioinformatic data
387 collection and gene expression analysis, accelerating further research in *Schistosoma*
388 transcriptomics.

389 **7. Conclusions**

390 In summary, the additional annotations and pathway analysis of public domain transcriptomic data
391 (Gobert, et al., 2009) has revealed numerous insights into the genes potentially contributing to
392 several important functions, within the lung-stage schistosomula of *S. japonicum*. Enriched
393 schistosome genes are relevant to immune evasion, energy metabolism and development,
394 demonstrating schistosomulum adaption to the mammalian host and their environment, which
395 could provide new interventions for disease control. The lung-stage schistosomula is recognised as

396 a target of vaccine development (Gobert, et al., 2007, Wilson, 2009, Wilson, 2012), therefore
397 targeting these enriched genes may prevent parasite development into the egg producing adult
398 stage, responsible for host pathology and disease transmission.

399

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