Transcriptomic responses of *Biomphalaria pfeifferi* to *Schistosoma mansoni*: Investigation of a neglected African snail that supports more *S. mansoni* transmission than any other snail species

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Abstract

**Background**

*Biomphalaria pfeifferi* is highly compatible with the widespread human-infecting blood fluke *Schistosoma mansoni* and transmits more cases of this parasite to people than any other snail species. For these reasons, *B. pfeifferi* is the world's most important vector snail for *S. mansoni*, yet we know relatively little at the molecular level regarding the interactions between *B. pfeifferi* and *S. mansoni* from early-stage sporocyst transformation to the development of cercariae.

**Methodology/Principal findings**

We sought to capture a portrait of the response of *B. pfeifferi* to *S. mansoni* as it occurs in nature by undertaking Illumina dual RNA-Seq on uninfected control *B. pfeifferi* and three intramolluscan developmental stages (1- and 3-days post infection and patent, cercariae-producing infections) using field-derived west Kenyan specimens. A high-quality, well-annotated *de novo* *B. pfeifferi* transcriptome was assembled from over a half billion non-*S. mansoni* paired-end reads. Reads associated with potential symbionts were noted. Some infected snails yielded fewer normalized *S. mansoni* reads and showed different patterns of transcriptional response than others, an indication that the ability of field-derived snails to support and respond to infection is variable. Alterations in transcripts associated with reproduction were noted, including for the oviposition-related hormone ovipostatin and enzymes involved in metabolism of bioactive amines like dopamine or serotonin. Shedding snails exhibited responses consistent with the need for tissue repair. Both generalized stress and immune factors immune factors (VIGLs, PGRPs, BGBP, complement C1q-like, chitinases) exhibited complex transcriptional responses in this compatible host-parasite system.
Significance

This study provides for the first time a large sequence data set to help in interpreting the important vector role of the neglected snail *B. pfeifferi* in transmission of *S. mansoni*, including with an emphasis on more natural, field-derived specimens. We have identified *B. pfeifferi* targets particularly responsive during infection that enable further dissection of the functional role of these candidate molecules.

Author summary

*Biomphalaria pfeifferi* is the world’s most important snail vector for the widespread human-infecting blood fluke *Schistosoma mansoni*. Despite this, we know relatively little about the biology of this highly compatible African snail host of *S. mansoni*, especially for specimens from the field. Using an Illumina-based dual-seq approach, we captured a portrait of the transcriptional responses of Kenyan snails that were either uninfected with *S. mansoni*, or that harbored 1-day, 3-day, or cercariae-producing infections. Responses to infection were influenced both by the extent of schistosome gene expression and infection duration. We note and discuss several alterations in transcriptional activity in immune, stress and reproduction related genes in infected snails and the *B. pfeifferi* symbionts detected. Several host genes were highly up-regulated following infection and these might comprise excellent candidates for disruption to diminish compatibility. This study provides for the first time a large sequence dataset to help in interpreting the important vector role of *B. pfeifferi* in transmission of *S. mansoni*, including with an emphasis on more natural, field-derived specimens.

Introduction

Schistosomiasis is one of the world’s most prevalent neglected tropical diseases with over 218 million people worldwide requiring preventive chemotherapy in 2015, 92% of those occurring in 41 countries in Africa [1]. Human schistosomiasis has a greater public health impact than usually appreciated [2], often with a disproportionate impact on children, in whom it can cause both cognitive and physical impairments [3–6]. There is a growing consensus that we need to supplement chemotherapy with other control methods, including control of the obligatory molluscan intermediate host of schistosomes [7–10]. Snail control has been identified as an important component of the most successful control programs [11].

Among the most important schistosome species infecting humans and the one with the broadest geographical range is *Schistosoma mansoni*. *Biomphalaria pfeifferi* is one of 18 *Biomphalaria* species known to transmit *S. mansoni*. *Biomphalaria pfeifferi* has a broad geographic distribution in sub-Saharan Africa where the majority of cases of *S. mansoni* occur and exhibits a high degree of susceptibility to *S. mansoni* [12–16]. For instance, *B. pfeifferi* typically shows high infection rates (50%+) following exposure to *S. mansoni* from locations throughout Africa, but even to isolates originating from the Americas [12]. For these reasons, it can be argued that *B. pfeifferi* is the world’s most important intermediate host for *S. mansoni*. Understanding the role of *B. pfeifferi* in human schistosomiasis transmission becomes more critical because expanding agriculture and water development schemes [17] and climate change...
[18,19] threaten to alter the geographic range of both this snail species and of S. mansoni as well.

Given B. pfeifferi’s importance in transmission of S. mansoni, it is surprising we lack even the most basic information at the molecular level about its interactions with, and responses to, S. mansoni. Such responses could be particularly interesting in the case of B. pfeifferi because it differs from other major S. mansoni-transmitting snail species in that it is a strong preferential selfing species, a characteristic potentially resulting in low genetic diversity within populations [20–23]. Our relative ignorance regarding B. pfeifferi reflects the simple fact that it is often difficult to maintain this species in the laboratory, in contrast to the Neotropical snail B. glabrata which has been the standard model laboratory snail host for S. mansoni for decades [24]. Biomphalaria glabrata surely remains an important intermediate host of S. mansoni in the Neotropics, but given that the vast majority of S. mansoni cases occur in sub-Saharan Africa, it is critical that we extend more attention to the relevant African snail, B. pfeifferi.

The advent of genomics approaches including high throughput sequencing techniques have lead over the past decade to several studies of Biomphalaria snails and their interactions with S. mansoni and other trematodes including echinostomes. All of these studies have been undertaken with B. glabrata and have been amply reviewed and discussed [25–36]. In addition, the report of the international consortium on the Biomphalaria glabrata genome has now been published [37]. Ironically, the African Biomphalaria species that are responsible for transmitting the most S. mansoni infections by far have been largely ignored with respect to application of modern high-throughput sequence-based tools.

Projects going beyond the study of individual genes or gene families of B. glabrata began with studies of expressed sequence tags [38–40], ORESTES studies [41,42], and then microarrays [43,44]. These studies showed B. glabrata has the capacity for more diverse immune responsiveness than previously known, including production of diversified molecules like FREPs (fibrinogen-related proteins) [28,45,46]. Hanington et al. [47] examined the transcriptional responses of B. glabrata during the intramolluscan development of both S. mansoni and Echinostoma paraensei, and showed snail defense-related transcripts were generally down-regulated starting shortly after infection. A later generation array including ~31,000 ESTs from B. glabrata provided new insights into how the APO or amebocyte-producing organ of B. glabrata responds to immune challenge [48], and to the effects on B. glabrata transcriptional responses of the molluscicide niclosamide that is commonly used for snail control operations [49].

Additional recent studies of the interactions between B. glabrata and S. mansoni have focused on genetic linkage studies to identify chromosome regions of interest that contain genes influencing resistance to infection [32,50,51]. Functional studies have also used RNAi to knock-down particular B. glabrata gene products shown to influence susceptibility to S. mansoni [30–32,52].

Relevant to the present study, Deleury et al. [53] published the first Illumina sequencing study with B. glabrata, and identified 1,685 genes that exhibited differential expression after immune challenge. More recent studies employing RNA-Seq have identified B. glabrata genes associated with a state of heightened innate immunity [54] or with differential response of FREPs in B. glabrata strains differing in their susceptibility to S. mansoni [34]. Despite the fairly extensive efforts with respect to gene and genomic sequencing, gene profiling, or transcriptomics for B. glabrata and to a lesser extent for Oncomelania hupensis [55,56], the snail host of Schistosoma japonicum, to date there have been no equivalent studies published for B. pfeifferi, or for other schistosome-transmitting planorbid snails, including species of Bulinus, several of which transmit members of the Schistosoma haematobium species group in Africa, southern Europe and southwest Asia.
With this in mind, we have undertaken an Illumina RNA-Seq study of *B. pfeifferi*, and of *B. pfeifferi* infected with *S. mansoni* for 1 or 3 days, or with naturally acquired cercariae-shedding or "patent" infections. The intramolluscan transcriptional responses of *S. mansoni* will be the subject of a separate paper. The challenge of parsing *S. mansoni* sequences from the aggregate of reads obtained from infected *B. pfeifferi* has been aided by availability of the *S. mansoni* genome [57] and stage-specific transcriptional studies for *S. mansoni* [58–60].

Our view of schistosome-snail encounters has also been largely formed by studies of lab-reared snails and schistosomes. RNA-Seq offers a way to bridge and expand upon these traditional views by revealing the detailed molecular and cellular mechanisms taking place in genetically diverse hosts and parasites. This is the first Illumina study performed on samples of both field-derived vector snails and their corresponding schistosome parasites, adding a unique perspective to our understanding of schistosome transmission "in the wild" in endemic regions. This approach also serves to remind us that the snails targeted for infection by schistosome miracidia in the field are best considered as holobionts with potentially complex sets of symbiotic associates [61,62]. Finally, we note that this study will add to the literature a considerable amount of new data for *B. pfeifferi*, an important neglected vector species that has hitherto been understudied. Included among the snail genes highlighted are several that relate to stress, immune or reproductive functions, or that may be key players in influencing the noteworthy widespread ability of this snail to support schistosomiasis transmission.

**Methods**

**Ethics and permissions statements**

We enrolled human subjects who provided fecal samples containing *Schistosoma mansoni* eggs that were hatched to obtain miracidia used to infect some of the *Biomphalaria pfeifferi* snails used in this study. Fecal samples were obtained and pooled from five *S. mansoni*-positive primary school children aged 6–12 years from Obuon primary school in Asao, Nyakach area, Nyanza Province, western Kenya (00°19'01"S, 035°00'22"E). Written and signed consent was given by parents/guardians for all children. The KEMRI Ethics Review Committee (SSC No. 2373) and the UNM Institution Review Board (IRB 821021–1) approved all aspects of this project involving human subjects. All children found positive for *S. mansoni* were treated with praziquantel following standard protocols. Details of recruitment and participation of human subjects for fecal collection are described in Mutuku et al. [15]. This project was undertaken with approval of Kenya’s National Commission for Science, Technology, and Innovation (permit number NACOSTI/P/15/9609/4270), National Environment Management Authority (NEMA/AGR/46/2014) and an export permit has been granted by the Kenya Wildlife Service (0004754).

**Sample collection and experimental exposures**

*Biomphalaria pfeifferi* used in Illumina sequencing were collected from Kasabong stream in Asembo Village, Nyanza Province, western Kenya (34.42037°E, 0.15869°S) in November 2013. Snails were transferred to our field lab at The Centre for Global Health Research (CGHR) at Kisian, western Kenya. Snails sized 6-9mm in shell diameter were placed into 24-well culture plates and exposed to natural light to check for the shedding of digenetic trematode cercariae, including cercariae of *S. mansoni* [15]. Snails found to be shedding cercariae of other digenetic trematode species were excluded from this study.

Snails shedding *S. mansoni* cercariae and non-shedding snails (controls) were separated and held for one day in aerated aquaria containing dechlorinated tap water and boiled leaf lettuce. After cleaning shells with 70% EtOH, whole shedding and control snails were placed
individually into 1.5ml tubes with 1ml of TRIzol (Invitrogen, Carlsbad CA) and stored at -80˚C until extraction.

*Biomphalaria pfeifferi* confirmed to be uninfected were exposed to *S. mansoni* using standard methods to hatch the parasite eggs [15]. Snails were individually exposed to 20 miracidia for 6 hours in 24-well culture plates and then returned to aquaria. At 1 and 3 days post-infection (d), snails were collected and stored in TRIzol as described above. We chose not to maintain the field-derived snails for longer intervals post-infection as we did not want them to lose their unique field-associated properties while maintained in laboratory aquaria.

In addition to the Illumina RNA-Seq samples indicated above and mentioned throughout this study, we have RNA-Seq data from *B. pfeifferi* obtained from two 454 GS FLX (Roche, Basel Switzerland) runs and six Illumina-sequenced *B. pfeifferi* exposed to molluscicide, all field-derived from Kenya (Table 1). These reads were used to aid assembly of the *B. pfeifferi de novo* transcriptome and were not included in expression studies.

### RNA extraction, library preparation, and sequencing

Individual snails stored in TRIzol were homogenized using plastic pestles (USA Scientific, Ocala FL). For each biological treatment (control, 1d, 3d, and shedding), total RNA was purified separately from three individual snails (each snail a biological replicate) using the TRIzol protocol provided by the manufacturer (Invitrogen, Carlsbad CA). RNA samples were further purified using the PureLink RNA Mini Kit (ThermoFisher Scientific, Waltham MA). Genomic DNA contamination was removed with RNase-free DNase I (New England BioLabs, Ipswich MA) at 37˚C for 10 minutes. This combination method based on the two RNA extraction assays had been developed in our lab and proved to produce a high quality of RNA from snail

| Field-collected samples | Replicate | Abbreviation | Paired-end reads mapping to *S. mansoni* genome ‡ | Paired-End Reads/Sample (post-quality filtering) |
|-------------------------|-----------|--------------|-----------------------------------------------|-----------------------------------------------|
| *B. pfeifferi* control  | 1         | control-R1   | 0.07%                                         | 28,903,992                                   |
|                         | 2         | control-R2   | 0.08%                                         | 34,318,971                                   |
|                         | 3         | control-R3   | 0.04%                                         | 27,557,936                                   |
| *B. pfeifferi* x *S. mansoni* 1 day post infection (1d) | 1         | 1d-R1        | 0.1%                                          | 36,450,649                                   |
|                         | 2         | 1d-R2        | 1.5%                                          | 33,634,117                                   |
|                         | 3         | 1d-R3        | 1.9%                                          | 30,932,207                                   |
| *B. pfeifferi* x *S. mansoni* 3 days post infection (3d) | 1         | 3d-R1        | 4.1%                                          | 30,648,913                                   |
|                         | 2         | 3d-R2        | 0.1%                                          | 26,445,297                                   |
|                         | 3         | 3d-R3        | 13.2%                                         | 31,159,822                                   |
| *B. pfeifferi* shedding *S. mansoni* (S) | 1         | shedding-R1  | 3.7%                                          | 32,200,842                                   |
|                         | 2         | shedding-R2  | 8.2%                                          | 33,570,583                                   |
|                         | 3         | shedding-R3  | 0.5%                                          | 27,569,638                                   |
| *B. pfeifferi* control x molluscicide | 1         | *            | *                                             | 35,289,769                                   |
|                         | 2         | *            | *                                             | 34,450,509                                   |
|                         | 3         | *            | *                                             | 25,652,418                                   |
| *B. pfeifferi* shedding *S. mansoni* x molluscicide | 1         | *            | *                                             | 30,587,208                                   |
|                         | 2         | *            | *                                             | 35,071,339                                   |
|                         | 3         | *            | *                                             | 28,843,961                                   |

*Samples used in the assembly but expression results not discussed in this paper

‡ See Methods for explanation of *S. mansoni* read mapping

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samples [47]. RNA quality and quantity was evaluated on a Bioanalyzer 2100 (Agilent Technologies, Santa Clara CA) and Nanodrop 2000 (ThermoFisher Scientific, Waltham MA).

Complementary DNA (cDNA) synthesis and Illumina Hi-Seq sequencing was performed at the National Center for Genome Resources (NCGR) in Santa Fe, NM. Most liquid handling was performed by a Sciclone G3 Automated Liquid Handling Workstation (Caliper Life Sciences, Hopkinton MA) with Multi TEC Control (INHECO, Martinsried Germany). Synthesis of cDNA and library preparation was prepared using Illumina TruSeq protocol according to the manufacturer’s instructions (Illumina, Carlsbad CA). Complementary DNA libraries were paired-end sequenced (2x 50 base reads) on a HiSeq2000 instrument (Illumina, Carlsbad CA).

Pre-processing of Illumina reads and isolation of *B. pfeifferi* reads

Sequencing adapters, nucleotides with a Phred quality score <20 within a sliding window of 4bp, and non-complex reads were removed using Trimmomatic v.0.3 [63]. Raw read quality control checks were performed before and after Trimmomatic filtering using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/).

To reduce assembly of chimeric transcripts, we created a novel pipeline to separate reads of related organisms when only one organism has a sequenced genome while also allowing for recovery of shared reads (Fig 1). First, all reads (including control samples) that passed quality filtering were aligned to the *S. mansoni* genome (GeneDB: *S. mansoni* v5.0) using STAR v.2.5 2-pass method [64] or Tophat v.2 [65] (see Table 1 for alignment percentages). From examination of the percentage values in Table 1, it may be interpreted that unexposed control actually harbor *S. mansoni*. However, the reads contributing to the positive percentage values for the controls are ones that we have found to be shared with either *B. glabrata* or another organism such that they represent a background level of sequence similarity obtained by chance. Although partial mapping of reads may occur, none appear to be expressed *S. mansoni* transcripts. None of the unexposed control reads mapping to the *S. mansoni* genome are unequivocally *S. mansoni*. By contrast, *S. mansoni*-exposed snails (1d, 3d, shedding) all expressed bona fide *S. mansoni* genes. Only in 1d, 3d, and shedding snails were transcripts clearly distinctive to *S. mansoni* found, such as venom allergen proteins (SmVal) (Accessions: AAY43182.1, AAY28955.1, AAZ04924.1, ABO09814.2), tegument allergen-like proteins (Accession: P14202), and cercarial stage-specific proteins (Accession: ABS87642.1), verifying the presence of a *S. mansoni* infection. This explanation also serves to verify that individual snails (such as 1dR2) with low *S. mansoni* percentages were indeed infected, such that they could be expected to be responsive to infection. Therefore, relatively low *S. mansoni* genome mapping, especially for shedding-R3, should not be interpreted that the infection was not successful, but rather as an indication of the transcriptional activity.

Reads that mapped to *S. mansoni* were also cross-examined by mapping to the version BglA1 of the *B. glabrata* genome (https://www.vectorbase.org/organisms/biomphalaria-glabrata) using STAR. Reads that first mapped to *S. mansoni* and then also to *B. glabrata* were determined to be shared reads and added to the reads destined for *B. pfeifferi* transcriptome de novo assembly.

One issue encountered was to deal with both paired- and single-end reads resulting from initial quality filtering and from discordant or single-mate mapping to the *S. mansoni* genome. Pseudo-mate reads were created to allow maximum read usage in all stages of analysis (details and script available at https://github.com/lijingbu/RNA-Seq-Tools). This tool, pseudoFastqMate.pl, creates pseudo mate reads for single reads in a fastq file by generating a string of N's the same length and quality score as its mate read. Reads entirely made up with Ns were ignored during the mapping process and have no impact on the final alignment and read counts.
De novo transcriptome assembly and annotation

Unaligned paired and unpaired reads, determined not to solely belong to *S. mansoni*, were assembled using Trinity v2.2 RNA-Seq *de novo* assembler [66,67]. Trinity *de novo* and *B. pfeifferi* transcriptomic expression from dual RNA-Seq data.
glabrata genome-guided assemblies were employed to maximize the chances of recovering unique B. pfeifferi transcripts. The de novo assemblies were concatenated and redundancy reduced using the EvidentialGene tr2aacsds pipeline [68]. EvidentialGene determines the best set of transcripts based on the coding potential of transcripts generated from multiple assemblies. Only primary transcripts, denoted in EvidentialGene as “okay” and “okalt” were used in further analysis. In silico translation of the transcriptome was done using TransDecoder v3.0 (https://transdecoder.github.io) [65] to extract long open reading frames (ORFs) and identify ORFs with homology to known proteins with blast and pfam searches.

Biomphalaria pfeifferi CDS were annotated based on their closest homologs and predicted functional domains in the following databases and tools: BLASTp with NCBI non-redundant protein database (sequence identity >30%, E-value <10^{-06}), BLASTn with NCBI nucleotide database (sequence identity >70%, E-value <10^{-06}), Gene Ontology [69], KEGG [70], and InterProScan5 [71]. For query CDS whose top hit was “uncharacterized”, “hypothetical”, or otherwise unknown, the consensus hit (of up to 20 hits that also meet minimum sequence identity and E-value requirements shown above is reported to help elucidate any putative function. Additionally, B. pfeifferi CDS were further scrutinized against molluscan transcripts and proteins identified in the literature.

Identification of non-snail and non-parasite reads

As a consequence of sequencing field-collected specimens, we expected some reads to be of non-B. pfeifferi and non-S. mansoni origin. Screening for the presence of third party symbionts was one of our motivations for investigating field-derived snails in the first place. We performed the de novo assembly pipeline without first removing non-snail or non-schistosome sequences to get a more complete view of the complex environment in which S. mansoni development takes place. CDS coverage, sequence identity, and E-value of BLASTn, BLASTp, and MEGABLAST results were all taken into consideration when determining organism identification. The BLASTn and MEGABLAST against the NCBI nucleotide database had minimum sequence identity of 70% and E-value <10^{-06} and the BLASTp against the NCBI protein database had a minimum sequence identity of 30% and E-value <10^{-06}. Query coverage (qcov) was also calculated in all BLASTs. When different BLASTs disagreed in their taxonomic assignment, the hit with highest percent query coverage, highest sequence identity, and lowest E-value was chosen, in that order. Although minimum parameters were set, nearly all CDS BLAST hits exceeded these bounds. BLASTp hits tended to have better quality hits because nucleotide sequences from the NCBI nucleotide database often contained non-coding regions that our CDS lack. CDS designated as “undetermined” had hits that did not meet minimum BLAST parameters. CDS that had a non-molluscan BLAST hit but still mapped to the B. glabrata genome (sequence identity >70%, E-value <10^{-06}) were considered “shared” sequences.

Non-B. pfeifferi and non-S. mansoni CDS were categorized into 14 broad taxonomic groups: Mollusca, Amoebozoa, SAR, Viruses, Plantae, Fungi, Bacteria, Rotifera, Platyhelminthes, Arthropoda, Annelida, Nematoda, Chordata, and Miscellaneous. Potential trematode CDS were further filtered to require a minimum of 70% query coverage. Genomes and CDS of specific symbionts of interest (if publicly available) were interrogated using BLASTn (>70% identity, E-value <10^{-06}, query coverage >70%).

Identification of toll-like receptors (TLR) and variable immunoglobulin lectins (VIgLs)

Given that a number of previous studies of Biomphalaria immunobiology have focused on molecules with TLR or immunoglobulin domains, we undertook an analysis of these groups of
molecules. *Biomphalaria pfeifferi* CDS with a BLASTp or BLASTn annotation as a toll-like receptor (TLR), were further screened for toll/interleukin-1 receptor (TIR), leucine-rich repeats (LRR), and transmembrane regions with InterProScan5 and TMHMM (Transmembrane helix prediction based on hidden Markov model) [72]. CDS identified as complete TLRs contained TIR, transmembrane, and LRR domains. Similarly, CDS annotated as a VgI-L (FREPs, CREPs, GREPs, and FREDs) were scanned for an immunoglobulin domain and a fibrinogen, C-type lectin, or galectin domain using InterProScan5. For CDS to be identified as a FREP, CREP, or GREP, they had to contain a lectin domain and at least one immunoglobulin domain.

**Transcriptome completeness**

To estimate the completeness of our *B. pfeifferi* transcriptome assembly and assess similar transcripts across related species, *B. pfeifferi* predicted ORFs were compared to other molluscan peptides (the cephalopod *Octopus bimaculoides*, the oysters *Crassostrea gigas* and *Pinnaquida fucata*, the owl limpet *Lottia gigantea*, the California sea hare *A. californica*, as well as two pulmonates: *B. glabrata* and *Radix balthica*) using BLASTp (sequence identity >30%, E-value <10^−06). ORFs with 100 or more amino acids were extracted from each transcriptome. To maximize sensitivity for retaining ORFs that may have functional significance, predicted ORFs were scanned for homology to known proteins in the Uniref90 database with a subsequent search using PFAM and hmmer3 to identify protein domains.

**Differential expression analyses**

Properly paired reads not filtered as *S. mansoni* were mapped to EvidentialGene-generated *B. pfeifferi* CDS with Bowtie2 [73]. Read abundance was quantified with RSEM (RNA-Seq by expectation maximization) [74]. Pairwise analyses for comparisons between control group and other infected groups were run in EBSeq [75]. Transcripts with a posterior probability of differential expression (PPDE) \( \geq 0.95 \) were considered significant. With the aim of detecting less abundant transcripts that may still have significant biologically effects (i.e. neuropeptides), we deliberately did not set a minimum read count threshold for detection of DE CDS in EBSeq.

**Variation among infected snails with respect to representation of *S. mansoni* reads, and testing among them for associated differences in host responses**

As noted above, field-collected specimens of both snails and schistosomes are naturally more genetically diverse than lab-reared counterparts, so variation in response among infected snails might be expected. In fact, by chance, for each of the time points studied, one of the 3 infected snails examined differed notably from the other two in having fewer normalized *S. mansoni* read counts (suggestive of less extensive parasite activity and/or more effective host limitation of parasite development). We hypothesized that the snail response is influenced by the extent of *S. mansoni* representation, as assessed by examining normalized parasite read counts from each infected snail. In addition to doing “3 controls vs. 3 infected” (3v3) comparisons, for each time point we also examined “3 control vs. 2 infected” (3v2) comparisons where the two snails harbored higher *S. mansoni* read counts to identify CDS whose responses were associated with *S. mansoni* abundance. We also performed “3 control vs. 1 infected” (3v1) comparisons where the one infected snail was the one with low *S. mansoni* read counts. The overall DE results
include all CDS that were differentially expressed in any of the three comparisons, the results for each comparison being separately singled out and enumerated.

Quantitative PCR validation of differential expression

cDNA was synthesized from 5μg of total RNA from the original samples by the SuperScript II First-Strand Synthesis Kit for RT-PCR (Invitrogen) in a 20μl reaction using random hexamers. Manufacturer directions were followed for the reaction profile. An additional 80μl of molecular grade water was added to the cDNA for a final volume of 100μl. qPCR target primer sequences were generated in Primer3 software [76] and details are shown in S1 Table. We tested probes for single-copy genes only and final selection of qPCR targets were chosen to highlight the variability between replicates. Primer testing verified one product was produced in traditional PCR amplification and in melt curve analyses. RT-qPCR reactions were performed in 20μl reactions according to manufacturer’s directions using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules CA) with 0.5μM primer concentration and 2μl cDNA. Reactions were denatured at 95°C for 2 minutes followed by 40 cycles of 95°C for 5 seconds and annealing/extension and plate read for 30 seconds. Melt curve analysis was performed from 65–95°C at 0.5°C increments for 5 seconds. All biological replicates were run in technical triplicate for each transcript on a Bio-Rad CFX96 system and analyzed with Bio-Rad CFX Manager software.

Results

Transcriptome sequencing, assembly, and annotation

To investigate the gene expression profiles of *B. pfeifferi* following infection with *S. mansoni*, we analyzed the transcriptome from Illumina sequencing of infected snails at 1-day (1d), 3-day (3d), and from shedding snails using three biological replicates each (Table 1). The raw and assembled sequence data are available at NCBI under BioProject ID PRJNA383396. The results and statistics describing the *B. pfeifferi* assembly are summarized in Table 2. Trinity *de novo* transcript assemblies and additional reads from two 454 runs resulted in 1,856,831 contigs. The EvidentialGene program generated a non-redundant *B. pfeifferi* transcriptome of 194,344 protein-coding sequences (CDS) that includes isoforms. From nucleotide sequence length histograms, we calculated that more than half of the CDS were between 300–499 nucleotides with 6.7% > = 1500 nucleotides (S1 Fig).

Five publicly available databases were used to annotate and obtain functional information for the CDS (S1 File; Table 3). The top 20 most common GO assignments are shown in S2 Fig. Six KEGG categories are shown with their constituent classes organized by abundance in S3 Fig. Altogether, 179,030 of 194,344 total (92.1%) CDS were annotated from at least one of the five databases shown in Table 3.

Identification of toll-like receptors (TLRs) and variable immunoglobulin lectins (VIGLs)

Pattern recognition receptors like TLRs and VIGLs (FREP, CREPS, and GREPs) are key components of the innate immune response and their involvement in the *B. glabrata* defense response has been documented [28,77]. The *B. glabrata* genome contains 56 TLR (toll-like receptor) genes, 27 of which encode complete TLRs [37]. Our *B. pfeifferi* transcriptome had 190 CDS annotated as a homolog to a *B. glabrata* TLR (Fig 2). Note that numbers assigned to TLRs in *B. glabrata* were assigned in the order they were identified and not by homology to vertebrate TLRs. The TLR numbers we refer to for *B. pfeifferi* match most closely the TLR with...
the corresponding number from *B. glabrata*. InterProScan5 analysis revealed 78 of *B. pfeifferi* TLR CDS contain a TIR (toll/interleukin receptor) domain and 118 have at least one LRR (leucine-rich repeat) domain. In total, we found 48 complete *B. pfeifferi* TLRs (TIR, transmembrane, LRR domains all present) and 142 partial homologs to *B. glabrata* TLRs (annotated as a TLR, but not all domains complete and/or confidently identified) in our transcriptional study. Others may certainly exist in the genome of *B. pfeifferi*.

There are 22 FREP genes in the *B. glabrata* genome [37,77] and all were represented in our *B. pfeifferi* transcriptome, at least in part. Our BLAST annotations identified 249 *B. pfeifferi* CDS homologous to *B. glabrata* FREPs and 12 of these were verified to be full-length FREP homologs (Fig 3). There were no full-length, complete GREPs identified in our transcriptome, but there were 5 CDS with a BLAST annotation homologous to one *B. glabrata* GREP identified by Dheilly *et al.* [77] (Fig 3). Four CREPs (C-type lectin protein) have been identified in *B. glabrata* [77] with 2 of the 14 full-length, complete *B. pfeifferi* CDS homologous to CREP 1 in *B. glabrata* (Fig 3).

### Sequence homology between related mollusc species

A BLASTp comparison between *B. pfeifferi* and *B. glabrata* shows high sequence similarity with 35,150 (95.8%) polypeptides shared between the two species (sequence identity >30%
and E-value $<1e^{-06}$ (Table 4). We found 1,525 *B. glabrata* polypeptides without homologs in our *B. pfeifferi* transcriptome. With respect to the 127,626 translated CDS that have homologs to *B. glabrata* polypeptides, more than half of these have a sequence identity greater than 90% (S4 Fig). To further assess the completeness and to enhance annotation of our *B. pfeifferi* transcriptome, we searched for homologous polypeptides from genomes of two additional gastropods (*Aplysia californica* and *Lottia gigantea* [78]), two bivalves (*Pinctada fucata* [79]) and *Crassostrea gigas* [80]), and one cephalopod (*Octopus bimaculoides* [81]) (Table 4). Shown in S5 Fig is one hypothesis of the phylogeny of molluscs, and mapped onto this are the mollusc genomes that are currently available [82]. Note that the percent identity of homologous sequences follows the general branching pattern. The California sea hare, *A. californica*, has 88.3% of its polypeptides homologous to *B. pfeifferi* peptides. The most distantly related

| Public Database                  | Annotation Summary                      |
|----------------------------------|-----------------------------------------|
| BLASTp x nr                      | 140,484 CDSs (72.3%)                     |
|                                  | 49,518 unique protein identities        |
| BLASTn x nt                      | 128,028 CDSs (65.9%)                     |
|                                  | 26,708 unique nt identities             |
| InterProScan                     | 137,778 (70.9%)                          |
| Gene Ontology (GO)               | 50,870 CDSs (26.2%)                     |
| Unique Molecular Function        | 3,246                                    |
| Unique Cellular Component        | 1,618                                    |
| Unique Biological Process        | 8,282                                    |
| KEGG                             | 145,197 CDSs (74.7%)                    |
| Unique KEGG orthologous groups   | 3,824                                    |
| Unique KEGG pathways             | 387                                      |
| Unique KEGG classes              | 46                                       |
| Unique KEGG categories           | 6                                        |
| Cellular Processes               | 13,845                                   |
| Environmental Information Process| 16,093                                   |
| Genetic Information Processing   | 13,722                                   |
| Human Diseases                   | 32,748                                   |
| Metabolism                       | 41,022                                   |
| Organismal Systems               | 27,767                                   |

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Fig 2. Identification of the innate immune recognition receptors TLRs in *B. pfeifferi*. Partial CDS counts had a BLAST hit against a known TLR but all necessary domains could not be confidently determined by InterProScan5.

![Fig 2. Identification of the innate immune recognition receptors TLRs in *B. pfeifferi*. Partial CDS counts had a BLAST hit against a known TLR but all necessary domains could not be confidently determined by InterProScan5.](https://doi.org/10.1371/journal.pntd.0005984.g002)
mollusc, the California two-spot octopus, *O. bimaculoides*, is 56.7% homologous at the protein level to *B. pfeifferi*.

**Other organismal sequences derived from the de novo assembly**

Of the 194,344 CDS assembled post-*S. mansoni* read filtering, 18,907 (9.73%) of these were determined to be of non-mollusc origin (Fig 4). Some of the non-*B. pfeifferi* transcripts found were bacteria with most belonging to the genera *Escherichia*, *Mycoplasma*, *Aeromonas*, and *Pseudomonas* (Fig 5). Among them, a CDS with homology to *Neorickettsia* *sp.*, a known obligatory symbiont of digenetic trematodes [83], was recovered and has read counts >10 in 2 of our samples that also had relatively high counts of *S. mansoni* (3d-R3 and shedding-R1) (Table 1; S2 File). In addition, there are three CDS assembled from the infected 454 *B. pfeifferi* sample that were identified as *Paenibacillus* spp. and were similar, but not identical, to the snail pathogen *Candidatus* *Paenibacillus glabratella* (S2 File) [84].

Among the eukaryotic sequences retrieved from generation of the de novo assembly, there are some familiar snail symbionts listed in S2 and S3 Tables including 1) *Chaetogaster* annelids, 2) *Trichodina* ciliates, and 3) *Capsaspora owczarzaki* [85] and 4) microsporidians [86–89] (see also S2 File and Discussion for further comments).

**Table 4. Number of polypeptides queried in various molluscs and matches with *B. pfeifferi* TransDecoder-predicted ORFs.**

| Reference                    | # Reference polypeptides | *B. pfeifferi* polypeptides matched to reference polypeptides | Download location                                                                 |
|------------------------------|--------------------------|---------------------------------------------------------------|----------------------------------------------------------------------------------|
| *Biomphalaria glabrata* v1.0 [37] | 36,675                   | 127,626                                                      | [https://www.ncbi.nlm.nih.gov/genome/annotation_euk/Biomphalaria_glabrata/100/](https://www.ncbi.nlm.nih.gov/genome/annotation_euk/Biomphalaria_glabrata/100/) |
| *Aplysia californica* v3.0*  | 27,591                   | 99,884                                                       | [http://www.ncbi.nlm.nih.gov/genome/annotation_euk/Aplysia_californica/101/](http://www.ncbi.nlm.nih.gov/genome/annotation_euk/Aplysia_californica/101/) |
| *Lottia gigantea* v1.0 [78]  | 188,590                  | 74,494                                                       | [http://genome.jgi.doe.gov/Lotgi1/Lotgi1.download.ftp.html](http://genome.jgi.doe.gov/Lotgi1/Lotgi1.download.ftp.html) |
| *Pinctada fucata* v2.0 [79]  | 31,477                   | 77,341                                                       | [http://marinegenomics.oist.jp/pearl/viewer/download?project_id=36](http://marinegenomics.oist.jp/pearl/viewer/download?project_id=36) |
| *Crassostrea gigas* v9 [80]  | 45,406                   | 80,505                                                       | [ftp://ftp.ncbi.nlm.nih.gov/organisms/Crassostrea_gigas/](ftp://ftp.ncbi.nlm.nih.gov/organisms/Crassostrea_gigas/) |
| *Octopus bimaculoides* v2.0 [81] | 38,585                  | 71,395                                                       | [http://genome.jgi.doe.gov/pages/dynamicOrganismDownload.jsf?organism=Metazome](http://genome.jgi.doe.gov/pages/dynamicOrganismDownload.jsf?organism=Metazome) |

*Genome is publicly available at link provided*  
[https://doi.org/10.1371/journal.pntd.0005984.t004](https://doi.org/10.1371/journal.pntd.0005984.t004)
In addition to prokaryotes and eukaryotes, nearly 1,300 of our assembled CDS were provisionally identified as viruses (Fig 4). Sample Control-R2 had the highest abundance of reads mapping to the viral sequences compared to the other samples, though some putative viral sequences were recovered from all 12 snails examined.

Lastly, even after the initial screening and removal of *S. mansoni* reads from the nine snails with known *S. mansoni* infections, some reads remained that were classified as platyhelminth in origin (Fig 4). Two individual snails in particular, control-R3 and 3d-R2, the latter a replicate with low *S. mansoni* read counts, had many platyhelminth reads (Fig 5). We sequenced a 28S rRNA gene from cDNA of control-R3 using digenean-specific primers [90] to determine if other digeneans were present in our sample. The resulting 28S sequence was identified as belonging to the genus *Ribeiroia*, members of which are known to occur in East Africa and to infect *Biomphalaria* [91]. Most of the platyhelminth CDS present in this sample were identified as “hypothetical” but CDS with the highest read abundance are involved in membrane transport and cell structural functions. For 3d-R2, *cox1* mitochondrial gene primers amplified an amphistome sequence that groups phylogenetically with an amphistome species (provisionally *Calicophoron sukari*) that uses *B. pfeifferi* from East Africa as a first intermediate host [92]. Like control-R3, CDS with the highest read abundance in 3d-R2 were membrane associated and structural with the addition of several myoglobins and surface glycoprotein CDS.

**Variation among infected snails with respect to the representation of *S. mansoni* reads, and associated responses**

The extent of representation of *S. mansoni* in the dual transcriptome as measured by read counts is variable among the three replicates for each development time sampled in shedding snails (Table 1). Normalized read abundance of *S. mansoni* housekeeping genes remained
consistently high across all samples, eliminating the possibility that S. mansoni read count variability was due to sampling effects. Because of this inherent variability, we performed additional DE comparisons to the traditional 3 control v 3 experimental (3v3) replicates isolating either the two snails that contained higher S. mansoni read counts (3v2 analysis) or the one snail with the fewest S. mansoni read counts of each triplicate time point (3v1). With respect to the overall response patterns of snails that yielded either high or low numbers of S. mansoni reads, in most cases, for both up- and down-regulated CDS, the majority of significantly differentially expressed CDS fell into the 3v3 comparison category (Fig 6), indicative of uniformity of response across infected snails. For up-regulated features, there were also substantial additional numbers of significant CDS in the 3v2 or 3v1 infected categories, with the latter being greater in 2 of 3 cases. By contrast, for the down-regulated features, at 1d, the snails with high or low S. mansoni read counts did not as clearly differentiate from one another, but the snails with low read counts for S. mansoni (3v1) clearly showed an additional allotment of down-
regulated features. For the other two time points, the snails with high and low *S. mansoni* read counts did separate from one another, and especially noteworthy is the relatively small proportion of down-regulated features in the 3v1 comparisons.

### B. pfeifferi CDS responsive during *S. mansoni* infection

*S3 File* provides a summary of all CDS retrieved in the DE analysis, *S4 File* summarizes those general, reproduction or immune system features that were most differentially expressed, and Tables 5 and 6 distill CDS (see Discussion also) that we feel are most worthy of further functional study in *B. pfeifferi*. Multidimensional scaling (MDS) plots show that for each of the three groups of infected snails, overall transcript expression of the experimental groups is distinct from the control groups (Fig 7). At 1d, snails showed a slight preponderance of down-regulated over up-regulated CDS, but in both 3d and shedding snails, the opposite trend was observed (Fig 8A and 8B). Overall, the most transcriptional activity was in the 3d snails. All three groups of infected snails (1d, 3d, shedding) showed distinct transcriptional profiles, suggesting the snail response is different at each time point (Fig 8C). Generally, each of the three groups has more unique responsive CDS than they do in common with one another. As anticipated, 1d and 3d snails have more shared transcripts both up- and down-regulated than either do with the shedding snails.

It should also be noted that 59 CDS were up-regulated, and 63 CDS down-regulated in common to all three groups of infected snails (Fig 8C). Those up-regulated across time points include hemocytin, CD209 antigen-like, DBH-like monooxygenase, and a fibrinolytic enzyme. Some ubiquitously down-regulated features include neural cell adhesion molecule 1-like, a TNF receptor, peroxiredoxin 5, F-box/LRR repeat protein 4-like, the cytoprotective hypoxia up-regulated protein 1-like that is triggered by oxygen deprivation and oxidative stress, glutathione-S-transferase omega 1-like, type 1 serotonin receptor 5HT-1Hel, a feeding circuit activating peptide that induces feeding behavior [93], and TLR 7.

In addition to identifying those CDS up- or down-regulated in common to all three groups of infected snails, we also identified CDS not known to be related to reproduction or defense that exhibited the highest fold expression changes in shedding snails. Snail CDS most highly up-regulated may represent molecules essential for the parasite to sustain a patent infection, or conversely, those most strongly down-regulated may otherwise interfere with parasite development in ways we do not presently understand. A selected few, that had an annotation and were consistently expressed compared to controls in each replicate, are shown in Table 5.

### Table 5. Highly up- or down-regulated *B. pfeifferi* CDS whose response may be required for maintaining a patent *S. mansoni* infection.

| B. pfeifferi CDS            | Annotation                       | Log$_2$FC 3v3 | Log$_2$FC 3v2 | Log$_2$FC 3v1 |
|---------------------------|----------------------------------|---------------|---------------|---------------|
| evgTRINITY_DN89401_c6_g1_i1 | GD13313-like                     | 9.14          | 9.58          | 6.75          |
| evgTRINITY_DN19832_c0_g1_i1 | deleted in malignant brain tumors 1 protein-like | 6.65          | 7.09          | 4.48          |
| evgTRINITY_DN95353_c0_g1_i1 | collagen alpha-3(VI) chain-like  | 6.41          | 6.80          | 4.48          |
| evglcIGWVJS02FGR88         | cAMP-dependent prot kinase catalytic subunit-like | 6.17          | 6.60          | 4.15          |
| evgTRINITY_DN84392_c3_g1_i1 | galactocerebrosidase-like        | 5.41          | 5.53          | 5.14          |
| evgTRINITY_DN104940_c0_g1_i1| cAMP-dependent prot kinase catalytic subunit-like | 5.32          | 5.79          | 3.06          |
| evgTRINITY_DN84179_c0_g1_i1 | uncharacterized transporter slc-17.2-like | 5.31          | 5.21          | 5.20          |
| evgTRINITY_DN16840_c0_g1_i1 | papilin-like                     | 5.05          | 5.55          | 5.55          |
| evgTRINITY_GG_14665_c0_g1_i2| ctenidin-3-like                  | -5.43         | -4.89         | -4.89         |
| evgTRINITY_DN92655_c9_g2_i1 | deoxyribonuclease-1-like         | -4.84         | -4.63         | -4.63         |
| evgTRINITY_DN68720_c0_g1_i1 | testisin-like                    | -4.69         | -4.18         | -4.18         |

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Table 6. Highlights of general, reproductive, and immune responses of *B. pfeifferi* in response to *S. mansoni* infection.

| One day post-infection (1d) | General | Reproductive | Immune |
|----------------------------|---------|--------------|--------|
| **UP-REGULATED**            |         |              |        |
| Overall                     | phospholipase A2s | Na and Cl dependent glycoside transporter 2-like | dermatopontins |
|                            | endoglucanases | neuropeptide Y receptor type 5-like | ficolin-like proteins |
|                            | Proteases and protease inhibitors | DBH-like monoxygenase protein 1 | macrophage man rec 1-like isoform X1 |
|                            | Guanine nucleotide-binding protein-like 3 | C-type lectin-6 member A-like | |
|                            | Translationally-controlled tumor protein | acidic mammalian chitinase-like | |
|                            |                      | chitinase-3-like protein 1-like | |
|                            |                      | hemocytin | |
|                            |                      | laccase-15-like | |
|                            |                      | laccase-1-like | |
|                            |                      | tyrosinase-1-like | |
| Two snails with higher *S. mansoni* read counts | FMRF-amide receptor-like | Cu, Zn superoxide dismutase | |
|                            |                      | Tyrosinase-like protein tyr-1 | GTPase IMAP family members 4 and 7 |
|                            |                      | beta 1.3 glucan-bind protein-like precursor | |
|                            |                      | complement C1q-like protein | |
|                            |                      | fibrinogen-related protein 2 (FREP2) | |
| One snail with least *S. mansoni* read counts | ATP synthase FO6 | macrophage expressed gene-1 | |
|                            |                      | spermine oxidase | |
|                            |                      | glutathione-S-transferase | |
|                            |                      | laccase-2-like | |
| **DOWN-REGULATED**          |         |              |        |
| Overall                     | glyceraldehyde-3-phosphate dehydrogenase | ovipostatin 2 | FREP12 and its precursors |
|                            | respiratory pigment hemoglobin | tyramine/dopamine β-hydroxylase-like | toll-like receptor 8 |
|                            | insulin-like peptide 7 --modestly down | FMRF-amide isoform X2 --modestly down | cytidine deaminase |
|                            | pedal peptide 2 | PTSP-like molecule | zinc metalloproteinase/disintegrin-like |
|                            | Na dependent nutrient aa transporter 1-like | pheromone Alb-1 | |
|                            | enterin | type 1 serotonin receptor | |
|                            | FMRF-amide isoform X2 | schistosomin | |
|                            | cytidine deaminase | soma ferritins | |
| **COMPLEX (MIXED RESPONSES)** |         |              |        |
|                            | collagens | | |
|                            | acidic mammalian chitinase-like proteins | | |
|                            | cytochrome c oxidases | | |
|                            | Mucins | | |
|                            | Cytochrome P450 family members | | |
| Three day post-infection (3d) |         |              |        |
| **UP-REGULATED**            |         |              |        |

(Continued)
Table 6. (Continued)

| Overall | Overall phospholipase A2s | Na and Cl dependent glycine transporter 2-like | GTPase IMAPs complex, but mostly up |
| --- | --- | --- | --- |
| Overall proteases and protease inhibitors | endogluccanases | temptin-like | beta-1,3-glucan binding proteins |
| Overall 17-beta hydroxysteroid dehydrogenase type 6 | 17-beta hydroxysteroid dehydrogenase type 6 | kynurenine 3-monoxygenase-like | complement C1q-like proteins |
| Overall betaine homocysteine-methyltransferase 1-like | betaine homocysteine-methyltransferase 1-like | probable serine carboxypeptidases (1–5) |
| Overall translationally controlled tumor protein homolog | translationally controlled tumor protein homolog | glutathione S-transferases |
| Overall hemoglobin type 1 | hemoglobin type 1 | laccase-2-like |

| Two snails with higher S. mansoni read counts | insulin-related peptide-3-like | Tyrosinase-like protein tyr-1 | dermatopontins |
| --- | --- | --- | --- |
| Two snails with higher S. mansoni read counts cytochrome b | Na- and Cl-dependent taurine transporter-like | ficolins |
| Two snails with higher S. mansoni read counts serine proteases alpha and beta | dopamine receptor 2-like | Cu-Zn superoxide dismutases |
| Two snails with higher S. mansoni read counts ADP, ATP carrier-like protein | ADP, ATP carrier-like protein | C-type lectin domain family 6, A-like |
| Two snails with higher S. mansoni read counts heparinase-like isoform X1 | heparinase-like isoform X1 | chitinase-3-like protein |
| Two snails with higher S. mansoni read counts serpin B6-like | serpin B6-like | alysiain-like proteins |
| Two snails with higher S. mansoni read counts | | FREP2, FREP5 |
| Two snails with higher S. mansoni read counts | | macrophage-expressed gene 1 protein-like |
| Two snails with higher S. mansoni read counts | | laccase-15-like |
| Two snails with higher S. mansoni read counts | | tyrosinase-1-like |

| One snail with least S. mansoni read counts | profilin | ovipostatin 6 | hemocytin |
| --- | --- | --- | --- |
| One snail with least S. mansoni read counts | cathepsin B and L1-like | yolk ferritin precursor | hemagglutinin/amoebocyte aggreg factor-like X1 |
| One snail with least S. mansoni read counts | neuroglobinase-like | DBH-like monoxygenase protein 1 | G-type lysozyme |
| One snail with least S. mansoni read counts | chymotrypsin-like elastase family member | sialate–O-acetylsterase-like protein |
| One snail with least S. mansoni read counts | histone transcription factor | peroxidase-like protein |
| One snail with least S. mansoni read counts | fibrinogen-like protein A | FREP 7 |
| One snail with least S. mansoni read counts | | peptidoglycan-recognition proteins SC2-like |
| One snail with least S. mansoni read counts | | LRR-containing 15-like, toll-like receptor 13 |

DOWN-REGULATED

| Overall | Overall glyceraldehyde-3-phosphate dehydrogenase | FMRF-amide-like isoform X2 – modestly down | caveolin-1-like |
| --- | --- | --- | --- |
| Overall aryl hydrocarbon receptor nuclear translocator-like | aryl hydrocarbon receptor nuclear translocator-like | tyramine/dopamine β-hydroxylase-like | disintegrin/metalloprotease containing prot17-like |
| Overall PTSP-like molecule | PTSP-like molecule | LRR contain G-prot coupled rec 5-like |
| Overall pheromone Alb-1 | pheromone Alb-1 | alpha-crystalline B chain-like |
| Overall type 1 serotonin receptor | type 1 serotonin receptor | toll-like receptors 4 and 6 |
| Overall schistosomin | schistosomin | cytidine deaminase |

| Two snails with higher S. mansoni read counts | tyramine/dopamine β-hydroxylase-like |

COMPLEX (MIXED RESPONSES)

(Continued)
Table 6. (Continued)

| Shedding                  | General                                                                 | Reproductive                                           | Immune                                                                 |
|---------------------------|-------------------------------------------------------------------------|--------------------------------------------------------|------------------------------------------------------------------------|
| **UP-REGULATED**          |                                                                         |                                                        |                                                                       |
| Overall                    | FMRF-amide receptor-like—modestly up                                    | dopamine beta hydroxylase-like                        | pcrotocadherein Fat 3 or 4-like                                        |
| small cardioactive peptides| FMRF-amide receptor-like                                               | ADAM family mig-17-like                               |                                                                        |
| phospholipases A2s         | ovipostatin 5                                                           | zinc metalloprotease nas 13- & 14-like                 |                                                                        |
| arginase-1-like isoform X2 | DBH-like monoxygenase protein                                           | ficolins                                               |                                                                        |
| reverse transcriptase      |                                                                        |                                                        |                                                                       |
| protease inhibitors BPTI Kunitz-domain class |                                          |                                                        |                                                                       |
| ubiquitin ISG15            |                                                                        |                                                        |                                                                       |
| Angiopoietin–1 receptor    |                                                                        |                                                        |                                                                       |
| Angiopoietin-related 2-like|                                                                        |                                                        |                                                                       |
| mucins—complex but most are up |                                                      |                                                        |                                                                       |
| soma ferritins             |                                                                        |                                                        |                                                                       |
| Two snails with higher S. mansoni read counts | endonuclease G mitochondrial-like | yolk ferritin-like and snail yolk ferritin molecules | aplysianin-A-like                                                      |
| zinc carboxypeptide A 1-like | Neuropeptide Y receptor type 5-like                                      | mammal ependymin-related prot 1- like                 |                                                                        |
| serpinB3-like protease inhibitor |                                                  | zinc carboxypeptidase A1-like                         |                                                                        |
| cystatin protease inhibitor |                                                                        | beta-1,3-glucan binding protein-like                   |                                                                        |
| putative amine-oxidases (copper containing) |                                    |                                                        | FREP 2, 7 and 14                                                      |
| One snail with least S. mansoni read counts | multiple epidermal growth factor-like domains |                                        | macrophage-expressed gene                                              |
| serine/threonine-protein kinase mos-like | C-type lectin -6 member A-like                                        |                                                        |                                                                        |
|                                          | chitinase-3-like-protein                                               |                                                        |                                                                        |
|                                          | LRR and Ig domain containing protein                                   |                                                        |                                                                        |
|                                          | toll-like receptor 3                                                   |                                                        |                                                                        |
| **DOWN-REGULATED**         |                                                                         |                                                        |                                                                       |
| Overall                    | insulin-like gr fact protein acid labile subunit                        | ovipostatin 2                                         | toll-like receptor 7                                                   |
| pedal peptide 2            | dopamine beta-hydroxylase-like                                          | galectin-6                                            |                                                                        |
| profilin-like isoform X1    |                                                                        | probable serine carboxypeptidase CPVL                 |                                                                        |
| neuroglobin-like            |                                                                        |                                                        |                                                                        |
| calreticulin-like           |                                                                        |                                                        |                                                                        |
| tyrosinase tyr-3            |                                                                        |                                                        |                                                                        |

(Continued)
Table 6. (Continued)

| Two snails with higher S. mansoni read counts | hemoglobin | dihydropyrimidinase |
|---------------------------------------------|------------|---------------------|
|                                             | collagen-related | macrophage man recep1-like protein |
|                                             | tyrosinase-3-like | tyrosinase-1-like |

| One snail with least S. mansoni read counts | tyrosinase-1-like |

COMPLEX (MIXED RESPONSES)

|                  |                  |
|------------------|------------------|
| collagen, mixed but mostly down |                  |
| cathepsins        |                  |
| tubulins          |                  |
| cytochromes       |                  |
| ankyrins          |                  |
| Rho GTPase-activity protein 1-like |                  |
| cytochrome P450 family members |                  |
| multidrug resistance proteins |                  |
| glutathione-S-transferases |                  |
| dermatopontins    |                  |
| GTPase IMAP family members |                  |
| thioredoxins—but mostly up |                  |

With respect to transcripts involved in reproduction and potentially associated with *S. mansoni*-induced parasitic castration, we identified homologs to more than 100 invertebrate neuropeptides, hormones, pheromones, and polypeptides involved in reproduction, most of which have been identified in *Lymnaea stagnalis*, the sea hare *Aplysia californica*, or in *B. glabrata* (S4 File; Fig 9, and see Discussion). We also searched for over 500 different genes identified from previous publications that are related to immune, defense or stress responses to various pathogens or environmental stressors (S4 File; Fig 10). Each gene of interest has been organized into one of six broad functional groups for ease of interpretation, although it must be noted that many of these genes have multiple roles and could belong in several functional categories. After 1d, the majority of immune, stress and defense features were up-regulated. Noteworthy from Fig 10B is that for snails with low reads counts for *S. mansoni* (3v1 comparison), proportionately more features were up-regulated than for snails with high *S. mansoni* reads counts.

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Fig 7. Multidimensional scaling (MDS) plots of pairwise comparisons of control versus 1d, 3d, and shedding replicates used for differential expression analyses.

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read counts. In two out of three comparisons, snails with low read counts for *S. mansoni* had fewer down-regulated genes than snails with high levels of *S. mansoni* read counts.

**Expression patterns validated by qPCR**

Quantitative RT-PCR (qPCR) was used to validate differential expression trends by quantifying mRNA transcripts of four single-copy genes (3 up-regulated and 1 down-regulated) that highlight varying expression patterns in 1d, 3d, and shedding snails. Overall expression patterns are similar between the qPCR and Illumina DE results (Fig 11) with the same variability in DE pattern between replicates echoed in the qPCR. The only difference seen was in the gene DAN4 where the shedding group was not considered significantly DE in the qPCR analysis but was in Illumina analysis.
Fig 9. Biomphalaria pfeifferi CDS identified as neuropeptides, hormones, or involved in reproduction that are differentially expressed in 1d, 3d, and shedding snails. Note that the 3v3 comparison includes all 3 infected snails within a time point, whereas 3v2 includes the two infected snails with the most S. mansoni reads and the 3v1 includes only the infected snail with the fewest S. mansoni reads.

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Fig 10. Differential expression of *Biomphalaria pfeifferi* defense-related CDS in 1d, 3d, and shedding snails. (A) Defense CDS in the 3v3 DE analysis. (B) Pie charts of proportions of CDS found to be DE in 3v3, 3v2, and 3v1 analyses. (C) Heat maps show expression levels from each of the three DE analyses highlighting the most relevant biological functional groups. Note that the 3v3 comparison includes all 3 infected snails within a time point, whereas 3v2 includes the two infected snails with the most *S. mansoni* reads and the 3v1 includes only the infected snail with the fewest *S. mansoni* reads.

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Discussion
Considerations regarding the dual-seq dataset and pipeline

This paper represents a novel pipeline for dual RNA-Seq studies where the genome of just one of the interacting partners, the parasite in this case, is available. It also highlights an advantage of using field specimens in RNA-Seq studies to reinforce the notion that individual snails are actually holobionts, and the symbiont species they carry with them may play a role in influencing susceptibility to schistosome infection or in modulating disease transmission. Also, variance among the individual snails within the groups examined presented challenges for traditional bioinformatics analyses but also revealed the heterogeneity that realistically exists among naturally diverse snails and schistosomes as they encounter one another in real-life settings in the field. We must also note that the identity and functional role for many of the CDS remain unknown thus posing rich opportunities for study for the future.
Considerations with respect to compatibility with *S. mansoni*

The specific *B. pfeifferi*-*S. mansoni* system studied here is noteworthy for the high degree of susceptibility shown by the snail to infection [15,16]. Compatibility with *S. mansoni* is characteristic of *B. pfeifferi* throughout its range [12]. As a consequence, all snails exposed to *S. mansoni* or known to be shedding *S. mansoni* cercariae contained transcripts contributed by *S. mansoni*. The extent of representation of *S. mansoni* in the dual transcriptome is variable among the replicates for each time sampled (Table 1). Given the effects of both genetic diversity in *S. mansoni* [94] and in Biomphalaria snail hosts [34,95] on the rate or extent of *S. mansoni* development, it is not surprising that field-derived representatives will differ with respect to extent of parasite development and transcriptional activity. Here it should be noted that read counts may not always be fully indicative of *S. mansoni* biomass in snails as the transcriptional activity of the parasite may vary temporally, both daily [96] and at longer time scales [97], and in response to other stimuli, as noted in the following section regarding symbionts.

**Recovered symbiont sequences**

Whole snail transcriptome sequencing gave us the opportunity to identify sequences of non-mollusc and non-schistosome origin, including viruses, bacteria and eukaryotes. These sequences provide evidence of symbionts that are found in or on *B. pfeifferi* and/or *S. mansoni*. Some of the symbionts identified are surely worthy of further future investigation and may offer potential in application of novel and as yet unforeseen control efforts.

With respect to viruses, in general the array of viruses found in invertebrates has recently been shown to be much more diverse than previously known, including in molluscs [98]. Of the nearly 1,300 of our assembled CDS identified provisionally as viruses, most have homology to Beihai paphia shell viruses, picorna-like viruses, and crawfish viruses. In terms of read abundance, the five most abundant viral CDS we found in *B. pfeifferi* had the most similarity to the Wenzhou picorna-like virus 33 from the channeled apple snail Pomacea canaliculata, Sanxia picorna-like virus 4 from a freshwater atyid shrimp, Beihai picorna-like virus 47 from a sesarmid crab, bivalve RNA virus G2 a picorna virus from the gills of a bivalve [99], and Beihai hypo-like virus 1 from a razor shell [98]. Picorna viruses have recently been described in both *B. glabrata* from South America and *B. pfeifferi* from Oman [100]. Three novel RNA viruses were reported in the *B. glabrata* genome, the first with similarities to an iflavirus, the second with similarities to a Nora virus or Picornavirales, and the third with similarities to several viruses [37]. Further study is required to confidently designate any of the putative viral sequences recovered as actual infectious entities of snails, or possibly of schistosomes or other digeneans. They might infect other potential hosts like rotifers or diatoms among the symbionts living in *B. pfeifferi*.

The recovery of a few sequences of the digenean-inhabiting *Neorickettsia* from two infected snails with relatively high percentages of *S. mansoni* reads (3d-R3 and shedding-R1) is suggestive of an association. *Neorickettsia* has been found from non-human schistosomes [101] but further study is needed to document the presence of *Neorickettsia* in human-infesting schistosomes. For example, the *Neorickettsia* might be associated with metacercariae of other digeneans that are commonly found encysted in *B. pfeifferi* from natural habitats.

With respect to eukaryotes, CDS representing the following groups were recovered: 1) *Chaetogaster* annelids which mostly colonize the external soft surfaces of freshwater snails and are known to ingest digenean miracidia and cercariae [102–105]; 2) *Trichodina* ciliates known to live on the soft surfaces of snails but with poorly characterized influence on their snail hosts [106]; 3) *Capsaspora owczarzaki*, a Filasterean amoeba-like symbiont known from *Biomphalaria glabrata* [107,108]; 4) Microsporidians, not surprising for *B. pfeifferi* considering...
microsporidians are known from both Biomphalaria and Bulinus [109]; 5) Perkinsea, an alveolate group of considerable commercial significance in marine bivalves, but with at least two reports suggesting their presence in freshwater habitats as well [110,111]; 6) Rotifers (possibly attached to the shell or ingested) and diatoms (probably ingested) were frequently recovered as well; 7) Four tardigrade CDS were recovered, two from the uninfected control 454-sequenced snail similar to Richtersius coronifer and two from the Illumina de novo assembly similar to Ramazzottius varieornatus. Control-R1 had read counts >10 for the two R. coronifer CDS and 1d-R3 had read counts >10 for a R. varieornatus CDS. It is unprecedented to find tardigrades associated with snails. Fox and García-Moll [112] identified the tardigrade Echiniscus molluscorum in the feces of land snails from Puerto Rico. Although the tardigrade may have been ingested along with food, the authors did not rule out the possibility that E. molluscorum may be a symbiont of the snail.

It was not surprising that two of our snails yielded several reads mapping to sequences from other digeneans. The first, control-R3, returned sequences consistent with Ribeiroia, representatives of which occur in East Africa and are known to infect Biomphalaria there [91]. It seems most likely this snail had an infection with Ribeiroia sporocysts and/or rediae, though the extent of this infection must have been minimal as the transcriptomics response of this snail was not unusual compared to the other control snails. It may also have been infected with Ribeiroia metacercariae which are most familiarly known to infect amphibians or fish [113,114], but have been recovered and sequence-verified in specimens of Biomphalaria spp. from Kenya (MR Laidemitt, personal communication, April 2017). The other snail, 3d-R2, yielded confirmed amphistome sequences, probably from the commonly recovered species Calicophoron sukari [91], so it may have harbored developing larvae of both S. mansoni and an amphistome, reflective of real-life circumstances in the habitat from which the snail was collected, so this may be an alternative explanation for the presence of amphistome reads in 3d-R2. The peculiar nature of infection in this snail further justifies our rationale for including it in the separate analyses (3v1) described in the results.

Some overall highlights of the response of infection

At 1d, snails showed proportionately more down-regulated CDS, possibly reflective of a strong parasite-induced immunomodulatory effect during the establishment phase of infection [54]. For the two additional time points examined, the majority of features in B. pfeifferi were up-regulated (Fig 8; S3 File). This pattern differed from a previous microarray-based expression studies for susceptible B. glabrata for which a predominant trend of down-regulation was noted from 2–32 days post-exposure to S. mansoni [47]. The more comprehensive transcriptional picture resulting from next-gen sequencing provides a different overview of responses following infection with S. mansoni (see also [54]).

Many host CDS responded uniformly across individual snails regardless of the number of S. mansoni reads recovered. However, at 1d and 3d, snails with fewer S. mansoni reads had higher proportions of up-regulated features than did snails with higher numbers of S. mansoni reads. Furthermore, for both 3d and shedding snails, snails with low S. mansoni read counts had smaller proportions of down-regulated features. These patterns are suggestive that up-regulated host responses might limit S. mansoni gene expression and that snails with less parasite
gene expression may be less vulnerable to gene down-regulation, but care in interpretation is required as alternative explanations may exist. For example, as noted above, replicate 3d-R2 also contained an amphistome infection. Negative interactions among the two digeneans which are known to occur from experimental studies (MR Laidemitt, personal communication, April 2017) may account for the limited number of \( S. mansoni \) reads.

At 1d, up-regulated responses, as exemplified by CDS for phospholipases, endoglucanases, and several proteases and protease inhibitors, were usually less pronounced than at 3d, suggesting it takes a few days to mobilize responses. Notable at 1d were down-regulation of CDS that might lower hemoglobin levels, and influence feeding behavior and heart beat rate. Infected snails exhibited complex mixed responses with respect to mucins, multidrug resistance proteins, glutathione-S-transferases and cytochrome P450 family members. Cytochrome P450s are part of the stress response shown by \( B. glabrata \) snails following exposure to molluscicides [49] and to biotic stressors [48]. For heat shock proteins, \( B. glabrata \) snails elaborated more complex up-regulated responses following exposure to molluscicides [49] than \( B. pfeifferi \) did following exposure to \( S. mansoni \). Complex patterns in stress response gene families were also noted for 3d and shedding snails. It is noteworthy that exposure to \( S. mansoni \), a specific extrinsic biotic stressor, also provokes components of a generalized stress response in \( B. pfeifferi \) and \( B. glabrata \) [115,116].

Snails with 3 day infections had the highest number of up-regulated CDS. Some of the features down-regulated at 1d were again down at 3d. Additionally, one CDS (aryl hydrocarbon receptor) associated with controlling circadian rhythm [117] was down-regulated. Daily feeding patterns of infected snails [119–121] or patterns of release of cercariae [96] could potentially be influenced by this CDS. Several gene families also showed complex patterns of responses at 3d. Among them were amine oxidases which, as noted by Zhang et al. [48], are involved in oxidation of amine-containing compounds including neurotransmitters, histamines and polyamines [122].

The overall responses of shedding snails were surprising in not being more dramatically altered relative to controls than they were. This is because snails with more advanced schistosome infections (28+ day infections) experience several noteworthy physiological changes, including altered feeding behavior, decreased locomotory activity, increased heartbeat rate [118–121,123] and castration (see section below). From our shedding snails, we noted up-regulated levels of FMRF-amide receptor and small cardioactive peptides that influence heart beat rate. Shedding snails also uniquely showed up-regulated levels of CDS involved in collagen synthesis or epithelial cell and blood vessel formation, processes involved in wound healing [49,123,124], of relevance to a snail experiencing the tissue damage associated with cercarial emergence. Other up-regulated features are indicative of stress. Modestly up-regulated levels of reverse transcriptase are of interest because of previous reports of enhanced RT activity in susceptible \( B. glabrata \) exposed to \( S. mansoni \) [115].

Down-regulated levels of features potentially helping to explain reduced growth rates [125,126], reduced motility [119,120,127,128] or depleted levels of hemoglobin [129] observed in shedding snails were noted (S3 File). Other down-regulated features of interest were noted including tyrosinase, which is involved in melanin synthesis (see also discussion of reproduction).

**Consequences of infection on host reproduction**

Snails infected with the proliferating larval stages of digenetic trematodes, including \( B. pfeifferi \) infected with \( S. mansoni \), suffer parasitic castration, marked by a sharp or complete reduction in production of eggs [121,125,130]. In \( B. pfeifferi \), egg-laying begins to decline 7–10 days
following exposure to *S. mansoni* and is complete in most snails by 14 days. The time course and extent of castration are influenced by the age of the snail at the time of exposure and by the dose of miracidia received [130,131]. In some cases, a slight increase in egg production compared to unexposed controls can be seen in the pre-shedding period, but this is followed by castration [125,130,131].

Studies of the reproductive physiology of freshwater gastropods have identified a number of peptides and non-peptide mediators (including biogenic monoamines) involved in neuroendocrine control of reproduction [132,133]. We found evidence for the presence and expression of homologs of over 50 of these neuropeptides in *B. pfeifferi* (S4 File; Fig 9) and several additional neuropeptide precursors. It has also been noted that in *B. glabrata* castrated by *S. mansoni*, repeated exposure to serotonin enabled snails to resume egg-laying [134]. Furthermore, dopamine is present in reduced levels in infected snails, and administration of this catecholamine stimulated the release of secretory proteins from albumen gland cultures of *B. glabrata* [135] and the related snail *Helisoma duryi* [136].

Although infections of 1 or 3 days duration are too young to manifest castrating effects, upregulation of some features with possible inhibitory effect on reproduction were noted at these times. Several features were also down-regulated at 1 day, including ovipostatin 2, a type 1 serotonin receptor (relevant because of serotonin’s ability to stimulate egg-laying), and schistosomin. Schistosomin has been implicated in *Lymanea stagnalis* in inhibiting hormones involved in stimulating egg-laying or the albumen gland [137]. A role for schistosomin in reproduction or trematode-mediated castration was not found in *B. glabrata* infected with *S. mansoni* [138] and we saw no change in its expression in *B. pfeifferi*. Kynurenine 3-monoxygenase-like transcripts were up-regulated in all snails with 3 day infections. By degrading tryptophan, this enzyme may limit concentrations of serotonin.

It was of interest to learn if the water-borne pheromones (temptin, enticin, seduction, and attractin) that favor aggregation in *Aplysia* [139] were expressed in *B. pfeifferi,* especially given its preference for self-fertilization. We found evidence only for the expression of temptin, which was up-regulated at 3d, but otherwise was not differentially expressed. Likewise, only temptin was isolated in proteins released from *B. glabrata* [37] and egg-mass proteins [140]. It has been shown to be an attractant for *B. glabrata* [141].

Our results with shedding snails are most pertinent with respect to parasitic castration. Several reproduction-related neuropeptides, including caudal dorsal cell hormone, and neuropeptides associated with production of egg and egg mass fluids such as snail yolk ferritin (vitellogenin), galactogen synthesis, lipopolysaccharide binding protein/bacterial permeability-increasing proteins (LBP/BPI) or aplysianin/achacin-like protein [140] were not strongly down-regulated as a consequence of infection. Some of the most obvious changes we noted were up-regulated levels of transcripts encoding dopamine beta-hydroxylase and especially dopamine beta-hydroxylase–like monooxygenase protein 1, both of which convert dopamine to noradrenaline so their enhanced expression may help to explain the declining levels of dopamine noted in *S. mansoni*-infected snails [134]. This may in turn help to explain diminished egg production given dopamine’s effect on release of albumen gland proteins. Tyrosinase-1, involved in production of melanin, is down-regulated in shedding snails and this may have the effect of preserving dopamine levels in these snails. At both earlier sampling points, tyrosinase-1 is strongly up-regulated especially in snails with abundant *S. mansoni* reads, and thus may mark an early phase in initiation of castration by diverting tyrosine to production of melanin as opposed to dopamine. Transcription of enzymes involved in dopamine metabolism are strongly affected in *S. mansoni*-infected snails. Tyrosinase-1 is also discussed in the next section regarding its potential involvement in defense responses.
There are numerous ovipostatins produced in *Biomphalaria* (we found 6 different versions in *B. pfeifferi*), with ovipostatin 5 being the most prominent responder in shedding snails. In *L. stagnalis*, ovipostatin is passed in seminal fluid from one individual to another during mating and inhibits oviposition in the recipient [132]. Although *B. pfeifferi* is predominantly a self-fertilizer [20], ovipostatin 5 could potentially down-regulate oviposition in ways not reliant on copulation. Neuropeptide Y inhibits egg-laying in *L. stagnalis* [142] and though we did not observe up-regulation of this neuropeptide, up-regulated transcripts for neuropeptide Y receptor type 5-like protein in our shedding snails is consistent with a possible enhanced inhibitory effect on reproduction of neuropeptide Y. Strong up-regulation of transcripts for yolk ferritin-like and snail yolk ferritin molecules (vitellogenins) in shedding snails was also observed and is somewhat paradoxical but may suggest they are diverted to the parasite for metabolism since it is known that schistosomes require iron stores for development [143]. Notably, the extent of up-regulation for yolk ferritin-like and snail yolk ferritin, ovipostatin 5, neuropeptide Y receptor type 5-like, and dopamine beta-hydroxylase-like, was the least in the shedding snail expressing the lowest number of normalized *S. mansoni* reads.

Wang et al. [133] recently used proteomics methods (liquid chromatography tandem mass spectrometry) to examine and identify neuropeptides in central nervous system (CNS) ganglia dissected from *B. glabrata*, either from control snails or snails at 12 days post infection with *S. mansoni*. They noted many reproductive neuropeptides, such as egg laying hormone 2, at significantly reduced levels at 12d compared to controls. They also reported an increase in some neuropeptides including FMRFamide, luqin, NKY, and sCAP in infected snail CNS. Based on predicted protein interaction networks, Wang et al. [133] suggested that snail-produced leucine aminopeptidase 2 (LAP2) interacts with several *S. mansoni* miracidia peptides so may be a key player in regulating parasite-induced changes in host physiology. A homolog to the *B. glabrata* LAP2 was present in our transcriptome but was not differentially expressed in any sample. When comparing our results to those of Wang et al. [133], it should be noted that our approach was transcriptome-centered, examined different time points post-infection, and was based on whole body extractions of *B. pfeifferi*, rather than *B. glabrata*. Our methods may bias against detection of changes in expression of potentially rare neuropeptide transcripts, but cast a wider net for potential downstream effects of castration, so provides a valuable complementary view to the approach taken by Wang et al. [133].

**Immune response of *B. pfeifferi* infected with *S. mansoni***

At 1d (S4 File; Fig 10), several immune-relevant CDS were up-regulated in all three snails including dermatopontins (frequently noted in *B. glabrata* studies), ficolins [48], and chitinase attacking enzymes [42,48]. For the two snails with the highest proportions of *S. mansoni* reads, up-regulated responses were observed for a number of additional immune features. Cu,Zn SOD is of particular interest because previous work has implicated high expression of certain alleles of Cu,Zn SOD with resistance to *S. mansoni* in the 13-16-R1 strain of *B. glabrata*, because of Cu,Zn SOD’s involvement in converting superoxide anion to schistosomicidal hydrogen peroxide [144–146]. Our study is in agreement with Hanington et al. [47] who noted up-regulated levels of Cu,Zn superoxide dismutase (SOD) at early time points following exposure of susceptible M line *B. glabrata* to either *S. mansoni* or *E. paraensei*. Hanington et al. [47] also found both FREP2 and FREP4 to be consistently up-regulated following exposure of M line *B. glabrata* to *S. mansoni* or *E. paraensei*, so much so either might be considered as markers of infection. Although a FREP2 homolog was consistently up-regulated following exposure of *B. pfeifferi* to infection, a FREP4 homolog was not expressed in *B. pfeifferi* at any of the time points we examined.
In contrast, among CDS more up-regulated in the 1 day infected snail with a low proportion of *S. mansoni* reads were macrophage expressed gene-1, known to be up-regulated in both abalone following bacterial infection [147] and from resistant and non-susceptible strains of *B. glabrata* in early exposure to *S. mansoni* [148]. Hemocytin was also up-regulated in the 1d snail with low proportion of *S. mansoni* reads. Hemocytin, a homolog for an insect humoral lectin with a role in hemocyte nodule formation [149], was consistently up-regulated in all *S. mansoni*-infected snails at all three time points, especially at 3d when it was increased over 8-fold in expression. For both 1 and 3d, hemocytin expression was highest in those snails with fewer *S. mansoni* reads. FREP3, previously implicated in resistance to *S. mansoni* in *B. glabrata* [52], was minimally responsive in this compatible *B. pfeifferi* system. It was modestly up-regulated only at 1d, in the snail with fewest *S. mansoni* reads.

Down-regulated immune features at 1d were relatively few but prominent among them were FREP12 and its precursors, toll-like receptor 8 and cytidine deaminase. FREP12 down-regulation has also been noted upon exposure of *B. glabrata* amebocyte-producing organs to fucoidan, a fucosyl-rich PAMP chosen to mimic the surface of *S. mansoni* sporocysts [48], and in *B. glabrata* exposed to *S. mansoni* [47]. A strain of *B. glabrata* resistant to *S. mansoni* exhibits higher levels of a TLR on its immune cells, and exposure to *S. mansoni* significantly enhances their expression, whereas compatible snails show little response following exposure to infection [31]. Our *B. pfeifferi* showed no conspicuously up-regulated TLR genes at 1d, and we found no *B. pfeifferi* TLR with strong homology to the TLR reported by Pila *et al.* [31], but the relatively strong down-regulation of TLR 8 in this model of compatibility is noteworthy. Although the immune role of cytidine deaminase is not clear, Bouchut *et al.* [150] associated higher levels of its expression with enhanced resistance to echinostome infections and Ittiprasert *et al.* [148] observed up-regulation of cytidine deaminase in resistant and non-susceptible *B. glabrata* in early exposure to *S. mansoni*. Down-regulation of cytidine deaminase might therefore be associated with lower responsiveness to *S. mansoni* infections, particularly early in infection (down-regulation also noted at 3d, but not in shedding snails).

The responses of putative immune factors were most extensive in snails at 3d and this is not surprising as this is a critical stage in the early establishment of the parasite. Several CDS mentioned with respect to the 1d response were again noted at 3d. Snails with more *S. mansoni* reads had high levels of several transcripts including for aplysianin-like proteins and FREP 5. Aplysianin, first described from *Aplysia*, is an L-amino oxidase that has tumoricidal and bactericidal effects [151], and a distinct aplysianin-like protein exists in egg mass fluids of *B. glabrata* [140]. Aplysianin-like transcripts were more abundant in echinostome-resistant than susceptible strains of *B. glabrata* [150]. FREP 5 was shown to be down-regulated in microarray studies of *B. glabrata* in response to successfully developing *S. mansoni* or *Echinostoma paraensei* [47].

The snail with relatively few *S. mansoni* reads at 3d revealed a different group of up-regulated transcripts, with hemocytin again being prominent. Also notable were distinctive CDS potentially involved in hemocyte aggregation [152], FREP 7, peptidoglycan-recognition proteins SC2-like (PGRPs), and TLR 13. PGRPs are well-known anti-bacterial factors and were found to be up-regulated following exposure of *B. glabrata* to LPS [153] and to bacteria [53]. Down-regulated features for snails with 3d infections again included cytidine deaminase, FREP12 precursors, and TLR 4 and 8 among others.

Laccases and tyrosinases are two groups of phenoloxidases observed to be responsive in early *S. mansoni* infection within *B. pfeifferi* (Table 6; S4 File). Tyrosinase has been isolated from *B. glabrata* egg masses with a presumptive immunoprotective effect for offspring [140,154]. As mentioned earlier with details of its reproductive consequences, tyrosinase-1 was up-regulated at 1d and 3d. Tyrosinase-1 was down-regulated in the shedding replicate with the
least *S. mansoni* reads and tyrosinase-3 was down-regulated in the two replicates with the most *S. mansoni* reads. Another type of phenoloxidase, laccase, was shown to have decreased activity in *B. glabrata* hemolymph beginning at 7 weeks post-infection with *S. mansoni* [155]. We found laccase-1-like was up-regulated in all three comparisons (3v3, 3v2, 3v1) at both 1d and 3d. Laccase-1-like was up-regulated in all three comparisons at 1d and laccase-2-like was up-regulated in all comparisons at 3d. Laccases were not significantly DE in shedding snails. In *B. pfeifferi*, the up-regulation of two phenoloxidases, tyrosinase and laccase, at 1d and 3d suggests an increase in the synthesis of early-stage reactions in the melanin pathway, however, further work is needed to determine if melanization is involved in schistosome killing, especially in the *B. pfeifferi* model characterized by its compatibility.

It is worth noting that members GTPase IMAP family (GIMAP) were found to be up-regulated in 1d and 3d (mostly up-regulated in 3d). The possible role of GIMAPs in immunity has not been realized in protostomes until it was shown that several GIMAPs were up-regulated in the amebocyte organ of *B. glabrata* following exposure to extrinsic stimuli [48]. This finding was reconfirmed by later work, which demonstrated that GIMAPs not only play a role in immunity, but are highly diverse in the eastern oyster *Crassostrea virginica* where they were down-regulated following exposure to bacterial infection. GIMAPs may promote hemocyte survival by inhibiting apoptosis [156].

Immune-related responses for shedding snails were surprising for being mostly up-regulated (Fig 10), with only a few features being modestly down-regulated, among them galectin-6. Galectins recognize carbohydrates associated with schistosome surfaces and are implicated as pattern recognition receptors for other pathogens as well [157]. Dihydropyrimidinase and cytidine deaminase, also down-regulated, are additional CDS potentially affecting pyrimidine levels in infected snails. Interestingly, in contrast to 1d and 3d responses, shedding snails did not show up-regulated Cu,Zn SOD levels.

Among those up-regulated features, shedding snails with high levels of *S. mansoni* reads had distinctly higher responses for aplysianin-A-like, beta-1,3-glucan binding protein-like, and FREPS 2, 7 and 14. By contrast, the snail with a low percentage of *S. mansoni* reads expressed higher levels of macrophage-expressed gene, chitinase-3-like-protein, a distinct CDS with a leucine rich repeat and immunoglobulin domain, and TLR 3.

Features highlighted in recent genetic linkage studies [32,50,51] including components of the “Guadeloupe Resistance Complex” were sought among *B. pfeifferi* transcripts. Most did not show strong patterns of up- or down regulation in this compatible species following exposure to *S. mansoni*, but zinc metalloproteinase/disintegrin-like was down-regulated at 1d and zinc metalloproteinase nas-13- and -14-like showed some up-regulation in shedding snails. Probable serine carboxypeptidases (versions 1–5) revealed a mixed pattern of expression at 3d, but were mostly up-regulated, whereas probable serine carboxypeptidase CPVL was down-regulated in shedding snails. Granulin, a growth factor that drives the proliferation of immune cells was up-regulated at both 1d and 3d [30,33].

**Genes showing either extraordinary up- or down-regulation following exposure to *S. mansoni* infection**

Unlike *B. glabrata* for which isolates or inbred lines are known that are resistant to *S. mansoni*, *B. pfeifferi* is a species typically discussed in the context of its high compatibility with many *S. mansoni* isolates. Although particular lineages of *B. pfeifferi* may certainly come to light that exhibit strong incompatibility, key factors that dictate compatibility might best be sought not among the putative immune factors that characterize the *B. glabrata* response, but among those genes that exhibit the strongest transcriptional responses, up or down, to *S. mansoni*.
exposure (Table 5; S3 File). Strongly up-regulated snail genes may be responsible for encoding proteins essential to *S. mansoni* development, and those strongly down-regulated may represent parasite-manipulated factors that if left un-manipulated would otherwise discourage parasite development. Certainly such a role has been proposed for schistosomes in altering expression of genes in compatible snails to their advantage [158–160].

Although many *B. pfeifferi* CDS that were highly altered in their expression are unknowns and thus represent intriguing subjects for future research, some did have homologs in the database and could also represent outstanding future targets for manipulation to discourage *S. mansoni* development. For example, we note the up-regulation of the protease inhibitor papillin-like and galactocerebrosidase-like. Galactocerebrosidase is an enzyme that removes galactose from galactocerebroside (a ceramide sphingolipid with a galactose residue) to form a ceramide, an important lipid signaling molecule that has been reported in *Crassostrea gigas* [161]. A transcript coding for deleted in malignant brain tumor 1 protein-like (DMBT1) was also highly up-regulated in all shedding snails. DMBT1 is a pattern recognition receptor in mammals that belongs to a group of secreted scavenger receptors involved in pathogen binding [162]. However, its role in invertebrate systems needs to be established [159].

In conclusion, provided here is a de novo assembled transcriptional database based on over half a billion paired-end reads for an understudied schistosome vector, *B. pfeifferi*, one that is probably responsible for transmission of more *S. mansoni* to people than any other *Biomphalaria* species. We have deliberately chosen to emphasize the study of field-derived *B. pfeifferi* and *S. mansoni* to provide a more realistic view of the context in which they live, and how they interact in the wild, including with third party symbionts. Our approach has revealed that the extent of *S. mansoni* transcriptional activity varies among snails and this is reflected in different transcriptional responses of the snails, suggestive of diverse trajectories in what is typically a highly compatible host-parasite model. We have highlighted several snail features warranting further study with respect to their roles in potentially supporting or enabling parasite development, that might limit the extent of development, and that might play a role in the diminished egg production typically shown by snails with shedding *S. mansoni* infections. Another generation of research exploiting the power of techniques like CRISPR-Cas, when it becomes available for snails, will enable further dissection of the functional role of these candidate molecules. A further challenge will then be to determine how the responses of compatible snails, or perhaps of the schistosome parasites within, can be exploited, ideally to prevent or suppress in a highly specific manner the development of schistosome parasites in snails.

**Supporting information**

S1 Table. qPCR primers used to validate differential expression trends. (DOCX)

S2 Table. *Biomphalaria pfeifferi* CDS identified as a symbiont of interest during annotation efforts and verified by MEGABLAST x NCBI nt database. (DOCX)

S3 Table. *Biomphalaria pfeifferi* CDS with a BLASTn hit against publicly available genomes and CDS from molluscan symbionts of interest. (DOCX)

S1 Fig. Nucleotide sequence length distribution of 194,344 assembled *B. pfeifferi* CDS from EvidentialGene. (TIFF)
S2 Fig. The top 20 most abundant Gene Ontology assignments for *B. pfeifferi* CDS in each molecular function, cellular component category, and biological process.

(TIF)

S3 Fig. All represented KEGG classes, organized by category and abundance, in the *B. pfeifferi* transcriptome.

(TIF)

S4 Fig. BLASTp distribution of *B. pfeifferi* ORFs that have homologs to *B. glabrata* predicted polypeptides.

(TIF)

S5 Fig. Relationships of selected molluscs with genomes and/or transcriptomes sequenced. Percentages next to organisms show the percent of proteins present in our *B. pfeifferi* transcriptome predicted by TransDecoder.

(TIF)

S1 File. Annotations for *B. pfeifferi* transcriptome.

(XLSX)

S2 File. Read counts for symbionts of interest.

(XLSX)

S3 File. Overall differential expression results for *B. pfeifferi*.

(XLSX)

S4 File. General, reproduction or immune system features that were most differentially expressed.

(XLSX)

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References

1. World Health Organization. Schistosomiasis Weekly epidemiological record. 2017. Geneva, Switzerland.
2. King CH, Dangerfield-Cha M. The unacknowledged impact of chronic schistosomiasis. Chronic Illness 2008; 4: 65–79. https://doi.org/10.1177/17423953070784407 PMID: 18322031
3. World Health Organization. Public health impact of schistosomiasis: disease and mortality. Bulletin of the World Health Organization. 1993; 71: 657–662. PMID: 8313484
4. World Health Organization. Prevention and control of schistosomiasis and soil-transmitted helminthiasis. World Health Organization technical report series. 2002. Geneva, Switzerland.
5. Zhou H, Ohtsuka R, He Y, Yuan L, Yamauchi T, Sleigh AC. Impact of parasitic infections and dietary intake on child growth in the schistosomiasis-endemic Dongting Lake Region, China. American Journal of Tropical Medicine and Hygiene. 2005; 72: 534–539.
6. Colley DG, Bustinduy AL, Secor WE, King CH. Human schistosomiasis. The Lancet. 2004; 383: 2253–2264. https://doi.org/10.1016/S0140-6736(13)61949-2 PMID: 24698483
7. King CH, Sturrock RF, Kariuki HC, Hamburger J. Transmission control for schistosomiasis—why it matters now. Trends in Parasitology. 2006; 22: 575–582. https://doi.org/10.1016/j.pt.2006.09.006 PMID: 17030017
8. World Health Organization. Preventive chemotherapy in human helminthiasis. 2006. Geneva, Switzerland.
9. Lelo AE, Mburu DN, Magoma GN, Mungai BN, Kihara JH, Mwangi IN, et al. No apparent reduction in schistosome burden or genetic diversity following four years of school-based mass drug administration in Mwea, central Kenya, a heavy transmission area. PLoS Neglected Tropical Diseases. 2014; 8: e3221–11. https://doi.org/10.1371/journal.pntd.0003221 PMID: 25299057
10. King CH, Bertsch D. Historical Perspective: Snail Control to Prevent Schistosomiasis. PLoS Neglected Tropical Diseases. 2015; 9: e0003657–6. https://doi.org/10.1371/journal.pntd.0003657 PMID: 25905621
11. Sokolow SH, Wood CL, Jones IJ, Swartz SJ, Lopez M, Hsieh MH, et al. Global assessment of schistosomiasis control over the past century shows targeting the snail intermediate host works best. PLoS Neglected Tropical Diseases. 2016; 10: e0004794–20. https://doi.org/10.1371/journal.pntd.0004794 PMID: 27441556
12. Frandsen F. Discussion of the relationships between Schistosoma and their intermediate hosts, assessment of the degree of host-parasite compatibility and evaluation of schistosome taxonomy. Z Parasitenkd.1979; 58: 275–296. https://doi.org/10.1002/bf00933934 PMID: 452646
13. Brown D. Freshwater snails of Africa and their medical importance, Department of Zoology, Natural History Museum. London: Taylor & Francis Ltd; 1994.
14. De Jong RJ, Morgan JA, Paraense WL, Pointier JP, Amarista M, Ayeh-Kumi PFK, et al. Evolutionary relationships and biogeography of Biomphalaria (Gastropoda: Planorbidae) with implications regarding its role as host of the human bloodfluke, Schistosoma mansoni. Molecular Biology and Evolution. 2001; 18: 2225–2239. https://doi.org/10.1093/oxfordjournals.molbev.a003769 PMID: 11719572
15. Mutuku MW, Dweni CK, Mwangi M, Kinuthia JM, Mwangi IN, Maina G, et al. Field-derived Schistosoma mansoni and Biomphalaria pfeifferi in Kenya: A compatible association characterized by lack of strong local adaptation, and presence of some snails able to persistently produce cercariae for over a year. Parasites & Vectors. 2014; 7: 485–13. https://doi.org/10.1186/s13071-014-0533-3 PMID: 25425455
16. Lu L, Zhang S-M, Mutuku MW, Mkoji GM, Loker ES. Relative compatibility of Schistosoma mansoni with Biomphalaria sudanica and B. pfeifferi from Kenya as assessed by PCR amplification of the S. mansoni/NDS gene in conjunction with traditional methods. Parasites & Vectors. 2016; 1–13. https://doi.org/10.1186/s13071-016-1457-x PMID: 27000855
17. Steinmann P, Keiser J, Bos R, Tanner M, Utzinger J. Schistosomiasis and water resources development: systematic review, meta-analysis, and estimates of people at risk. Lancet Infectious Diseases 6. 2006;411–425. https://doi.org/10.1016/S1473-3099(06)70521-7 PMID: 16790382

18. Altizer S, Ostfeld RS, Johnson PTJ, Kutz S, Harvell CD. Climate change and infectious diseases: From evidence to a predictive framework. Science. 2013; 341: 514–519. https://doi.org/10.1126/science.1239401 PMID: 23908230

19. Stensgaard A-S, Utzinger J, Younatsou P, Hürlimann E, Schur N, Saarnak CFL, et al. Large-scale determinants of intestinal schistosomiasis and intermediate host snail distribution across Africa: Does climate matter? Acta Tropica. 2013; 128: 378–390. https://doi.org/10.1016/j.actatropica.2011.11.010 PMID: 22142789

20. Charbonnel N, Angers B, Rasatavonijazy R, Brémond P, Debain C, Jarne P. The influence of mating system, demography, parasites and colonization on the population structure of Biomphalaria pfeifferi in Madagascar. Molecular Ecology. 2002; 11: 2213–2228. https://doi.org/10.1046/j.1365-294X.2002.01586.x PMID: 12406234

21. Charbonnel N, Rasatavonijazy R, Sellin E, Brémond C, Jarne P. The influence of genetic factors and population dynamics on the mating system of the hermaphroditic freshwater snail Biomphalaria pfeifferi. Oikos. 2005; 108: 283–296.

22. Campbell G, Noble LR, Rollinson D, Southgate VR, Webster JP, Jones CS. Low genetic diversity in a snail intermediate host (Biomphalaria pfeifferi Krass, 1848) and schistosomiasis transmission in the Senegal River Basin. Molecular Ecology. 2009; 19: 2213–2228. https://doi.org/10.1111/j.1365-294X.2009.04453.x PMID: 20025653

23. Nguema RM, Langand J, Galinier R, Idris MA, Shaban MA, Al Yafae S, et al. Genetic diversity, fixation and differentiation of the freshwater snail Biomphalaria pfeifferi (Gastropoda, Planorbiidae) in arid lands. Genetics. 2013; 141: 171–184. https://doi.org/10.1007/s11070-013-9715-8 PMID: 23543205

24. Eveland LK, Haseeb MA. Laboratory rearing of Biomphalaria glabrata snails and maintenance of larval schistosomines in vivo and in vitro. In Biomphalaria snails and larval trematodes. Toledo R. and Fried B, eds. Springer, pp. 33–55. 2011.

25. Lockyer AE, Jones CS, Noble LR, Rollinson D. Trematodes and snails: an intimate association. Canadian Journal of Zoology. 2004; 82: 251–269. https://doi.org/10.1139/z03-215

26. Loker ES, Adema CM, Zhang S-M, Kepler TB. Invertebrate immune systems–not homogeneous, not simple, not well understood. Immunological Reviews. 2004; 198: 10–24. https://doi.org/10.10111/j.0105-2896.2004.0117.x PMID: 15199951

27. Bayne CJ. Successful parasitism of vector snail Biomphalaria glabrata by the human blood fluke (trematode) Schistosoma mansoni: A 2009 assessment. Molecular & Biochemical Parasitology. 2009; 165: 8–18. https://doi.org/10.1016/j.molbiopara.2009.01.005 PMID: 19393158

28. Loker ES. Gastropod immunobiology. Advances in Experimental Medicine and Biology. 2010; 708: 17–43. https://doi.org/10.1007/978-1-4419-8059-5_2 PMID: 21528691

29. Coustau C, Gourbal B, Duval D, Yoshino TP, Adema CM, Mitta G. Advances in gastropod immunity from the study of the interaction between the snail Biomphalaria glabrata and its parasites: A review of research progress over the last decade. Fish and Shellfish Immunology. 2015; 46: 5–16. https://doi.org/10.1016/j.fsi.2015.01.036 PMID: 25662712

30. Pila EA, Gordy MA, Phillips VK, Kabore AL, Rudko SP, Hanington PC. Endogenous growth factor stimulation of hemocyte proliferation induces resistance to Schistosoma mansoni challenge in the snail host. Proceedings of the National Academy of Sciences. 2016; 113: 5305–5310. https://doi.org/10.1073/pnas.1521239113 PMID: 27114544

31. Pila EA, Tarrabain M, Kabore AL, Hanington PC. A novel toll-Like receptor (TLR) influences compatibility between the gastropod Biomphalaria glabrata, and the digenean trematode Schistosoma mansoni. PLoS Pathogens. 2016; 12: e1005513–23. https://doi.org/10.1371/journal.ppat.1005513 PMID: 27015424

32. Allan ERO, Tennessen JA, Bollmann SR, Hanington PC, Bayne CJ, Blouin MS. Schistosome infectivity in the snail. Biomphalaria glabrata, is partially dependent on the expression of Grctm6, a Guadalupe Resistance Complex protein. PLoS Neglected Tropical Diseases. 2017; 11: e0005362–15. https://doi.org/10.1371/journal.pntd.0005362 PMID: 28158185

33. Mitta G, Gourbal B, Grunau C, Knight M, Theron A. The compatibility between Biomphalaria glabrata snails and Schistosoma mansoni: an increasingly complex puzzle. Advances in Parasitology. 2017; 97: 111–145. https://doi.org/10.1016/bs.appar.2016.08.006 PMID: 28325369

34. Galinier R, Roger E, Moné Y, Duval D, Portet A, Pinaud S, et al. A multistrain approach to studying the mechanisms underlying compatibility in the interaction between Biomphalaria glabrata and Schistosoma mansoni. PLoS Neglected Tropical Diseases. 2017; 11: e0005398–25. https://doi.org/10.1371/journal.pntd.0005398 PMID: 28253264
Pila EA, Li H, Hambrook JR, Wu X, Hanington PC. Schistosomiasis from a snail's perspective: advances in snail immunity. Trends in Parasitology. 2017; In press. https://doi.org/10.1016/j.pt.2017.07.006 PMID: 28803793

Portet A, Pinaud S, Tetreau G, Galinier R, Cosseau C, Duval D, et al. Integrated multi-omic analyses in Biomphalaria-Schistosoma dialogue reveal the immunobiological significance of FREP-SmPoMuc interaction. 2017; 75:16–27. https://doi.org/10.1016/j.dci.2017.02.025 PMID: 28257854

Adema CM, Hillier LW, Jones CS, Loker ES, Knight M, et al. Whole genome analysis of a schistosomi-asis-transmitting freshwater snail. Nature Communications. 2017; 8: 1–11.

Nowak TS, Woodards AC, Jung Y, Adema CM, Loker ES. Identification of transcripts generated during the response of resistant Biomphalaria glabrata to Schistosoma mansoni infection using suppression subtractive hybridization. Journal of Parasitology. 2004; 90: 1034–1040. https://doi.org/10.1645/GE-193R1 PMID: 15562603

Mitta G, Galinier R, Tisseynne P, Allienne J, Girerdchamba Y, Guillou F, et al. Gene discovery and expression analysis of immune-relevant genes from Biomphalaria glabrata hemocytes. Developmental & Comparative Immunology. 2005; 29: 393–407. https://doi.org/10.1016/j.dci.2004.10.002 PMID: 15707661

Guillou F, Mitta G, Galinier R, Coustau C. Identification and expression of gene transcripts generated during an anti-parasitic response in Biomphalaria glabrata. Developmental & Comparative Immunology. 2007; 31: 657–671. https://doi.org/10.1016/j.dci.2006.10.001 PMID: 17166585

Lockyer AE, Spinks JN, Walker AJ, Kane RA, Noble LR, Rollinson D, et al. Biomphalaria glabrata transcriptome: Identification of cell-signalling, transcriptional control and immune-related genes from open reading frame expressed sequence tags (ORESTES). Developmental & Comparative Immunology. 2007; 31: 763–782. https://doi.org/10.1016/j.dci.2006.11.004 PMID: 17206299

Hanelt B, Lun C-M, Adema CM. Comparative ORESTES-sampling of transcriptomes of immune-challenged Biomphalaria glabrata snails. Journal of Invertebrate Pathology. 2008; 99: 192–203. https://doi.org/10.1016/j.jip.2008.06.002 PMID: 18590737

Lockyer AE, Spinks J, Kane RA, Hoffmann KF, Fitzpatrick JM, Rollinson D, et al. Biomphalaria glabrata transcriptome: cDNA microarray profiling identifies resistant- and susceptible-specific gene expression in haemocytes from snail strains exposed to Schistosoma mansoni. BMC Genomics. 2008; 9: 634–17. https://doi.org/10.1186/1471-2164-9-634 PMID: 19114004

Adema CM, Hanington PC, Lun C-M, Rosenberg GH, Rosenberg GH, Aragon AD, et al. Differential transcriptomic responses of Biomphalaria glabrata (Gastropoda, Mollusca) to bacteria and metazoan parasites, Schistosoma mansoni and Echinostoma paraensei (Digenea, Platyhelminthes). Molecular Immunology. 2010; 47: 849–860. https://doi.org/10.1016/j.molimm.2009.10.019 PMID: 19962194

Zhang SM, Adema CM, Kepler TB, Loker ES. Diversification of Ig superfam ily genes in an invertebrate. Science. 2004; 305: 251–254. https://doi.org/10.1126/science.1088069 PMID: 15247481

Hanington PC, Zhang S-M. The primary role of fibrinogen-related proteins in invertebrates is defense, not coagulation. Journal of Innate Immunology. 2011; 3: 17–27. https://doi.org/10.1159/000321882 PMID: 21063081

Hanington PC, Lun C-M, Adema CM, Loker ES. Time series analysis of the transcriptional responses of Biomphalaria glabrata throughout the course of intramolluscan development of Schistosoma mansoni and Echinostoma paraensei. International Journal for Parasitology. 2010; 40: 819–831. https://doi.org/10.1016/j.ijpara.2009.12.005 PMID: 20083115

Zhang S-M, Loker ES, Sullivan JT. Pathogen-associated molecular patterns activate expression of genes involved in cell proliferation, immunity and detoxification in the amebocyte-producing organ of the snail Biomphalaria glabrata. Developmental & Comparative Immunology. 2016; 56: 25–36. https://doi.org/10.1016/j.dci.2015.11.008 PMID: 26592964

Zhang S-M, Buddenberg SK, Adema CM, Sullivan JT, Loker ES. Altered gene expression in the schistosome-transmitting snail Biomphalaria glabrata following exposure to niclosamide, the active ingredient in the widely used molluscicide Bayluscide. PLoS Neglected Tropical Diseases. 2015; 9: e0004131–21. https://doi.org/10.1371/journal.pntd.0004131 PMID: 26452273

Tennessen JA, Bonner KM, Bollmann SR, Johnston JA, Yeh J-Y, Marine M, et al. Genome-wide scan and test of candidate genes in the snail Biomphalaria glabrata reveal new locus influencing resistance to Schistosoma mansoni. PLoS Neglected Tropical Diseases. 2015; 9: e0004077–19. https://doi.org/10.1371/journal.pntd.0004077 PMID: 26372103

Tennessen JA, Théron A, Marine M, Yeh J-Y, Rognon A, Blouin MS. Hyperdiverse gene cluster in snail host conveys resistance to human schistosome parasites. PLoS Genetics. 2015; 11: e1005067–21. https://doi.org/10.1371/journal.pgen.1005067 PMID: 25775214

Hanington PC, Forys MA, Loker ES. A somatically diversified defense factor, FREP3, is a determinant of snail resistance to schistosome infection. PLoS Neglected Tropical Diseases. 2012; 6: e1591. https://doi.org/10.1371/journal.pntd.0001591 PMID: 22479663
53. Deleury E, Dubreuil G, Elangoovan N, Wajnberg E, Reichhart J-M, Gourbal B, et al. Specific versus non-specific immune responses in an invertebrate species evidenced by a comparative de novo sequencing study. PLoS ONE. 2012; 7: e32512–15. https://doi.org/10.1371/journal.pone.0032512 PMID: 22427848

54. Pinaud S, Portela J, Duval D, Nowacki FC, Olive M-A, Allienne JF, et al. A shift from cellular to humoral responses contributes to innate immune memory in the vector snail Biomphalaria glabrata. PLoS Pathogens. 2016; 12: e1005361–18. https://doi.org/10.1371/journal.ppat.1005361 PMID: 26735307

55. Wang H, Zhao QP, Nie P, Jiang MS, Song J. Identification of differentially expressed genes in Oncomelania hupensis chronically infected with Schistosoma japonicum. Experimental Parasitology. 2012; 130: 374–383. https://doi.org/10.1016/j.exppar.2012.02.004 PMID: 22343044

56. Zhao QP, Xiong T, Xu XJ, Jiang MS, Dong HF. De novo transcriptome analysis of Oncomelania hupensis after molluscicide treatment by Next-Generation Sequencing: Implications for Biology and Future Snail Interventions. PLoS ONE. 2015; 10: e0118673–16. https://doi.org/10.1371/journal.pone.0118673 PMID: 25775015

57. Berriman M, Haas BJ, LoVerde PT, Wilson RA, Dillon GP, Cerqueira GC, et al. The genome of the blood fluke Schistosoma mansoni. Nature. 2009; 460: 352–358. https://doi.org/10.1038/nature08160 PMID: 19606141

58. Verjovski-Almeida S, DeMarco R, Martins EAL, Guimarães PEM, Ojopi EPB, Paquola ACM, et al. Transcriptome analysis of the acelomate human parasite Schistosoma mansoni. Nature Genetics. 2003; 35: 148–157. https://doi.org/10.1038/ng1237 PMID: 12937950

59. Vermeire JJ, Taft AS, Hofmann KF, Fitzpatrick JM, Yoshino TP. Schistosoma mansoni: DNA microarray gene expression profiling during the miracidium-to-mother sporocyst transformation. Molecular & Biochemical Parasitology. 2006; 147: 39–47. https://doi.org/10.1016/j.molbiopara.2006.01.006 PMID: 16483678

60. Taft AS, Vermeire JJ, Bernier J, Birkeland SR, Cipriano MJ, Papa AR, et al. Transcriptome analysis of Schistosoma mansoni larval development using serial analysis of gene expression (SAGE). Parasitology. 2009; 136: 469–87. https://doi.org/10.1017/S0031182009006735 PMID: 19265565

61. van Horn DJ, Garcia JR, Loker ES, Mitchell KR, Mkoji GM, Adema CM, Takacs-Vesbach CD. Complex intestinal bacterial communities in three species of planorbid snails. Journal of Molluscan Studies. 2012; 78: 74–80. https://doi.org/10.1083/mollus/eyr038

62. Silva TM, Melo ES, Lopes ACS, Veras ACS, Brayer FA. Characterization of the bacterial microbiota of Biomphalaria glabrata (Say, 1816) (Mollusca: Gastropoda) from Brazil. Letters in Applied Microbiology. 2013; 57: 19–25. https://doi.org/10.1111/lam.12068 PMID: 23488866

63. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. 2014; 30: 2114–2120. https://doi.org/10.1093/bioinformatics/btu170 PMID: 24959404

64. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics. 2013; 29: 15–21. https://doi.org/10.1093/bioinformatics/bts655 PMID: 23104886

65. Kim D, Pertea G, Trapnell C, Pimentel H, Kelley R, Salzberg SL. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biology. 2013; 14: R36–R36. https://doi.org/10.1186/gb-2013-14-4-r36 PMID: 23618408

66. Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, et al. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nature Biotechnology. 2011; 29: 644–652. https://doi.org/10.1038/nbt.1883 PMID: 21572440

67. Haas BJ, Papanicolaou A, Yassour M, Grabherr M, Blood PD, Bowden J, et al. De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. Nature Protocols. 2013; 8: 1494–1512. https://doi.org/10.1038/nprot.2013.084 PMID: 23859625

68. Gilbert D. Gene-omes built from mRNA seq not genome DNA. 7th Annual Arthropod Genomics Symposium. Notre Dame. 2013. http://arthropods.eugenics.org/EvidentialGene/about/EvigenesRNA2013poster.pdf and http://globalhealth.nd.edu/7th-annual-arthropod-genomics-symposium/

69. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, et al. Gene Ontology: tool for the unification of biology. Nature Genetics. 2000; 25: 25–29. https://doi.org/10.1038/75556 PMID: 10802651

70. Kanehisa M, Sato Y. KEGG: Kyoto Encyclopedia of Genes and Genomes. Nucleic Acid Research. 2000; 28: 27–30. https://doi.org/10.1093/nar/28.1.27

71. Jones P, Binns D, Chang HY, Fraser M, Li W, McAnulla C, et al. InterProScan S: genome-scale protein function classification. Bioinformatics. 2014; 30: 1236–1240. https://doi.org/10.1093/bioinformatics/btu331 PMID: 24451626
72. Krogh A, Larsson B, von Heijne G, Sonnhammer ELL. Predicting transmembrane protein topology with a hidden Markov model: Application to complete genomes. Journal of Molecular Biology. 2001; 305: 567–580. https://doi.org/10.1006/jmbi.2000.4315 PMID: 11152613

73. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nature Methods. 2012; 9: 357–359. https://doi.org/10.1038/nmeth.1923 PMID: 22388286

74. Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics. 2011; 12: 323–323. https://doi.org/10.1186/1471-2105-12-323 PMID: 21816040

75. Leng N, Dawson JA, Thomson JA, Ruotti V, Rissman AI, Smits BMG, et al. EBSeq: an empirical Bayes hierarchical model for inference in RNA-seq experiments. Bioinformatics. 2013; 29: 1035–1043. https://doi.org/10.1093/bioinformatics/btt087 PMID: 23428641

76. Rozen S, Skaletsky HJ. Primer3. 1998. http://www-genome.wi.mit.edu/genome_software/other/primer3.html. Accessed February 2017.

77. Dheilly NM, Duval D, Mouahid G, Emans R, Allienne JF, Galinier R, et al. A family of variable immunoglobulin and lectin domain containing molecules in the snail Biomphalaria glabrata. Developmental & Comparative Immunology. 2015; 48: 234–243. https://doi.org/10.1016/j.dci.2014.10.009 PMID: 25451302

78. Simakov O, Marletza F, Edsinger-Gonzales E, Havlak P, Hellsten U, Kuo D-H, et al. Insights into bilateral evolution from three spiralian genomes. Nature. 2013; 493: 526–531. https://doi.org/10.1038/nature11696 PMID: 23254933

79. Takeuchi T, Kawashima T, Koyanagi R, Gyoja F, Tanaka M, Ikuta T, et al. The oyster genome reveals stress adaptation and complexity of shell formation. Nature. 2012; 490: 49–54. https://doi.org/10.1038/nature11413 PMID: 22992520

80. Albertin CB, Simakov O, Mitros T, Yan Wang Z, Pungor JR, Edsinger-Gonzales E, et al. The octopus genome and the evolution of cephalopod neural and morphological novelties. Nature. 2013; 524: 220–224. https://doi.org/10.1038/nature14668 PMID: 26266193

81. Kocot KM, Cannon JT, Todt C, Citarella MR, Kohn AB, Meyer A, et al. Phylogenomics reveals deep molluscan relationships. Nature. 2011; 477: 452–456. https://doi.org/10.1038/nature10382 PMID: 21892190

82. Greiman SE, Vaughan JA, Elmahy R, Adisakwattana P, Van Ha N, Fayton TJ, et al. Real-time PCR detection and phylogenetic relationships of Neorickettsia spp. in digeneans from Egypt, Philippines, Thailand, Vietnam and the United States. Parasitology International. 2017; 66: 1003–1007. https://doi.org/10.1016/j.parint.2016.08.002 PMID: 27510768

83. Tkach VV, Kudlai O, Kostadinova A. Molecular phylogeny and systematics of the Echinostomatoidea Looss, 1899 (Platyhelminthes: Digenea). International Journal for Parasitology. 2016; 46: 171–185. https://doi.org/10.1016/j.ijpara.2015.11.001 PMID: 26699402
91. Wilson WD, Johnson PT, Sutherland DR, Moné H, Loker ES. A molecular phylogenetic study of the genus *Ribeiraia* (Digenea): trematodes known to cause limb malformations in amphibians. Journal of Parasitology. 2005; 91: 1040–1045. https://doi.org/10.1645/GE-465R.1 PMID: 16419746

92. Laidemitt MR, Zawadzki ET, Brant SV, Mutuku MW, Mkoji GM, Loker ES. Loads of trematodes: Discovering hidden diversity of paramphistomoids in Kenyan ruminants. Parasitology. 2017; 144: 131–147. https://doi.org/10.1017/S0031182016001827 PMID: 27762185

93. Sweedler JV, Li L, Rubakhin SS, Alexeeva V, Dembrow NC, Dowling O, et al. Identification and characterization of the feeding circuit-activating peptides, a novel neuropeptide family of *Aplysia*. Journal of Neuroscience. 2002; 22: 7797–7808. PMID: 12196603

94. Davies CM, Webster JP, Woolhouse MEJ. Trade-offs in the evolution of virulence in an indirectly transmitted macroparasite. Proceeding of the Royal Society B. 2001; 268: 251–257. https://doi.org/10.1098/rspb.2000.1367 PMID: 11217894

95. Tavaire HF, Blouin MS, Steinauer ML. Genotypic variation in host response to infection affects parasite reproductive rate. International Journal for Parasitology. 2016; 46: 123–131. https://doi.org/10.1016/j.ijpara.2015.10.001 PMID: 26552016

96. Steinauer ML, Hanet B, Mwangi IN, Maina GM, Agola EL, Kinuthia JM, et al. Introggressive hybridization of human and rodent schistosomes parasites in western Kenya. Molecular Ecology. 2008; 17: 5062–5074. https://doi.org/10.1111/j.1365-294X.2008.03957.x PMID: 18992007

97. Theron A. Dynamics of larval populations of *Schistosoma mansoni* in *Biomphalaria glabrata*. Annals of Tropical Medicine and Parasitology. 1981; 75: 71–77. https://doi.org/10.1080/00034983.1981.11720796 PMID: 7271357

98. Shi M, Lin X-D, Tian J-H, Chen L-J, Chen X, Li C-X, et al. Redefining the invertebrate RNA virosphere. Virus Research. 2016; 1–10. https://doi.org/10.1016/j.virusres.2016.10.009 PMID: 27769778

99. Galinier R, Tetreau G, Portet A, Pinaud S, Duval G, Gourbal B. First characterization of viruses from freshwater snails of the genus *Biomphalaria*, the intermediate host of the parasite *Schistosoma mansoni*. Acta Tropica. 2017; 167: 196–203. https://doi.org/10.1016/j.actatropica.2016.12.021 PMID: 28012902

100. Park B-K, Kim M-J, Kim E-H, Kim M-S, Na D-G, Chae J-S. Identification of trematode cercariae carrying *Neorickettsia risticii* in freshwater stream snails. Annals of the New York Academy of Sciences. 2003; 990: 239–247. https://doi.org/10.1111/j.1749-6632.2003.tb07371.x PMID: 12860634

101. Michelson E. The protective action of *Chaetogaster limnaei* on snails exposed to *Schistosoma mansoni*. Journal of Parasitology. 1964; 50: 441–444. https://doi.org/10.2307/3275851 PMID: 14169541

102. Rodgers JK, Sandiland GJ, Joyce SR, Minchella DJ. Multi-species interactions among a commensal (*Chaetogaster limnaei limnaei*), a parasite (*Schistosoma mansoni*), and an aquatic snail host (*Biomphalaria glabrata*). Journal of Parasitology. 2005; 91: 709–712. https://doi.org/10.1645/GE-421R PMID: 16108575

103. Ibrahim MM. Population dynamics of *Chaetogaster limnaei* (Oligochaeta: Naididae) in the field populations of freshwater snails and its implications as a potential regulator of trematode larval community. Parasitology Research. 2007; 101: 25–33. https://doi.org/10.1007/s00436-006-0436-0 PMID: 17252272

104. Zimmermann MR, Luth KE, Esch GW. Complex interactions among a nematode parasite (*Daubaylia potomaca*), a commensalistic annelid (*Chaetogaster limnaei limnaei*), and trematode parasites in a snail host (*Helisoma aniceps*). Journal of Parasitology. 2011; 97: 788–791. https://doi.org/10.1645/GE-2733.1 PMID: 21506797

105. Hertel LA, Barbosa CS, Santos RAAL, Loker ES. Molecular identification of symbionts from the pulmonate snail *Biomphalaria glabrata* in Brazil. Journal of Parasitology. 2004; 90: 759–763. https://doi.org/10.1645/GE-223R PMID: 15357095

106. Hertel LA, Bayne CJ, Loker ES. The symbiont *Capsaspora owczarzaki*, nov. gen. nov. sp., isolated from three strains of the pulmonate snail *Biomphalaria glabrata* is related to members of the Mesomyzozoecia. International Journal for Parasitology. 2002; 32: 1183–1189. https://doi.org/10.1017/S0020-7519(02)00066-8 PMID: 12117501

107. Ruiz-Trillo I, Inagaki Y, Davis LA, Sperstad S, Landfald B, Roger AJ. *Capsaspora owczarzaki* is an independent opisthokont lineage. Current Biology. 2004; 14: R946–7. https://doi.org/10.1016/j.cub.2004.07.037 PMID: 15568489

108. McLymont EH, Dunn AM, Terry RS, Rollinson D, Littlewood DTJ, Smith JE. Molecular data suggest that microsporidian parasites in freshwater snails are diverse. International Journal for Parasitology. 2005; 35: 1071–1078. https://doi.org/10.1016/j.ijpara.2005.05.008 PMID: 16023122
110. Chambouvet A, Gower DJ, Jirků M, Yabsley MJ, Davis AK, Leonard G, et al. Cryptic infection of a broad taxonomic and geographic diversity of tadpoles by Perkinsinella protists. Proceedings of the National Academy of Sciences. 2015; 112: E4743–E4751. https://doi.org/10.1073/pnas.1500163112 PMID: 26261337

111. Bráte JBA, Logares R, Berney CED, Kee DK, Klaaveness D, Jakobsen KS, Shalchian-Tabrizi K. Freshwater Perkinsinella and marine-freshwater colonizations revealed by pyrosequencing and phylogeny of environmental rDNA. The ISME Journal. 2010; 4: 1144–1153. https://doi.org/10.1038/ismej.2010.39 PMID: 20393574

112. Fox I, Garcia-Moll I. Echiniscus molluscorum, new tardigrade from the feces of the land snail, Bulimulus exilis (Gmelin) in Puerto Rico (Tardigrada: Scutechiniscidae). The Journal of Parasitology. 1962; 48: 177–181.

113. Basch PF, Sturrock RF. Life History of Ribeiroia marini (Faust and Hoffman, 1934) comb. n. (Trematoda: Cathaenidae). Journal of Parasitology. 1969; 55: 1180.

114. Johnson PTJ, Hoverman JT. Heterogeneous hosts: how variation in host size, behaviour and immunity affects parasite aggregation. Journal of Animal Ecology. 2014; 83: 1103–1112. https://doi.org/10.1111/1365-2656.12215 PMID: 24548254

115. Ittiprasert W, Nene R, Miller A, Raghaven N, Lewis F, Hodgson J, Knight M. Schistosoma mansoni infection of juvenile Biomphalaria glabrata induces a differential stress response between resistant and susceptible snails. Experimental Parasitology. 2009; 123: 203–211. https://doi.org/10.1016/j.exppara.2009.07.015 PMID: 19860454

116. Knight M, Ittiprasert W, Arican-Goktas HD, Bridger JM. Epigenetic modulation, stress and plasticity in susceptibility of the snail host, Biomphalaria glabrata, to Schistosoma mansoni infection. International Journal for Parasitology. 2016; 46:389–394. https://doi.org/10.1016/j.ijpara.2016.03.003 PMID: 27056272

117. Perrigault M, Tran D. Identification of the molecular clockwork of the oyster Crassostrea gigas. PLoS ONE. 2017; 12: e0169790–21. https://doi.org/10.1371/journal.pone.0169790 PMID: 28072861

118. Lee FO, Cheng TC. Schistosoma mansoni Infection in Biomphalaria glabrata: alterations in heart rate and thermal tolerance in the host. Journal of Invertebrate Pathology. 1971; 18: 412–418. https://doi.org/10.1016/0022-2011(71)90047-4 PMID: 5151914

119. Williams CL, Gilbertson DE. Altered feeding response as a cause for the altered heartbeat rate and locomotor activity of Schistosoma mansoni-infected Biomphalaria glabrata. Journal of Parasitology. 1983; 69: 671–676. https://doi.org/10.2307/3281138 PMID: 6631636

120. Boissier J, Rivera ER, Moné H. Altered behavior of the snail Biomphalaria glabrata as a result of Schistosoma mansoni infection. Journal of Parasitology. 2003; 89: 429–433. https://doi.org/10.1645/0022-3395(2003)089[0429:ABOTS B]2.0.CO;2 PMID: 12880237

121. Humphries J. Effects of larval schistosomes on Biomphalaria snails. In Biomphalaria snails and larval trematodes. Toledolo R. and Fried B, eds. Springer, pp. 103–125. 2011.

122. Finney J, Moon H-J, Ronnebaum T, Lantiz M, Mure M. Human copper-dependent amine oxidases. Archives of Biochemistry and Biophysics. 2014; 546: 19–32. https://doi.org/10.1016/j.abb.2013.12.022 PMID: 24407025

123. Lee JO, Cheng TC. Increased heart rate in Biomphalaria glabrata parasitized by Schistosoma mansoni. Journal of Invertebrate Pathology. 1970; 16: 148–149. PMID: 5449737

124. Dzik JM. Evolutionary roots of arginase expression and regulation. Frontiers in Immunology. 2014; 5: 544. https://doi.org/10.3389/fimmu.2014.00544 PMID: 25426114

125. Sturrock BM. The influence of infection with Schistosoma mansoni on the growth rate and reproduction of Biomphalaria pfeifferi. Annals of Tropical Medicine and Parasitology. 1966; 60: 187–97. https://doi.org/10.1080/00034983.1966.11686405 PMID: 6006924

126. Sturrock RF, Sturrock RM. Observations on some factors affecting growth rate and fecundity of Biomphalaria glabrata (Say). Annals of Tropical Medicine and Parasitology. 1970; 64: 349. https://doi.org/10.1080/00034983.1970.11686704 PMID: 5500110

127. Gérard C. Energy constrains exerted by the parasite Schistosoma mansoni on the locomotion of its snail host, Biomphalaria glabrata. Canadian Journal of Zoology. 1996; 74: 594–598. https://doi.org/10.1139/z96-068

128. Ibrahim MM. Energy allocation patterns in Biomphalaria alexandrina snails in response to cadmium exposure and Schistosoma mansoni infection. Experimental Parasitology. 2006; 112: 31–36. https://doi.org/10.1016/j.exppara.2005.08.012 PMID: 16256110

129. Lee FO, Cheng TC. Schistosoma mansoni: Alterations in total protein and hemoglobin in the hemolymph of infected Biomphalaria glabrata. Experimental Parasitology. 1972; 31: 203–216. PMID: 5016592
130. Meuleman EA. Host-Parasite Interrelationships Between the Freshwater Pulmonate Biomphalaria pfeifferi and the Trematode Schistosoma mansoni. Netherlands Journal of Zoology. 1971; 22: 355–427. https://doi.org/10.1163/002829672X00013

131. Ibikounlé M, Mouahid G, Mintsa Nguema R, Sakitì NG, Kindé-Gasard D, Massougbodji A, Moné H. Life-history traits indicate local adaptation of the schistososome parasite, Schistosoma mansoni, to its snail host, Biomphalaria pfeifferi. Experimental Parasitology. 2012; 132: 501–507. https://doi.org/10.1016/j.exppara.2012.09.020 PMID: 23031799

132. Koene JM. Neuro-endocrine control of reproduction in hermaphroditic freshwater snails: mechanisms and evolution. Frontiers in Behavioral Neuroscience. 2010; 4: 1–17.

133. Wang T, Zhao M, Liang D, Bose U, Kaur S, McManus DP, Cummins SF. Changes in the neuropeptide Santhanagopalan V, Yoshino TP. Monoamines and their metabolites in the freshwater snail Biomphalaria glabrata, 2017: 10: 1–13.

134. Manger P, Li J, Christensen BM, Yoshino TP. Biogenic monoamines in the freshwater snail, Biomphalaria glabrata: influence of infection by the human blood fluke, Schistosoma mansoni. Comparative Biochemistry and Physiology Part A: Physiology. 1996; 114: 227–234. https://doi.org/10.1016/0300-9629(95)02131-0

135. Santhanagopalan V, Yoshino TP. Monoamines and their metabolites in the freshwater snail Biomphalaria glabrata. Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology. 2000; 125: 469–478.

136. Mukai ST, Kienh L, Saleuddin AS. Dopamine stimulates snail albumen gland glycoprotein secretion through the activation of a D1-like receptor. Journal of Experimental Biology. 2004; 207: 2507–2518. https://doi.org/10.1242/jeb.01052 PMID: 15184522

137. Dictus WJ, de Jong-Bring M, Boer HH. A neuropeptide (Calfluxin) is involved in the influx of calcium into mitochondria of the albumen gland of the freshwater snail Lymnaea stagnalis. General and Comparative Endocrinology. 1987; 65: 439–450.

138. Zhang S-M, Nian H, Wang B, Loker ES, Adema CM. Schistosomiasis from the snail Biomphalaria pfeifferi transcriptomic response to Schistosoma mansoni. Journal for Parasitology. 2013; 43: 51–55. https://doi.org/10.1163/002829672X00013

139. Goodall CP, Bender RC, Broderick EJ, Bayne CJ. Constitutive differences in Cu/Zn superoxide dismutase mRNA levels and activity in hemocytes of Biomphalaria glabrata (Mollusca) that are either susceptible or resistant to Schistosoma mansoni (Trematoda). Molecular & Biochemical Parasitology. 2004; 137: 321–328. https://doi.org/10.1016/j.molbiopara.2004.06.011

140. Blouin MS, Bonner KM, Cooper B, Amarasinghe V, O’Donnell RP, Bayne CJ. Three genes involved in the oxidative burst are closely linked in the genome of the snail, Biomphalaria glabrata. International Journal for Parasitology. 2013; 43: 51–55. https://doi.org/10.1016/j.ijpara.2012.10.020 PMID: 23207063

141. Pila EA, Peck SJ, Hanington PC. The protein pheromone temptin is an attractant of the gastropod Biomphalaria glabrata. Journal of Comparative Physiological A. 2017: 1–12. https://doi.org/10.1007/s00359-017-1198-0

142. Hathaway JJM, Adema CM, Stout BA, Mobarak CD, Loker ES. Identification of protein components of egg masses indicates parental investment in immunoprotection of offspring by Biomphalaria glabrata (Gastropoda, Mollusca). Developmental & Comparative Immunology. 2010; 34: 425–435. https://doi.org/10.1016/j.dci.2009.12.001 PMID: 19995576

143. Pila EA, Peck SJ, Hanington PC. The protein pheromone temptin is an attractant of the gastropod Biomphalaria glabrata. Journal of Comparative Physiological A. 2017: 1–12. https://doi.org/10.1007/s00359-017-1198-0

144. Jones MK, McManus DP, Sivadorai P, Gianfield A, Moertel L, Belli SI, Gobert GN. Tracking the fate of iron in early development of human blood flukes. The International Journal of Biochemistry and Cell Biology. 2007; 39: 1646–1658. https://doi.org/10.1016/j.biocel.2007.04.017 PMID: 17556009

145. Goodall CP, Bender RC, Brooks JK, Bayne CJ. Biomphalaria glabrata cytosolic copper/zinc superoxide dismutase (SOD1) gene: Association of SOD1 alleles with resistance/susceptibility to Schistosoma mansoni. Molecular & Biochemical Parasitology. 2006; 147: 207–210. https://doi.org/10.1016/j.molbiopara.2006.02.009

146. Bathige SDNK, Umasuthan N, Whang I, Lim B-S, Won SH, Lee J. Antibacterial activity and immune responses of a molluscan macrophage expressed gene-1 from disk abalone, Haliotis discus discus.
148. Ittiprasert W, Miller A, Myers J, Nene V, El-Sayed NM, Knight M. Identification of immediate response genes dominantly expressed in juvenile resistant and susceptible Biomphalaria glabrata snails upon exposure to Schistosoma mansoni. Molecular and Biochemical Parasitology. 2010; 169: 27–39. https://doi.org/10.1016/j.molbiopara.2009.09.009 PMID: 19815034

149. Arai I, Ohta M, Suzuki A, Tanaka S, Yoshizawa Y, Sato R. Immunohistochemical analysis of the role of hemocytin in nodule formation in the larvae of the silkworm, Bombyx mori. Journal of Insect Science. 2013; 13: 125–13. https://doi.org/10.1673/031.013.12501 PMID: 24766322

150. Takamatsu N, Shiba T, Muramoto K, Kamiya H. Molecular cloning of the defense factor in the albumen gland of the sea hare Aplysia kurodai. FEBS Letters. 1995; 377: 373–376. https://doi.org/10.1016/0014-5793(95)01375-X PMID: 8549758

151. Fujii N, Minetti T, Casa T, Nakashi HI, Chen SW, Barbehenn E, et al. Isolation, cDNA cloning and characterization of an 18 kDa hemagglutinin and amebocyte aggregation factor from Lymus polyphemus. Journal of Biological Chemistry. 1992; 267: 22452–22459 PMID: 1429596

152. Zhang S-M, Zeng Y, Loker ES. Characterization of immune genes from the schistosome host snail Biomphalaria glabrata that encode peptidoglycan recognition proteins and gram-negative bacteria binding protein. Immunogenetics. 2007; 59: 883–898. https://doi.org/10.1007/s00251-007-0245-3 PMID: 17805526

153. Bai G, Brown JF, Watson C, Yoshino TP. Isolation and characterization of phenoloxidase from egg masses of the gastropod mollusc, Biomphalaria glabrata. Comparative Biochemistry and Physiology — Part B: Biochemistry & Molecular Biology. 1997; 118: 463–9. https://doi.org/10.1016/S0305-0491(97)00159-4

154. Le Clec'h W, Anderson TJ, Chevalier F. Characterization of hemolymph phenoloxidase activity in two Biomphalaria snail species and impact of Schistosoma mansoni infection. Parasites and Vectors. 2016; 9. https://doi.org/10.1186/s13071-016-1319-6 PMID: 26797101

155. McDowell IC, Modak TH, Lane CE, Gomez-Chiarri M. Multi-species protein similarity clustering reveals novel expanded immune gene families in the eastern oyster Crassostrea virginica. Fish and Shellfish Immunology. 2016; 53: 13–23. https://doi.org/10.1016/j.fsi.2016.03.157 PMID: 27033806

156. Yoshino TP, Dinguirard N, Kunert J, Hokke CH. Molecular and functional characterization of a tandem-repeat galectin from the freshwater snail Biomphalaria glabrata, intermediate host of the human blood fluke Schistosoma mansoni. Gene. 2008; 411: 46–58. https://doi.org/10.1016/j.gene.2008.01.003 PMID: 18280060

157. Yoshino TP, Rayne CG. Mimicry of snail host antigens by miracidia and primary sporocysts of Schistosoma mansoni. Parasite Immunology. 1983; 5: 317–328. https://doi.org/10.1111/j.1365-3024.1983.tb00747.x PMID: 6191266

158. Knight M, Arican-Goktas HD, Ittiprasert W, Odoemelam EC, Miller AN, Bridger JM. Schistosomes and snails: a molecular encounter. Frontiers in Genetics. 2014; 5: 230. https://doi.org/10.3389/fgene.2014.00230 PMID: 25101114

159. Timmins-Schiffman E, Roberts S. Characterization of genes involved in ceramide metabolism in the Pacific oyster (Crassostrea gigas). BMC Research Notes. 2012; 5: 202.