

RESEARCH ARTICLE

Proteomic Analysis of the *Schistosoma mansoni* Miracidium

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Citation: Wang T, Zhao M, Rotgans BA, Strong A, Liang D, Ni G, et al. (2016) Proteomic Analysis of the *Schistosoma mansoni* Miracidium. PLoS ONE 11(1): e0147247. doi:10.1371/journal.pone.0147247

Editor: Dongmei Li, University of Rochester, UNITED STATES

Received: November 17, 2015

Accepted: January 3, 2016

Published: January 22, 2016

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Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: The authors thank the following for support for this work: University of the Sunshine Coast internal grant and Australian Research Council (FT110100990); University of the Sunshine Coast, URG 13/11. This research was undertaken with the assistance of resources from the National Computational Infrastructure (NCI), which is supported by the Australian government. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

Despite extensive control efforts, schistosomiasis continues to be a major public health problem in developing nations in the tropics and sub-tropics. The miracidium, along with the cercaria, both of which are water-borne and free-living, are the only two stages in the life-cycle of *Schistosoma mansoni* which are involved in host invasion. Miracidia penetrate intermediate host snails and develop into sporocysts, which lead to cercariae that can infect humans. Infection of the snail host by the miracidium represents an ideal point at which to interrupt the parasite's life-cycle. This research focuses on an analysis of the miracidium proteome, including those proteins that are secreted. We have identified a repertoire of proteins in the *S. mansoni* miracidium at 2 hours post-hatch, including proteases, venom allergen-like proteins, receptors and HSP70, which might play roles in snail-parasite interplay. Proteins involved in energy production and conservation were prevalent, as were proteins predicted to be associated with defence. This study also provides a strong foundation for further understanding the roles that neurohormones play in host-seeking by schistosomes, with the potential for development of novel anthelmintics that interfere with its various life-cycle stages.

Introduction

The schistosome life cycle is complex, involving a number of different stages including larval cercariae, shed from freshwater intermediate host snails which subsequently penetrate the skin of their human or other mammalian definitive hosts. On successful invasion, cercariae develop into schistosomula and mature into adult male and female worms which pair up and migrate to the portal mesenteric system of the mammalian host, where the females lay eggs. Some of the eggs leave the host and hatch in water as free-living miracidia. The miracidia seek out intermediate host snails, thus completing the schistosome life cycle.

The publication of the genomes for *Schistosoma japonicum* and *Schistosoma mansoni* have significantly advanced our understanding of schistosome biology [1,2]. Most interest to date has been devoted to using these data to interpret the mechanisms of interplay between

Competing Interests: The authors have declared that no competing interests exist.

schistosomes and their mammalian hosts, and the development of new control interventions including vaccines or additional therapies to praziquantel [3,4,5,6]. However, insufficiency in controlling the disease by chemotherapy alone has been increasingly noticed [7]; in addition, the possibility of the emergence of drug resistant parasites has been raised [8,9]. A feasible alternative is to decipher the process in which miracidia invade their intermediate snail hosts to develop new strategies to prevent attraction and/or invasion of snails, thereby preventing transmission. With this goal in mind, an international consortium was initiated to sequence the genome of *Biomphalaria glabrata*, the intermediate host of *S. mansoni* [10]. As such, *S. mansoni* miracidia and *B. glabrata* represent a highly tractable model for analysis of the snail host-parasite relationship. The interaction between *B. glabrata* and water-borne free-living schistosome miracidia is multifaceted. From the perspective of snail, studies have suggested that macromolecular glycoconjugates released by snails seem to be miracidium attractants [11,12]. Following invasion, *B. glabrata* often fails to activate an appropriate immune response against *S. mansoni*. The parasite consequently develops successfully in these susceptible snails, resulting in the release of infective cercariae several weeks post-exposure.

The proteomic analysis of different subset proteins of Schistosome relevant to the infection process of snail, including immature egg [13,14], egg secreted proteins/secretome [15,16], egg contents [13,17], excretory-secretory proteins of miracidia *in vitro* [18] and sporocysts [19,20], has been advancing the progress of unveiling the snail-parasite interaction mechanism. Mucins of *S. mansoni* were recognised as key factors effecting the interaction between miracidia/sporocysts and host snail [17]. Chaperones, such as Hsp70, 60 and 90, were identified as the most abundant protein family in egg development, while defence proteins were enriched in the hatch fluid [13]. Cytoskeletal proteins were with similar expression levels in the immature eggs and mature eggs contents [13]. The synthetic activity of mature eggs was found to depend on the secreted proteins, which included two proteases [15]; a later study identified 188 proteins from the secretome of eggs, playing roles in redox balance, development, protein folding and molecular chaperoning, and so forth [16]. At the *in vitro* miracidium-to-sporocyst stage, 127 identified larval transformation proteins mainly composed of proteases/protease inhibitors, small HSPs, redox/antioxidant enzymes, ion-binding proteins, and venom allergen-like (VAL) proteins [20].

The characterisation of the proteome of the *S. mansoni* miracidium has not been reported, possibly due to the difficulty in purifying eggs of *S. mansoni* out of the debris of mammalian organs, which results in low quality proteomic data for miracidia. The present study utilised a protocol to obtain *S. mansoni* eggs with minimal mammalian host contamination, and characterised the proteome of miracidium at the stage of 2hrs post-hatch using high-throughput LC-MS/MS screening. At about 2hrs post-hatch the miracidium has completed development, initiates host finding and meanwhile starts to die [21,22]. Secreted proteins and chemosensory receptors are at the forefront of snail-miracidia interaction, and were therefore of particular interest for investigation. This information furthers our understanding of the complete schistosome proteome, and provides the foundation for further investigations into the molecular basis of schistosome modulation of snail-host immunity and host finding. This increases the possibility of identifying those factors that promote parasite resistance in *B. glabrata*, and may aid the development of new approaches to control snail infection, so that schistosomiasis can be tackled from a new angle.

Materials and Methods

Ethics statement

The conduct and procedures involving animal experimentation were approved by the Animal Ethics Committee of the QIMR Berghofer Medical Research Institute (project number P242).

This study was performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Collection of *Schistosoma mansoni* miracidia and protein extraction

We used ARC Swiss, outbred mice which were from the Animal Resource Centre in Western Australia. The Puerto Rican strain of *S. mansoni* is maintained, under permit from the Australian Department Agriculture, Fisheries and Forestry Biosecurity (DAFF), in ARC Swiss mice and *Biomphalaria glabrata* snails at QIMR-Berghofer Medical Research Institute (QIMR-B) from stock originating from the National Institute of Allergy and Infectious Diseases Schistosomiasis Resource Centre, Biomedical Research Institute (Rockville, Maryland, USA).

Isolation of Schistosome eggs. Mice were euthanized with CO₂ gas and their livers were perfused with chilled PBS. Eggs of *S. mansoni* were collected during perfusion of mice. Four infected mouse livers were sliced with scalpel blades and blended to a smooth consistency in 50 mL phosphate buffered saline (PBS). *S. mansoni* eggs formed a very firm pellet at the bottom of the tubes, which was incubated with collagenase B (20 mg) and 110 μ L Pen/strep (or 10 μ g penicillin and 20 μ g streptomycin) with at 37°C in a shaker overnight [23].

The pellet was washed twice using PBS with centrifugation at 400 x g for 5 min, in a final volume of 25 mL PBS. This mixture was filtered successively through 250 μ m and 150 μ m sieves and subjected to centrifugation at 400 x g for 5 min, and the pellet resuspended in 10 mL PBS. This mixture was placed onto a Percoll column (8 mL Percoll + 32 mL of 0.25 M sucrose in 50 mL tube), which was centrifuged at 800 x g for 10 min. Liver cells were removed from the top of the Percoll column with a Pasteur pipette.

The egg pellet was retained within 25 mL PBS containing 1 mM EDTA, 1 mM EGTA, centrifuged at 30 x g for 3 min, then the discarded supernatant was mixed with 10 mL of the same buffer and centrifuged at 30 x g for 3 min, and the procedure was repeated. The egg pellet was resuspended in 5 mL PBS and applied on to a second Percoll column (2.5 mL Percoll + 7.5 mL 0.25 M sucrose in a 15 mL tube), followed by centrifugation at 800 g for 10 min. The supernatant was removed and the egg pellet washed with 10 mL PBS and then centrifuged at 30 x g for 3 min. The washing with PBS was repeated three times.

Isolation of miracidia. Eggs were transferred into a 200 mL hatching measuring cylinder wrapped completely in light-blocking black tape with the exclusion of the top 4 cm from the lip, thereby producing a light-gradient. The hatching cylinder was topped with conditioned water (Milli-Q water stored with calcium carbonate chips until pH \approx 7) until above the tape-covered area \sim 1.5 cm and exposed to bright light at 27°C. Eggs were incubated for 2 h post-hatch before collection of the top 10 mL of miracidium-containing water (MCW) above the light-blocking region. Hatched miracidia were collected by centrifugation at 8,000 x g for 1 min at 4°C, and were then washed twice with water.

Protein extraction from miracidia. Miracidia pellets at 2h post-hatch were used for protein extraction using urea/thiourea buffer [7 M urea, 2 M thiourea, and 4% CHAPS, in 30 mM Tris-HCl (pH 8.5)] with a protease inhibitor cocktail (GE Healthcare Life Sciences), and homogenised. The lysate was allowed to settle at room temperature for 10 min; subsequently, the sample lysate was centrifuged at 15,000 x g for 15 min. The supernatant was collected for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE and trypsin digestion

Lysate proteins were size fractionated by 1D SDS-PAGE with a 4–12% polyacrylamide gradient gel (Amersham ECL Gel, GE Healthcare Life Sciences) according to the manufacturer's instructions. The gel was stained with Coomassie Brilliant Blue R250 (Sigma-Aldrich). Two

lanes of biological replicates (about 150 miracidia each) containing protein were excised (equally cut into 51 pieces in each lane) and processed for LC-MS/MS. Briefly, proteins within each gel piece were subjected to reduction (10 mM dithiothreitol, DTT, 45 min at 56°C) and alkylation (iodoacetamide, IAA, 30 min, RT, in the dark) followed by Sequencing Grade Modified Trypsin (Promega) for 16 h at 37°C. Peptides were then extracted from the gel pieces as described [24], desalted and concentrated by Ziptip C18 (Millipore).

NanoHPLC-ESI-TripleTOF Analysis

Purified extracts (after Ziptip) were analysed by LC-MS/MS on a Shimadzu Prominane Nano HPLC (Japan) coupled to a Triple ToF 5600 mass spectrometer (ABSCIEX, Canada) equipped with a nano electrospray ion source. The protocol has been detailed elsewhere [25]. Briefly, approximately 6 µL of each extract was injected and de-salted on the trap column before entering a nano HPLC column (Agilent Technologies, Australia) for mass spectrometry analysis. The mass spectrometer acquired 500 ms full scan TOF-MS data followed by 20 by 50 ms full scan product ion data. Full scan TOFMS data was acquired over the mass range 350–1800 and for product ion MS/MS 100–1800. Ions observed in the TOF-MS scan exceeding a threshold of 100 counts and a charge state of +2 to +5 were set to trigger the acquisition of product ion. The data were acquired and processed using Analyst TF 1.5.1 software (ABSCIEX, Canada).

Protein identification

The *S. mansoni* protein sequence database (ASM23792v2.27) was downloaded from the *Schistosoma mansoni* database of EnsemblMetazoa website (http://metazoa.ensembl.org/Schistosoma_mansoni/Info/Index), with a total number of 11,774 sequences. To improve protein identification and coverage, four search engines were comparatively used to analyse the mass spectrometric data, including PEAKS (Bioinformatics Solutions Inc., Waterloo, ON, Canada, version 7.0), X! Tandem [26] (version 2009.04.01), MS Amanda [27] (version 1.0.0.5242) and OMSSA [28] (version 2.1.7). A composite target–decoy database was built with the forward and reverse sequences for calculating the false discovery rate.

All searches used the following search parameters: precursor ion mass tolerance, 0.1 Da; fragment ion mass tolerance, 0.1 Da; fully tryptic enzyme specificity; two missed cleavage; the number of unique peptide ≥ 1 ; monoisotopic precursor mass (PEAKS and OMSSA); monoisotopic fragment ion mass (OMSSA); a fixed modification of cysteine carbamidomethylation; and variable modifications included methionine oxidation, conversion of glutamine and glutamic acid to pyroglutamic acid, and deamidation of asparagine. For PEAKS, *de novo* sequencing, database search and characterising unspecific post-translational modifications (PTMs) were used to maximise the identifications; false discovery rate (FDR) was set to $\leq 1\%$, and the individual peptide ion score $[-10 \cdot \log(p)]$ was calculated accordingly, where p is the probability that the observed match is a random event. An additional parameter unique to OMSSA was used that requires that one of a variable number of the most intense fragment ions match those of the theoretical peptide; in this case the parameter was set to 5. For other three engines except PEAKS, the confidence was set to greater than 95%.

Gene ontology, KEGG pathway and prediction of secreted proteins

Identified proteins were firstly subject to BLASTp and tBLASTn using non-redundant protein sequences and nucleotide collection of NCBI, respectively. Then, BLAST results were combined and imported to BLAST2GO [29] (version 3.1), to perform gene ontology (GO) and KEGG pathway analysis. Fisher's exact test was carried out to evaluate the enrichment of GO terms in miracidium proteome (test dataset) against the annotation of all *S. mansoni* proteins

(reference dataset). The significant GO terms with $p < 0.01$ were considered as over-represented, and FDRs were calculated from p-values using the Benjamini-Hochberg procedure [30].

Protein N-terminal signal sequences were predicted using the SignalP 4.1 [31] and Predisi [32], with the transmembrane domains predicted by TMHMM [33]. For SignalP predictions, positive identifications were made when both neural network and hidden Markov model algorithms gave coincident estimations; D-cutoff values were set to 0.34 (to increase sensitivity) for both SignalP-noTM and TM networks. Herein, a protein was designated as secreted, only when it met the criteria of both SignalP and Predisi, and did not have a transmembrane domain predicted by TMHMM.

Identification of neuropeptide genes

To identify target sequences, previously identified platyhelminth (flatworm) neuropeptide genes were used as query (BLASTn and BLASTp) against the *S. mansoni* genome that was imported into the CLC genomics workbench (version 7.0.3). Six target neuropeptide genes were compared to known platyhelminth neuropeptides using multi-sequence alignments constructed in the Molecular Evolutionary Genetics Analysis (MEGA) software version 6.0.5 [34]. Similar homology comparisons were utilized through the NCBI database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) using BLASTp and BLASTx searches. Sequence presentation and shading of multiple sequence alignments was performed using the LaTeX TEXshade package [35]. Relative gene expression of target neuropeptide genes between cercariae, 3S, 24S and adult schistosomes (as relative normalized reads) were obtained from SchistoDB [36] (<http://SchistoDB.net>) for *S. mansoni*.

To identify neuropeptide gene expression in miracidia, total RNAs were prepared from the 2 h post-hatch larvae using TRIzol reagent (Invitrogen), following the manufacturer's protocol. Purity and quantity was measured using a UV spectrophotometer (NanoDrop ND-1000) at 260 and 280 nm. First-strand cDNA was generated from miracidia total RNA using random hexamer primers and the Superscript Preamplification System for First-strand Synthesis (Invitrogen). RT-PCR was performed using primers designed on CLC genomic workbench (version 7.0.3) (Table 1), with actin used as a positive control, inclusive of amplification without cDNA as negative control. Samples were heated at 94°C (2 min), amplified for 35 cycles (repetition of 30 s at 95°C, 30s at 50°C and 1 min at 72°C) and extended at 72°C (5 min). Samples were separated on agarose gel (2%, ethidium bromide) for visualisation.

Results

S. mansoni miracidia proteomic analysis and protein identification

The overall experimental procedure to map and annotate miracidia proteins encoded by the *S. mansoni* genome is outlined in Fig 1A. Miracidia proteins were extensively fractionated by 1D SDS-PAGE, followed by in-gel digestion. All samples were subjected to high-accuracy mass spectrometry, and the raw data were rigorously analysed using available informatics tools. Based on the genome of *S. mansoni* published in 2009, it was estimated that there are at least 11,809 genes (encoding 13,197 transcripts in mixed-sex cercariae), of which certain features were described, including unusual intron size distribution and new families of micro-exon genes that undergo frequent alternative splicing [1]. The protein database in our study contained 11,774 different sequences, from which we have identified a total of 1,910 *S. mansoni* proteins integrating the results of four search engines with high confidence (one or more unique peptides with an FDR less than 1%) in miracidia (S1 Table). The numbers of proteins identified by four search engines are compared in Fig 1B. Overall, there are 643 proteins

Table 1. Primers used for RT-PCR of neuropeptide genes.

Gene	Sequence 5' to 3'
GFVRIamide	
Forward:	AAACAGTGATGGCTTACCAA
Reverse:	TGAAACAACACTGACTACCGAA
RGMIIamide	
Forward:	CTACAACAACAATCACCCAC
Reverse:	TAAACCACTTCTGCAAAAC
PWamide	
Forward:	TTTCCCACCATCATCATC
Reverse:	CTTACATTTACCTTCAGTC
RLamide	
Forward:	ATCACCAACCAACATCATAG
Reverse:	AGTTCAGCAACATTATCACC
Neuropeptide F (NPF)	
Forward:	TCCATTTCTTCTCTTCTCTCT
Reverse:	TCTCCTGACGTTGTTCTACT
Actin	
Forward:	CTCTCACTCAATCACTAACTC
Reverse:	ACATAGCTGGCACATTAAC

doi:10.1371/journal.pone.0147247.t001

mutually identified by all engines; PEAKS have 420 sequences uniquely identified, compared to 70, 66 and 36 by MS Amanda, X! Tandem and OMSSA. There are a number of proteins are crossed identified among different engines. This offers the highest number of proteins identified from miracidia, providing ~16.2% of the total proteins predicted from the *S. mansoni* genome (11,774 entries, Jul. 2015). As it has been reported that around 6000–7000 genes are expressed in each *S. mansoni* stage [37], then the number of proteins we have identified may cover approximately 32–45% of those expressed proteins.

Gene ontology analysis

The distributions of the GO term (the second level) of proteins identified for the three categories of biological process, molecular function and cellular component are shown in Fig 2A, while the distributions of the GO term at all levels of the LC-MS/MS identified proteins for the same three categories are recorded in S2 Table. There is also a large category comprised of proteins without any GO term prediction (15.4%, Table 2), including 41 novel proteins (no BLAST result meeting the eValue cutoff). Cellular and metabolic processes are the two most highly represented biological process terms in the *S. mansoni* miracidium proteome, each containing over 800 proteins. There was also a high presence of miracidial proteins implicated for roles in basic regulation and responses specifically relevant to single organisms, as well as for responding to stimuli, probably a requirement for movement post-hatch, seeking out a host snail and host penetration. Approximately 198 proteins are associated with signalling and cell-cell signalling, respectively (S2 Table); these are characteristic components of those systems requiring multicellular processes and communication.

GO of proteins related to molecular function show that the majority of proteins identified in this study are involved in binding (61.3%) and catalytic activity (42.0%) (Fig 2A). Within the binding group, organic cyclic compound binding (34.3%), ion binding (32.4%), and protein binding (29.4%) and are the top three categories. Meanwhile, within the catalytic activity group,

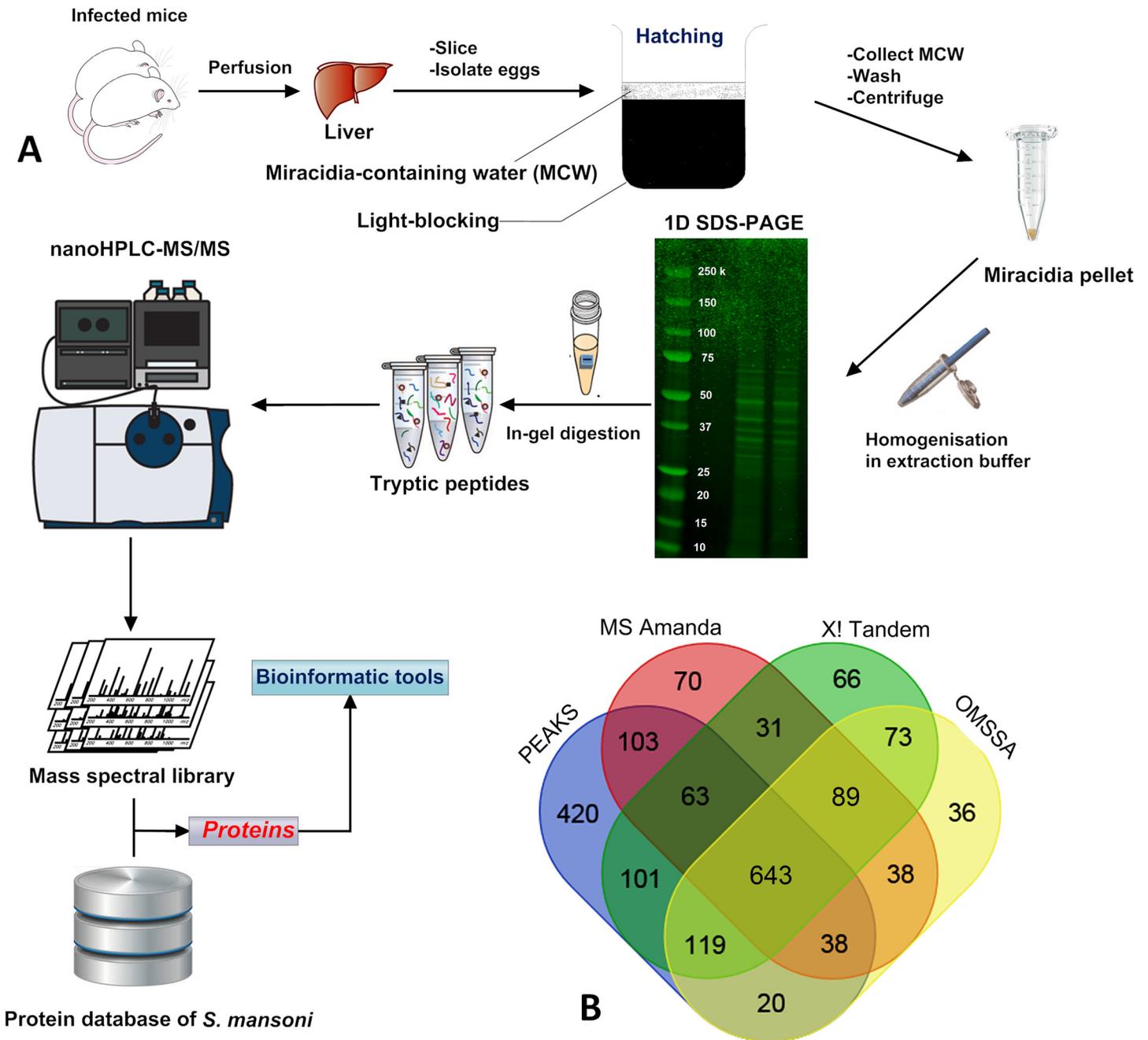


Fig 1. (A) Overall workflow for *S. mansoni* egg isolation, protein extraction and identification from miracidia. Miracidium proteins were extensively fractionated followed by identification with high-accuracy nano-LC TripleTOF MS. **(B) Protein identification results of different search engines** (see [Methods](#) for search parameter settings).

doi:10.1371/journal.pone.0147247.g001

hydrolase activity (20.3%) and transferase activity (13.3%) are the two categories containing largest number of sequences ([S2 Table](#)). Most hydrolase activity-type and transferase activity-type proteins participate in the catalysis of glycosylation hydrolysis, as well as in transferring phosphorus-containing and glycosyl groups. Miracidium proteins with GO under the cellular component category included those with cell (46.5%), organelle (33.9%), macromolecular

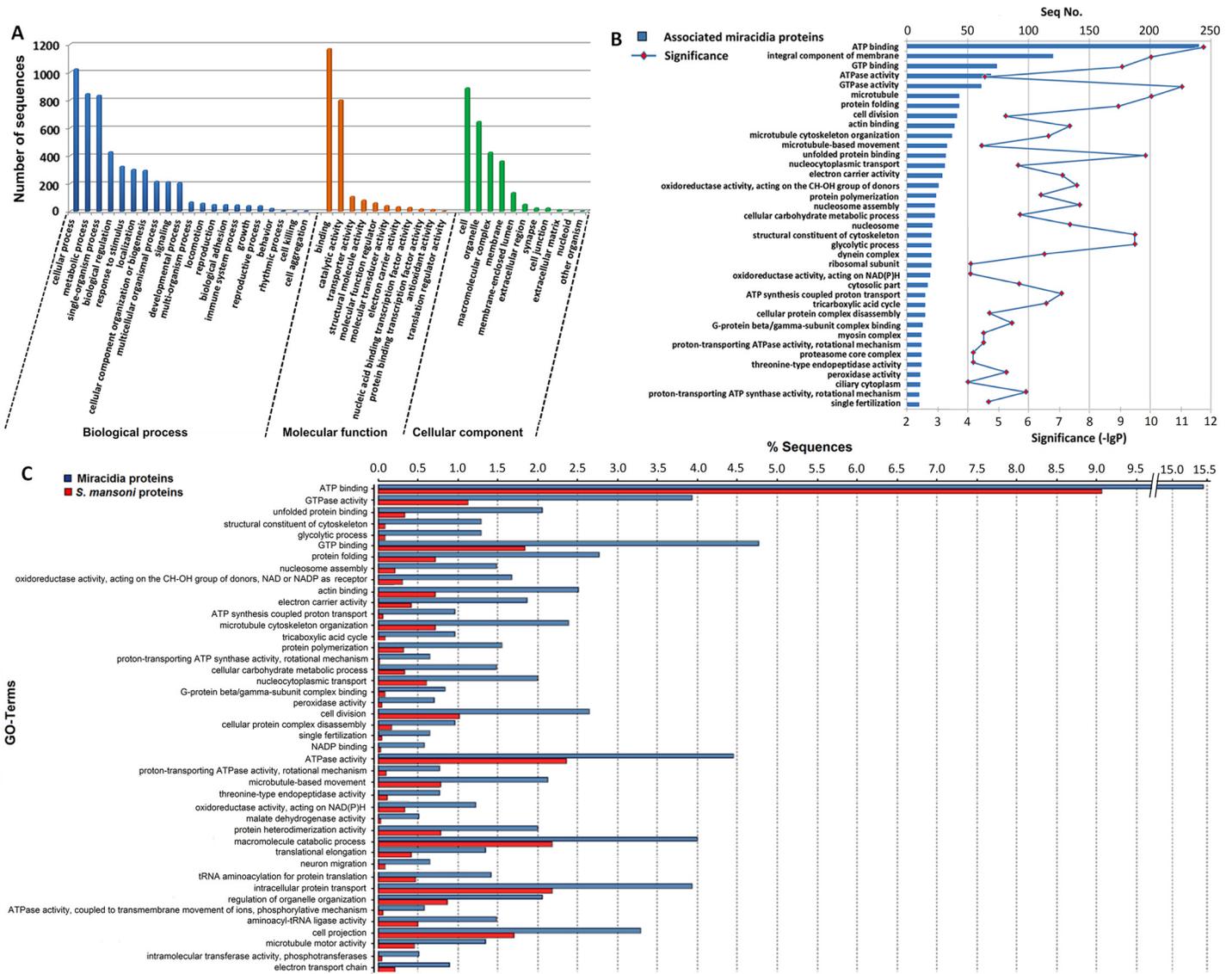


Fig 2. Gene ontology annotation of miracidia proteins. The bar chart (A) describes the GO distribution of GO term on level 2 from the proteome. When one protein was known to be localised in more than one cellular compartment, all of the localisations were counted non-exclusively. (B) All identified miracidia proteins were compared with the whole protein database of *S. mansoni*. Significantly over-represented GO terms ($p < 0.01$) are shown. The number of proteins and their levels of significance are also shown. (C) GO terms of biological process and molecular function over-represented in miracidia compared to the whole *S. mansoni* proteins, in terms of the ratio of protein numbers.

doi:10.1371/journal.pone.0147247.g002

Table 2. Categories of proteins identified from *S. mansoni* miracidia, annotations with/without GO terms, predicted as secreted proteins and receptors

Sequence number	Miracidia
Identified, in total	1910
With GO term(s)	1616
Without GO term(s)	250
Secreted	75
Receptors	37

doi:10.1371/journal.pone.0147247.t002

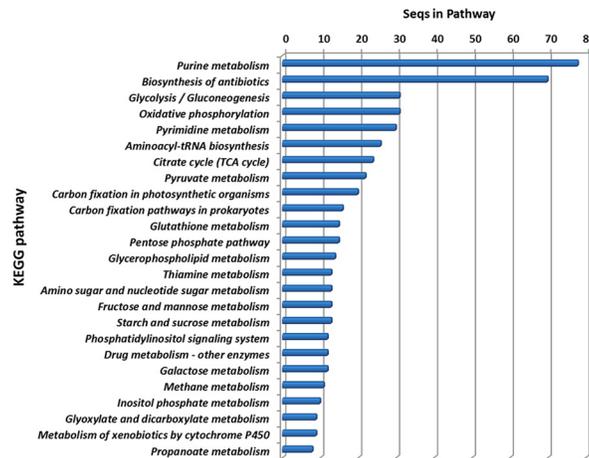


Fig 3. KEGG analysis of miracidium proteins showing the top 25 highly represented KEGG pathways (see S2 Table for the list of all KEGG pathways identified).

doi:10.1371/journal.pone.0147247.g003

complex (22.4%), membrane (19.1%), membrane-enclosed lumen (7.2%), extracellular region (2.8%), cell junction (1.4%), synapse (1.4%) and extracellular matrix (0.1%) activity. In addition, there are 294 proteins that do not match to any GO category (S2 Table).

The fisher’s exact test of the GO is displayed in Fig 2B, with enriched terms listed in S3 Table. It can be seen that the processes of carbohydrate breakdown ($p = 3.18E-10$) are significant in miracidia 2h post-hatch. Specific events such as protein folding ($p = 1.10E-9$) and nucleosome assembly ($p = 2.07E-8$) are also enriched. These processes are important for energy production, protein functionalisation and DNA replication. We also found significant overrepresentation of proteins in ATP synthesis coupled proton transport. In terms of molecular function, statistical analysis shows a very significant enrichment of ATP/GTP binding and ATPase/GTPase activity. In comparison to the pooled *S. mansoni* database, we found a high ratio of the proteins involved in cell projection, intracellular protein transport, macromolecule catabolism, microtubule-based movement and cytoskeleton organisation, cell division, nucleocytoplasmic transport and protein polymerisation (Fig 2C). The under-represented molecular function in miracidia included G-protein coupled receptor activity and sequence-specific DNA binding transcription factor activities (S3 Table).

KEGG pathway analysis

Using the KEGG pathway database, the *S. mansoni* miracidium proteins identified can be classified into 95 pathways (S1 Fig and S2 Table), the top 25 of which are shown in Fig 3. Purine metabolism is the top pathway represented, followed by the protein processing pathway of biosynthesis of antibiotics, which includes 70 sequences. There are also a significant number of proteins present that relate to pathways involved in energy procurement, e.g., 31 sequences playing roles in glycolysis/gluconeogenesis, oxidative phosphorylation and pyrimidine metabolism, respectively. Besides, a number of proteins relate to sugar metabolism, including the metabolism of fructose and mannose (13 seqs), starch and sucrose metabolism (13), amino and nucleotide sugar (13), and galactose (12) (Fig 3). In addition, proteins were identified that are associated with inositol phosphate metabolism and the citrate cycle (TCA cycle), which could play crucial roles in a diverse number of cellular functions, such as cell growth, apoptosis, cell migration, endocytosis, and cell differentiation [38]. The TCA cycle is the final pathway of metabolism of three nutrients—carbohydrates, lipids, and amino acids, so the presence of these

Table 3. List of identified secreted proteins with $-10 \times \text{Log}(P) \geq 50$ from *S. mansoni* miracidia (see S2 Table for the full list).

Sequence name	Sequence description	$-10 \times \text{Log}(P)$	#Peptides	Mass (kDa)
Smp_049550.1:pep	78 kda glucose-regulated protein	335.14	31	71.2
Smp_179250.1:pep	alpha-galactosidase, alpha-n-acetylgalactosaminidase	275.55	25	47.8
Smp_079770.1:pep	protein disulfide isomerase-associated 3 precursor	251.47	15	54.4
Smp_056760.1:pep	protein disulfide-isomerase	207.21	14	54.2
Smp_030300.4:pep	endoplasmic	183.37	20	239.5
Smp_089670.1:pep	alpha-2-macroglobulin-like protein 1	181.61	13	229.9
Smp_021730.2:pep	cytochrome c oxidase subunit Vb: cox4	171.7	9	25.1
Smp_150240.1:pep	secretory glycoprotein k5 precursor	168.2	7	31.2
Smp_098420.1:pep	hypothetical protein (<i>function unknown</i>)	159.57	8	36.5
Smp_112110.1:pep	interleukin-4-inducing protein precursor	155.09	4	15.4
Smp_154290.1:pep	glipr1-like protein 1 precursor	152.75	7	20.7
Smp_143150.1:pep	elongation factor 2	151.28	10	60.7
Smp_020340.1:pep	lysosomal alpha-glucosidase	137.23	7	101.6
Smp_032670.2:pep	egg protein c122-like	137.1	4	17.2
Smp_030370.1:pep	calreticulin	132.13	7	45.4
Smp_172110.1:pep	protein disulfide-isomerase	120.47	3	40.2
Smp_017730.1:pep	GPI anchored surface glycoprotein	117.25	6	186.5
Smp_180530.2:pep	phosphoglucomutase	114.35	3	62.7
Smp_160560.1:pep	iron dependent peroxidase	113.15	5	79.5
Smp_145300.1:pep	peptidylglycine alpha-hydroxylating monooxygenase	110.24	2	25.4
Smp_199860.1:pep	6-phosphogluconate decarboxylating	109.65	3	17.6
Smp_155890.1:pep	alkaline phosphatase	90.67	4	46.3
Smp_089640.2:pep	succinate dehydrogenase	86.78	4	32.3
Smp_159800.1:pep	hypothetical protein (<i>function unknown</i>)	78.58	1	9.1
Smp_000260.1:pep	proactivator polypeptide	78.41	1	104.7
Smp_005710.1:pep	egg protein cp391s-like	76.57	3	41.7
Smp_056200.1:pep	isocitrate dehydrogenase	65.77	2	43.5
Smp_088720.1:pep	nadh:ubiquinone oxidoreductase complex I intermediate-associated protein-containing protein	63	3	27.4
Smp_179560.1:pep	multiple inositol polyphosphate phosphatase	54.7	2	59.2

doi:10.1371/journal.pone.0147247.t003

pathway proteins may be attributed to the process of nutrient synthesis and storage in miracidia.

In addition, 15 proteins can be attributed to glutathione metabolism. Glutathione (GSH) works as an antioxidant molecule in plants, animals, fungi and some bacteria and archaea, preventing damage to important cellular components [39]. A similar role is likely for this group of proteins in the miracidium. So far, several glutathione metabolism relevant proteins have been considered as antischistosomal drug candidates, such as thioredoxin glutathione reductase (TGR) [40,41] and glutathione-S-transferase [42], both of which were identified in this study.

Secreted proteins in *S. mansoni* miracidia

Of the 1,910 miracidium proteins identified, 75 are predicted by SignalP, Predisi and TMHMM to be secreted (Table 3 and S2 Table). Secreted proteins may be involved in a variety of physiological processes in the miracidium, including cell signalling and cell-cell communication. We identified various members of the venom allergen-like (VAL) protein family (Table 4), and VAL 2, 3, 9, 26, 27 and 28 are predicted to be secretory.

Receptors and neuropeptides in *S. mansoni* miracidia

Approximately 43 receptors and receptor-associated proteins were identified and annotated in the proteome analysis, as shown in [S2 Table](#), represented by ionotropic, G-protein coupled, kinase-related and nuclear receptors. Most of these receptors have low sequence coverage supported by LCMS spectra, which could be attributed to the limitation of protein extraction method in purifying membrane proteins. The mitochondrial import receptors (TOM34 and 40) were most abundant.

Neuropeptide and neurotransmitter receptors were also found, including an acetylcholine receptor subunit α -2 and 4 that belongs to a superfamily of ligand-gated ion channels, allowing for the flow of sodium and potassium across the plasma membrane in response to ligands such as acetylcholine and nicotine. Also an FMRFamide receptor was found by X! Tandem ([S2 Table](#)).

No neuropeptides were identified from the proteome analysis, however, neuropeptide precursor genes were obtained from public databases for several platyhelminth species, and then used as a query to perform *in silico* BLASTp search of the *S. mansoni* genome, from which 5 *S. mansoni* neurohormone genes were identified. Translated sequences encoded RGMIIamide, GFVRIamide, PWamide, RLamide and Neuropeptide F (NPF) neuropeptides ([Fig 4](#) and [Table 5](#)). Compared with *S. japonicum* and *S. haematobium* homologs, there appears to be high amino acid identity. Our RT-PCR determined that all five *S. mansoni* neurohormone genes were expressed at the 2h post-hatch stage. Besides the miracidia stage, GGVRI, RGMII, PWamide and NPF gene transcripts have been identified in other life-stages, including cercariae, somule and adult worms ([Table 5](#) and [S2 Fig](#)).

Discussion

In this study, to provide a comprehensive overview of the miracidia proteome, we combined the results of four search engines to assess the MS/MS spectral data. The outcome indicated distinct variations in protein identification dependent on the search engine (see [Fig 1B](#)), which could be attributed to differences in search algorithms and associated parameter settings [43]. This is supported by a recent benchmark study that used a pool of 20,103 synthetic peptides to evaluate peptide-spectrum matches (PSMs) using two different LCMS systems followed by the analysis with 1,800 different search engine and parameter set combinations [43]. That study found that the choice of parameter settings had a large influence on the identification performance of the search engine. Thus, we recognise that the downstream statistical analysis we used to filter PSMs could potentially generate true/false positives or negatives, as different search engines provide various fractions of correct and incorrect PSM-assignments, numbers and types of correct assignments.

Table 4. Venom allergen-like (VAL) proteins identified by from *S. mansoni* miracidia (also see [S1 Table](#) for more details).

Accession	-10*Log(P)/Confidence	Coverage (%)	#Peptides	Mass (kDa)	Description
Smp_120670.1:pep	153.53	25	7	16.4	VAL5
Smp_154290.1:pep	152.75	35	7	20.7	VAL27
Smp_176160.1:pep	151.87	34	7	20.6	VAL26
Smp_154260.1:pep	151.87	34	7	20.6	VAL28
Smp_176180.1:pep	105.61	25	7	21.0	VAL9
Smp_179480.1:pep	88.87	15	4	31.2	VAL5
Smp_070250.1:pep	73.94	10	3	31.2	VAL15
Smp_002630.1:pep	21.22	4	1	26.6	VAL2
Smp_078490.1:pep	96.89 (X! Tandem)	7	1	24.6	VAL14

doi:10.1371/journal.pone.0147247.t004

GFVRI amides: (Smp_042120)

MTRYSLGIIQLFISYQIFCLLFICAHGSSRCTTTVNSDGLPILTCDDHRLKVQSHFDIYN

DEQNELIPESDYSNDPLQSQSAEYIMPS**KKAFVRLGKRGFVRIGKRGFVRIGR***

S. mansoni MTRYSLGIIQLFISYQIFCLLFICAHGSSRCTTTVNSDGLPILTCDDHRLKVQSHFDIYNDEQNELIPESDYSNDPLQSQSAEYIMPSKKAFVRLGKRGFVRIGKRGFVRIGR 115
S. japonicum MTRYSFYGIQLFTTYQIFCLLFISTYGFSRCAITVSSDGLPILTCDDHRSKTESHFPLYDDEQNELIPESVNTNDQLRVQQLPEYIVPDKKAFVRLGKRGFVRIGKRGFVRIGR 115
S. haematobium MTRYSLGIIQLFTTYQIFCLLFICAHGFSRCTTTVNSDGLPILTCDDHRSKAQSHFDLYDDEPNELIPESDYLNDPLQSQQLSEYIIPSKRAFVRLGKRGFVRIGKRGFVRIGR 115

RGMI amides: (Smp_154970)

MFYKRINDYILLCMLIAFLTISISRAKYFKCNVAACERCVLSEQISLNECCSNPSIHDVCN

FCDQNSETEIDISDCLLTYFRIML**KRRGMIGKRRGMIG***

S. mansoni MFYKRINDYILLCMLIAFLTISISRAKYFKCNVAACERCVLSEQISLNECCSNPSIHDVCNFCNQNSETEIDISDCLLTYFRIMLKRRGMIGKRRGMIG 99
S. japonicum MFLKQINFLFLICMLFAFYTMSTIKAKYSKCNVACERCMMSELTTLNECCSQPSIHEVCSYCDQNSETEIDISDCLSTYFRVMLKRRGMIGKRRGMIG 99
S. haematobium MFYKRINDYILLCMLIAFLTISISRAKYFKCNVAACERCVLSEQISLNECCSNPSIHDVCNFCNQNSETEIDISDCLSTYFRIMLKRRGMIGKRRGMIG 99

PWamides: (Smp_052880)

MNMLPFQKLSFVILTTLYLSLPWIISMVNTLPATNNNDPNILYRFPSSSSGSLSHQNGV

ERVYLPEELLAQLSNDPYFNMYL**PKRAAYMDLPWGKRALAKRAAYIDLWPWC***

S. mansoni MNMLPFQKLSFVILTTLYLSLPWIISMVNTLPATNNNDPNILYRFPSSSSGSLSHQNGV GSLSHQNGV ERVYLPEELLAQLSNDPYFNMYLPKRAAYMDLPWGKRALAKRAAYIDLWPWC 111
S. japonicum MLTTHKLTFCVLTTLFLSLPWIIFTVNTLPATNNNDPNILYRFLS SAPHQNG ERVYLPEELLAQLSNDPYFNMYLPKRAAYMDLPWGKRALAKRAAYIDLWPWC 102
S. haematobium MNMFLQKLSFVILTTLYLSLPWIISMVNTLPATNNNDPNILYRFLS SAPHQNG ERVYLPEELLAQLSNDPYFNMYLPKRAAYMDLPWGKRALAKRAAYIDLWPWC 119

RLamides: (Smp_070100)

MFGKIQLIFLLSTIMFYMQTANKINGEPMRRWYPLGVEAPRKASFSQGDNVAELEF

S. mansoni MFGKIQLIFLLSTIMFYMQTANKINGEPMRRWYPLGVEAPRKASFSQGDNVAELEFPYNGDQYDSEVMKRAVRLMRLG 60
S. japonicum MFGKIQLIFLLSTIMFYMQTANKINGEPMRRWYPLGVEAPRKASFSQGDNVAELEFPYNGDQYDSEVMKRAVRLMRLG 60
S. haematobium MFGKIQLIFLLSTIMFYMQTANKINGEPMRRWYPLGVEAPRKASFSQGDNVAELEFPYNGDQYDSEVMKRAVRLMRLG 60

Neuropeptide F: (Smp_088360)

MYSYMYGQYSFNWNVKSMSILLILLKFIHQNSIFINADELNLNTNNNLSNIPN

DQQAQALAKLSLFTYSDAFNKYMNLDAYYML**RGRPRFGKRN**YNPIKDNNDLMK

NDLINNYLRQKLLQKYLIDNYNNDGDENFLRN*

S. mansoni MYSYMYGQYSFNWNVKSMSILLILLKFIHQNSIFINADELNLNTNNNLSNIPNDQQAQALAKLSLFTYSDAFNKYMNLDAYYMLRGRPRFGKRNYNPIKDNNDLMKNDL 110
S. japonicum MHCYGRVFPSSKWNVKSMSILLILLKFIHQNSIFINADELNLNTNNNLSNIPNDQQAQALAKLSLFTYSDAFNKYMNLDAYYMLRGRPRFGKRNYNPIKDNNDLMKNDL 110
S. haematobium MSYHMYRKCSLWNVKSMSILLILLKFIHQNSIFINADELNLNTNNNLSNIPNDQQAQALAKLSLFTYSDAFNKYMNLDAYYMLRGRPR 97
 INNYLRQKLLQKYLIDNYNNDGDENFLRN 147
 LNSLLRQKLLRFPFDNLNDGDENFLRN 146

Fig 4. Characterisation of neuropeptides within *Schistosoma mansoni* miracidia. Shown is *S. mansoni* annotated precursor neuropeptides, including signal peptide (yellow), cleavage sites (red), glycine for amidation (green) and bioactive peptide (light blue). Also, comparison with *S. japonicum* and *S. haematobium* precursor protein homologs. Dark blue shading indicates amino acid conservation.

doi:10.1371/journal.pone.0147247.g004

Numerous *de novo* only peptides (peptide sequences that don't have any match against the database in use) resulted from *de novo* sequenced peptide segment from the MS/MS data have been provided in this work. For example, PEAKS engine derived a total of 3,790 *de novo* only peptides with high average local confidence greater than 80% (S1 Table), which could be attributed to: i) the incompleteness of the protein database due to the highly repetitive *S. mansoni*

Table 5. Summary of *S. mansoni* neuropeptide genes identified in this study.

Name	Full length (aa) ^a	Signal (aa) ^b	Mass (kDa) ^c	Miracidia	Cercariae	Adult
GFVRIamide	115	28	13.2	Yes	Yes	Yes
RGMIIamide	99	26	11.5	Yes	Yes	Yes
Pwamide	111	30	12.7	Yes	Yes	Yes
RIamide	80	23	9.3	Yes	Yes	No
Neuropeptide F	147	37	17.6	Yes	Yes	Yes

^a size in amino acids of precursor neurohormone

^b predicted length of signal sequence based on SignalP

^c full-length precursor

doi:10.1371/journal.pone.0147247.t005

genome, ii) short/alternative open reading frame (s/altORF) encoded peptides that were overlooked during genome annotation, and iii) contaminating mouse liver tissue attached to the isolated *S. mansoni* eggs that could have further attached to hatched miracidium. Herein, we provide the sequences of *de novo* only peptides as a data source for future investigation of possibilities i) and ii). Particularly for ii), the existence of s/altORF encoded peptides has been widely described in viruses [44,45,46,47] and increasingly found in humans with potential functions [48,49,50,51,52,53], including targets of anti-tumor responses in several type of cancers [50].

The enrichment analysis emphasises the importance of proteins playing roles in energy production, transportation and conversion in miracidia (Fig 2). It is possible that the overrepresentation of proteins in energy-related processes is due to the active development and more importantly host-seeking movement of miracidium. Thus, the signalling blockade of the key proteins on these pathways might inhibit its maturation and interfere with the snail infection. The biosynthesis of antibiotics is the KEGG pathway with large number of proteins in miracidium (70 relevant proteins with 49 enzymes), which approximately include 50% of sequences on this pathways identified from entire *S. mansoni* proteome [1], suggesting a complex defence mechanism existing in it despite the small body size.

Amongst the 1,910 miracidium proteins identified, the most abundant proteins were house-keeping/motor proteins (tubulin, actin, myosin etc) and enzymes (reductases, transferases, proteases and peptidases) (S2 Table). A putative rootletin protein and ciliary outer arm dynein are present at relatively high abundance, which likely originates from the miracidia cilia. A number of putative glycoproteins with high abundance were also identified, including both secreted and non-secreted proteins; these include secretory glycoprotein k5 precursor, nuclear pore membrane glycoprotein, GPI-anchored surface glycoprotein, and so forth.

One of well represented protein identified by LC-MS/MS is the *S. mansoni* protein 40 (Smp-40), also known as heat shock protein-HSP20, which is in accordance with a recent report showing that this protein can contribute to 15% of the soluble proteins of miracidia [13]. Smp-40 is one of a number of previously identified soluble egg antigens (SEA) and soluble worm antigen proteins (SWAP) [54], that has been investigated for its role in blastogenic reactions and granuloma responses, and as serodiagnostic target. As a member of the small heat shock protein (sHSP) family, it helps to capture unfolded proteins that form stable complexes [55], resulting in efficient disaggregation of the protein aggregates. The amphitropic sHSPs have been shown to associate with membranes, despite the lack of transmembrane domains or signal sequences. Proteins homologous to Smp-40 have previously been identified in *S. japonicum* [56], as well as the tapeworms *Taenia saginata* [57] and *Echinococcus multilocularis* [58], and the lung fluke *Paragonimus westermani* [59]. In addition to HSP20, HSP16, 27 and 40

were also identified in this study. A set of sHSPs are highly upregulated in the 'dauer' larvae of *Caenorhabditis elegans* [60], which is a stage induced by unfavourable growth conditions. The dauer state is resistant to environmental stresses by significantly reducing metabolic activity, yet a functional chemosensory system and rapid responsive movement can be maintained [61,62,63]. Miracidia face similar physiological stresses after hatching and during host-finding; thus, the abundance of sHSP could offer the miracidium protection against stress. Moreover, it has been suggested that the sHSPs work in an ATP-independent manner [64,65], ensuring that glucose reserves are not overused.

A 78 kDa glucose-regulated protein (GRP78) was also identified with high abundance, which is a member of the heat shock protein 70 (HSP70) multigene family (Table 3). This finding extends upon a previously reported study which found that chaperones are the most abundant proteins in the *S. mansoni* egg [13]. The expression of HSP70 has been noticed throughout the development of egg, which suggests its multi-functional roles, including protein maturation in the reticulum of embryogenic processes [66] and in mitochondrial biogenesis [67]. GRP78 is involved in the folding of nascent proteins within the endoplasmic reticulum and the translocation of secretory proteins [68], thus it could play a similar role in stabilising macromolecular structure for miracidium proteins destined for secretion. The GRP78 itself has also been found to be released from cells and into the peripheral circulation [69]. We also identified the predicted secreted protein alkaline phosphatase in the miracidia proteome (see Table 3). Alkaline phosphatase in *S. mansoni* sporocysts has been speculated to function in nutrient uptake and might serve as a marker of infection of *B. glabrata* snails [70].

Two mucin-like proteins were identified, with N-terminal domains containing a variable number of tandem repeats. Polymorphic Mucins (*SmPoMucs*) act as key strain markers since they vary between the strains of *S. mansoni* known to infect *B. glabrata* [71]. Their proposed function is to interact with the host snails Fibrinogen Related Proteins (FREPs) [72], and other proteins associated with the secondary immune response of the snail [73,74]. Of note, it has been observed that the *SmPoMuc* genes are only expressed in stages that interact with the host snail [17] and that each individual miracidium shows significant sequence differences [75].

The secreted glycoprotein kappa-5 (k5) was first characterised as an immunogenic egg glycoprotein from *S. mansoni*, and is probably a key mediator of host-parasite interactions since it triggers a pronounced IgE response in the human host [76]. Similarly, its role as an authentic granuloma-inducing component in snail hosts is worthy of study, and to compare with its similar function in the mammalian host [77]. The secreted calreticulin-like protein is a Ca^{2+} binding/storage protein, which is highly conserved, being found in a number of different animal taxa. It has recently been considered as a novel antigen for the detection of anti-*S. mansoni* antibodies as it possesses the advantage of cheaper and easier production compared with the soluble egg antigen that is traditionally used [78].

Previously, four VAL protein members have been found by the transcriptomic analysis of different life stages of *S. mansoni* [37], and later a further 24 members were discovered [79]. Although the biological function of VAL proteins still remains elusive, it has been proposed that may play an important role in the host-parasite interaction [80]. Gene expression of some VAL proteins (VAL6 and 7) is restricted to the oral and ventral suckers of adult worms and in the oesophageal gland [81], while VAL 2, 3, 5 and 9 have inflammation-inducing properties [16]. The VAL 9 protein has additionally been found in soluble egg products and miracidia/sporocysts, and is potentially involved in tissue reorganisation/extracellular matrix remodelling [82].

We found a GPI-anchored surface glycoprotein within the miracidia proteome, however, its role in miracidia remains unclear. The GPI-anchored surface glycoproteins are also found in adult *S. mansoni*, localised to the outer surface [83,84]. A saposin protein was identified,

displaying the characteristic six conservative cysteines that form three disulphide bridges, previously reported within the gastrodermis of *S. mansoni* adult and cercariae [85]. Saposins exist widely in most animals; their function varies but seems to relate to interactions with lipids. In *Clonorchis sinensis*, it has been shown to lyse ingested host cells for nutritional purposes [86], while in the case of *Fasciola hepatica*, it works externally to the parasite as a secreted product [87], thus implicating it in a feeding role.

The interleukin-4-inducing protein precursor was first identified with high expression in the eggs of *S. mansoni*, where it can stimulate the mammalian host to rapidly release large amounts of interleukin-4 (IL-4) [88]. IL-4 is a key promoter of a Th2 response, via an IgE-dependent but non-antigen-specific mechanism. We found that this protein is present in abundance in miracidia (Table 3), which contrasts a report that this protein is produced exclusively by the eggs [89]. As a secreted glycoprotein, it could play a similar role in the body of the intermediate snail host.

Cathepsins D and L were also identified in our miracidia proteome. It has been suggested, from an evolutionary perspective, that cathepsins D and L are two key proteases (others include cathepsins B, C and legumain) that allow for efficient digestion in early metazoans. In addition, studies of parasitic helminths have suggested that these proteases function as digestive enzymes in vertebrates earlier than the evolution of the pancreas and the serine [90]. The aspartic protease cathepsin D has been found in the schistosome gut as an apical enzyme in digesting hemoglobin released from ingested erythrocytes [91]. The reduction of cathepsin D transcripts leads to significant development retardation *in vitro*, aspartic protease enzyme activity suppression and the malfunction of the digestion of host hemoglobins in the gut of schistosome adults [92]. Vaccination of sheep and cattle with purified *F. hepatica* cathepsin L elicits protection (50–73%) and anti-fecundity effects against challenge infection [93]. Thus, these two proteases might play certain roles in miracidia metabolism, for example, at post-infection they may regulate the digestion of host snail's haemoglobin [94].

The identification of abundant mitochondrial import receptors may correlate with the high energy requirement for miracidium growth, development and extensive host-seeking activity within the first 5–6 h post-hatch. TOM34 was found to act as an important component of the translocation machinery of the outer mitochondrial membrane [95], while TOM40 forms hydrophilic channel for the transport of preproteins [96]. The presence of epidermal growth factor, netrin and ribosome receptors in miracidia additionally supports the requirement for active growth. Human low-density lipoprotein (LDL) binds to the surface of schistosomula adult and cercariae via LDL receptor [97]; the identification of LDL receptor in miracidium indicates a similar scenario might take place between miracidium and host snail.

Neuropeptides are used by neurons to communicate with cells. Despite little understanding of their role in schistosome miracidia, we expected that they would be important as key endogenous mediators during host-seeking behaviours, required for locomotion, sensory responses and basic catabolism of endogenous glycogen stores. In this study, although no neuropeptides were identified from the miracidia proteome analysis, 5 neurohormone genes were identified by RT-PCR. Of the 5 neurohormones, NPF has been the most extensively studied across the phylum Platyhelminthes. The neuropeptide Y superfamily has important functions in both vertebrate and invertebrate taxa with two distinguishable types of peptides, neuropeptide F-like (NPF), most notably found in invertebrates, and NPY-like peptides found typically in vertebrate species [98]. However, analysis of recent planarian genomes has shown that a specific family of NPY (specifically NPY-1,4,9) genes could encode for both NPY-like and NPF-like neuropeptides, suggesting an evolutionary relationship between vertebrate NPY and invertebrate NPF peptides [98]. Neuropeptide F within invertebrates has been linked to feeding, various locomotion roles, reproduction and stress responses [99]. In adult worms of *S. mansoni*

NPF functions in modulation of sensory systems, motor function, reproduction, and feeding [100]. However, as the two latter functions can be excluded from miracidium, with neither the need to feed, nor reproduce, it can be predicted to play roles in locomotion and in varying sensory modulation [100].

A report has indicated possible inter-species differences in the functions of GFVRIamide, which fall into the broad I/Lamide family, due to the apparent lack of conservation and difference in expression [101]. Recently described, the novel GFVRIamide has been linked to potential neuromuscular and interneuronal communication roles in the adult worm, suggesting a putative role also in the miracidium [101]. RLamide and RGMamide are newly described novel peptides that fall into the same I/Lamide family, and along with the PWamides all have unknown phenotypical expression across the phylum Platyhelminthes. A high level of inter-species conservation, which was seen within the multi sequence alignment, suggests potential putative roles for these neuropeptides, with neuropeptides such as NPF, having similar and consistent functions across invertebrate species. However, obtaining a next-generation transcriptome is recommended so as to identify further novel neuropeptides and associated receptors, where functions of expressed and novel neuropeptides can be determined using gene knockout techniques. Species conserved neuropeptide receptor systems make for potential novel drug targets, and further exploration of the function of these receptors systems at the miracidial level is recommended, with future studies to elicit the expression of each neuropeptide throughout the miracidium life-span to analyse neuropeptide expression patterns.

Conclusions

Our study has revealed the proteome of the *S. mansoni* miracidium, uncovering important molecules in biological pathways that play key roles, such as in glutathione metabolism, phenylalanine metabolism, phenylpropanoid biosynthesis and the TCA cycle. The biological processes related to energy regulation are highly enriched at this stage, with a large ratio of proteins having functions of ATP/GTP relevance; in addition, the enrichment of protein folding/polymerisation and microtubule-related processes further indicates the active development of miracidia. It has highlighted the principal secreted proteins the miracidium releases, some of which are possibly involved in environment adaption, host seeking and invasion, including sHSP, HSP70, mucins, VALs, interleukin 4, GPI-anchored glycoproteins and others; in addition, identified receptors also suggest that active biological processes occur in miracidia, including immune response, phosphorylation and epithelial cell growth. The neuropeptides, including RGMamide, GFVRIamide, PWamide, RLamide and NPF, were identified through gene expression analysis. These are highly conserved between schistosome species.

Supporting Information

S1 Fig. The distribution of all KEGG pathways identified from miracidia proteins.
(TIF)

S2 Fig. Graphs show relative mRNA concentration of neuropeptide at cercariae (C), somulae (3S and 24S) and adult schistosome (A) stages.
(TIF)

S1 Table. Identification of *Schistosoma mansoni* miracidia proteins by PEAKS, MS Amanda, X! Tandem and OMSSA, including summary of proteins, supporting peptides and *de novo* only peptides identified by LC-MS/MS, see [Methods](#) for more details.
(XLSX)

S2 Table. Summary of annotation of identified proteins, predicted secretory proteins and receptors.

(XLSX)

S3 Table. Summary of gene ontology analysis at all levels, Fisher's exact test result and KEGG analysis.

(XLSX)

Acknowledgments

We acknowledge the help of Ms Mary Duke of QIMR Berghofer with the egg hatching and the miracidia processing and harvesting. We gratefully thank Dr Alun Jones (Institute for Molecular Bioscience, the University of Queensland) for advice and assistance with the LC-MS/MS. DPM is a National Health and Medical Research Council Senior Principal Research Fellow and Senior Scientist at QIMR Berghofer.

Author Contributions

Conceived and designed the experiments: TW SFC. Performed the experiments: TW MZ BAR AS DL GN. Analyzed the data: TW MZ SFC. Contributed reagents/materials/analysis tools: DPM SFC. Wrote the paper: TW DPM SFC YL PR.

References

1. Berriman M, Haas BJ, LoVerde PT, Wilson RA, Dillon GP, Cerqueira GC, et al. (2009) The genome of the blood fluke *Schistosoma mansoni*. *Nature* 460: 352–358. doi: [10.1038/nature08160](https://doi.org/10.1038/nature08160) PMID: [19606141](https://pubmed.ncbi.nlm.nih.gov/19606141/)
2. Zhou Y, Zheng H, Chen Y, Zhang L, Wang K, Guo J, et al. (2009) The *Schistosoma japonicum* genome reveals features of host–parasite interplay. *Nature* 460: 345–351. doi: [10.1038/nature08140](https://doi.org/10.1038/nature08140) PMID: [19606140](https://pubmed.ncbi.nlm.nih.gov/19606140/)
3. Aly HF, Mantawy MM (2013) Efficiency of ginger (*Zingbar officinale*) against *Schistosoma mansoni* infection during host-parasite association. *Parasitology International* 62: 380–389. doi: [10.1016/j.parint.2013.04.002](https://doi.org/10.1016/j.parint.2013.04.002) PMID: [23583317](https://pubmed.ncbi.nlm.nih.gov/23583317/)
4. Danso-Appiah A, Olliaro PL, Donegan S, Sinclair D, Utzinger J (2013) Drugs for treating *Schistosoma mansoni* infection. *Cochrane Database of Systematic Reviews*.
5. Odegaard JI, Hsieh MH (2014) Immune responses to *Schistosoma haematobium* infection. *Parasite Immunology* 36: 428–438. doi: [10.1111/pim.12084](https://doi.org/10.1111/pim.12084) PMID: [25201406](https://pubmed.ncbi.nlm.nih.gov/25201406/)
6. Pitta MGD, Pitta MGD, Rego M, Galdino SL (2013) The Evolution of Drugs on *Schistosoma* Treatment: Looking to the Past to Improve the Future. *Mini-Reviews in Medicinal Chemistry* 13: 493–508. PMID: [23373654](https://pubmed.ncbi.nlm.nih.gov/23373654/)
7. King CH (2009) GLOBAL HEALTH Toward the Elimination of Schistosomiasis. *New England Journal of Medicine* 360: 106–109. doi: [10.1056/NEJMp0808041](https://doi.org/10.1056/NEJMp0808041) PMID: [19129524](https://pubmed.ncbi.nlm.nih.gov/19129524/)
8. Pinheiro CS, Martins VP, Assis NR, Figueiredo BC, Morais SB, Azevedo V, et al. (2011) Computational vaccinology: an important strategy to discover new potential *S. mansoni* vaccine candidates. *Journal of Biomedicine and Biotechnology*: 503068. doi: [10.1155/2011/503068](https://doi.org/10.1155/2011/503068) PMID: [22013383](https://pubmed.ncbi.nlm.nih.gov/22013383/)
9. Prichard RK, Basanez MG, Boatman BA, McCarthy JS, Garcia HH, Yang GJ, et al. (2012) A research agenda for helminth diseases of humans: intervention for control and elimination. *PLoS Neglected Tropical Diseases* 6: e1549. doi: [10.1371/journal.pntd.0001549](https://doi.org/10.1371/journal.pntd.0001549) PMID: [22545163](https://pubmed.ncbi.nlm.nih.gov/22545163/)
10. Gregory TR (2003) Genome size estimates for two important freshwater molluscs, the zebra mussel (*Dreissena polymorpha*) and the schistosomiasis vector snail (*Biomphalaria glabrata*). *Genome* 46: 841–844. PMID: [14608401](https://pubmed.ncbi.nlm.nih.gov/14608401/)
11. Kalbe M, Haberl B, Haas W (1997) Miracidial host-finding in *Fasciola hepatica* and *Trichobilharzia ocellata* is stimulated by species-specific glycoconjugates released from the host snails. *Parasitology Research* 83: 806–812. PMID: [9342748](https://pubmed.ncbi.nlm.nih.gov/9342748/)
12. Haberl B, Korner M, Spengler Y, Hertel J, Kalbe M, Haas W (2000) Host-finding in *Echinostoma caproni*: miracidia and cercariae use different signals to identify the same snail species. *Parasitology* 120: 479–486. PMID: [10840977](https://pubmed.ncbi.nlm.nih.gov/10840977/)

13. Mathieson W, Wilson RA (2010) A comparative proteomic study of the undeveloped and developed *Schistosoma mansoni* egg and its contents: The miracidium, hatch fluid and secretions. *International Journal for Parasitology* 40: 617–628. doi: [10.1016/j.ijpara.2009.10.014](https://doi.org/10.1016/j.ijpara.2009.10.014) PMID: [19917288](https://pubmed.ncbi.nlm.nih.gov/19917288/)
14. Curwen RS, Ashton PD, Johnston DA, Wilson RA (2004) The *Schistosoma mansoni* soluble proteome: a comparison across four life-cycle stages. *Molecular and Biochemical Parasitology* 138: 57–66. PMID: [15500916](https://pubmed.ncbi.nlm.nih.gov/15500916/)
15. Ashton PD, Harrop R, Shah B, Wilson RA (2001) The schistosome egg: development and secretions. *Parasitology* 122: 329–338. PMID: [11289069](https://pubmed.ncbi.nlm.nih.gov/11289069/)
16. Cass CL, Johnson JR, Califf LL, Xu T, Hernandez HJ, Stadecker MJ, et al. (2007) Proteomic analysis of *Schistosoma mansoni* egg secretions. *Mol Biochem Parasitol* 155: 84–93. PMID: [17644200](https://pubmed.ncbi.nlm.nih.gov/17644200/)
17. Roger E, Gourbal B, Grunau C, Pierce RJ, Galinier R, Mitta G (2008) Expression analysis of highly polymorphic mucin proteins (Sm PoMuc) from the parasite *Schistosoma mansoni*. *Molecular and Biochemical Parasitology* 157: 217–227. doi: [10.1016/j.molbiopara.2007.11.015](https://doi.org/10.1016/j.molbiopara.2007.11.015) PMID: [18187213](https://pubmed.ncbi.nlm.nih.gov/18187213/)
18. Guillou F, Roger E, Mone Y, Rognon A, Grunau C, Theron A, et al. (2007) Excretory-secretory proteome of larval *Schistosoma mansoni* and *Echinostoma caproni*, two parasites of *Biomphalaria glabrata*. *Molecular and Biochemical Parasitology* 155: 45–56. PMID: [17606306](https://pubmed.ncbi.nlm.nih.gov/17606306/)
19. Roger E, Mitta G, Mone Y, Bouchut A, Rognon A, Grunau C, et al. (2008) Molecular determinants of compatibility polymorphism in the *Biomphalaria glabrata/Schistosoma mansoni* model: new candidates identified by a global comparative proteomics approach. *Molecular and Biochemical Parasitology* 157: 205–216. PMID: [18083248](https://pubmed.ncbi.nlm.nih.gov/18083248/)
20. Wu XJ, Sabat G, Brown JF, Zhang M, Taft A, Peterson N, et al. (2009) Proteomic analysis of *Schistosoma mansoni* proteins released during in vitro miracidium-to-sporocyst transformation. *Molecular and Biochemical Parasitology* 164: 32–44. doi: [10.1016/j.molbiopara.2008.11.005](https://doi.org/10.1016/j.molbiopara.2008.11.005) PMID: [19095013](https://pubmed.ncbi.nlm.nih.gov/19095013/)
21. Maldonado JF, Acosta-Matienzo J (1948) Biological studies on the miracidium of *Schistosoma mansoni*. *The American journal of tropical medicine and hygiene* 1: 645–657.
22. Schreiber FG, Schubert M (1949) Experimental infection of the snail *Australorbis glabratus* with the trematode *Schistosoma mansoni* and the production of cercariae. *The Journal of parasitology*: 91–100. PMID: [18111996](https://pubmed.ncbi.nlm.nih.gov/18111996/)
23. Dalton JP, Day SR, Drew AC, Brindley PJ (1997) A method for the isolation of schistosome eggs and miracidia free of contaminating host tissues. *Parasitology* 115 (Pt 1): 29–32. PMID: [9226954](https://pubmed.ncbi.nlm.nih.gov/9226954/)
24. Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M (2006) In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nature Protocols* 1: 2856–2860. PMID: [17406544](https://pubmed.ncbi.nlm.nih.gov/17406544/)
25. Adamson KJ, Wang T, Rotgans B, Kuballa AV, Storey KB, Cummins SF (2015) Differential peptide expression in the central nervous system of the land snail *Theba pisana*, between active and aestivated. *Peptides*.
26. Craig R, Beavis RC (2004) TANDEM: matching proteins with tandem mass spectra. *Bioinformatics* 20: 1466–1467. PMID: [14976030](https://pubmed.ncbi.nlm.nih.gov/14976030/)
27. Dorfer V, Pichler P, Stranzl T, Stadlmann J, Taus T, Winkler S, et al. (2014) MS Amanda, a universal identification algorithm optimized for high accuracy tandem mass spectra. *Journal of Proteome Research* 13: 3679–3684. doi: [10.1021/pr500202e](https://doi.org/10.1021/pr500202e) PMID: [24909410](https://pubmed.ncbi.nlm.nih.gov/24909410/)
28. Geer LY, Markey SP, Kowalak JA, Wagner L, Xu M, Maynard DM, et al. (2004) Open mass spectrometry search algorithm. *Journal of Proteome Research* 3: 958–964. PMID: [15473683](https://pubmed.ncbi.nlm.nih.gov/15473683/)
29. Conesa A, Gotz S, Garcia-Gomez JM, Terol J, Talon M, Robles M (2005) Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* 21: 3674–3676. PMID: [16081474](https://pubmed.ncbi.nlm.nih.gov/16081474/)
30. Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society Series B (Methodological)*: 289–300.
31. Petersen TN, Brunak S, von Heijne G, Nielsen H (2011) SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nature methods* 8: 785–786. doi: [10.1038/nmeth.1701](https://doi.org/10.1038/nmeth.1701) PMID: [21959131](https://pubmed.ncbi.nlm.nih.gov/21959131/)
32. Hiller K, Grote A, Scheer M, Munch R, Jahn D (2004) PrediSi: prediction of signal peptides and their cleavage positions. *Nucleic Acids Research* 32: W375–379. PMID: [15215414](https://pubmed.ncbi.nlm.nih.gov/15215414/)
33. Krogh A, Larsson B, von Heijne G, Sonnhammer EL (2001) Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *Molecular and Biochemical Parasitology* 305: 567–580.
34. Tamura K, Stecher G, Peterson D, Filipowski A, Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular biology and evolution* 30: 2725–2729. doi: [10.1093/molbev/mst197](https://doi.org/10.1093/molbev/mst197) PMID: [24132122](https://pubmed.ncbi.nlm.nih.gov/24132122/)

35. Beitz E (2000) TEXshade: shading and labeling of multiple sequence alignments using LATEX2 epsilon. *Bioinformatics* 16: 135–139. PMID: [10842735](#)
36. Zerlotini A, Aguiar ER, Yu F, Xu H, Li Y, Young ND, et al. (2013) SchistoDB: an updated genome resource for the three key schistosomes of humans. *Nucleic Acids Research* 41: D728–731. doi: [10.1093/nar/gks1087](#) PMID: [23161692](#)
37. Verjovski-Almeida S, DeMarco R, Martins EAL, Guimaraes PEM, Ojopi EPB, Paquola ACM, et al. (2003) Transcriptome analysis of the acelomate human parasite *Schistosoma mansoni*. *Nature Genetics* 35: 148–157. PMID: [12973350](#)
38. Steger DJ, Haswell ES, Miller AL, Wente SR, O'Shea EK (2003) Regulation of chromatin remodeling by inositol polyphosphates. *Science* 299: 114–116. PMID: [12434012](#)
39. Pompella A, Visvikis A, Paolicchi A, De Tata V, Casini AF (2003) The changing faces of glutathione, a cellular protagonist. *Biochemical Pharmacology* 66: 1499–1503. PMID: [14555227](#)
40. Kuntz AN, Davioud-Charvet E, Sayed AA, Califf LL, Dessolin J, Arnér ES, et al. (2007) Thioredoxin glutathione reductase from *Schistosoma mansoni*: an essential parasite enzyme and a key drug target. *PLoS Medicine* 4: e206. PMID: [17579510](#)
41. Sharma M, Khanna S, Bulusu G, Mitra A (2009) Comparative modeling of thioredoxin glutathione reductase from *Schistosoma mansoni*: A multifunctional target for antischistosomal therapy. *Journal of Molecular Graphics and Modelling* 27: 665–675. doi: [10.1016/j.jmglm.2008.10.009](#) PMID: [19070522](#)
42. Luo Q-L, Qiao Z-P, Zhou Y-D, Li X-Y, Zhong Z-R, Yu Y-J, et al. (2009) Application of signaling protein 14-3-3 and 26kDa glutathione-S-transferase to serological diagnosis of *Schistosomiasis japonica*. *Acta tropica* 112: 91–96. doi: [10.1016/j.actatropica.2009.06.011](#) PMID: [19560432](#)
43. Quandt A, Espona L, Balasko A, Weisser H, Brusniak M-Y, Kunszt P, et al. (2014) Using synthetic peptides to benchmark peptide identification software and search parameters for MS/MS data analysis. *EuPA Open Proteomics* 5: 21–31.
44. Branch AD, Stump DD, Gutierrez JA, Eng F, Walewski JL (2005) The hepatitis C virus alternate reading frame (ARF) and its family of novel products: the alternate reading frame protein/F-protein, the double-frameshift protein, and others. *Semin Liver Dis* 25: 105–117. PMID: [15732002](#)
45. McFadden N, Bailey D, Carrara G, Benson A, Chaudhry Y, Shortland A, et al. (2011) Norovirus regulation of the innate immune response and apoptosis occurs via the product of the alternative open reading frame 4. *PLoS Pathog* 7: e1002413. doi: [10.1371/journal.ppat.1002413](#) PMID: [22174679](#)
46. Vassilaki N, Friebe P, Meuleman P, Kallis S, Kaul A, Paranhos-Baccala G, et al. (2008) Role of the hepatitis C virus core+1 open reading frame and core cis-acting RNA elements in viral RNA translation and replication. *Journal of Virology* 82: 11503–11515. doi: [10.1128/JVI.01640-08](#) PMID: [18799568](#)
47. Johnson CR, Griggs TF, Gnanandarajah J, Murtaugh MP (2011) Novel structural protein in porcine reproductive and respiratory syndrome virus encoded by an alternative ORF5 present in all arteriviruses. *Journal of General Virology* 92: 1107–1116. doi: [10.1099/vir.0.030213-0](#) PMID: [21307222](#)
48. Freson K, Jaeken J, Van Helvoirt M, de Zegher F, Wittevrongel C, Thys C, et al. (2003) Functional polymorphisms in the paternally expressed XLalphas and its cofactor ALEX decrease their mutual interaction and enhance receptor-mediated cAMP formation. *Human Molecular Genetics* 12: 1121–1130. PMID: [12719376](#)
49. Oh S, Terabe M, Pendleton CD, Bhattacharyya A, Bera TK, Epel M, et al. (2004) Human CTLs to wild-type and enhanced epitopes of a novel prostate and breast tumor-associated protein, TARP, lyse human breast cancer cells. *Cancer Research* 64: 2610–2618. PMID: [15059918](#)
50. Ho O, Green WR (2006) Alternative translational products and cryptic T cell epitopes: expecting the unexpected. *The Journal of Immunology* 177: 8283–8289. PMID: [17142722](#)
51. Rosenberg SA, Tong-On P, Li Y, Riley JP, El-Gamil M, Parkhurst MR, et al. (2002) Identification of BING-4 cancer antigen translated from an alternative open reading frame of a gene in the extended MHC class II region using lymphocytes from a patient with a durable complete regression following immunotherapy. *The Journal of Immunology* 168: 2402–2407. PMID: [11859131](#)
52. Slavoff SA, Mitchell AJ, Schwaid AG, Cabili MN, Ma J, Levin JZ, et al. (2013) Peptidomic discovery of short open reading frame-encoded peptides in human cells. *Nature Chemical Biology* 9: 59–64. doi: [10.1038/nchembio.1120](#) PMID: [23160002](#)
53. Andrews SJ, Rothnagel JA (2014) Emerging evidence for functional peptides encoded by short open reading frames. *Nature Reviews Genetics* 15: 193–204. doi: [10.1038/nrg3520](#) PMID: [24514441](#)
54. Hernandez HJ, Stadecker MJ (1999) Elucidation and role of critical residues of immunodominant peptide associated with T cell-mediated parasitic disease. *The Journal of Immunology* 163: 3877–3882. PMID: [10490987](#)

55. Nakamoto H, Vigh L (2007) The small heat shock proteins and their clients. *Cellular and Molecular Life Sciences* 64: 294–306. PMID: [17187175](#)
56. Liu F, Lu J, Hu W, Wang SY, Cui SJ, Chi M, et al. (2006) New perspectives on host-parasite interplay by comparative transcriptomic and proteomic analyses of *Schistosoma japonicum*. *Plos Pathogens* 2: 268–281.
57. Benitez L, Harrison LJS, Parkhouse RME, Garate T (1998) Sequence and preliminary characterisation of a *Taenia saginata* oncosphere gene homologue of the small heat-shock protein family. *Parasitology Research* 84: 423–425. PMID: [9610643](#)
58. Merckelbach A, Wager M, Lucius R (2003) Analysis of cDNAs coding for immunologically dominant antigens from an oncosphere-specific cDNA library of *Echinococcus multilocularis*. *Parasitology Research* 90: 493–501. PMID: [12827505](#)
59. Kappe G, Aquilina JA, Wunderink L, Kamps B, Robinson CV, Garate T, et al. (2004) Tsp36, a tapeworm small heat-shock protein with a duplicated alpha-crystallin domain, forms dimers and tetramers with good chaperone-like activity. *Proteins-Structure Function and Bioinformatics* 57: 109–117.
60. Burnell AM, Houthoofd K, O'Hanlon K, Vanfleteren JR (2005) Alternate metabolism during the dauer stage of the nematode *Caenorhabditis elegans*. *Experimental Gerontology* 40: 850–856. PMID: [16221538](#)
61. Leroux MR, Ma BJ, Batelier G, Melki R, Peter E, Candido M (1997) Unique structural features of a novel class of small heat shock proteins. *Journal of Biological Chemistry* 272: 12847–12853. PMID: [9139746](#)
62. Albert PS, Riddle DL (1988) Mutants of *caenorhabditis-elegans* that form dauer-like larvae. *Developmental Biology* 126: 270–293. PMID: [3350212](#)
63. Cassada RC, Russell RL (1975) Dauerlarva, a post-embryonic developmental variant of nematode *caenorhabditis elegans*. *Developmental Biology* 46: 326–342. PMID: [1183723](#)
64. Fu X (2014) Chaperone function and mechanism of small heat-shock proteins. *Acta Biochimica et Biophysica Sinica (Shanghai)* 46: 347–356.
65. Garrido C, Paul C, Seigneuric R, Kampinga HH (2012) The small heat shock proteins family: the long forgotten chaperones. *The International Journal of Biochemistry & Cell Biology* 44: 1588–1592.
66. ElRidi R, Velupillai P, Harn DA (1996) Regulation of schistosoma egg granuloma formation: Host-soluble L-selectin enters tissue-trapped eggs and binds to carbohydrate antigens on surface membranes of miracidia. *Infection and Immunity* 64: 4700–4705. PMID: [8890228](#)
67. Voos W, Rottgers K (2002) Molecular chaperones as essential mediators of mitochondrial biogenesis. *Biochimica Et Biophysica Acta-Molecular Cell Research* 1592: 51–62.
68. Rao RV, Bredesen DE (2004) Misfolded proteins, endoplasmic reticulum stress and neurodegeneration. *Current Opinion in Cell Biology* 16: 653–662. PMID: [15530777](#)
69. Delpino A, Castelli M (2002) The 78 kDa glucose-regulated protein (GRP78/BIP) is expressed on the cell membrane, is released into cell culture medium and is also present in human peripheral circulation. *Bioscience Reports* 22: 407–420. PMID: [12516782](#)
70. Ballen D, Theron A, Pointier JP, Coustau C, Cesari IM (2002) *Schistosoma mansoni*: identification of a possible daughter sporocyst alkaline phosphatase. *Experimental Parasitology* 101: 164–167. PMID: [12427471](#)
71. Mitta G, Adema CM, Gourbal B, Loker ES, Theron A (2012) Compatibility polymorphism in snail/schistosoma interactions: From field to theory to molecular mechanisms. *Developmental & Comparative Immunology* 37: 1–8.
72. Mone Y, Gourbal B, Duval D, Du Pasquier L, Kieffer-Jaquinod S, Mitta G (2010) A large repertoire of parasite epitopes matched by a large repertoire of host immune receptors in an invertebrate host/parasite model. *PLoS Neglected Tropical Diseases* 4. e813 doi: [10.1371/journal.pntd.0000813](#) PMID: [20838648](#)
73. Hanington PC, Forys MA, Dragoo JW, Zhang SM, Adema CM, Loker ES (2010) Role for a somatically diversified lectin in resistance of an invertebrate to parasite infection. *Proceedings of the National Academy of Sciences USA* 107: 21087–21092.
74. Zhang SM, Zeng Y, Loker ES (2008) Expression profiling and binding properties of fibrinogen-related proteins (FREPs), plasma proteins from the schistosome snail host *Biomphalaria glabrata*. *Innate Immunity* 14: 175–189. doi: [10.1177/1753425908093800](#) PMID: [18562576](#)
75. Cosseau C, Azzi A, Rognon A, Boissier J, Gourbière S, Roger E, et al. (2010) Epigenetic and phenotypic variability in populations of *Schistosoma mansoni*—a possible kick-off for adaptive host/parasite evolution. *Oikos* 119: 669–678.

76. Schramm G, Hamilton J, Balog C, Wuhrer M, Gronow A, Beckmann S, et al. (2009) Molecular characterisation of kappa-5, a major antigenic glycoprotein from *Schistosoma mansoni* eggs. *Molecular and biochemical parasitology* 166: 4–14. doi: [10.1016/j.molbiopara.2009.02.003](https://doi.org/10.1016/j.molbiopara.2009.02.003) PMID: [19428667](https://pubmed.ncbi.nlm.nih.gov/19428667/)
77. Van de Vijver KK, Deelder AM, Jacobs W, Van Marck EA, Hokke CH (2006) LacdiNAc- and LacNAC-containing glycans induce granulomas in an in vivo model for schistosome egg-induced hepatic granuloma formation. *Glycobiology* 16: 237–243. PMID: [16282603](https://pubmed.ncbi.nlm.nih.gov/16282603/)
78. El Aswad BEDW, Doenhoff MJ, El Hadidi AS, Schwaeble WJ, Lynch NJ (2011) Use of recombinant calreticulin and cercarial transformation fluid (CTF) in the serodiagnosis of *Schistosoma mansoni*. *Immunobiology* 216: 379–385. doi: [10.1016/j.imbio.2010.06.014](https://doi.org/10.1016/j.imbio.2010.06.014) PMID: [20691496](https://pubmed.ncbi.nlm.nih.gov/20691496/)
79. Chalmers IW, McArdle AJ, Coulson RM, Wagner MA, Schmid R, Hirai H, et al. (2008) Developmentally regulated expression, alternative splicing and distinct sub-groupings in members of the *Schistosoma mansoni* venom allergen-like (SmVAL) gene family. *BMC genomics* 9: 89. doi: [10.1186/1471-2164-9-89](https://doi.org/10.1186/1471-2164-9-89) PMID: [18294395](https://pubmed.ncbi.nlm.nih.gov/18294395/)
80. Farias LP, Rodrigues D, Cunna V, Rofatto HK, Faquim-Mauro EL, Leite LC (2012) *Schistosoma mansoni* venom allergen like proteins present differential allergic responses in a murine model of airway inflammation. *PLoS neglected tropical diseases* 6: e1510. doi: [10.1371/journal.pntd.0001510](https://doi.org/10.1371/journal.pntd.0001510) PMID: [22347513](https://pubmed.ncbi.nlm.nih.gov/22347513/)
81. Rofatto HK, Parker-Manuel SJ, Barbosa TC, Tararam CA, Alan Wilson R, Leite LC, et al. (2012) Tissue expression patterns of *Schistosoma mansoni* Venom Allergen-Like proteins 6 and 7. *International Journal for Parasitology* 42: 613–620. doi: [10.1016/j.ijpara.2012.04.008](https://doi.org/10.1016/j.ijpara.2012.04.008) PMID: [22575701](https://pubmed.ncbi.nlm.nih.gov/22575701/)
82. Yoshino TP, Brown M, Wu X-J, Jackson CJ, Ocadiz-Ruiz R, Chalmers IW, et al. (2014) Excreted/secreted *Schistosoma mansoni* venom allergen-like 9 (SmVAL9) modulates host extracellular matrix remodelling gene expression. *International journal for parasitology* 44: 551–563. doi: [10.1016/j.ijpara.2014.04.002](https://doi.org/10.1016/j.ijpara.2014.04.002) PMID: [24859313](https://pubmed.ncbi.nlm.nih.gov/24859313/)
83. Martins VP, Pinheiro CS, Figueiredo BC, Assis NR, Morais SB, Caliar MV, et al. (2012) Vaccination with enzymatically cleaved GPI-anchored proteins from *Schistosoma mansoni* induces protection against challenge infection. *Clinical and Developmental Immunology* 962538.
84. Araujo-Montoya BO, Rofatto HK, Tararam CA, Farias LP, Oliveira KC, Verjovski-Almeida S, et al. (2011) *Schistosoma mansoni*: molecular characterization of Alkaline Phosphatase and expression patterns across life cycle stages. *Experimental Parasitology* 129: 284–291. doi: [10.1016/j.exppara.2011.07.008](https://doi.org/10.1016/j.exppara.2011.07.008) PMID: [21784070](https://pubmed.ncbi.nlm.nih.gov/21784070/)
85. Don TA, Bethony JM, Loukas A (2008) Saposin-like proteins are expressed in the gastrodermis of *Schistosoma mansoni* and are immunogenic in natural infections. *International Journal of Infectious Diseases* 12: e39–e47. doi: [10.1016/j.ijid.2007.10.007](https://doi.org/10.1016/j.ijid.2007.10.007) PMID: [18571965](https://pubmed.ncbi.nlm.nih.gov/18571965/)
86. Lee J-Y, Cho P-Y, Kim TY, Kang S-Y, Song K-Y, Hong S-J (2002) Hemolytic activity and developmental expression of pore-forming peptide, clonorin. *Biochemical and biophysical research communications* 296: 1238–1244. PMID: [12207906](https://pubmed.ncbi.nlm.nih.gov/12207906/)
87. Espino AM, Hillyer GV (2003) Molecular cloning of a member of the *Fasciola hepatica* saposin-like protein family. *Journal of Parasitology* 89: 545–552. PMID: [12880256](https://pubmed.ncbi.nlm.nih.gov/12880256/)
88. Schramm G, Falcone FH, Gronow A, Haisch K, Mamat U, Doenhoff MJ, et al. (2003) Molecular characterization of an interleukin-4-inducing factor from *Schistosoma mansoni* eggs. *Journal of Biological Chemistry* 278: 18384–18392. PMID: [12624091](https://pubmed.ncbi.nlm.nih.gov/12624091/)
89. Schramm G, Gronow A, Knobloch J, Wippersteg V, Grevelding CG, Galle J, et al. (2006) IPSE/alpha-1: a major immunogenic component secreted from *Schistosoma mansoni* eggs. *Molecular and Biochemical Parasitology* 147: 9–19. PMID: [16480783](https://pubmed.ncbi.nlm.nih.gov/16480783/)
90. Delcroix M, Sajid M, Caffrey CR, Lim K-C, Dvořák J, Hsieh I, et al. (2006) A multienzyme network functions in intestinal protein digestion by a platyhelminth parasite. *Journal of Biological Chemistry* 281: 39316–39329. PMID: [17028179](https://pubmed.ncbi.nlm.nih.gov/17028179/)
91. Koehler JW, Morales ME, Shelby BD, Brindley PJ (2007) Aspartic protease activities of schistosomes cleave mammalian hemoglobins in a host-specific manner. *Memórias do Instituto Oswaldo Cruz* 102: 83–85. PMID: [17294005](https://pubmed.ncbi.nlm.nih.gov/17294005/)
92. Morales ME, Rinaldi G, Gobert GN, Kines KJ, Tort JF, Brindley PJ (2008) RNA interference of *Schistosoma mansoni* cathepsin D, the apical enzyme of the hemoglobin proteolysis cascade. *Molecular and Biochemical Parasitology* 157: 160–168. PMID: [18067980](https://pubmed.ncbi.nlm.nih.gov/18067980/)
93. Stack C, Dalton JP, Robinson MW (2011) The phylogeny, structure and function of trematode cysteine proteases, with particular emphasis on the *Fasciola hepatica* cathepsin L family. *Cysteine proteases of pathogenic organisms*: Springer. pp. 116–135.
94. Yoshino TP, Lodes MJ, Rege AA, Chappell CL (1993) Proteinase activity in miracidia, transformation excretory-secretory products, and primary sporocysts of *Schistosoma mansoni*. *J Parasitol* 79: 23–31. PMID: [8437057](https://pubmed.ncbi.nlm.nih.gov/8437057/)

95. Chewawiwat N, Yano M, Terada K, Hoogenraad NJ, Mori M (1999) Characterization of the novel mitochondrial protein import component, Tom34, in mammalian cells. *The Journal of Biochemistry* 125: 721–727. PMID: [10101285](#)
96. Hill K, Model K, Ryan MT, Dietmeier K, Martin F, Wagner R, et al. (1998) Tom40 forms the hydrophilic channel of the mitochondrial import pore for preproteins [see comment]. *Nature* 395: 516–521. PMID: [9774109](#)
97. Chiang CP, Caulfield JP (1989) Human lipoprotein binding to schistosomula of *Schistosoma mansoni*. Displacement by polyanions, parasite antigen masking, and persistence in young larvae. *The American Journal of Pathology* 135: 1015–1024. PMID: [2596569](#)
98. McVeigh P, Mair GR, Atkinson L, Ladurner P, Zamanian M, Novozhilova E, et al. (2009) Discovery of multiple neuropeptide families in the phylum Platyhelminthes. *International journal for parasitology* 39: 1243–1252. doi: [10.1016/j.ijpara.2009.03.005](#) PMID: [19361512](#)
99. Hřčkova G, Velebný S, Halton DW, Day TA, Maule AG (2004) Pharmacological characterisation of neuropeptide F (NPF)-induced effects on the motility of *Mesocostoides corti* (syn. *Mesocostoides vogae*) larvae. *International journal for parasitology* 34: 83–93. PMID: [14711593](#)
100. Nässel DR, Wegener C (2011) A comparative review of short and long neuropeptide F signaling in invertebrates: Any similarities to vertebrate neuropeptide Y signaling? *Peptides* 32: 1335–1355. doi: [10.1016/j.peptides.2011.03.013](#) PMID: [21440021](#)
101. McVeigh P, Mair GR, Novozhilova E, Day A, Zamanian M, Marks NJ, et al. (2011) Schistosome I/Lamides—A new family of bioactive helminth neuropeptides. *International journal for parasitology* 41: 905–913. doi: [10.1016/j.ijpara.2011.03.010](#) PMID: [21554884](#)