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Schistosomiasis in the Wild: A Transcriptomics Perspective on Field-Derived Biomphalaria pfeifferi and Schistosoma mansoni, and their Interactions

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SCHISTOSOMIASIS IN THE WILD: A TRANSCRIPTOMICS PERSPECTIVE ON FIELD-DERIVED *BIOMPHALARIA PFEIFFERI* AND *SCHISTOSOMA MANSONI,* AND THEIR INTERACTIONS

by

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B.S., Microbiology, Michigan State University, 2008
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ABSTRACT

Schistosomiasis, caused by trematodes in the genus *Schistosoma*, is a widespread neglected tropical disease with the species *S. mansoni* infecting over 100 million people. We aimed to better understand the snail host and parasite responses during intramolluscan stages of infection by performing dual RNA-Seq on field-collected snails with natural infections from western Kenya. We collected uninfected *Biomphalaria pfeifferi, B. pfeifferi* with a patent cercariae-producing *S. mansoni* infection, and *B. pfeifferi* exposed to field-collected *S. mansoni* at 1 and 3d (days post infection).

We first created a high-quality *B. pfeifferi* transcriptome to identify the snail response to *S. mansoni* infection. As reported in Chapter 2 and published in *PLoS NTD*, *B. pfeifferi* individuals show different patterns of transcriptional response, indicating 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 oviposition-related hormones and enzymes involved in metabolism of bioactive monoamines. Both generalized stress and immune factors immune factors exhibited complex transcriptional responses.

Chapter 3 explores *S. mansoni* transcriptomic activity during intramolluscan stages. The core metabolic transcriptome includes transporters required for glucose, amino acid, and nucleoside acquisition from *B. pfeifferi*. Proteases were expressed at all stages including elastases. Transcripts associated with GPCRs,

and stress and defense responses were well represented. We noted transcripts homologous to planarian anti-bacterial factors, neuropeptides and receptors associated with schistosome germinal cell maintenance that could also impact host reproduction. The presence of another trematode species (amphistome) in one snail was associated with repressed *S. mansoni* transcriptional activity.

Chapter 4 looks at the combined responses of the snail and parasite to the molluscicide, niclosamide. The parasite maintains expression of >80% of transcripts expressed in shedding stages with little evidence of a lethal effect from niclosamide. Conversely, niclosamide provokes a dramatic response in *B. pfeifferi*, with a majority of features up-regulated, including those for xenobiotic processing. The response of *B. pfeifferi* to both niclosamide and patent *S. mansoni* infection was greater than to either stressor alone with evidence of apoptosis, reduced protein synthesis, reduced production of detoxification enzymes, and diminished innate immune function.

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CHAPTER ONE
INTRODUCTION

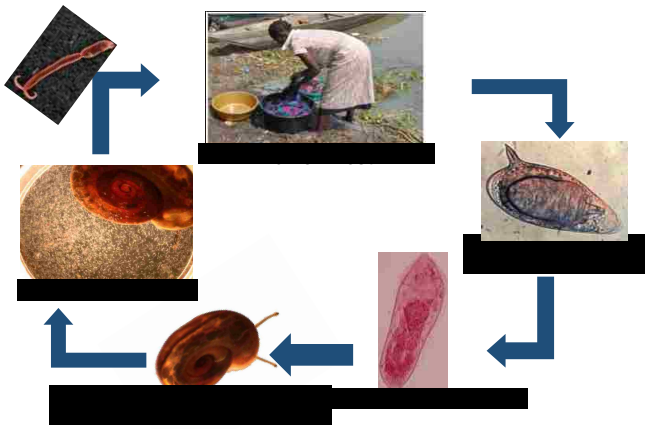
Schistosome primer

More than 25-50 thousand species of digenetic trematodes (also known as digeneans, or flukes) may exist (Cribb et al. 2002). Digeneans are remarkable for their complex life cycles involving sexual reproduction in (usually) a vertebrate definitive host, and asexual reproduction (almost always) in a molluscan intermediate host. The vast majority of digeneans require a gastropod intermediate host, occasionally infecting a bivalve or scaphopod mollusc, with a very few species developing in marine annelids. Among the most noteworthy digeneans are members of the family Schistosomatidae, which are unusual in several regards: they are dioecious with separate male and female worms; they live within the vasculature of the definitive host, hence they are called “blood flukes”; and several species in the genus *Schistosoma* are responsible for causing human schistosomiasis, one of the great neglected tropical diseases that still infects over 200 million people.

Schistosoma mansoni causes intestinal schistosomiasis, mostly in Africa but it is also found in SW Asia and South America and on some Caribbean islands. Male and female adult worms in the human host pair, copulate, and within the females a distinctive spined egg is produced that is typically deposited in the intestinal wall. Within each egg is found a developing miracidium (Figure 1). The eggs are passed in the feces and once they reach freshwater, they hatch. From each egg a free-living miracidium emerges and locates a snail intermediate host. This species is remarkable for its dependence on particular species of snails within a particular genus, *Biomphalaria*, for their successful intramolluscan development. In Kenya, *Biomphalaria pfeifferi* exhibits a high degree of susceptibility to *S. mansoni* (Mutuku et al. 2014). The miracidium penetrates *B. pfeifferi* and transforms into a mother sporocyst. One mother sporocysts can give rise to several daughter sporocysts that, following their migration to the digestive gland and ovotestis of the snail, can produce thousands of human-infective

cercariae. When fully developed in their snail hosts, schistosome sporocysts can occupy 65% of the snail's digestive gland (Gérard et al. 1993). Furthermore, infected snails are almost always partially or wholly castrated as a result of infection, this comprising one of the most widespread yet still poorly understood examples of parasite manipulation of host biology (Humphries 2011). Eventually cercariae are released from the snail, swim through the water and locate a human host to infect.

Figure 1. *Schistosoma mansoni* life cycle



One of the goals of my studies was to examine interactions between *S. mansoni* and *B. pfeifferi*. This is because *B. pfeifferi*, in spite of being the major species of snail transmitting *S. mansoni* over much of Africa, has rarely been the subject of molecular study and many aspects of its basic biology remain poorly known. This is particularly unfortunate because greater than 90% of the world's *S. mansoni* is now found in Africa. Although the Neotropical snail *B. glabrata* has been and will be the model host for *S. mansoni* in most studies, and we have learned a great deal about the biology of *B. glabrata* (Adema et al. 2017), it is important to understand how *S. mansoni* interacts with *B. pfeifferi*, because *S. mansoni* evolved in Africa and *B. pfeifferi* or a snail related to it, may well have been its ancestral host. Also, there are some fundamental differences in the basic biology between *B. glabrata* and *B. pfeifferi*. The former snail is a preferential outcrosser whereas the latter has been shown time and again to be a

preferential self-fertilizer. Thus its interactions with *S. mansoni* may prove to be quite unique.

Another goal of my study was to refocus attention on fresh isolates of *S. mansoni* and *B. pfeifferi* from the field in Kenya (see below), rather than on strains that have been maintained for convenience in the laboratory for decades. We wished to look at more genetically diverse participants, to get a feel for how variable the responses might be for both snail and schistosome. We also were interested in having these interactions occur in snails that also harbored natural populations of symbionts that might influence the outcome of interactions with *S. mansoni*.

Schistosomiasis in Kenya

In 2016, 206.5 million people required preventive treatment for schistosomiasis with >90% of these occurring in sub-Saharan Africa (WHO 2017). Since 2001, the main global strategy of prevention and control of schistosomiasis has been mass-administration of the anthelmintic drug praziquantel to school-aged children (WHO 2001). Arguments against the exclusive use of mass-drug administration are the emergence of parasite resistance to praziquantel (PZQ) and that transmission remains uninterrupted (Danso-Appiah and De Vlas 2002; Lawn et al. 2003; Smits 2009). Within Kenya, isolates of *S. mansoni*, from previously treated patients in the city of Kisumu, have shown lower susceptibility to PZQ (Melman et al.2009). A more appropriate and effective control strategy for *S. mansoni* would be the use of integrated control combining preventive chemotherapy, sanitation, provision of clean water, and breaking the complicated life cycle to eliminate transmission (Utzinger et al. 2009).

Biomphalaria pfeifferi used in this study were collected from Kasabong stream in Asembo Village, Nyanza Province, western Kenya (34.42037°E, 0.15869°S). We enrolled human subjects who provided fecal samples containing *S. mansoni* eggs that were hatched to obtain miracidia used to infect some of the *B. pfeifferi* snails. Fecal samples were obtained from *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).

With an eye on revealing patterns in gene expression relating to both host immunity and reproduction, we analyzed an Illumina RNA-seq data set of *B. pfeifferi* with and without natural *S. mansoni* infections. The following six groups were sequenced in biological triplicate: uninfected *B. pfeifferi, B. pfeifferi* with a patent natural *S. mansoni* infection (32+ days post exposure), *B. pfeifferi* exposed to field-collected *S. mansoni* at 1 and 3d (days post exposure), *B. pfeifferi* exposed to a sublethal dose of commercial molluscicide, and patently infected *B. pfeifferi* exposed to a sublethal dose of molluscicide.

Using RNA-Seq to understand *S. mansoni-B. pfeifferi* interactions

RNA-Seq offers a previously unavailable in-depth approach for looking at *S. mansoni-B. pfeifferi* molecular associations. By looking at the transcripts the snail is expressing during this parasitic infection, we can better understand the complicated interaction that is happening between the two organisms. It is well known the *B. pfeifferi* exhibits a high degree of susceptibility to *S. mansoni*, raising another important question of how the immune system of the snail is taken over. In general, gastropod immunobiology is still poorly understood. This project aimed to better characterize known and novel snail immune transcripts, especially in their level of involvement in response to trematode infections. Also, as typical of trematode-snail interactions in general, proliferating *S. mansoni* sporocysts largely replace the snail digestive gland and ovotestis, resulting in castration. Therefore, we had a specific focus in discovering transcript expression patterns relating to both host immunity and reproduction.

By sequencing both the host and parasite simultaneously, we are able to obtain a comprehensive picture of what *S. mansoni* is producing to effect changes in *B. pfeifferi*. Using a wealth of information available from existing genome and transcriptome analyses on adult and *in vitro S. mansoni* stages (i.e. Verjovski-Almeida et al.2003; Fitzpatrick et al. 2005; Vermieire et al. 2006; Berriman et al. 2009; Protasio et al. 2012), we can better understand the

parasite's development and its ability to maintain a long-term antagonistic relationship within its obligatory snail host. We also consulted the *B. glabrata* genome (Adema et al. 2017)

I also freely acknowledge that there is only so much we can learn from an examination of transcriptional profiles, so the work here described, by providing extensive lists of genes that are expressed in both *B. pfeifferi* and *S. mansoni* - many of them never before implicated in snail-schistosome interactions - will serve as a trove of information both for future functional studies and for follow-up attempts to exploit what we have learned to disrupt the interactions between *B. pfeifferi* and *S. mansoni*.

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CHAPTER TWO

The material in this chapter has been published and has the following citation:

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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, BGBPs, 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'S 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.

Table 1. Samples used for the study with total read numbers and the percent of reads mapping to the *S. mansoni* genome that were filtered prior to *de novo* assemblies.

Field-collected samples	Replicate	Abbreviation	Paired-end reads mapping to *S. mansoni* genome‡	Paired-end reads (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

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 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 BglaB1 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. 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 tr2aacds 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 VIgL (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 *Pinctada 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) >=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).

Fig 1. Overview of novel bioinformatics pipeline developed to isolate and analyze *B. pfeifferi* transcriptomic expression from dual RNA-Seq data

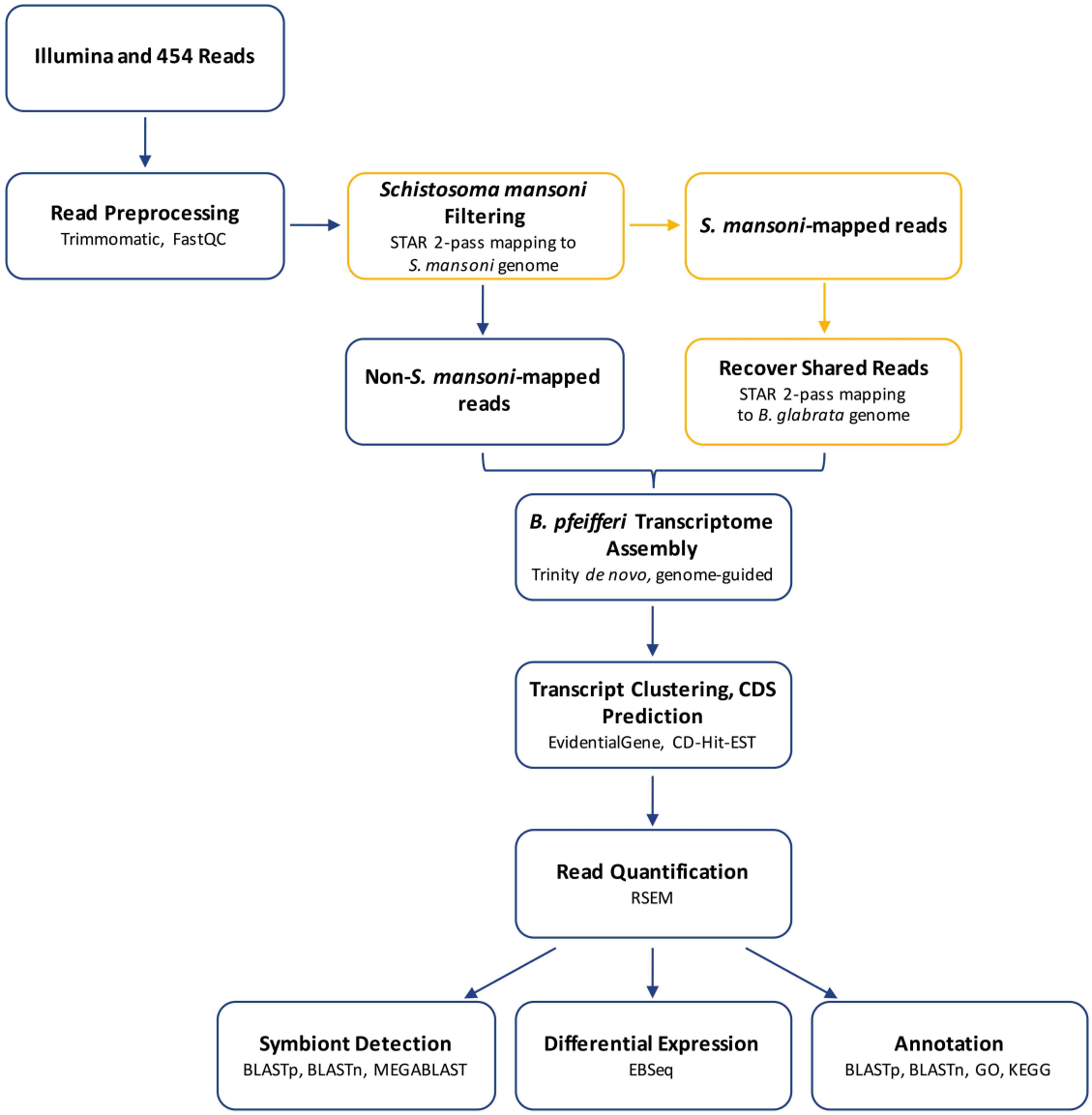


Table 2. Illumina sequencing and *B. pfeifferi de novo* transcriptome assembly summary metrics.

Raw Illumina data	
Number of paired-end reads sequenced	563,288,171
Number of reads sequenced	1,126,576,342
Reads surviving quality filtering and trimming	1,120,661,048
Reads surviving *S. mansoni* filtering	1,048,936,142
Filtered reads used in *de novo* assemblies	**1,048,936,142**
Assembled contigs	**1,805,496**
Trinity *de novo* Illumina	201,573
Trinity *de novo* Illumina including shared reads	225,929
Genome-guided Trinity *de novo* Illumina	62,682
Genome-guided Trinity *de novo* 454	71,199
Additional 454 reads	1,244,113
EvidentialGene clustering	
Okay + Okay alternate coding sequences (CDS)	**194,344**
% GC	44.24
N_{50}	654
Longest CDS length	28,302
Median CDS length	447
Average CDS length	634.57
Clusters >=1Kb	24,802
% positive strand orientation	53.2%
% negative strand orientation	46.8%
TransDecoder-predicted open reading frames (ORFs)	
Total predicted ORFs (minimum length=100 aa)	166,921
Longest ORF length (aa)	9,434
Median ORF length (aa)	157
Average ORF length (aa)	232.07
Average ORF size of 1,000 longest CDS	2014.1

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.

Table 3. CDS and predicted protein annotations using publicly available databases.

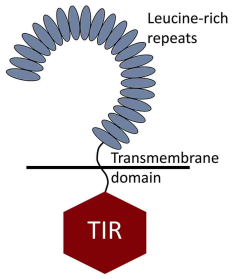
Public Database	Annotation Summary
BLASTp x nr	140,484 CDSs (72.3%) 49,518 unique protein identities
BLASTn x nt	128,028 CDSs (65.9%) 26,708 unique nt identities
InterProScan	137,778 (70.9%)
Gene Ontology (GO)	50,870 CDSs (26.2%)
Unique Molecular Function	3,246
Unique Cellular Component	1,618
Unique 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 Processing	16,093
Genetic Information Processing	13,722
Human Diseases	32,748
Metabolism	41,022
Organismal Systems	27,767

Identification of Toll-Like Receptors (TLRs) and Variable Immunoglobulin Lectins (VIgLs)

Pattern recognition receptors like TLRs and VIgLs (FREPs, 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*.

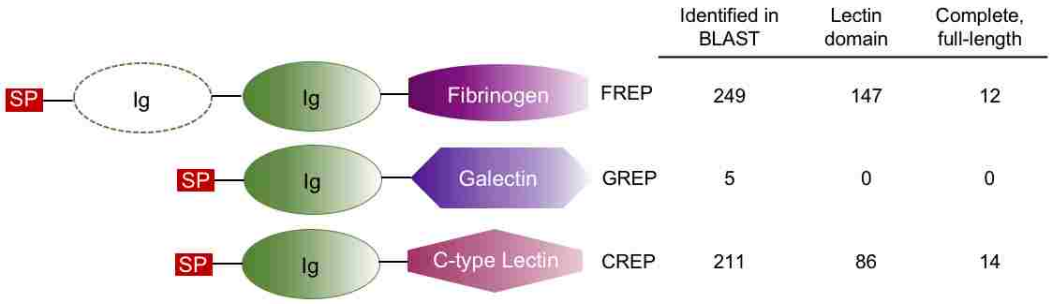
Fig 2. Identification of the innate immune recognition receptors TLRs in *B. pfeifferi.* Partial CDS counts had a BLAST hit against a known TLR but all necessary domains could not be confidently determined by InterProScan5.



Identified in BLAST	TIR domain	LRR domain(s)	Complete
190	78	118	48

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).

Fig 3. Identification of the innate immune recognition receptors VIgLs in *B. pfeifferi* with initial BLAST annotation and then verification of protein domains in InterProScan5.



	Identified in BLAST	Lectin domain	Complete, full-length
FREP	249	147	12
GREP	5	0	0
CREP	211	86	14

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 mollusc, the California two-spot octopus, *O. bimaculoides,* is 56.7% homologous at the protein level to *B. pfeifferi*.

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/
Aplysia californica v3.0*	27,591	99,884	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
Pinctada fucata v2.0 [79]	31,477	77,341	http://marinegenomics.oist.jp/pearl/viewer/download?project_id=36
Crassostrea gigas v9 [80]	45,406	80,505	ftp://ftp.ncbi.nlm.nih.gov/genomes/Crassostrea_gigas/
Octopus bimaculoides v2.0 [81]	38,585	71,395	http://genome.jgi.doe.gov/pages/dynamicOrganismDownload.jsf?organism=Metazome

*Genome is publicly available at link provided

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].

Fig 4. Identification of all *de novo* assembled transcripts after *S. mansoni* read filtering.

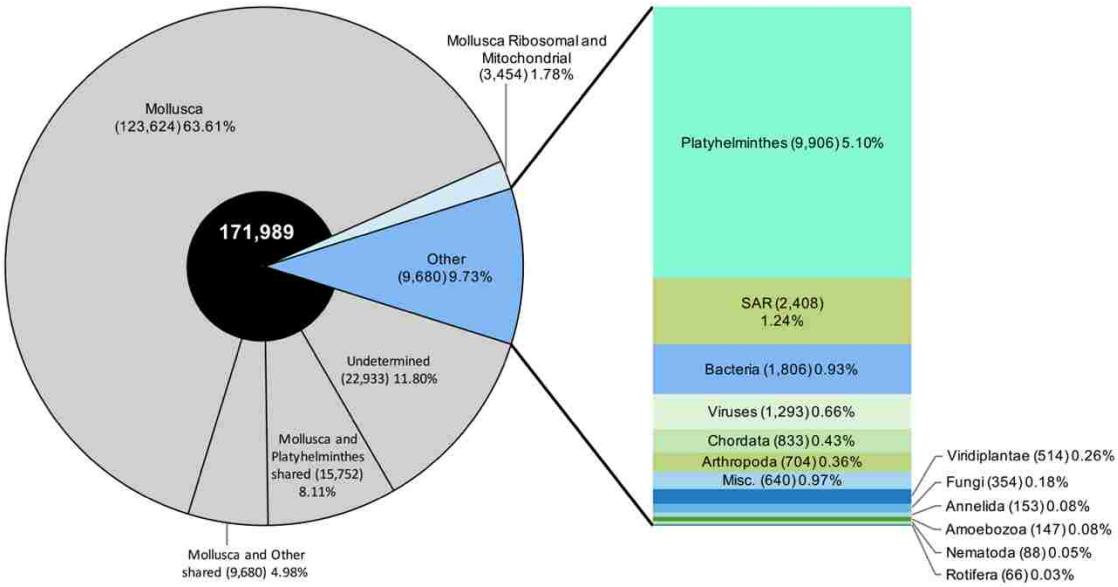
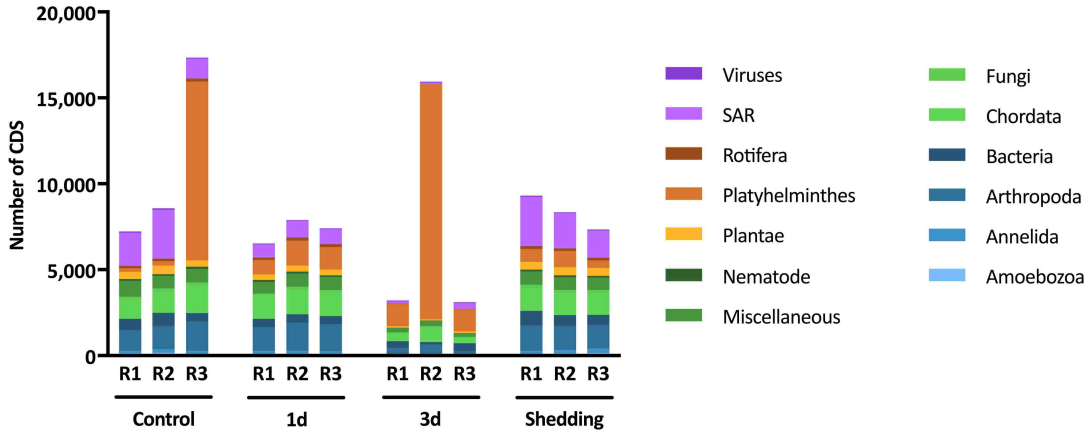


Fig 5. Sum of non-*B. pfeifferi de novo* assembled CDS for each replicate. CDS were counted as present if read count >0.



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).

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.

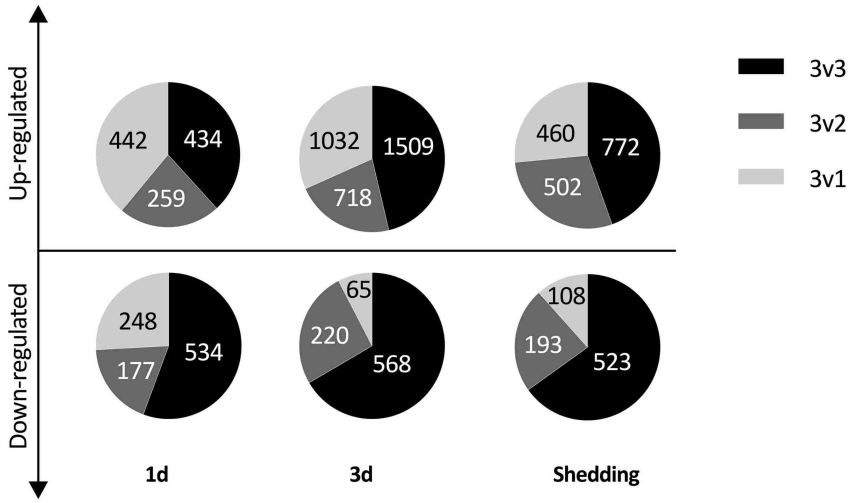
Lastly, even after the initial screening and removal of *S. mansoni* reads from the nine snails with known *S. mansoni* infections, some reads remained that were classified as platyhelminth in origin (Fig 4). Two individual snails in particular, control-R3 and 3d-R2, the latter a replicate with low *S. 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.

Fig 6. Pie charts of unique CDS found to be differentially expressed in 3v3, 3v2, and 3v1 EBSeq analyses.



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,B). 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.

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

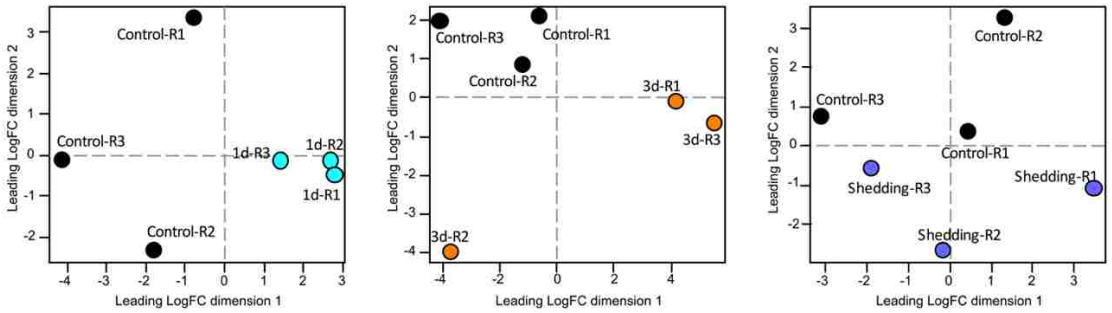
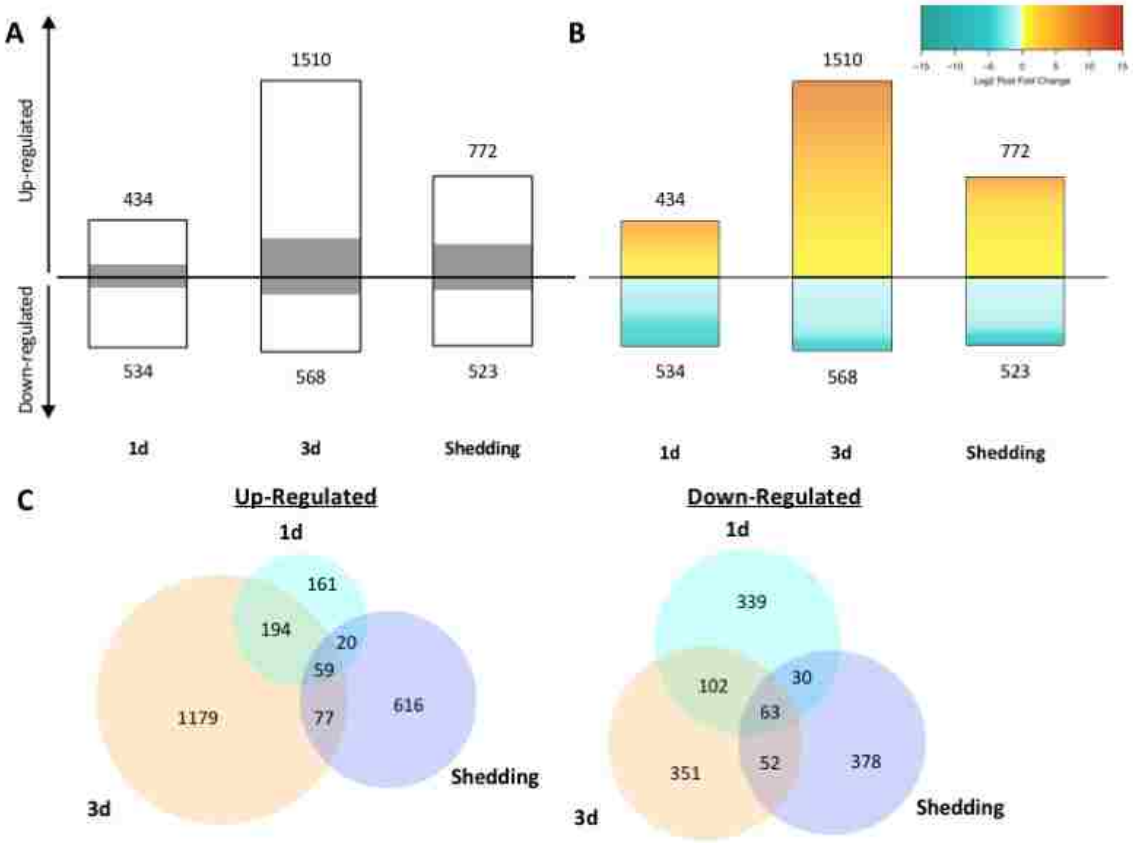


Fig 8. *Biomphalaria pfeifferi* differential expression profiles in 1d, 3d, and shedding snails. (A) Overall expression profiles for up- and down-regulated *B. pfeifferi* CDS in the 3v3 DE analysis with proportions shown for CDS with annotation known (white) and without annotation (gray) from one of the 5 databases searched (Table 3). Numbers by bars refer to numbers of up- and down-regulated features. (B) Heat map of differentially expressed *B. pfeifferi* CDS. (C) Up- and down-regulated *B. pfeifferi* CDS shared between 1d, 3d, and shedding snail groups in the 3v3 DE analysis are shown.



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_2FC 3v3**	**Log_2FC 3v2**	**Log_2FC 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	
evglcl|G0WVJSS02FGR88	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	
evgTRINITY_GG_14665_c0_g1_i2	ctenidin-3-like	-5.43	-4.89	
evgTRINITY_DN92655_c9_g2_i1	deoxyribonuclease-1-like	-4.84	-4.63	
evgTRINITY_DN68720_c0_g1_i1	testisin-like	-4.69	-4.18	

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* 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.

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.

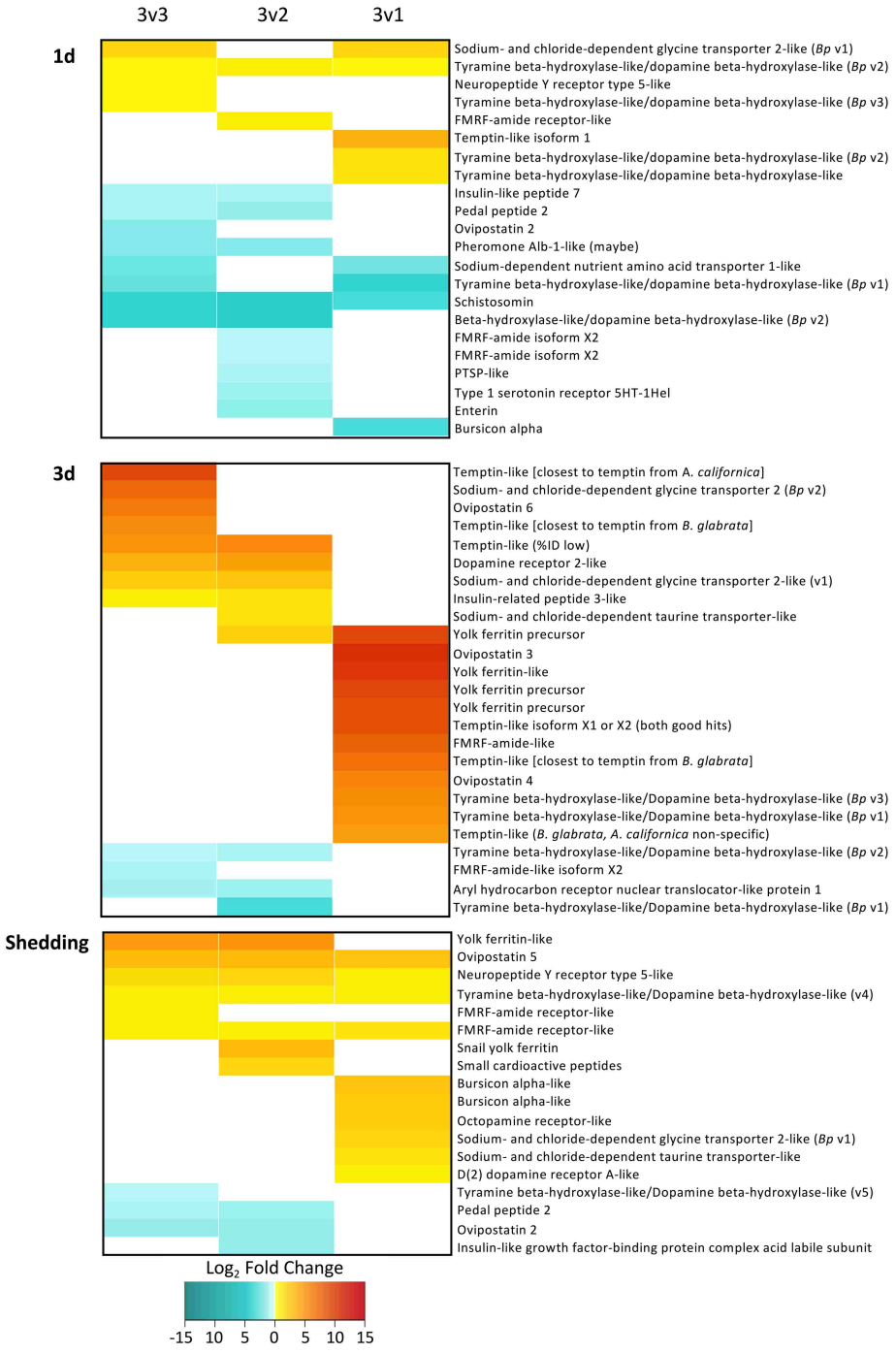
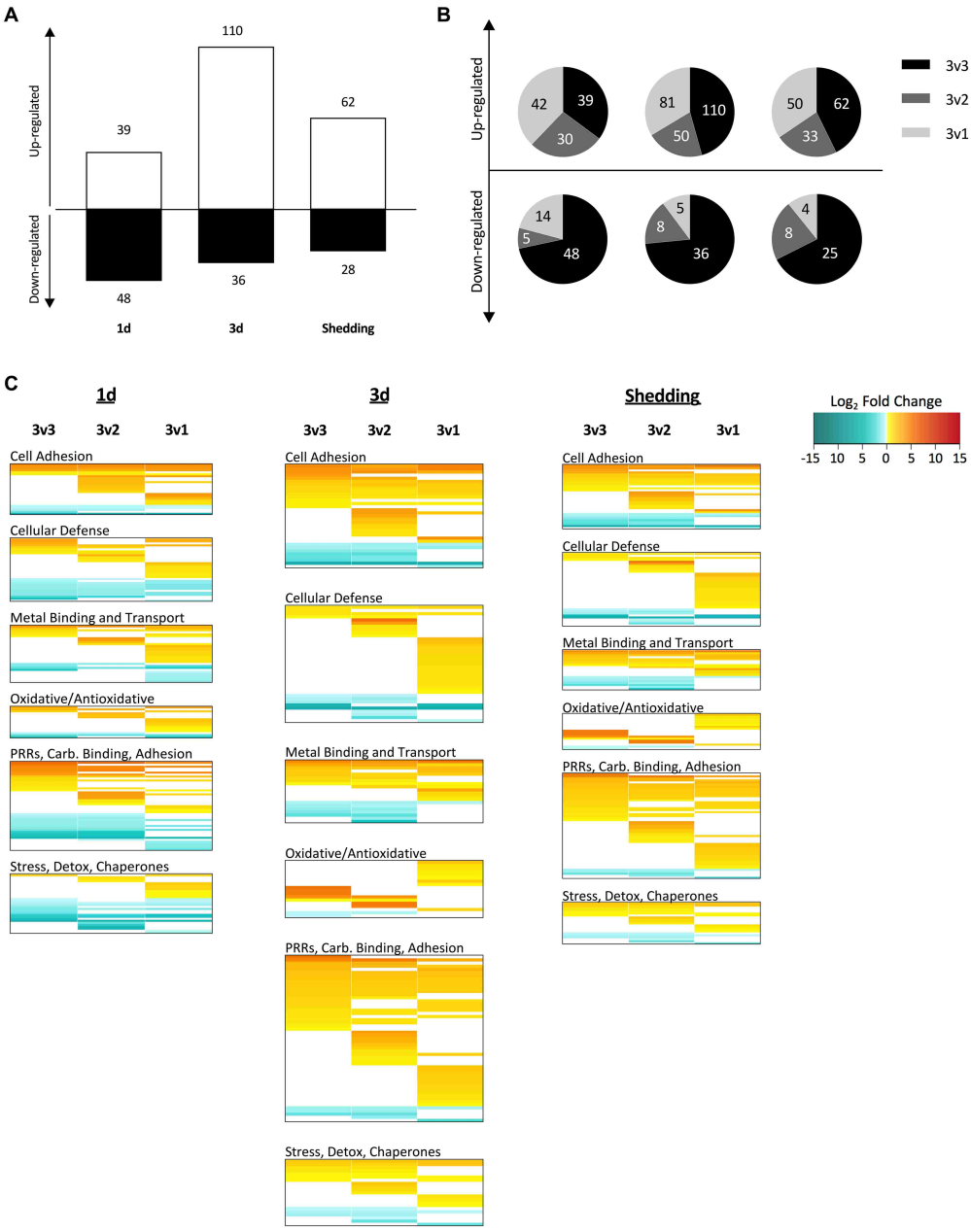


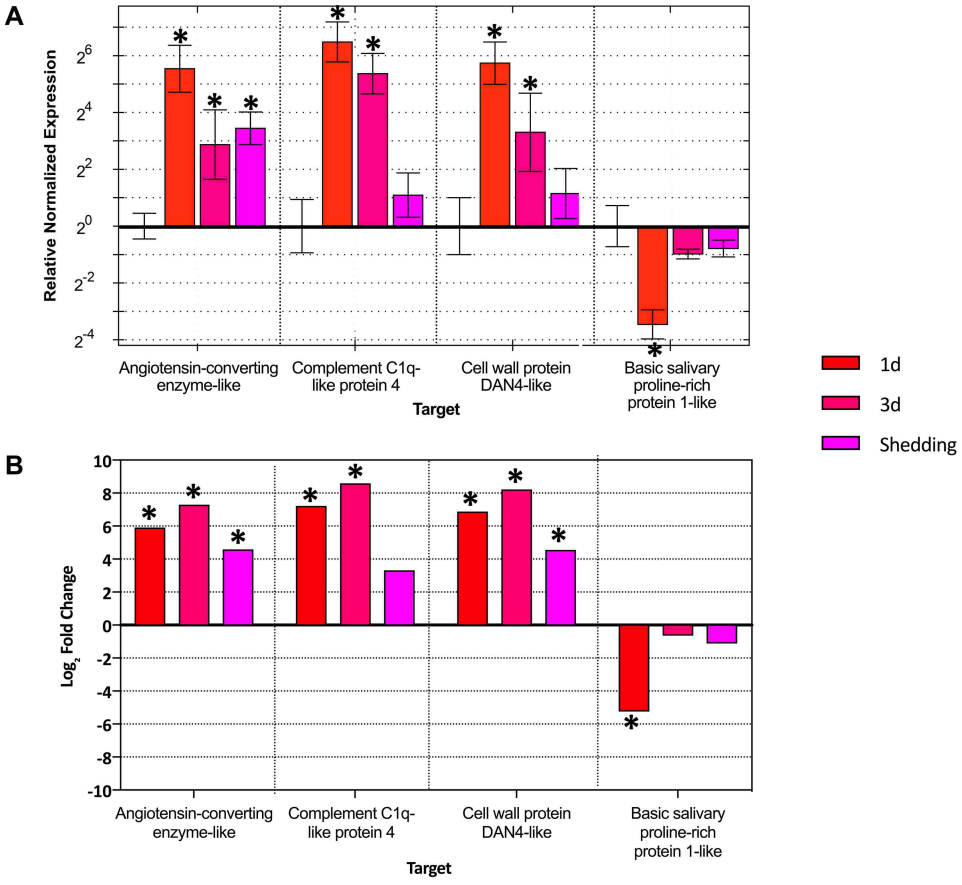
Fig 10. Differential expression of *Biomphalaria pfeifferi* defense-related CDS in 1d, 3d, and shedding snails. (A) Defense CDS in the 3v3 DE analysis. (B) Pie charts of proportions of CDS found to be DE in 3v3, 3v2, and 3v1 analyses. (C) Heat maps show expression levels from each of the three DE analyses 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.



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 11. qPCR results validate Illumina RNA-Seq differential expression results. (A) Quantitative real-time PCR verifies Illumina trends among biological replicates in 1d, 3d, and shedding samples. (B) Corresponding Illumina DE results for the four genes tested. Asterisks indicate genes that are significantly DE.



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-infecting 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 not 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 of origin where this amphistome species is the most common digenean to infect *B. pfeifferi* [92]. This co-infection may help to explain the relatively low numbers of *S. mansoni* reads recovered from this snail relative to 3d-R1 and 3d-R3. It has also been noted that *B. pfeifferi* ingests amphistome metacercariae (A Gleichsner, personal communication, June 2017) which are abundant on the submerged vegetation 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; Supplementary File 3). 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 neuro-endocrine control of reproduction [132,133]. We found evidence for the presence and expression of homologs of over 50 of these neuropeptides in *B. pfeifferi* (Supplementary File 4; 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, up-regulation 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 *Lymnaea 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-monooxygenase-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 (Supplementary File 4; 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; Supplementary File 4). 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-15-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.

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 endoglucanases Proteases and protease inhibitors Guanine nucleotide-binding protein-like 3 Translationally-controlled tumor protein	Na and Cl dependent glycine transporter 2-like neuropeptide Y receptor type 5-like DBH-like monooxygenase protein 1	dermatopontins ficolin-like proteins macrophage man rec 1-like isoform X1 C-type lectin -6 member A-like 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 Tyrosinase-like protein tyr-1	Cu, Zn superoxide dismutase 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 respiratory pigment hemoglobin insulin-like peptide 7 – modestly down pedal peptide 2 Na dependent nutrient aa transporter1-like enterin FMRF-amide isoform X2 cytidine deaminase soma ferritins	ovipostatin 2 tyramine/dopamine β-hydroxylase-like FMRF-amide isoform X2 – modestly down PTSP-like molecule pheromone Alb-1 type 1 serotonin receptor schistosomin	FREP12 and its precursors toll-like receptor 8 cytidine deaminase zinc metalloproteinase /disintegrin-like
COMPLEX (MIXED RESPONSES)			
	collagens acidic mammalian chitinase-like proteins cytochrome c oxidases Mucins Cytochrome P450 family members Multidrug resistance proteins Some heat shock proteins		

Three day post-infection (3d)

	General	Reproductive	Immune
UP-REGULATED			
Overall	phospholipase A2s endoglucanases Proteases and protease inhibitors 17-beta hydroxysteroid dehydrogenase type 6 betaine homocysteine-methyltransferase 1-like translationally controlled tumor protein homolog hemoglobin type 1	Na and Cl dependent glycine transporter 2-like temptin-like kynurenine 3-monooxygenase-like	GTPase IMAPs- complex, but mostly up beta-1,3-glucan binding proteins complement C1q-like proteins probable serine carboxypeptidases (1-5) glutathione S-transferases laccase-2-like
Two snails with higher *S. mansoni* read counts	insulin-related peptide-3-like cytochrome b serine proteases alpha and beta ADP, ATP carrier-like protein heparinase-like isoform X! serpin B6-like	Tyrosinase-like protein tyr-1 Na- and Cl-dependent taurine transporter-like dopamine receptor 2-like	dermatopontins ficolins Cu-Zn superoxide dismutases C-type lectin domain family 6, A-like chitinase-3-like protein chitotriosidase-1-like aplysianin-like proteins FREP2, FREP5 macrophage-expressed gene 1 protein-like laccase-15-like tyrosinase-1-like
One snail with least *S. mansoni* read counts	profilin cathepsin B and L1-like neuroglobinase-like chymotrypsin-like elastase family member histone transcription factor	ovipostatin 6 yolk ferritin precursor DBH-like monooxygenase protein 1	hemocytin hemagglutinin/amoebocyte aggreg factor-like X1 G-type lysozyme sialate–O-acetylesterase-like protein peroxidase-like protein fibrinogen-like protein A FREP 7 peptidoglycan-recognition proteins SC2-like LRR-containing 15-like, toll-like receptor 13
DOWN-REGULATED			
Overall	glyceraldehyde-3-phosphate dehydrogenase aryl hydrocarbon recep nucl translocator-like	FMRF-amide-like isoform X2 – modestly down tyramine/dopamine β-hydroxylase-like FMRF-amide isoform X2 – modestly down PTSP-like molecule pheromone Alb-1 type 1 serotonin receptor schistosomin	caveolin-1-like disintegrin/metalloprot containing prot t17-like FREP12 precursors LRR contain G-prot coupled rec 5-like alpha-crystalline B chain-like toll-like receptors 4 and 8 cytidine deaminase
Two snails with higher *S. mansoni* read counts		tyramine/dopamine β-hydroxylase-like	
COMPLEX (MIXED RESPONSES)			
	ornithine decarboxylase, actins, collagens, tubulins, mucins, cytochrome p450 members (mostly up), multidrug resistance proteins, HSPs, thioredoxins, annexins, putative copper-containing amine oxidases, soma ferritins		

Shedding

	General	Reproductive	Immune
UP-REGULATED			
Overall	FMRF-amide receptor-like – modestly up small cardioactive peptides phospholipases A2s arginase-1-like isoform X2 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	dopamine beta hydroxylase-like FMRF-amide receptor-like ovipostatin 5 DBH-like monooxygenase protein	pcrotocadherein Fat 3 or 4-like ADAM family mig-17-like zinc metalloproteinase nas 13- & 14-like ficolins
Two snails with higher *S. mansoni* read counts	endonuclease G mitochondrial-like zinc carboxypeptide A 1-like serpinB3-like protease inhibitor cystatin protease inhibitor putative amine-oxidases (copper containing)	yolk ferritin-like and snail yolk ferritin molecules Neuropeptide Y receptor type 5-like	aplysianin-A-like mammal ependymin-related prot 1- like zinc carboxypeptidase A1-like beta-1,3-glucan binding protein-like FREP 2, 7 and 14
One snail with least *S. mansoni* read counts	multiple epidermal growth factor-like domains serine/threonine-protein kinase mos-like		macrophage-expressed gene 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 pedal peptide 2 profilin-like isoform X1 neuroglobin-like calreticulin-like tyrosinase tyr-3	ovipostatin 2 dopamine beta-hydroxylase-like	toll-like receptor 7 galectin-6 probable serine carboxypeptidase CPVL
Two snails with higher *S. mansoni* read counts	hemoglobin collagen-related		dihydropyrimidinase macrophage man recep1-like protein tyrosinase-3-like
One snail with least *S. mansoni* read counts			tyrosinase-1-like
COMPLEX (MIXED RESPONSES)			
	collagens, 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		

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 papilin-like and galactocerebrosidase-like. Galactocerebrosidase is an enzyme that removes galactose from galactocerebrocide (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.

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SUPPORTING INFORMATION

S1 Table. qPCR primers used to validate differential expression trends.

	TARGET GENE	TARGET *B. pfeifferi* CDS	NAME	TEMP	E	R^2	SEQUENCE (5'-3')
Reference	40S ribosomal protein S2	evgTRINITY_DN94486_c0_g1_i1	S2_99-F	58°C	99.2%	0.999	GCCATCCGAGGAGCTATC
			S2_99-R				GCAGGGAACAGTGTGAGG
	elongation factor 1-alpha	evgTRINITY_DN94461_c0_g1_i1	EF1_124-F	58°C	104.7%	0.997	CCAGTCGGCAGAGTTGAG
			EF1_124-F				GGGTGGCTTCCTGAAGAG
Experimental	basic salivary proline-rich protein 1-like isoform X2	evgTRINITY_DN93030_c30_g1_i1	Sal_148-F	56°C	90.0%	0.989	TGGCTGTCTTCCTTACAAGC
			Sal_148-R				TTGCTCGAGCTTTTGGTC
	complement C1q-like protein 4, partial	evgTRINITY_GG_443_c0_g2_i1	C1q4_151-F	58°C	101.8%	0.995	GCTCTACCTGTTCCAGTTCC
			C1q4_151-R				AGCCAGCTTCAGGATTACAG
	cell wall protein DAN4-like	evgTRINITY_DN88408_c0_g1_i1	DAN4_156-F	56°C	101.4%	0.998	GATGTTGTCCAGATGCAGTG
			DAN4_156-R				GAGTCGATGTTTCCTCAACC
	angiotensin-converting enzyme-like, partial	evglcl|G0WVJSS02GCJC6	Ang_190-F	54°C	99.5%	0.998	TGCTTGCTGGAGTCAGTAGTC
			Ang_190-R				AACTGATCCCACTTGAGCTG

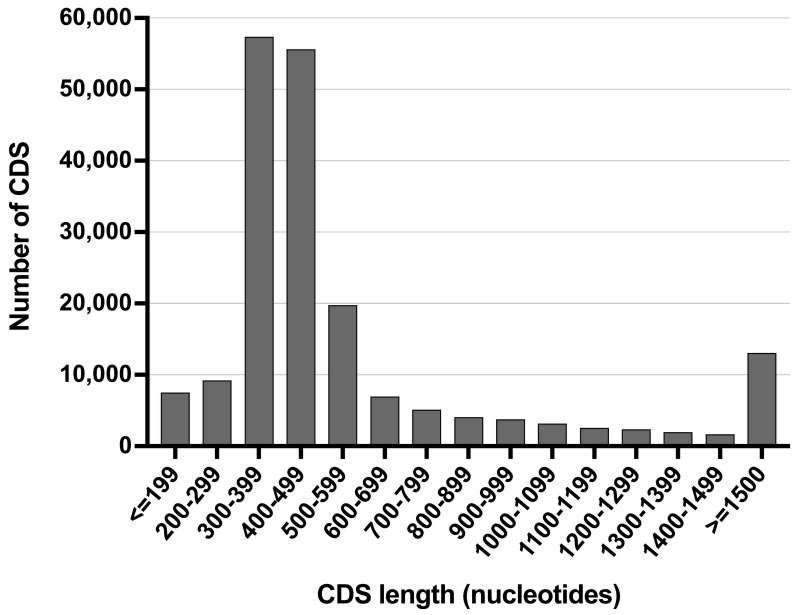
S2 Table. *Biomphalaria pfeifferi* CDS identified as a symbiont of interest during annotation efforts and verified by MEGABLAST x NCBI nt database.

MEGABLAST IDENTIFICATION	GI	*B. pfeifferi* CDS	% ID	% Query Coverage
Chaetogaster diastrophus 28S rRNA	GQ355444.1	evglcl|HJ4YRIA02J27K8	93%	100%
Chaetogaster diastrophus 28S rRNA	GQ355444.1	evglcl|HJ4YRIA02GT5FU	91%	100%
Chaetogaster diastrophus 28S rRNA	GQ355444.1	evglcl|HJ4YRIA01CKT7N	91%	100%
Chaetogaster diastrophus 28S rRNA	GQ355444.1	evglcl|HJ4YRIA02IR0J2	91%	99%
Chaetogaster diastrophus 28S rRNA	GQ355444.1	evglcl|HJ4YRIA02FUU8R	90%	100%
Chaetogaster diastrophus 28S rRNA	GQ355444.1	evglcl|HJ4YRIA01ALBT8	92%	93%
*Trichodina sp.*18S rRNA	KP295473.1	evglcl|HJ4YRIA02F8S6H	100%	93%
Trichodina sp. 18S rRNA	KP295473.1	evgTRINITY_GG_454Inf_840_c12_g1_i1	99%	100%
Trichodina sp. 18S rRNA	AY363960.1	evgTRINITY_DN54951_c0_g1_i1	99%	100%
Paenibacillus sp.	CP00928.1	evglcl|HJ4YRIA02HHYCK	90%	95%
Paenibacillus sp.	CP018620.1	evglcl|HJ4YRIA01AG2P6	82%	99%
Paenibacillus sp.	CP018620.1	evglcl|HJ4YRIA01AHR45	81%	99%
Neorickettsia sp. 3225 16S rRNA	KX818103.1	evglcl|HJ4YRIA02JQFT3	99%	100%
Microsporidium sp. BIOB 16S rRNA, ITS1	AJ871391.1	evglcl|HJ4YRIA02GDN25	100%	100%
Microsporidium sp. BIOPC 16S rRNA	AJ871390.1	evglcl|HJ4YRIA02FYGW4	99%	100%
Microsporidium sp. BIOPC 16S rRNA	AJ871390.1	evglcl|HJ4YRIA02H8J2W	99%	100%
Microsporidium sp. BUL 16S rRNA	AJ871392.2	evgTRINITY_GG_454Inf_715_c1_g1_i1	99%	99%
Microsporidium sp. BUL 16S rRNA	AJ871392.2	evglcl|HJ4YRIA02ID8PW	99%	100%
Microsporidium sp. BUL 16S rRNA	AJ871392.2	evgTRINITY_GG_454Inf_415_c15_g1_i1	100%	100%
Microsporidium sp. BUL 16S rRNA	AJ871392.2	evglcl|HJ4YRIA01B442V	99%	100%
Microsporidium sp. BUL 16S rRNA	AJ871392.2	evglcl|HJ4YRIA01ATBOD	99%	100%
Microsporidium sp. BUL 16S rRNA	AJ871392.2	evglcl|HJ4YRIA02GPVDH	99%	100%
Microsporidium sp. HEM-2006MCD 16S rRNA	AM411637.1	evglcl|HJ4YRIA02FZ7N4	97%	99%
Microsporidium sp. HEM-2006MCD 16S rRNA	AM411637.1	evglcl|HJ4YRIA01B7MY3	98%	100%
Microsporidium sp. HEM-2006MCD 16S rRNA	AM411637.1	evglcl|HJ4YRIA01C1BLC	99%	100%

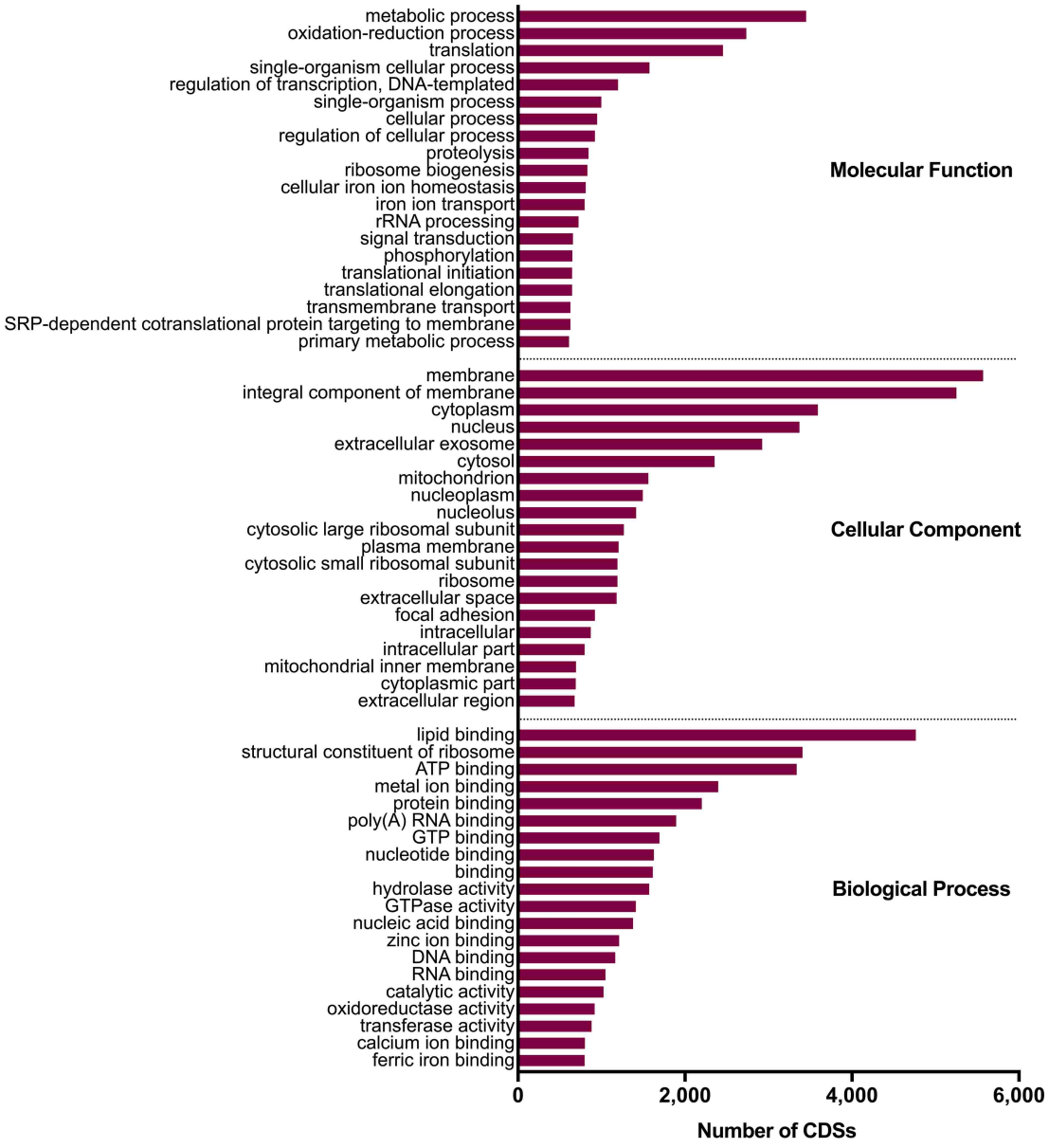
S3 Table. *Biomphalaria pfeifferi* CDS with a BLASTn hit against publicly available genomes and CDS from molluscan symbionts of interest.

	Species of Interest	Data File	# *B. pfeifferi* CDS
	Capsaspora owczarzaki	Capsaspora_owczarzaki_atcc_30864.C_owczarzaki_V2.cds.all [85]	103
	Capsaspora owczarzaki	Capsaspora_owczarzaki_atcc_30864.C_owczarzaki_V2.dna.toplevel [85]	216
	Perkinsus marinus	Perkinsus_marinus_atcc_50983.JCVI_PMG_1_0.cds.all	121
	Perkinsus marinus	Perkinsus marinus ATCC 50983 genome	257
Microsporidians [86-89]	*Anncalliia algerae*	anncaliia_algerae_pra109_4	1
	Anncalliia algerae	anncaliia_algerae_pra339_2	1
	Edhazardia aedis	edhazardia_aedis_usnm_41457_1	13
	Encephalitozoon cuniculi	encephalitozoon_cuniculi_eci	26
	Encephalitozoon cuniculi	encephalitozoon_cuniculi_ecii	36
	Encephalitozoon cuniculi	encephalitozoon_cuniculi_eciii	6
	Encephalitozoon cuniculi	encephalitozoon_cuniculi_gb-m1	3
	E. intestinalis	encephalitozoon_intestinalis_atcc_50506	1
	Nematocida sp	nematocida_sp1_ertm2_1	4
	Nematocida sp	nematocida_sp1_ertm6_2	4
	Vavraia culicis	vavraia_culicis_floridensis_1	12

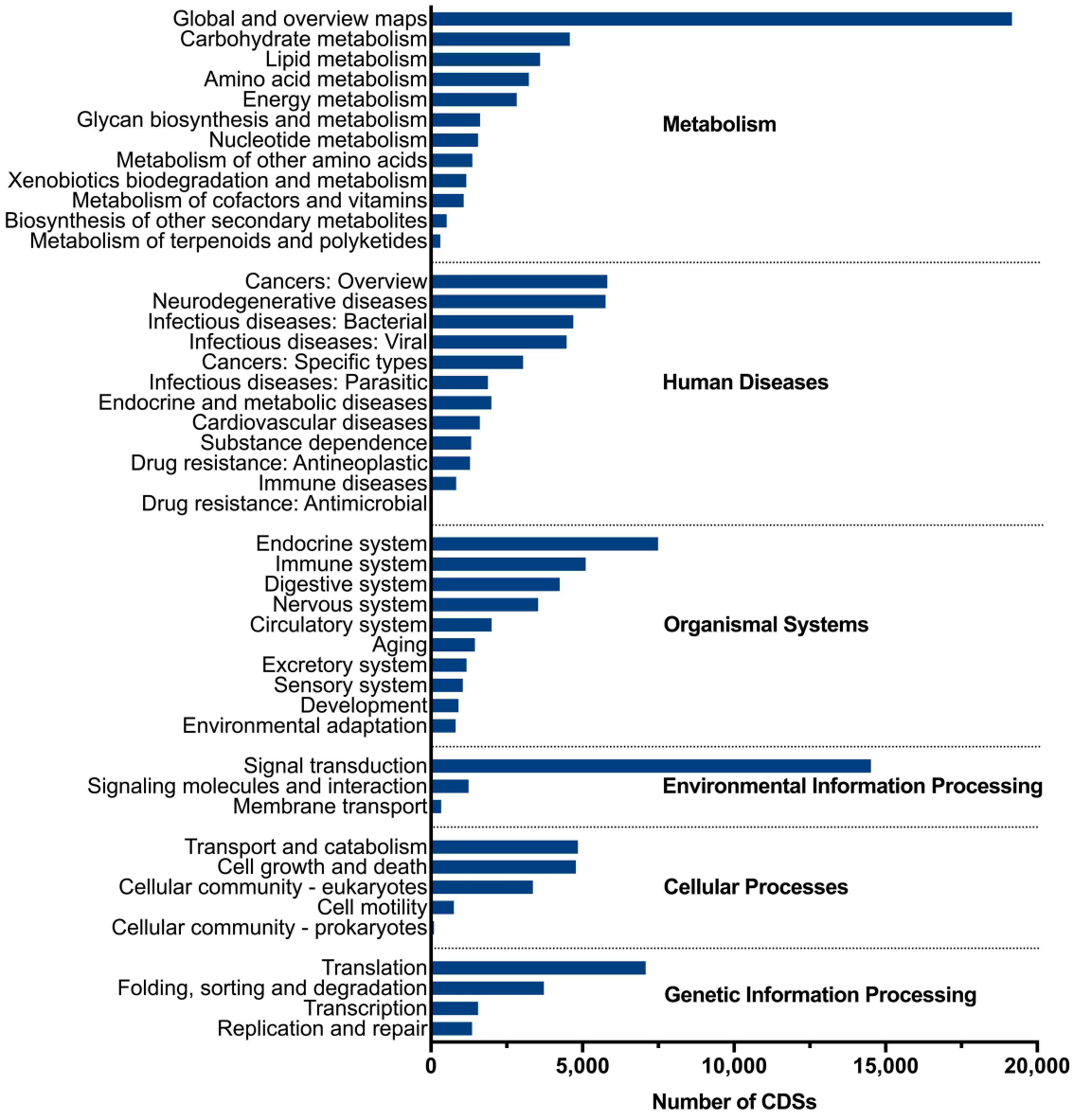
S1 Fig. Nucleotide sequence length distribution of 194,344 assembled *B. pfeifferi* CDS from EvidentialGene.



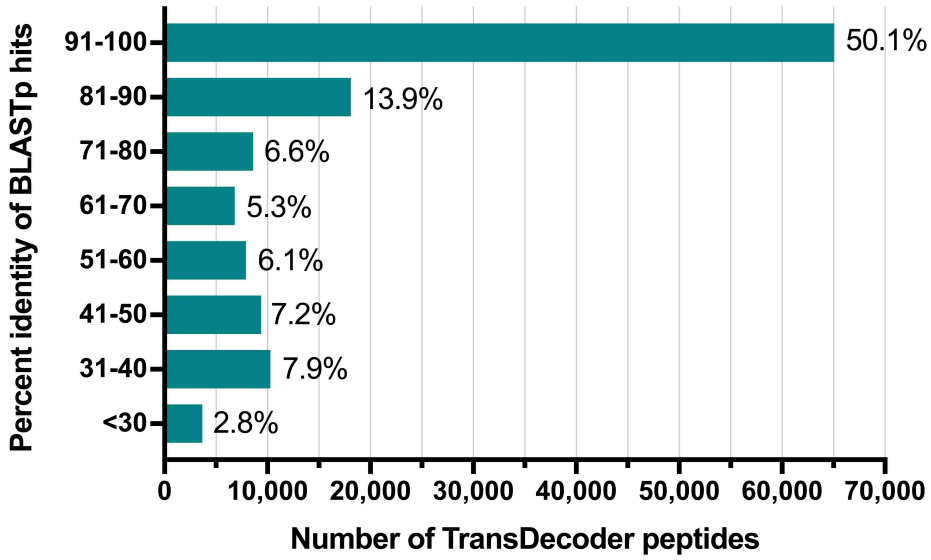
S2 Fig. The top 20 most abundant Gene Ontology assignments for *B. pfeifferi* CDS in each molecular function, cellular component category, and biological process.



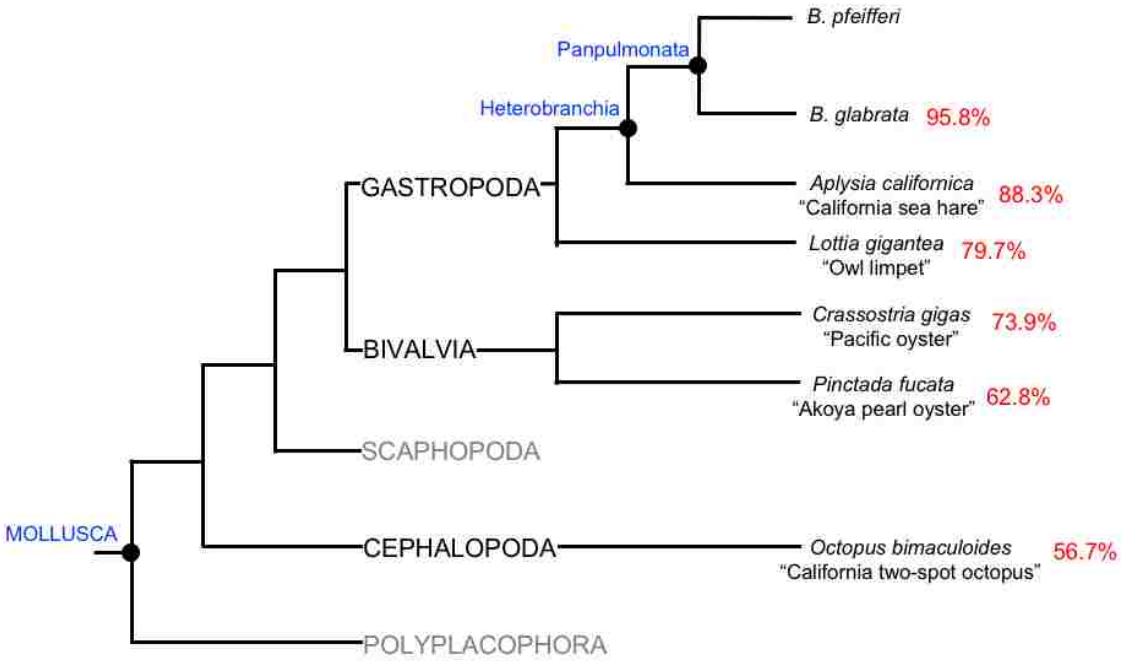
S3 Fig. All represented KEGG classes, organized by category and abundance, in the *B. pfeifferi* transcriptome.



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



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.



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CHAPTER THREE

The *in vivo* transcriptome of *Schistosoma mansoni* in two prominent vector species, *Biomphalaria pfeifferi* and *B. glabrata*

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ABSTRACT

Background

The full scope of the genes expressed by schistosomes during intramolluscan development has yet to be characterized. Understanding the gene products deployed by larval schistosomes in their snail hosts will provide insights into their establishment, maintenance, asexual reproduction, ability to castrate their hosts, and their prolific production of human-infective cercariae. Using the Illumina platform, the intramolluscan transcriptome of *Schistosoma mansoni* was investigated in field-derived specimens of the prominent vector species *Biomphalaria pfeifferi* at 1 and 3 days post infection (d) and from snails shedding cercariae. These *S. mansoni* samples were derived from the same snails used in our complementary *B. pfeifferi* transcriptomic study. We supplemented this view with microarray analyses of *S. mansoni* from *B. glabrata* at 2d, 4d, 8d, 16d, and 32d.

Principal Findings

Transcripts representing at least 7,740 (66%) of known *S. mansoni* genes were expressed during intramolluscan development, with the greatest number expressed in snails shedding cercariae. Many transcripts were constitutively expressed throughout development featuring membrane transporters, and metabolic enzymes involved in protein and nucleic acid synthesis and cell division. Several proteases and protease inhibitors were expressed at all stages, including some proteases usually associated with cercariae. Transcripts associated with G-protein coupled receptors, germ cell perpetuation, and stress responses and defense were well represented. We noted transcripts homologous to planarian anti-bacterial factors, several neural development or neuropeptide transcripts including neuropeptide Y, and receptors that may be associated with schistosome germinal cell maintenance and that could also impact host reproduction. In at least one snail the presence of larvae of another digenean species (an amphistome) was associated with repressed *S. mansoni* transcriptional activity.

Conclusions/Significance

This *in vivo* study, particularly featuring field-derived snails and schistosomes, provides a distinct view from previous studies of development of cultured intramolluscan stages from lab-maintained organisms. We found many highly represented transcripts with suspected or unknown functions, with connection to intramolluscan development yet to be elucidated.

INTRODUCTION

The vast majority of the estimated 18,000 species of digenetic trematodes depend on a molluscan host, usually a gastropod, in which to undertake the complex developmental program characterized by extensive asexual reproduction and production of numerous cercariae (Erasmus 1972; Basch 1991; Olson et al. 2003). The extent to which this large lineage of parasites has remained true to its dependence on molluscs, and the evident success achieved by digeneans - including by some species responsible for causing human disease - pose fundamental questions of interest for parasitologists, evolutionary biologists, ecologists, developmental biologists and comparative immunologists.

There is much about digenean-gastropod associations worthy of study: the host specificity often shown; the manner by which digeneans establish intimate infections without provoking destructive host responses; the ability of digeneans to affect and manipulate the energy and resource budgets of their hosts, including to achieve host castration; the intricate developmental program featuring multiple stages, asexual reproduction and the perpetuation of the germinal cell lineage; and finally the tendency for some infections to persist for long periods of time, implying protection of the snail-digenean unit that might involve contributions by the parasite to promote its perpetuation. These common and enduring relationships are also targeted and exploited by other organisms, including competing digenean species. One way forward to gain a deeper understanding of all these processes is to acquire a comprehensive overview of the genes expressed by host snails and larval digeneans during the course of

infection. This in turn sets the stage for eventually learning more about these two sets of gene products interact (the interactome) to influence the outcome of this interaction.

Because *S. mansoni* causes intestinal schistosomiasis in an estimated 166 million people in the Neotropics, Africa and Southwest Asia, it has long been intensively studied, in part because it is relatively easily maintained in a laboratory setting (Lee and Lewert 1956; Lewis et al. 1986). Many molecular tools, including the current fifth version of an improving genome sequence and assembly are available for *S. mansoni* (Berriman et al. 2009; Protasio et al. 2012). Additionally, *B. glabrata*, the most important host for *S. mansoni* in the Neotropics, has become a model gastropod host, including with a recently available genome sequence (Adema et al. 2017). In Africa, several *Biomphalaria* species play an important role in transmission, with the most important being *B. pfeifferi*. The latter species occurs widely across sub-Saharan Africa, where >90% of the world's cases of schistosomiasis now occur. *B. pfeifferi* is probably responsible for transmitting more *S. mansoni* infections than any other snail species (Brown 1994).

With respect to the intramolluscan development of *S. mansoni*, following penetration of miracidia usually into the tentacles or head-foot of the snail, there is a 24 hour period of transformation as miracidial ciliated epidermal plates, apical papilla, and sensory papillae are shed and a syncytial tegument is formed around the developing mother (or primary) sporocyst (Schutte 1974; Pan 1996). This early period can be thought of as one of parasite transition and establishment. The miracidium has carried into the snail a series of germinal cells that are destined to give rise to the daughter (or secondary) sporocysts (Schutte 1974). Mitotic division of germinal cells begins as early as 24 hours and germinal cells proliferate notably in an enlarging mother sporocyst (Wang et al. 2013). By 6 days after infection, all mother sporocysts have germinal balls (daughter sporocyst embryos) which occupy nearly the entire body cavity (Maldonado and Acosta-Matienzo 1947; Wajdi 1966; Schutte 1974). The embryonic daughter sporocysts, of which there are an average of 23 produced per mother sporocyst

(Schutte 1974), continue to grow and elongate. Daughter sporocysts exit mother sporocysts at 12-14 days and start their migration to the digestive gland and ovotestis region of the snail (Maldonado and Acosta-Matienzo 1947; Pan 1964; Schutte 1974). Upon release of daughter sporocysts, mother sporocysts collapse and typically do not continue to produce daughter sporocysts, but they nonetheless persist in the head-foot of the snail. By 15-20 days, daughter sporocysts undergo a remarkable transformation, losing their definitive vermiform shape to become amorphous and are wedged between lobules of the digestive gland. Within them, cercarial embryos begin to develop, passing through 10 characteristic developmental stages culminating in the production of a muscular tail and a body dominated by gland cells that are filled with lytic enzymes (Cheng and Bier 1972; Schutte 1974). Once again, a separate allotment of germinal cells is sequestered in the cercarial body and these are destined to become the gonads and reproductive cell lineages of adult worms. Around 32 days post-exposure, cercariae exit from daughter sporocysts, migrate through the snail's body and emerge into the water, usually through hemorrhages in the mantle. Daughter sporocysts occupy a significant proportion of the snail host's body; 65% of the snail's digestive gland can be occupied by daughter sporocysts in a patent, cercariae-producing infection (Gerard et al. 1993).

The timing of these events is dependent on temperature but cercarial shedding can occur as early as 19 days post-miracidial penetration (Frandsen 1979). Also, in some situations, daughter sporocysts will produce granddaughter sporocysts in lieu of cercariae (Jourdane et al. 1980). So, beginning with the penetration of a single miracidium and proceeding through at least two distinct phases of asexual reproduction, thousands of cercariae can ultimately be produced (Colley et al. 2014). A typical consequence of infection is that snails are partially or totally castrated, the extent depending on whether they were infected before or after achieving maturity (Pan 1964; Humphries 2011). This interaction is remarkable in that some *Biomphalaria* snails can survive for over a year shedding cercariae daily (Colley et al. 2014), although there is considerable variability in the duration of survival of infected snails. The productivity of

infections within snails has no doubt contributed greatly to the success of all digenetic trematodes, and in the case of human-infecting schistosomes, is a major factor complicating their control.

Despite the significant immunobiological, physiological, and reproductive changes inflicted upon infected *Biomphalaria* snails (Buddenborg et al. 2017), we still lack a comprehensive picture of what the parasite is producing to effect such changes. Verjovski-Almeida et al. (2003) recovered 16,715 ESTs from early-developing cercarial germ balls derived from snails with patent *S. mansoni* infections. These ESTs were distinctive from those noted for miracidia, cercariae, schistosomulae, eggs, and adults. A first-generation *S. mansoni* microarray containing 7,335 features was used to monitor expression changes of miracidia and *in vitro*-cultured 4d mother sporocysts (Vermeire et al. 2006). Of the 7,335 features, 273 (6%) of these were expressed only in sporocysts. Gene products with antioxidant activity, oxidoreductases, and intermolecular binding activity were represented in mother sporocyst-specific genes. Proteomic analyses of products released *in vitro* by miracidia of *S. mansoni* transforming into mother sporocysts revealed 127 proteins produced, 99 of which could be identified (Wu et al. 2009). Among these were proteases, protease inhibitors, heat shock proteins, redox/antioxidant enzymes, ion-binding proteins, and venom allergen-like (SmVAL) proteins. Wang et al. (2016) also provided an analysis of proteins released by *S. mansoni* miracidia and noted several of the same features, and also provided a foundation for further study of neurohormones produced by *S. mansoni* larvae. Cultured mother sporocysts were a component of SAGE tag generation by Williams et al. (2007). Highlights of 6d and 20d cultured mother sporocysts transcript expression were an up-regulation of HSP 70, HSP 40, egg protein, and trypsinogen 1-like all exclusive to miracidia and sporocyst stages. LongSAGE was utilized by Taft et al. (2009) to identify transcripts from miracidia, or 6d or 20d cultured mother sporocysts grown in medium conditioned by sporocysts or by products derived from the Bge (*B. glabrata* embryo) cell line. Amongst the groups studied, 432 transcripts were differentially expressed (DE),

which was also dependent on whether or not the sporocysts had been conditioned in medium with Bge cell products.

A comprehensive *S. mansoni* microarray was developed with 37,632 features derived from 15 life cycle stages including cercariae and two intramolluscan stages (2d mother sporocyst cultured *in vitro* and migrating daughter sporocyst dissected from snails at 15d) (Fitzpatrick et al. 2009). Between cercariae and 15d daughter sporocysts, a large number of genes were DE (2,535) and 1,971 features were DE between 2d mother sporocysts and 15d daughter sporocysts. Wang et al. (2013) in a functional study of germinal cells in intramolluscan stages of *S. mansoni* noted similarities in molecular signatures with the neoblast stem cells produced by planarians.

Here, our primary focus is on presentation of RNA-Seq results for *S. mansoni* from the same field-derived Kenyan snails that comprised the *B. pfeifferi* transcriptomic study of Buddenborg et al. (2017). Briefly, field-derived snails found negative upon isolation and shedding were exposed experimentally to *S. mansoni* miracidia hatched from eggs from fecal samples from local schoolchildren. These exposed snails were harvested 1 or 3 days later. Additionally, field snails found to be naturally shedding *S. mansoni* cercariae were chosen for study. Our goal was to provide *in vivo* views of establishment of early mother sporocyst development and shedding stages for snails and parasites taken directly from natural transmission sites. We did not investigate longer exposure intervals following experimental exposures because we did not want these snails to lose their field characteristics. Additionally, we supplemented these observations with results from a set of independent microarray experiments of *S. mansoni* in *B. glabrata* acquired at 2, 4, 8, 16, and 32d. These time points cover some additional stages in development including production, release and migration of daughter sporocysts. Our approach is distinctive in its focus on *in vivo* life cycle stages, the inclusion of both snails naturally infected from an endemic area in western Kenya and of laboratory-maintained snails, and the use of two transcriptome technologies for co-validation of expression data. Using Gene Ontology (GO), and KEGG (Kyoto Encyclopedia of Genes and

Genomes) analysis, we grouped functionally similar transcripts and provide a general overview of *S. mansoni* intramolluscan development. We then examined specific groups of transcripts to gain distinctive insights on intramolluscan development. The database we provide should also provide helpful information in eventually achieving a deeper understanding of the interactome that is the essence of this dynamic host-parasite interaction.

METHODS

Ethics statement and sample collection

Details of recruitment and participation of human subjects for procurement of *S. mansoni* eggs from fecal samples collection are described in Mutuku et al. (2014) and Buddenborg et al. (2017). The Kenya Medical Research Institute (KEMRI) Ethics Review Committee (SSC No. 2373) and the University of New Mexico (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. 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).

Snail exposures for RNA-Seq experiments are described in detail in Buddenborg et al. (2017). Briefly, field-collected *B. pfeifferi* were simultaneously exposed to 20 miracidia each from pooled fecal samples (5 individuals) for 1d and 3d. Field-collected, cercariae-producing snails were used for the shedding sample group. Biological triplicates were sequenced for each sample group using Illumina HiSeq 2000 (Illumina, Carlsbad CA) at the National Center for Genome Resources (NCGR) in Santa Fe, NM. In addition, one naturally shedding *B. pfeifferi* snail was sequenced on a 454 sequencer (Roche, Basel Switzerland) to improve *S. mansoni* transcript assembly but these sequences were not used for quantification. See Buddenborg et al. (2017) for RNA extraction, library preparation, sequencing procedures, and sequencing summaries.

S. mansoni microarray experiments

The M-line strain of *B. glabrata* infected with *S. mansoni* PR1 strain was used in the microarray experiments to monitor parasite transcriptional changes that occur during infection. Both snail and trematode were maintained at UNM as previously described (Stibbs et al. 1979). Snails were exposed to 10 miracidia each of *S. mansoni* for 2d, 4d, 8d, 16d, or 32d (shedding snails), with biological triplicate replicates for each time point. An uninfected *B. glabrata* group was also used to account for cross-hybridization from mixed snail-trematode samples. Total RNA was extracted as previously described (Hines-Kay et al. 2011) and treated with DNAse I (Ambion UK) to remove gDNA contamination. RNA was quantified on a NanoDrop ND-1000 spectrophotometer and quality-assessed using an Agilent 2100 bioanalyzer. cDNA synthesis, amplification, labeling, and hybridization were performed as previously described (Hines-Kay et al. 2011).

A publicly available *S. mansoni* microarray (NCBI GEO accession GPL6936) representing 19,244 unique *S. mansoni* contigs (38,460 total experimental probes) was used with the following modification: all array probes were duplicated to allow for an added level of replicability. The transcript probes contained on the array were designed to profile 15 different developmental stages. Thus, many of the molecules likely important to larval development are present as well. Microarray images were recovered from a GenePix 4100A (Axon Instrument Inc.) dual channel laser scanner.

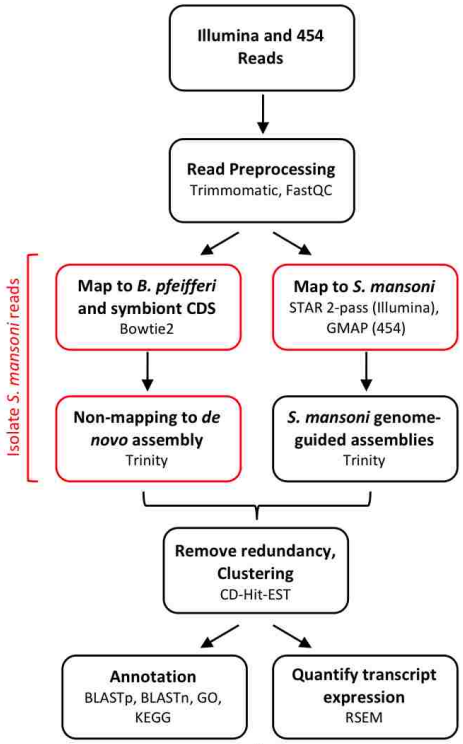
Raw data was averaged from replicates in each experimental group (2d, 4d, 8d, 16d, 32d), and for replicates in the uninfected snail group (Bg-only). For each experimental group, the mean and standard deviation were calculated, and values falling below one standard deviation from the mean were removed from further analysis. Features that were non-reactive for any of the groups used in this study, amounting to 26,581 probes, were removed as well as those that were cross-reactive with the Bg-only group (787 probes). The average Bg-only value was subtracted from experimental groups for each probe. Calculated expression values less than 1 were removed from the analysis and the remaining values were transformed by log base 2.

An updated annotation of array features was performed using BLASTn with the NCBI nucleotide database (sequence identity >70%, E-value $<10^{-06}$), BLASTp with the NCBI non-redundant protein database (sequence identity >40%, E-value $<10^{-06}$). Array features were matched to their homologous *S. mansoni* transcript by BLASTn against the assembled *S. mansoni* transcripts. These homologous transcripts were used for analyses comparing across Illumina and array samples.

S. mansoni transcriptome assembly and annotation

An overview of our analysis pipeline is shown in Figure 1. After pre-processing all Illumina reads, those from Illumina and 454 sequencing that did not map back to the *B. pfeifferi* transcriptome or identified symbionts were assembled into contigs (assembled, overlapping reads). The separation of host, parasite, and symbiont reads is described in detail in Buddenborg et al. (2017). We employed Trinity v2.2 RNA-Seq *de novo* assembler (Grabherr et al. 2011; Haas et al. 2013) for *de novo* and genome-guided transcriptome assembling using paired-end reads only. The *S. mansoni de novo* assembly consisted of reads that did not map to the *B. pfeifferi* transcriptome or symbionts after alignment with Bowtie2 v2.2.9 (Langmead et al. 2012). The genome-guided transcriptome assembly was performed using STAR v.2.5 2-pass alignment (Dobin et al. 2012) to the *S. mansoni* genome (GeneDB: *S. mansoni* v5.2).

Figure 1. Overall assembly and differential expression pipeline for *S. mansoni* reads in dual RNA-Seq.



Schistosoma mansoni genome-guided and *de novo* Trinity assemblies were concatenated and redundancy reduced using CD-Hit-EST at 95% similarity (Fu et al. 2012). The resulting sequences were screened against *B. glabrata* (VectorBase: BglaB1) and *S. mansoni* genomes, peptides, and mRNAs using BLASTx and BLASTn (sequence identity >70%, E-value < 10^{-12}). Sequences with blast results to *B. glabrata* were removed and remaining *S. mansoni*-specific sequences are henceforth referred to as transcripts.

All assembled transcripts 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 >40%, E-value <10^{-06}), BLASTn with NCBI nucleotide database (sequence identity >70%, E-value < 10^{-06}), BLASTn consensus of top 50 hits (sequence identity >70%, E-value < 10^{-06}), Gene Ontology (Ashburner et al. 2000), KEGG (Kanehisa and Goto, 2000), and InterProScan5 (Jones et al. 2014).

Schistosoma mansoni transcript-level quantification was calculated with RSEM (RNA-Seq by expectation maximization) (Li and Dewey 2011) and TPM (Transcripts Per kilobase Million) values were used for downstream analyses. TPM is calculated by normalizing for transcript length and then by sequencing depth ultimately allowing us to compare the proportion of reads that mapped to a specific transcript (Li et al. 2010; Shalek et al. 2013). Full Blast2Go (Götz et al. 2008) annotations were performed on all assembled *S. mansoni* transcripts.

RESULTS AND DISCUSSION

Illumina-derived *S. mansoni* transcriptomic characteristics

Throughout this discussion, a "transcript" is defined as assembled *S. mansoni* contigs formed from overlapping reads with the understanding that this includes both full-length transcripts, partial transcripts, and isoforms. For our Illumina-based study, a total of 23,602 transcripts made up our combined genome-guided and *de novo* assembled *S. mansoni* intramolluscan transcriptome. All raw data and tables associated with figures can be found in Supplementary File 1 with an index provided there. *Schistosoma mansoni* assembly metrics are provided in Table 1. When all raw reads from each infected snail were mapped to the *S mansoni* transcripts, 1d, 3d, and shedding replicates' mapping percentages ranged from 4.01-5.46, 1.48-4.05, and 4.36-9.72, respectively (Figure 2). The principal component analysis (PCA) plot (Figure 3) shows that the percentage of *S. mansoni* reads varies, and that 1d and 3d groups show more variation between replicates than do shedding replicates. It is not surprising that the transcriptional responses among early replicates at 1 an 3d are more variable in this natural system involving both genetically variable snails and schistosomes, especially as compared to shedding snails which have reached a steady state of continued cercarial production. Also, because the parasite stages at 1 and 3d are small relative to their hosts, uniform sampling of their contributions may be harder to achieve.

Table 1. RNA-Seq statistics and *S. mansoni de novo* assembly metrics

Filtered reads used in *S. mansoni de novo* assemblies	**222,593,797**
***De novo* assembled contigs**	
Trinity *de novo* Illumina	18,860
Genome-guided Trinity *de novo* Illumina	26,993
Genome-guided Trinity *de novo* 454	5,767
***S. mansoni* transcripts**	**23,602**
% GC	35.40%
N_{50}	1,412
Median transcript length	479
Average transcript length	841.92
Transcripts ≥500nt	11,419 (48.4%)

Figure 2. (A) Read mapping statistics for each replicate of our *S. mansoni* transcriptome. Percentage of all reads mapped are graphed on the left y-axis in dark bars and overall number of reads graphed on the right y-axis in light bars. (B) Number of transcripts expressed above ≥1 Log_2 (TPM) in each Illumina sample replicate.

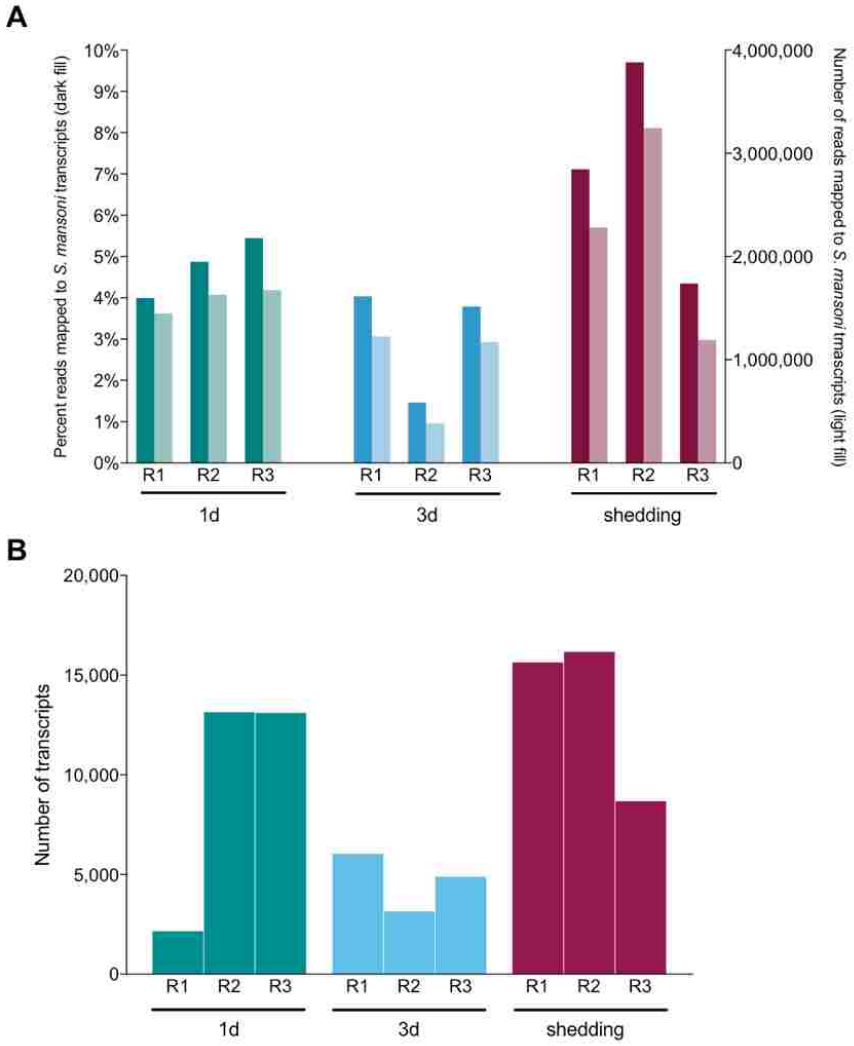
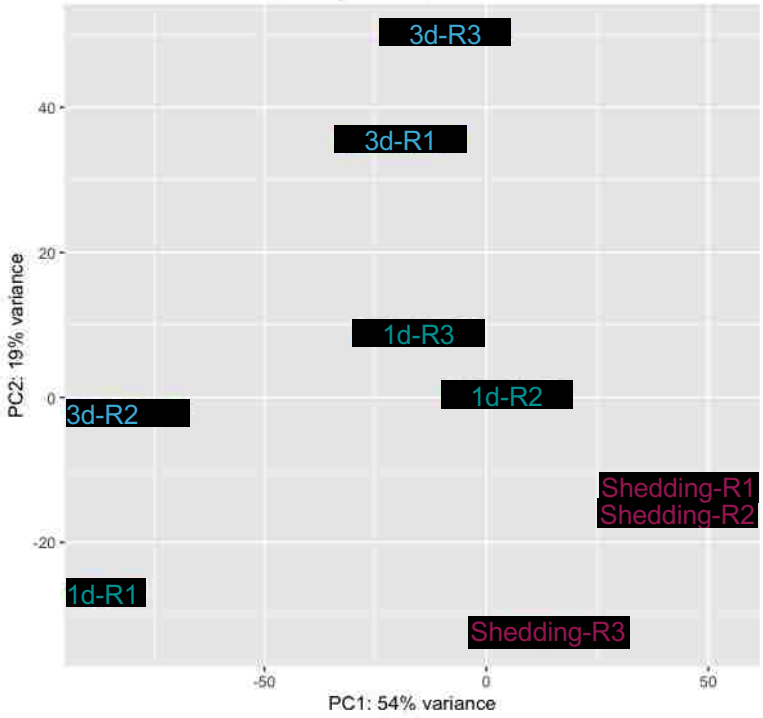


Figure 3. PCA plot of all transcripts expressed in replicates from 1d, 3d, and shedding Illumina groups.

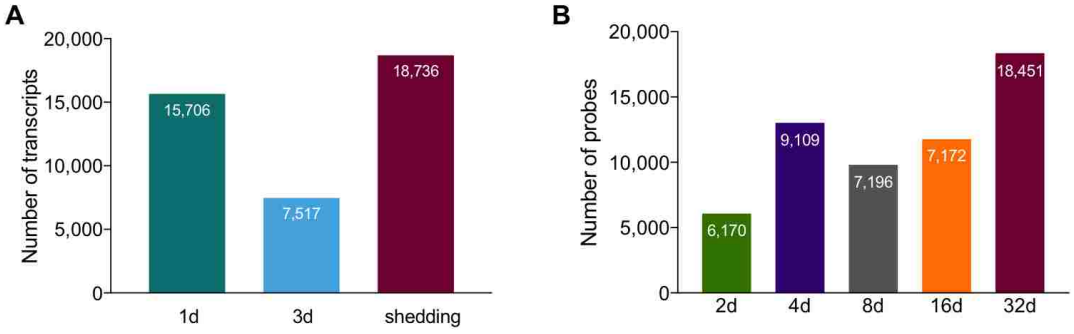


Overall Illumina and microarray *S. mansoni* transcript expression

Transcripts with ≥1 Log_2 TPM (Transcripts Per kilobase Million) in at least one replicate per group in Illumina samples and features with fluorescence ≥ 1 in microarrays were considered for expression analyses. Based on these cutoffs, over fifteen thousand different transcripts representing 7,252 *S. mansoni* genes were detected in 1d *S. mansoni* infections. Following a dip in 3d samples, even more *S. mansoni* transcripts were expressed in shedding snails (Figure 4a). The decline noted in the 3d Illumina samples may reflect that at least one replicate returned fewer *S. mansoni* reads in general or may simply reflect a sampling consideration due to the small size of the parasites relative to the snail at this time point. Sustained expression of a large number of transcripts with a general trend towards higher expression in shedding snails was also noted in the microarray data (Figure 4b). Particularly for the Illumina results, some of the transcripts enumerated represent different portions of the same original full-length mRNAs as well as different isoforms, so the actual numbers of expressed

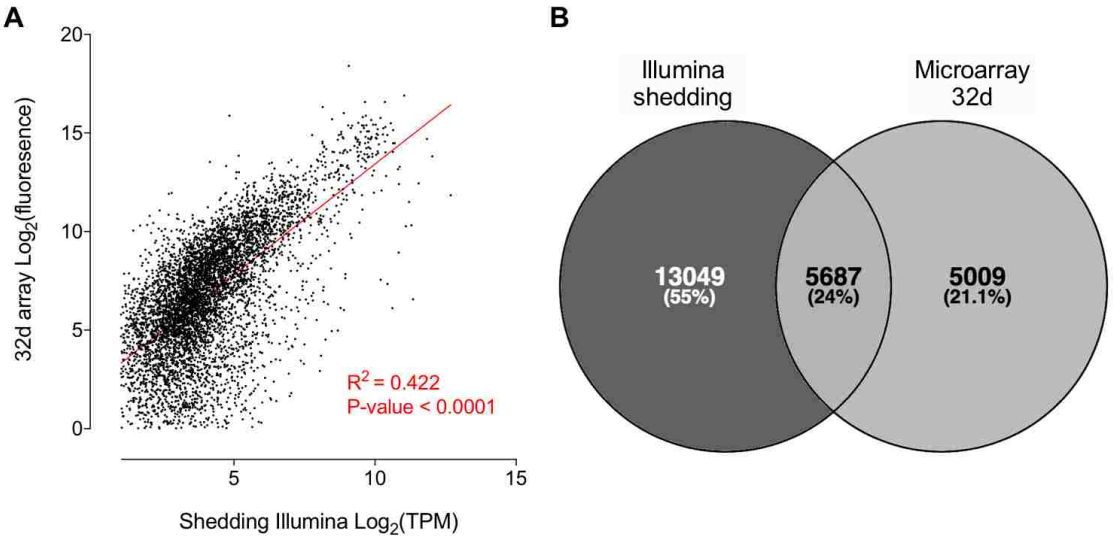
genes is approximately half as many as the number of recorded transcripts. Nonetheless, the variety produced is impressive and generally supported by our microarray results as well (at least 6,000 features expressed at all time points).

Figure 4. (A) *Schistosoma mansoni* transcripts with ≥1 Log_2 TPM in at least one replicate per group in Illumina samples. (B) Array features with fluorescence ≥1 in microarray analyses.



Our datasets generated by Illumina and microarray analyses might be expected to return different results for at least four different reasons: 1) the two methods are totally different in approach; 2) the host snail species and *S. mansoni* strains differed; 3) the time points sampled differed; and 4) the transcripts represented on the array are more limited than whole transcriptomic sequencing provided by Illumina. However, they also provide independent views of the same basic process, so some comparisons are warranted, especially so for shedding snails when the same developmental stage could be compared between techniques. Figure 5 shows for shedding snails the positive correlation of microarray fluorescence in averaged replicates with ≥1 fluorescence (then Log_2 transformed) and Illumina RNA-Seq taking the average of replicates with ≥1 Log_2 TPM. The positive correlation between the two platforms at 32d is a likely indication of the steady state of transcription achieved by *S. mansoni* at the stage of ongoing cercarial production. The venn diagram (Figure 5B) serves as a reminder of the greater overall coverage that is achieved in the Illumina samples.

Figure 5. (A) Linear regression of 32d array probes measured in log_2 fluoresence and shedding Illumina expression results measured in log_2 TPM. Only homologous probes and transcripts were included in the scatterplot. (B) Venn diagram of shared and unique features expressed between microarray and Illumina time points (32d).



Particularly noteworthy in both Illumina and array samples was that a large number of transcripts was shared across all time points (Figure 6). Among Illumina groups, >15% of all transcipts are expressed constitutively and among all microarray groups, >34% of all probes were expressed constitutively. This is suggestive of a core transcriptome required of schistosomes living in snails (see below for more details as to what comprises this core transcriptome). When comparing both early time points (1-4d) and shedding time points from both Illumina and array methods, venn diagrams not surprisingly indicate that Illumina RNA-Seq detects more *S. mansoni* transcripts (Figure 7). In addition, Figure 7 shows less overlap in expression profiles between Illumina and array methods at early time points than for shedding snails, again suggestive of more variation amongst the sampled early time points for reasons already stated above.

Figure 6. (A) Venn diagrams of *S. mansoni* replicates from Illumina sample groups. (B) Venn diagram of *S. mansoni* transcripts with ≥1 Log_2(TPM) in at least one replicate per group. (C) Venn diagram of expressed unique *S. mansoni* probes with >1 log_2 fluorescence in the microarray.

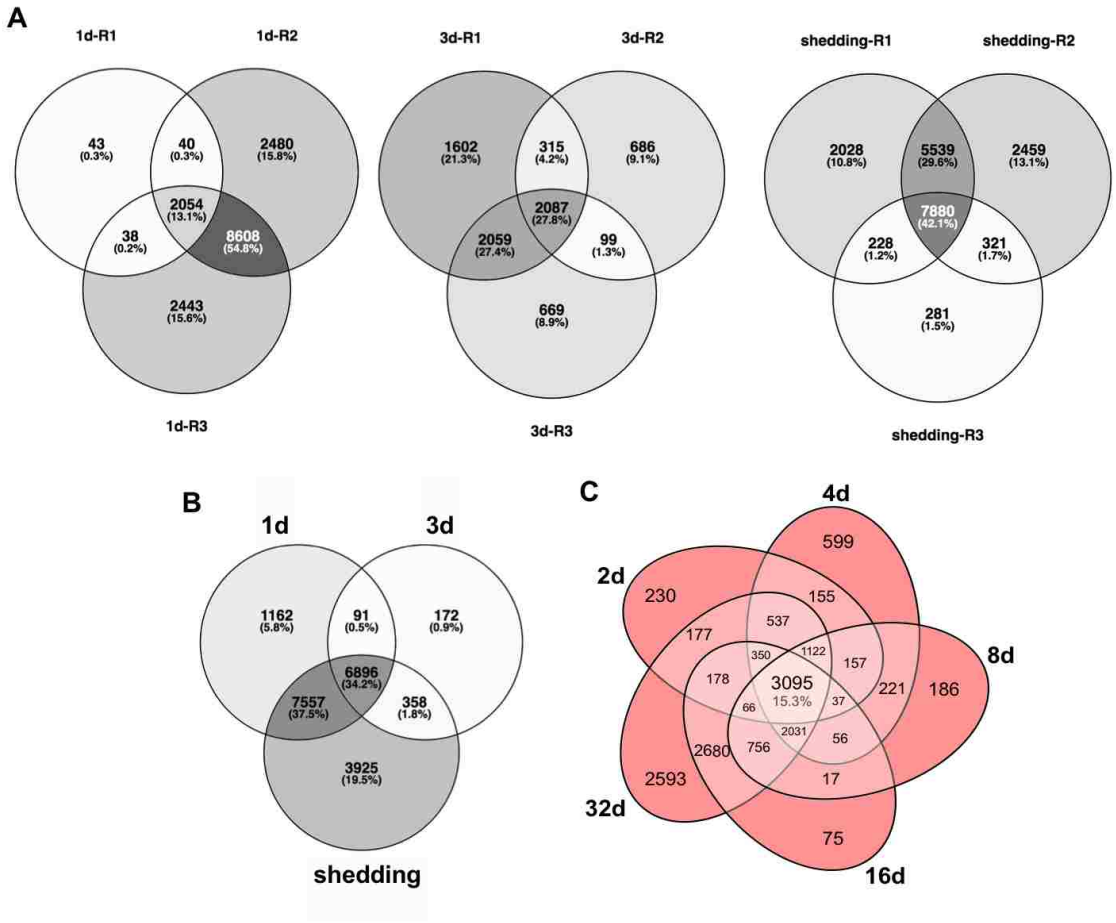
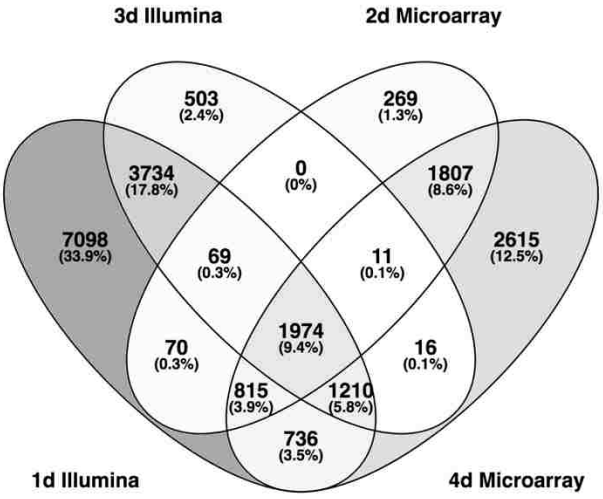


Figure 7. Venn diagram comparing expression results of early *S. mansoni* development

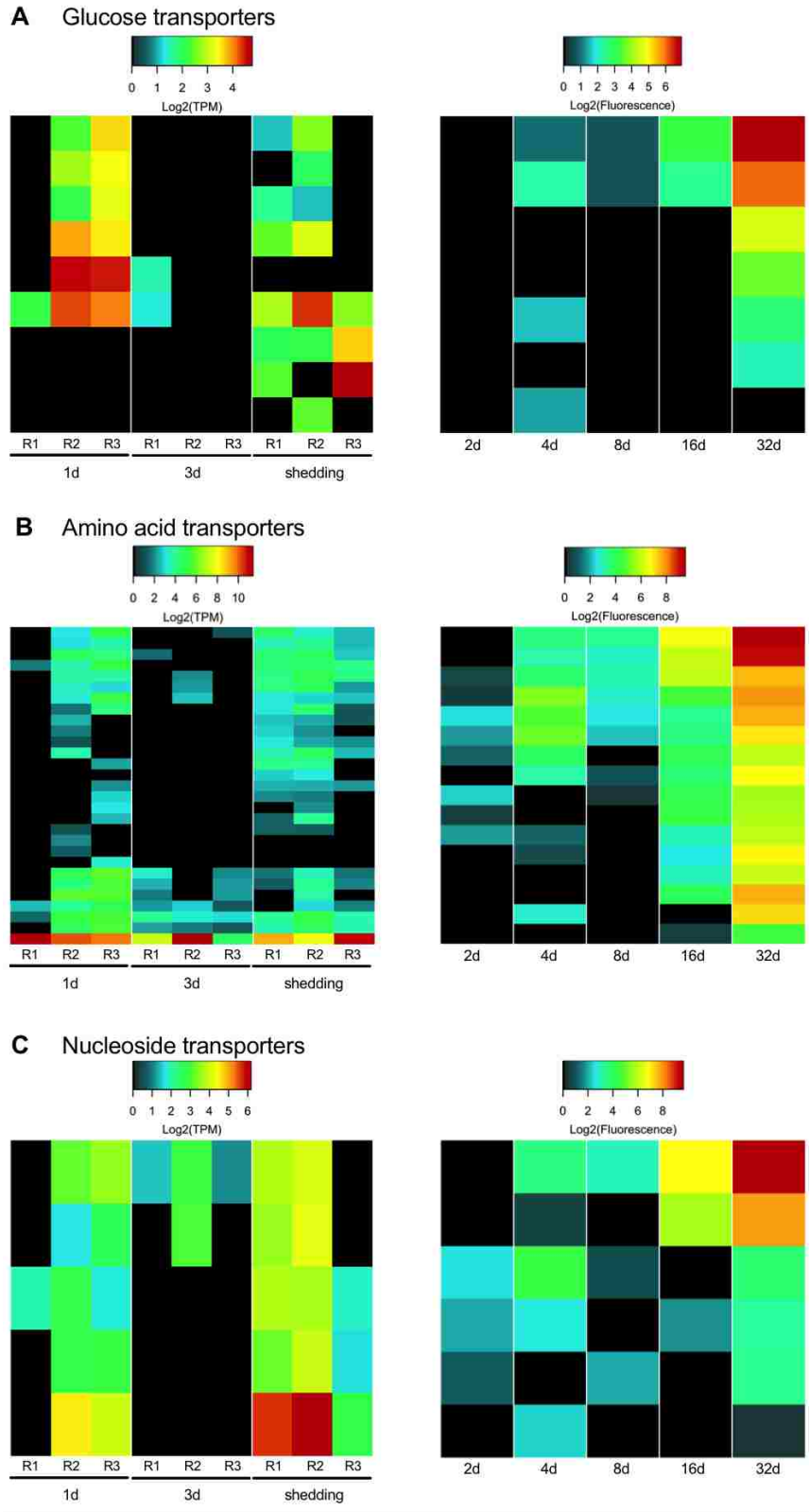


The intra-molluscan metabolic landscape

After successful penetration of the snail host, digeneans alter their metabolism to depend completely on the resources available in the molluscan host and shift their energy budget towards sporocyst and/or rediae development. One of the unique evolutionary innovations of the Neodermata is the syncytial tegument, a vital aspect of digenean biology providing both protection and a highly efficient route to acquire nutrients from the host species (Laumer et al. 2015). Through the tegument, schistosomes acquire most nutrients and other key molecules via facilitated or active transport using transmembrane transporters (Yoshino et al. 2017). Glucose transporters are expressed in both adult and larval stages of *S. mansoni* (Zhong et al. 1995; Boyle et al. 2003). While miracidia in water employ aerobic energy metabolism, after 24 hours of *in vitro* cultivation, sporocysts shift their metabolism towards lactate production (Tielens et al. 1992). Expression of glucose transporters is particularly important in initial establishment (1d) and in shedding snails (Figure 8A). By the 3d day post infection, the parasite up-regulates metabolic processes that are part of the purine salvage pathway and nucleotide biosynthesis, highlighting its transition to reproduction processes and mitosis (see list of enzymes and expression in Supplementary File 1). This is concurrent with a down-regulation of

phosphorylation and general mitochondrial metabolic activities. This highlights the transition to the less aerobic regime within the host, and the shift to a tightly regulated reproductive program rather than active migration within the host or the environment. It has also been observed that daughter sporocysts have fewer mitochondria (Basch and DiConza 1974, Smith and Chernin 1974). This shift to anaerobic mode of energy production is reversed by the presence of fully-formed cercariae developing in the sporocysts of actively shedding snails and is corroborated by the fact that aerobic respiration is especially active in the tails of cercariae (Skelly and Shoemaker 1995). This is expected due to the fact that cercariae, once released from the snail, have an active lifestyle and must generate enough energy from a limited amount of stored glycogen. Oxidative phosphorylation, aided by the greater availability of oxygen in the aquatic environment, helps cercariae fulfill their demanding energy requirements.

Figure 8. Glucose, amino acid, and nucleoside transmembrane transporters present in intramolluscan *S. mansoni* stages.



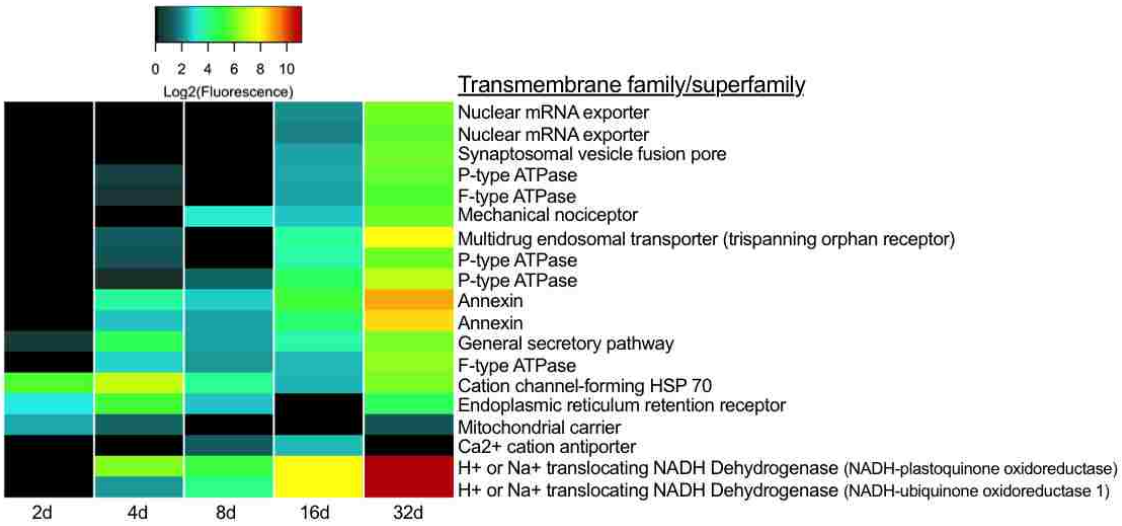
While obtaining organic carbon from the host fulfills the energetic requirements of the parasite, any actual growth is nitrogen dependent. Acquiring amino acids and other important building-block molecules is thus paramount to the parasite's fitness. Tegumental amino acid transporters have not been previously been reported in *S. mansoni* sporocysts (Yoshino et al. 2017) but here we provide evidence of the expression of several amino acid transporters across all intramolluscan time points, some of which may be tegumental. A glutamate transporter was the highest expressed amino acid transporter across all replicates in the Illumina samples. This is concurrent with an increase of amino acid biosynthesis by 3d which continues, but to a lesser degree in shedding snails (e.g. Alanine transaminase EC: 2.6.1.2, Glutamate Synthase EC 1.4.1.13). Nucleoside transporters were also abundantly expressed, especially in shedding snails, as noted both by Illumina and microarray results.

Components of receptor-mediated endocytosis are present in the transcriptome of free-living and adult stages of *S. mansoni* (Verjovski-Almeida et al. 2003). Transcripts necessary for clathrin-mediated endocytosis, including clathrin assembly proteins, low-density lipoproteins, and adapter complex Ap2 were present in our intramolluscan transcriptome. The regulator of endocytosis, dynamin, was also present. These transcripts may be used in endocytosis to bring in lipids needed to make membranes. Expression of transcripts involved in receptor-mediated endocytosis, and possibly also in exocytosis, was high immediately upon miracidial transformation in 1d *S. mansoni* mother sporocysts.

We identified additional putative transmembrane transporters using the Transporter Classification Database (www.tcdb.org) (Saier et al. 2016). For microarray samples at 16d and 32d, two transmembrane NADH oxidoreductases and two annexins were most highly expressed (Figure 9). For Illumina samples, the most abundantly expressed transporters at 1d were AAA-ATPase and protein kinase superfamilies whereas the nuclear pore complex, H+ or translocating NADH dehydrogenase, and endoplasmic reticular retrotranslocon families were dominant in 3d and shedding groups. ABC transporters were present in all Illumina samples, with 27 ABC transporter transcripts expressed in shedding

snails. Three transcripts, identified as an ATP-binding cassette sub-family F member 2-like isoform X1, isoform X2, and ATP-binding cassette sub-family E member 1-like were highly expressed in every Illumina replicate. It has been suggested that ABC transporters serve an excretory function for adult schistosomes, playing a role in the removal of xenobiotics and/or influencing interactions with the definitive host (Kusel et al. 2009). The high expression of ABC transporters in intramolluscan stages, particularly in shedding snails, suggests they have an important and as yet not fully appreciated role in development. Perhaps they play a role in elimination of wastes associated with production of cercariae or facilitate release of factors that modify the immediate environment of the daughter sporocysts to favor their continued productivity of cercariae.

Figure 9. Transmembrane families/superfamilies represented in microarray samples.

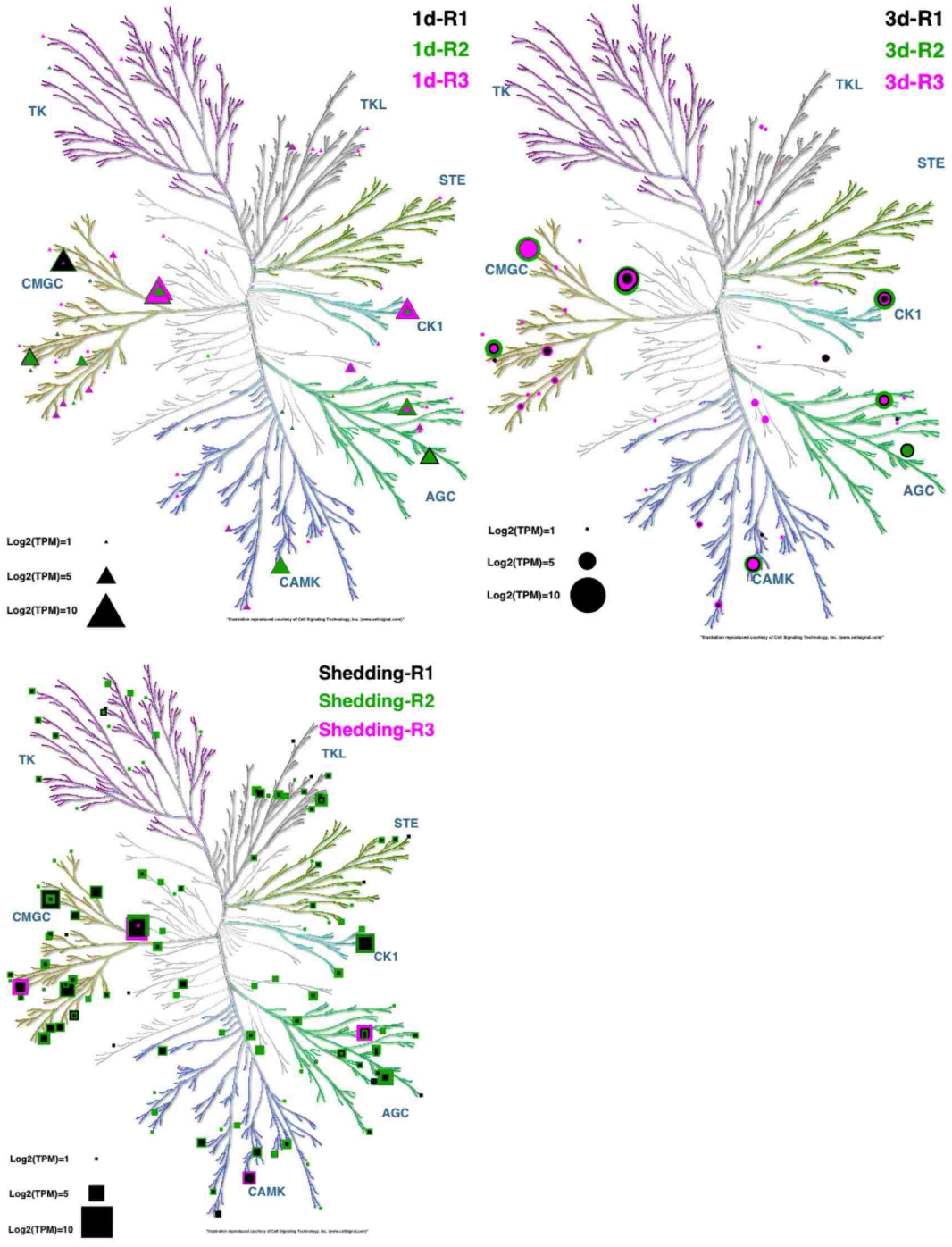


Protein kinases phosphorylate intracellular proteins in order to alter gene expression and are responsible for many basic cellular functions. In schistosomes, kinases are predicted to play a role in host invasion, sensory behavior, growth, and development (Walker et al. 2014). Because of their importance, kinases have been used as potential pharmaceutical targets against *S. mansoni* (Dissous et al. 2007). A BLASTx homology search of kinases from

Kinase SARfari (https://www.ebi.ac.uk/chembl/sarfari/kinasesarfari) confirmed the representation of 19 kinases from 4 different superfamilies on the microarray, and 154 kinases belonging to 7 different superfamilies expressed in Illumina 1d, 3d, and shedding samples (Figure 10). The highest expressed protein kinases are members of the group CMGC which includes MAPK growth and stress response kinases, cell cycle cyclin dependent kinases, and kinases for splicing and metabolic control.

Figure 10. *Schistosoma mansoni* kinases identified at 1d, 3d, and shedding time points, organized by kinase family.

TK: phosphorylate tyrosine residues; TKL: “tyrosine kinase-like” serine-threonine protein kinases; STE: mostly protein kinases involved in MAP (mitogen-activated protein) kinase cascades; CK1: casein kinases; AGC: cyclic-nucleotide-dependent family (PKA, PKG), PKC, and relatives; CAMK: calcium/calmodulin modulation activity; CMGC: cyclin-dependent kinases, MAP kinases, glycogen synthase kinases, and CDK-like kinases. The figure was generated using KinomeRender.

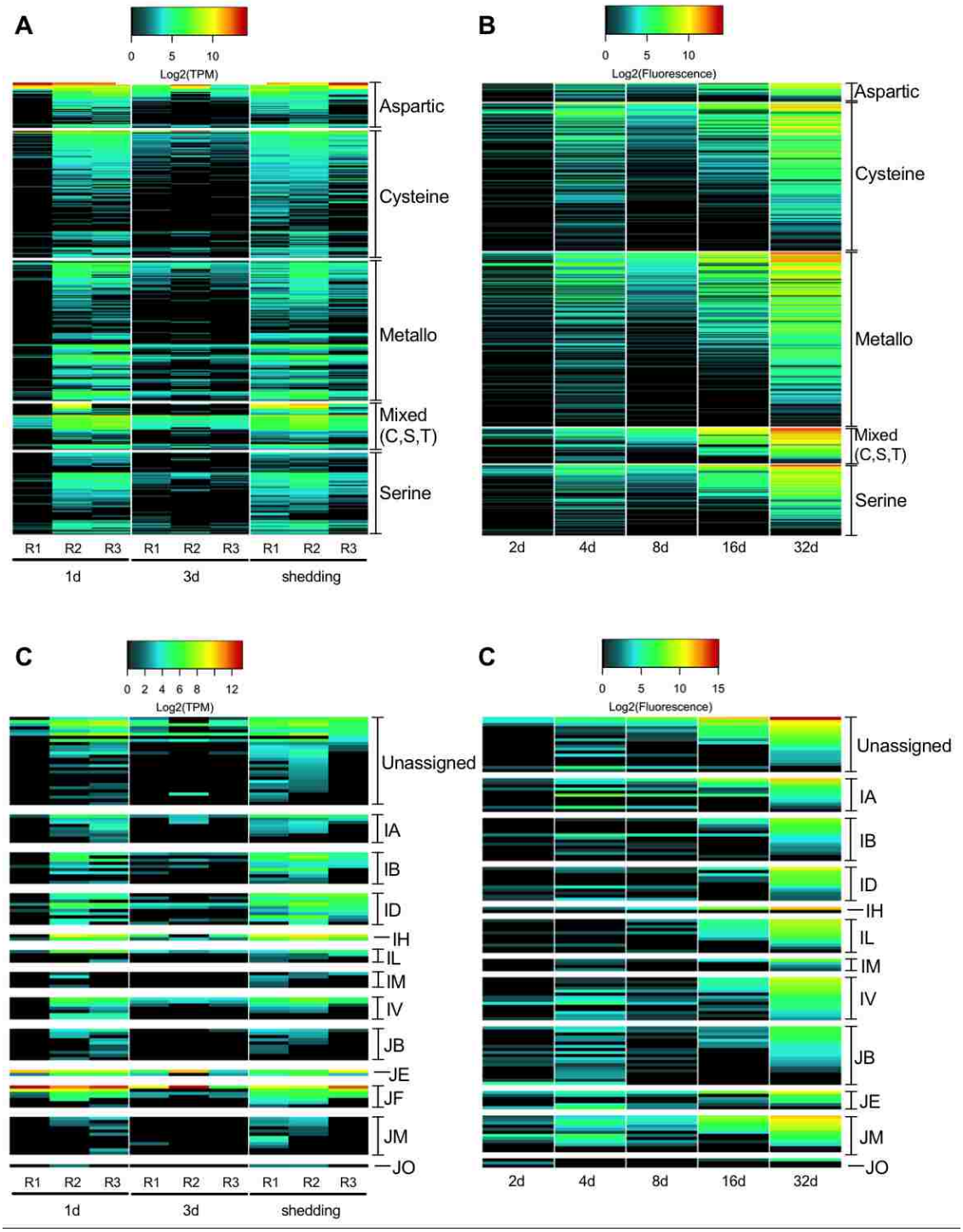


Protease and protease inhibitor transcripts expressed at different stages of parasite development

The protease-encoding genes of parasitic helminths have undergone gene duplication and divergence, and by enabling helminths to process diverse proteinaceous substrates are believed to be critical to establishment and perpetuation of infection (Tort et al. 1999; Dalton et al. 2006). Helminth proteases and protease inhibitors have proven useful as markers for diagnostics purposes, or as targets for drugs or vaccines (Dalton et al. 2006; Ranasinghe and McManus 2017; Tandon et al. 2017). In the snail host, larval schistosomes use proteases for nutrient acquisition, to create the space needed for their expansive growth, and for defense functions, potentially destroying or inhibiting lytic host proteases (Yoshino et al. 1993). Miracidia release proteases to facilitate entry into the snail host, often into dense tissue of the head-foot (Grevelding 2006). *In vitro* studies of cultured mother sporocysts have revealed secretion of proteases facilitating degradation of snail hemolymph proteins such as hemoglobin (Yoshino et al. 1993).

We observed that intramolluscan *S. mansoni* devotes considerable effort to making proteases and protease inhibitors with 397 protease transcripts and 77 protease inhibitor transcripts represented in at least one time point (Figure 11). Replicates of each Illumina time point with the lowest percentage of *S. mansoni* reads (1d-R1, 3d-R2, shedding-R3) also had the least abundant number of transcripts identified as proteases and protease inhibitors. One-day infections (see 1d-R2 and 1d-R3) with higher read counts indicative of robust development show expression of a gamut of *S. mansoni* proteases that somewhat surprisingly resemble those produced by *S. mansoni* in shedding snails. Coincidentally, we noted the snail host up-regulated expression of protease inhibitors especially during larval establishment at 1d and 3d (Buddenborg et al. 2017).

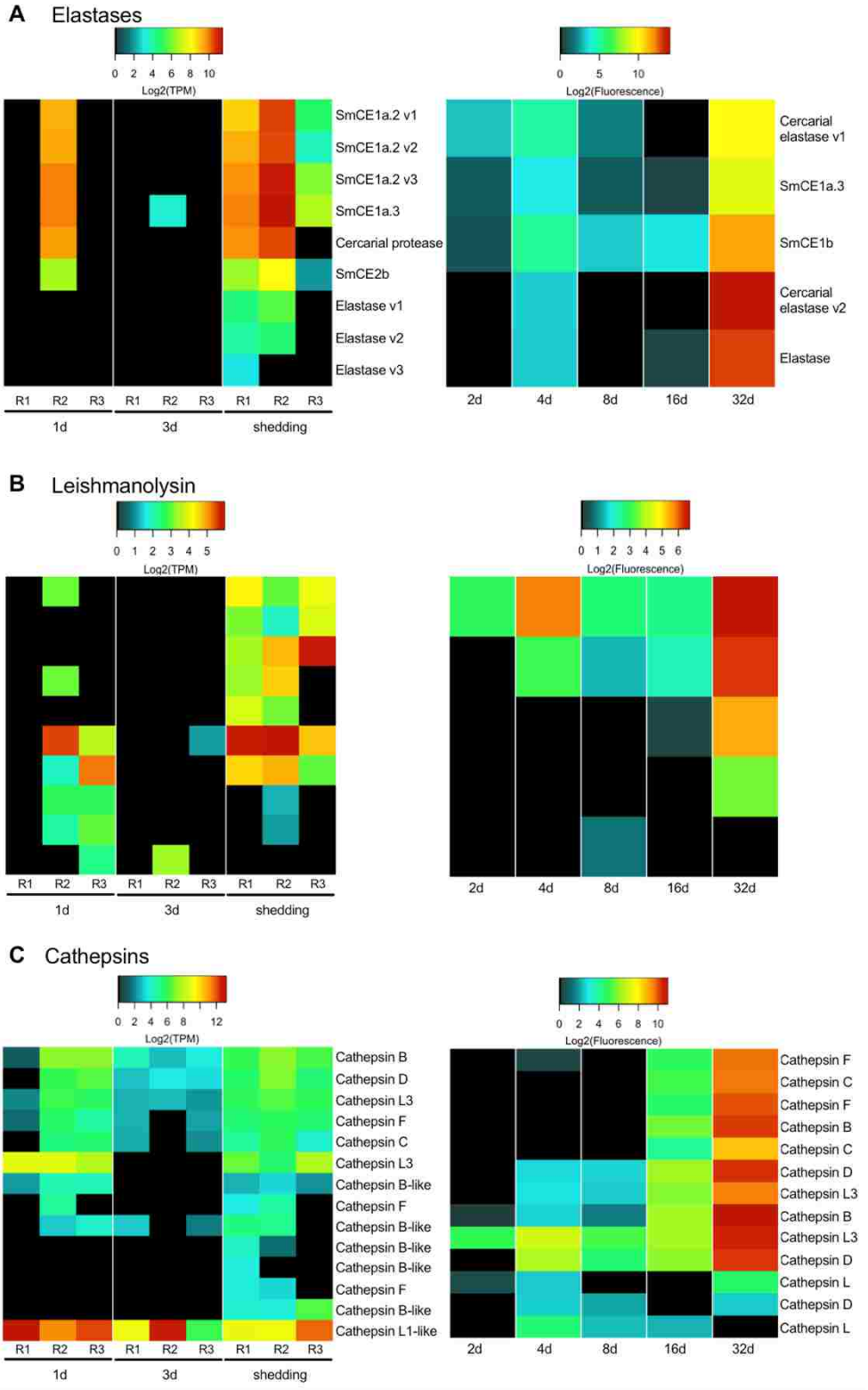
Figure 11. Intramolluscan expression of *S. mansoni* proteases and protease inhibitors organized by catalytic binding site for the proteases or MEROPS database clan for the protease inhibitors. Individual protease inhibitor clans contain inhibitors that have arisen from a single evolutionary origin. See https://www.ebi.ac.uk/merops/inhibitors/ for details.



At all time points more *S. mansoni* proteases were present than protease inhibitors and, in general, protease inhibitors and proteases increased in abundance and expression as infection progressed. For both Illumina and microarray samples, shedding snails had both the greatest number of proteases and protease inhibitors expressed relative to other time points, and the highest expression levels of proteases and protease inhibitors.

As expected, elastases, an expanded family of serine proteases in *S. mansoni* (Ingram et al. 2012), were the most highly expressed proteases in *S. mansoni* from both *B. glabrata* and *B. pfeifferi* shedding snails (Figure 12A). We identified 9 elastase transcripts including those previously designated as cercarial elastases 1a and 2b and found in daughter sporocysts and cercariae (Ingram et al. 2012). Although elastases are known to be used in definitive host skin penetration, active translation of SmCE2b into protein sequences is seen prior to exiting the snail and was postulated to be involved in facilitating egress from the snail (Ingram et al. 2012).

Figure 12. Prominent proteases of interest include elastases (A), leishmanolysins (B), and cathepsins (C).



Our data not only corroborate the presence of SmCE2b in shedding snails, but also reveal this and other *S. mansoni* elastases to be present at all time points we examined, in contrast to microarray results previously reported with early stage sporocysts from *in vitro* cultures (Fitzpatrick et al. 2009). For example, even at 1d (see 1d-R2) we found high expression of six *S. mansoni* elastases, some of which are those noted prominently in cercariae (Ingram et al. 2012). Our microarray samples also show expression of several elastases at all time points. It is not unusual to think that early-stage larval *S. mansoni* would express protease activity as they too must implement host penetration. Wu et al. (2009) noted a conspicuous absence of elastase proteins in *in vitro* larval transformation products but other proteases present suggested an obvious degree of overlap between cercarial versus larval protease repertoires.

Leishmanolysin (also called invadolysin), a metalloprotease, is the second most abundant type of secreted protease of cercariae after elastases (Curwen et al. 2006). Functional studies of leishmanolyin in larval *S. mansoni* suggested this protease is capable of interfering with the migration of *B. glabrata* hemocytes and may influence the establishment of infection (Kabore 2016). Leishmanolysin has also been detected among the proteins accompanying transformation of miracidia to mother sporocysts (Wu et al. 2009). We detected leishmanolysin transcripts at all time points, and they were most abundant in shedding snails, likely indicative of their representation in developing cercariae (Figure 12B).

Cathepsins are papain-like cysteine proteases and have been identified in the *S. mansoni* miracidia proteome, transforming miracidia, and mother sporocysts (Yoshino et al. 1993; Wang et al. 2016) and are implicated in tissue penetration, digestion and immune evasion in the definitive host (Sajid and McKerrow 2002; Caffrey et al. 2002; Dalton et al. 2006; Delcroix et al. 2006; Kašný et al. 2009). Cathepsins take the place of tissue-invasive elastases in the cercariae of avian schistosomes (Dolečková et al. 2010). Of two cathepsin B transcripts we noted, we found one expressed in all replicates except from 1d-R1 and 3d-R2, the early-stages replicates noted to have lower *S. mansoni* read counts (Figure 12C). *Schistosoma mansoni* expresses cathepsin B in the flame cells of cercariae

where they are believed to play a role in osmoregulation and/or secretion (Caffrey and Ruppel 1997). Cathepsin C, involved in acquisition of oligopeptides and free amino acids by larval schistosomes (Skelly and Shoemaker 2001), was also identified by Illumina at 1d, 3d, and in shedding snails, with the exception of replicate 3d-R2 which had a pre-patent amphistome infection. Cathepsins L1 and L3 were highly expressed by mother sporocyst stages (2d, 3d, 4d, 8d samples) in the microarray samples. At 16d, when daughter sporocysts are migrating through host tissue and hemolymph to the digestive gland, the proteases produced most closely resemble those from 32d shedding infections, including cathepsin C.

In contrast to proteases, there is relatively little information about protease inhibitors and their roles in parasite development and survival (see Ranasinghe and McManus 2017 for a thorough review of schistosome protease inhibitors). One of the better-characterized groups is the serine protease inhibitors (serpins; MEROPS clan ID, family I4) that may play a role in both post-translational regulation of schistosome proteases and defense against host proteases (Quezada and McKerrow 2011). Serpins were expressed in all the time points sampled but we observed the highest expression of serpins at 1d and in shedding snails. The most abundant protease inhibitors in the Illumina samples (1d, 3d, shedding) were those that belong to the JF clan which is interesting because it is by no means the most abundantly represented clan, comprised of only one family called cytotoxic T-lymphocyte antigen-2-alpha (CTLA-2α), known to induce apoptosis of T-lymphoma cells in schistosome-infected mice (Zhang et al. 2011). This gene homolog is not represented on the *S. mansoni* microarray which accounts for its absence in those samples. The homologous CTLA-2α transcripts expressed in the intramolluscan stages of *S. mansoni* may play a similar role in apoptosis or immunomodulation in snails to facilitate maintenance of long-term infections.

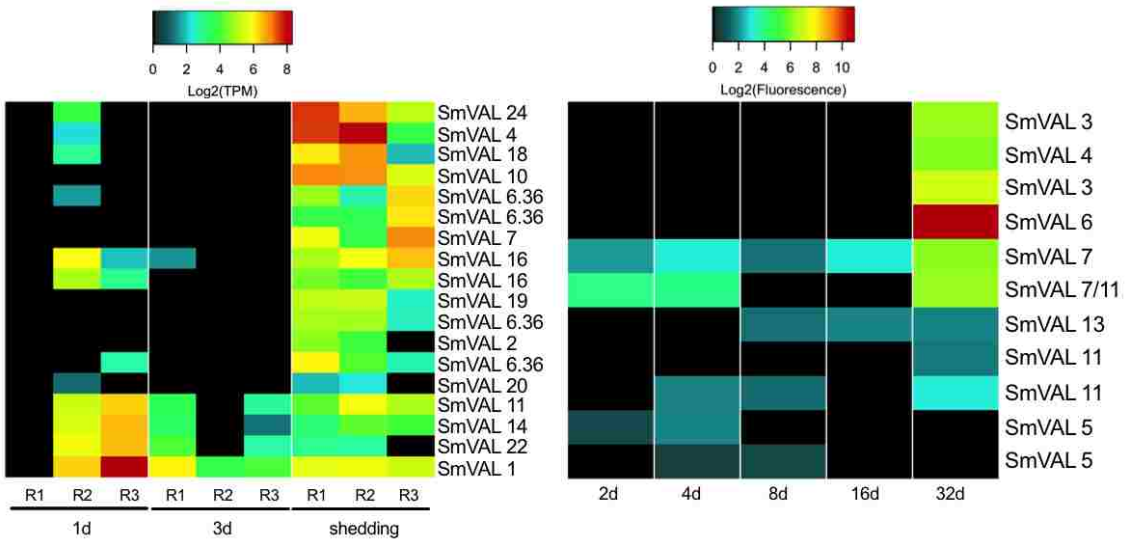
Transcripts identified as the protease inhibitor aprotinin (IB clan), a trypsin inhibitor, were moderately expressed in Illumina 1d-R2 and R3 replicates and in all replicates of shedding snails. In the plasma of *Biomphalaria*, the phenoloxidase enzyme laccase, whose activity is enhanced by trypsin, induces a

negative impact on late (7-9 week) *S. mansoni* infections (Le Clec'h et al. 2016). We noted an up-regulation of snail-produced trypsins in *B. pfeifferi* shedding *S. mansoni* cercariae (Buddenborg et al. 2017) as compared to uninfected controls. By inhibiting snail-produced trypsins, *S. mansoni* daughter sporocysts and/or developing cercariae within may disable an important snail defense strategy.

The *S. mansoni* venom allergen-like proteins (SmVALs)

The provocatively named venom allergen-like proteins (SmVAL2, 3/23, 9, 15, 26/28, and 27) have been identified as secreted larval transformation proteins (Wu et al. 2009). SmVAL proteins can be found throughout miracidia and sporocyst parenchymal cell vesicles and in germinal cells with evidence for involvement in larval tissue remodeling and development by regulating snail matrix metalloproteinases (Yoshino et al. 2014). One and 3d Illumina samples showed variable expression of SmVALs 1, 11, 14, and 22 (Figure 13). Replicates from shedding snails had more consistent SmVAL profiles, with 14 different SmVAL homologs found among Illumina replicates and 9 SmVAL homologs in the 32d microarray samples. SmVAL1 was ubiquitously expressed across 1d, 3d, and shedding Illumina samples. Chalmers et al. (2008) also noted abundant SmVAL transcripts in the infective stages of *S. mansoni*, namely miracidia and cercariae. SmVALs 4 and 24 transcripts, localized to the preacetabular glands of developing cercariae (Fernandes et al. 2017) were also the highest expressed SmVAL transcripts we found in shedding *S. mansoni*. SmVAL16 was localized close to the neural ganglia of adult male worms (Fernandes et al. 2017); we detected its expression at 1d, 3d, and shedding time points. The repertoire of SmVAL proteins secreted during transformation may differ from the SmVAL transcripts being produced and this may account for the differences in the SmVAL transcripts we report here versus previously published proteomic findings.

Figure 13. The venom allergen-like proteins of intramolluscan *S. mansoni*



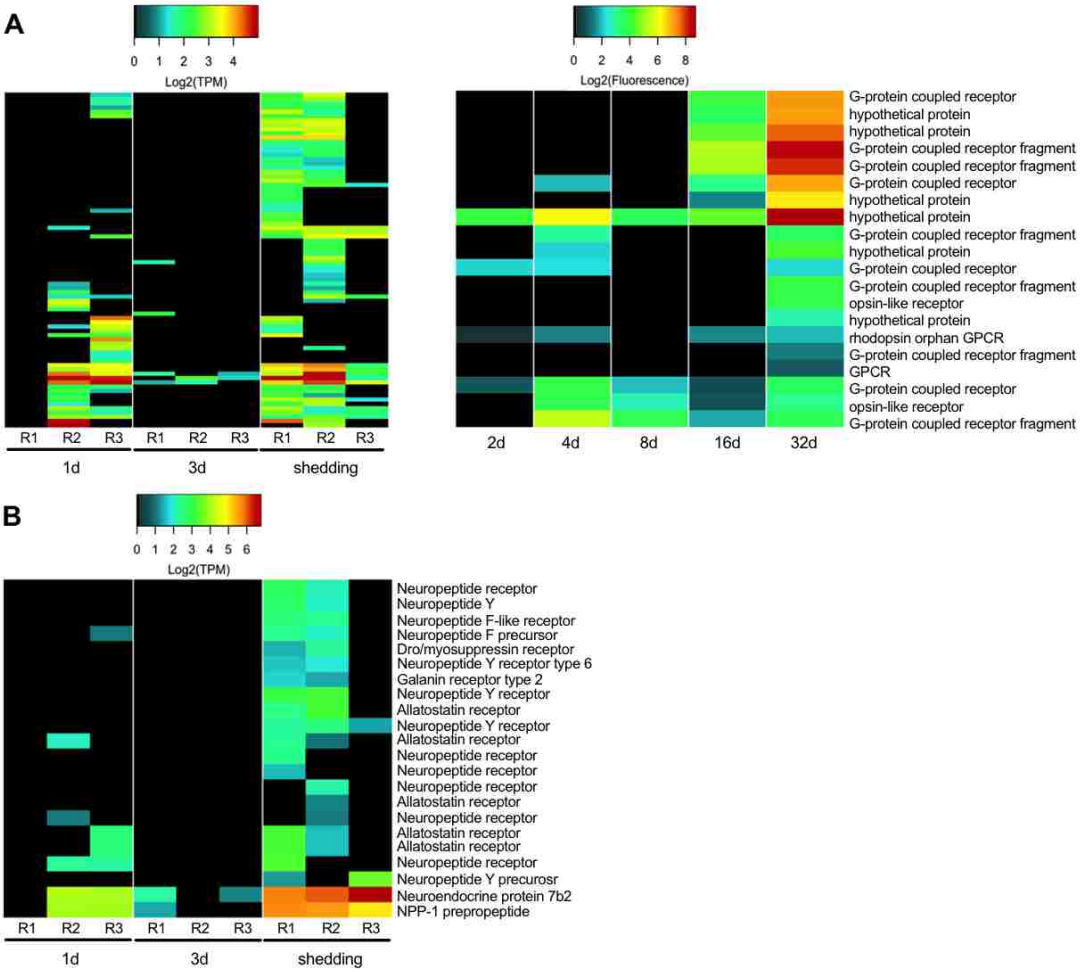
S. mansoni intramolluscan G-protein coupled receptors (GPCRs)

G-protein coupled receptors or GPCRs are the largest superfamily of transmembrane proteins in eukaryotes responsible for facilitating signaling affecting various downstream functions like development, reproduction, neuronal control of musculature and more (Patocka et al. 2014; Farran 2017). As receptors, GPCRs are involved in mediating a variety of processes critical to schistosome survival including mediating host-parasite interactions, reproduction, and mating (Liang et al. 2016). Praziquantel has been identified as a GPCR ligand acting to modulate serotoninergic signaling (Chan et al. 2017). Several *in silico* studies identifying and characterizing the *S. mansoni "*GPCRome" (Zamanian et al. 2011; Campos et al. 2014) culminated in the classification of a broad range of phylogenetically distinct clades/classes of GPCRs (Hahnel et al. 2018). *S. mansoni* microarray studies have reported diverse expression patterns of individual GPCRs, with the overall highest expression occurring in 3-7 week worms, indicating that they are associated with complex stage-specific roles (Fitzpatrick et al. 2009; Hahnel et al. 2018).

In intramolluscan stages, we identified 78 GPCR transcripts from our Illumina samples, and 26 probes from microarray samples (Figure 14A). Many (38%) of the Illumina GPCR transcripts were A FLPR-like, a GPCR class containing

receptors similar to FMRFamide GPCRs that invoke muscle fiber contractions in schistosomes by increasing calcium transport across voltage-gated calcium channels (Novozhilova et al. 2010). Shedding snails had the most diverse representation of GPCRs. One transcript, homologous to an identified *S. mansoni* GPCR (Smp_193810) with unknown function, was expressed at all time points with markedly high expression in both Illumina and microarray samples from shedding snails. A GPCR sensing the biogenic amine 5HT (Smp_126730) and known to be distributed throughout the adult worm's nervous system (Patocka et al. 2014), was expressed at 1d and shedding Illumina samples (no homologous probe was found on the microarray). Its presence in intramolluscan stages suggests that serotonin-stimulated movement is essential throughout the life cycle of schistosomes. At 1d and shedding, a type 1 serotonin receptor is down-regulated in *B. pfeifferi* and at 3d, kynurenine 3-monooxygenase (important for its ability to degrade tryptophan and limit concentrations of serotonin) is up-regulated (Buddenborg et al. 2017). Serotonin is a molecule of relevance to both the snail and parasite, and interference with its levels may be relevant to castration of snails (see concluding comments).

Figure 14. G-protein coupled receptors expressed in Illumina and microarray *S. mansoni* intramolluscan time points.



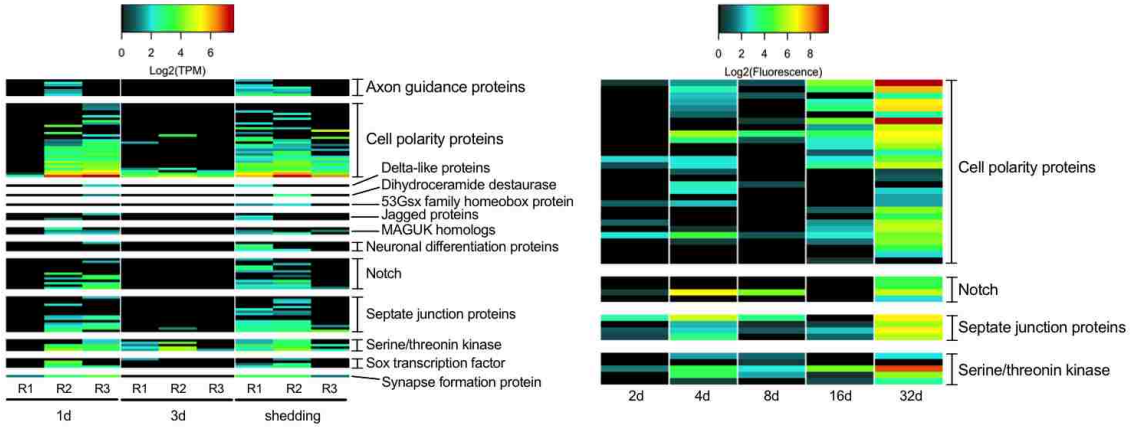
Neuropeptides and neural development

Studies on neuropeptides (peptide hormones) in planarian flatworms and their homologs in *S. mansoni* have identified their influence in locomotion, feeding, host location, regeneration, and development (Collins et al. 2010; McVeigh et al. 2009). Lu et al. (2016) reported the expression of putative neuropeptides and transcripts suspected to be involved in neural development from paired and unpaired female and male adult worms. Seventeen transcripts were identified as neuropeptide receptors from the Illumina transcriptome, all of which were GPCRs (Figure 14B). Neuropeptides and their receptors were mostly absent at 1d and 3d but abundant in shedding-R1 and R2. In shedding-R3, the replicate with a muted *S. mansoni* response, only one neuropeptide

receptor (neuropeptide Y receptor) was expressed. Shedding-R3 was curious in that protein 7b2 and NPP-1 (GFVRIamide) prepropeptide were highly expressed. Only one putative neuropeptide precursor (NPP-1 prepropeptide) was identified on the microarray with only ~1 Log_2 fluorescence at 32d and was not present in any other sample. In adult worms, GFVRIamide is localized to neurons that run along the cerebral commissure towards the oral sucker (McVeigh et al. 2009). Allatostatin receptor, a GPCR with ovary-specific transcription in adult *S. mansoni* (Hahnel et al. 2018), is important for reproductive development in *Schistosoma japonicum* adult females (Wang et al. 2017). Four transcripts homologous to allatostatin receptor were expressed primarily in shedding *S. mansoni* replicates. Our results indicate expanded roles for neuropeptides and neural development transcripts previously uncharacterized in intramolluscan stages of *S. mansoni*.

We identified 33 of the 39 genes found to be involved in neural development by Lu et al. (2016) in our Illumina *S. mansoni* transcriptome (Figure 15). Cell polarity proteins were the highest expressed transcripts involved in neural development at 1d, 3d, 16d, and shedding snails. 2d array *S. mansoni* showed little activity of transcripts related to neural development. In 4d and 8d samples, notch and septate junction transcripts were the most highly expressed neural development transcripts. Notch transcripts are highly expressed in eggs but not in cercariae and are thought to be mainly involved in *S. mansoni* oogenesis and embryogenesis within the vertebrate host (Magalhães et al. 2016) but have been implicated in neurogenesis (Verjovski-Almeida et al. 2003). Lu et al. (2016) found SOX to be transcribed in the ovary of paired and unpaired females and its expression in germ balls has also been established (Parker-Manuel et al. 2011). Three transcripts homologous to the *S. mansoni* SOX transcription factor were present predominantly in 1d and shedding time points reinforcing the role of SOX transcription in embryonic and germinal cell development.

Figure 15. Transcripts involved in neural development as detected by Lu et al. (2016)



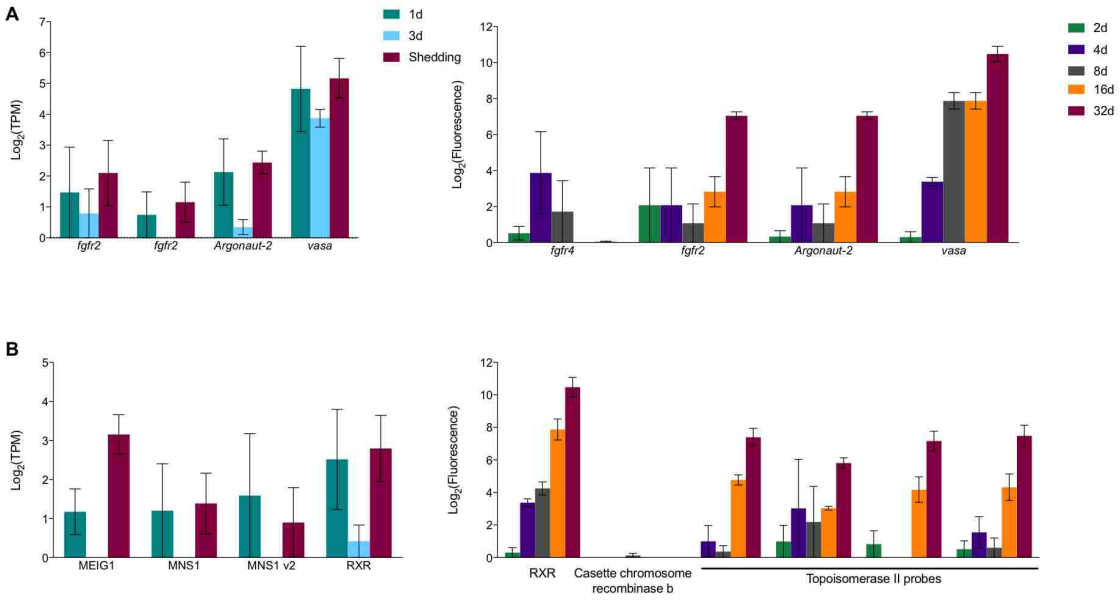
Transcripts associated with germinal cells and asexual reproduction of schistosomes in snails

A prominent feature of the complex developmental program of sporocysts in snails is the presence of germinal cells that give rise to embryos that come to contain both the somatic cells that eventually divide to comprise the bodies of either sporocysts or cercariae and more germinal cells. These germinal cells are then poised to give rise to the next generation. None of this asexual polyembryonic process involves the formation of gametes or evidence of fertilization. Germinal cells in *S. mansoni* sporocysts have been shown to share common molecular features with planarian neoblasts or stem cells, prompting the suggestion that the digenetic nature of the life cycle of schistosomes and other digenetic trematodes may have evolved because of the adaptation of a system of preservation of these stem cell-like germinal cells (Wang et al. 2013).

Consistent with Wang et al. (2013) we observed expression of fibroblast growth factor receptors (*fgfr*), *vasa*, *argonaute2* (*ago2*), and *nanos* transcripts shown to be associated with long-term maintenance of neoblast stem cells (Figure 16A). Expression of *fgfr2, argonaut-2* and especially *vasa* are expressed in all samples, suggestive of their importance in intramolluscan development. The microarray had an additional *fgfr* feature (*fgfr4*) that was not detected in the Illumina transcriptome. Our results are in agreement with Wang et al. (2013) that

nanos-1 is not expressed in sporocysts, consistent with their suggestion that *nanos-1* expression is exclusive in adult *S. mansoni* (Wang and Collins 2016). *Nanos-2* expression was observed in every replicate of every time point with the exception of the 2d microarray sample. It has been proposed that there are two populations of germinal cells, *nanos*$^{+}$ and *nanos*$^{-}$, with the latter population proliferating much more rapidly (Wang et al. 2013). *Vasa* is needed for proliferation of both *nanos*$^{+}$ and *nanos*$^{-}$ stem cell populations and *ago2* is required for proliferation of only *nanos*$^{-}$ cells. It is hypothesized that the two populations exist for different purposes: one a more undifferentiated stem cell-like population and the other a more differentiated one ready to enter embryogenesis (Wendt and Collins 2016).

Figure 16. Transcripts associated with maintenance of neoblast stem cells in platyhelminthes (A) and transcripts potentially involved in meiosis and/or homologous recombination in asexually reproducing *S. mansoni* (B).



The proliferation of sporocysts and then cercariae by digenetic trematodes in snails is now generally considered to be an asexual process, one that does not involve gamete formation or fertilization (Whitfield and Evans, 1983), and it is frequently assumed that the progeny produced from a single miracidium are

genetically the same. However, there are also persistent claims that the process is best considered as apomictic parthenogenesis (Galaktionov and Dobrovlskjj 2013). Some observations indicate that the *S. mansoni* cercariae arising from a single miracidium are not genetically identical but exhibit some variation with respect to representation of repetitive elements that has been attributed to mitotic recombination (Grevelding et al. 1999; Bayne and Grevelding 2003). Khalil and Cable (1968) examined germinal development in rediae of *Philopthalmus megalurus* and concluded the process was diploid parthenogenesis. They observed the presence of cells interpreted to be oögonia entering meiotic prophase I up to the stage of diakinesis that was then followed by the cell returning to interphase rather than proceeding through meiosis. Such a process might also allow for some recombination among the progeny produced during intramolluscan development.

Although the preponderance of evidence is surely against the occurrence of meiosis, gamete formation or fertilization during intramolluscan development (Whitfield and Evans, 1983), there may be peculiar remnants of these processes represented, especially considering that most accounts of the evolution of digenetic trematodes favor the interpretation that the ancestral state was likely the sexually reproducing adult worm which was followed at a later time by the addition of asexual proliferative larval development in molluscs (Cribb et al. 2003). Might there then be peculiar remnant signatures of meiosis in intramolluscan larvae? We identified homologs to two known meiosis prophase-specific transcripts in our Illumina samples (Figure 16B), which were originally characterized in mice: meiosis express protein 1 (MEIG1) known to be involved in chromosome/chromatin binding in meiosis (Zhang et al. 2004) and highly expressed during meiosis prophase 1 (Steiner et al. 1999), and meiosis-specific nuclear structural protein 1 (MNS1). Anderson et al. (2015) identified a MEIG transcript expressed in adult male and female *S. mansoni* with a potential role in gamete production but no possible functional role was suggested to explain its high expression in eggs. MNS1 is specifically expressed in mice during the pachytene stage of prophase 1 of meiosis. Retinoic acid (RA) initiates meiosis

and although retinoic acid is not implicated in development of *S. mansoni*, we did see expression of retinoic acid receptor RXR. Of the putative meiosis stage-specific homologs, only RXR was present as a feature on the *S. mansoni* microarray, and it showed increasing expression as intramolluscan development progressed. Further study is warranted to learn if the transcripts we observed from intramolluscan stages are perhaps indicative of some tendency for occasional formation of bivalents without associated gamete formation or fertilization, or of a general repurposing of these molecules for use in many kinds of cellular reproduction, including asexual reproduction.

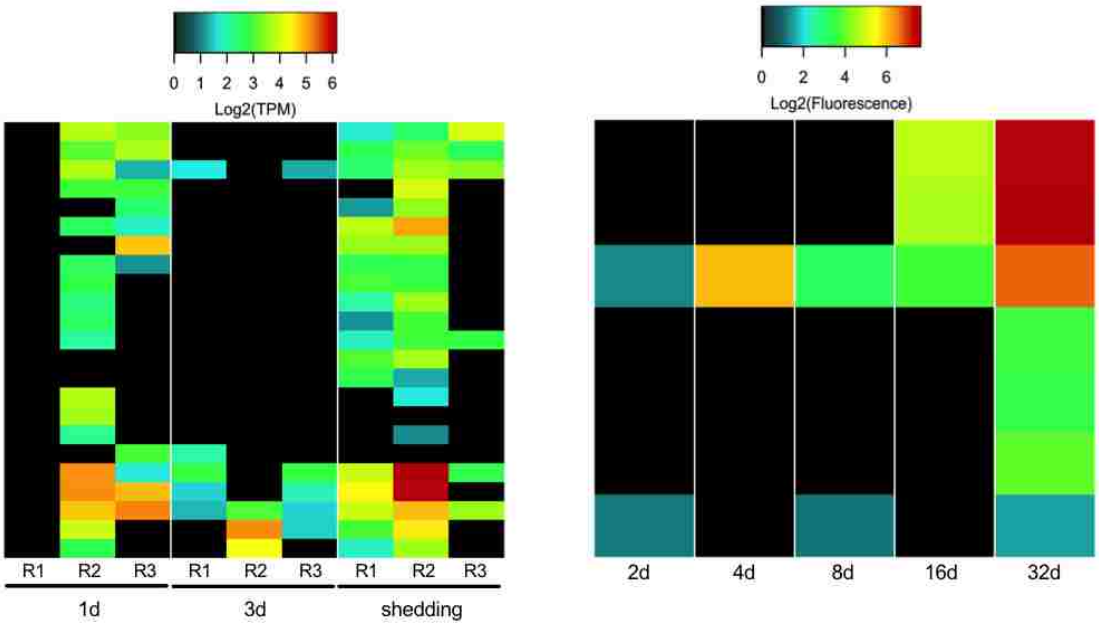
Six recombinase transcripts were expressed in our Illumina samples: three RAD51 homologs, two cassette chromosome recombinase b homologs, and one trad-d4 homolog. Recombinases like RAD51 are up-regulated in the testis and ovary of adult *S. mansoni* as compared to whole worm controls (Nawaratna et al. 2011) and in female adult *S. japonicum* when compared to males (Cai et al. 2016). Recombinases can repair breaks in DNA as a result of DNA damage or that occur during homologous recombination during meiosis. At least one transcript of another recombinase, topoisomerase II, was expressed in every time point. Among other functions, topoisomerase II interacts with the meiosis-specific RecA-like protein Dmc1 or RAD51 to facilitate pairing of homologous chromosomes during chromosome strand exchange (Iwabata et al. 2005).

Glycosyltransferase expression in intramolluscan stages

Molecular mimicry has been an area of interest with respect to schistosome-snail interactions since the early 1960s with the hypothesis that parasites express host-like molecules to evade host immune responses (Damien 1964). Several studies have highlighted antigenic similarities between miracidia and mother sporocysts and *B. glabrata* hemolymph proteins (Yoshino and Bayne 1983; Yoshino and Boswell 1986), and the glycans on *S. mansoni* glycoproteins and glycolipids have been extensively studied, including for their potential role in mediating host mimicry (Nyame et al. 2002; Lehr et al. 2007, 2008; Peterson et al. 2009; Johnston and Yoshino 2001; Wu et al. 2009). Yoshino et al. (2012)

showed that antibodies to *S. mansoni* glycotopes bound more extensively to cell-free hemolymph (plasma) from snails susceptible to infection than plasma from resistant strains, and suggested host-mimicking glycotopes could be a determining factor in compatibility during early larval stages. Consequently, we were interested in examining *S. mansoni* glycosyltransferases because of the role they play in generation of glycan moieties on lipids and proteins. Among others, one group of glycosyltranferases we found to be prominent in *S. mansoni* intramolluscan stages were fucosyltransferases (FTRs). Several of the surface membrane glycoconjugates of *S. mansoni* that interact with *B. glabrata* are fucosylated (Castillo et al. 2007) and are suspected to be involved in host mimicry (Nyame et al. 2002). We found 22 unique Illumina-assembled FTRs transcripts and 8 fucosyl-transferase-specific probes represented on the microarray (Figure 17).

Figure 17. Fucosyltransferases of intramolluscan *S. mansoni*



Fitzpatrick et al. (2009) observed two major clades of FTRs expressed by *S. mansoni,* those expressed in miracidia and mother sporocyst stages (alpha 1,6 fucosyltransferases D and E) and those expressed primarily in sexually mature adults (alpha 1,3 fucosyltransferases B, L, F). We did not observe an obvious

stage-specific demarcation in FTRs expression but rather observed a broad range of FTR transcripts, including those Fitzpatrick et al. (2009) observed primarily in sexually mature adults. They were expressed at all time points with highest diversity being at 1d and in shedding snails. Alpha 1,6 fucosyltransferase H was expressed ubiquitously across all Illumina and microarray samples. We also saw expression of five O-fucosyltransferase transcripts exclusively at 1d and in shedding snails. O-fucosyltransferases add a fucose residue to the oxygen on a side chain of either serine or threonine residues in a glycoprotein. Heavy expression in shedding snails was not surprising because cercariae possess a prominent fucose-rich glycocalyx (Řimnáčová et al. 2017).

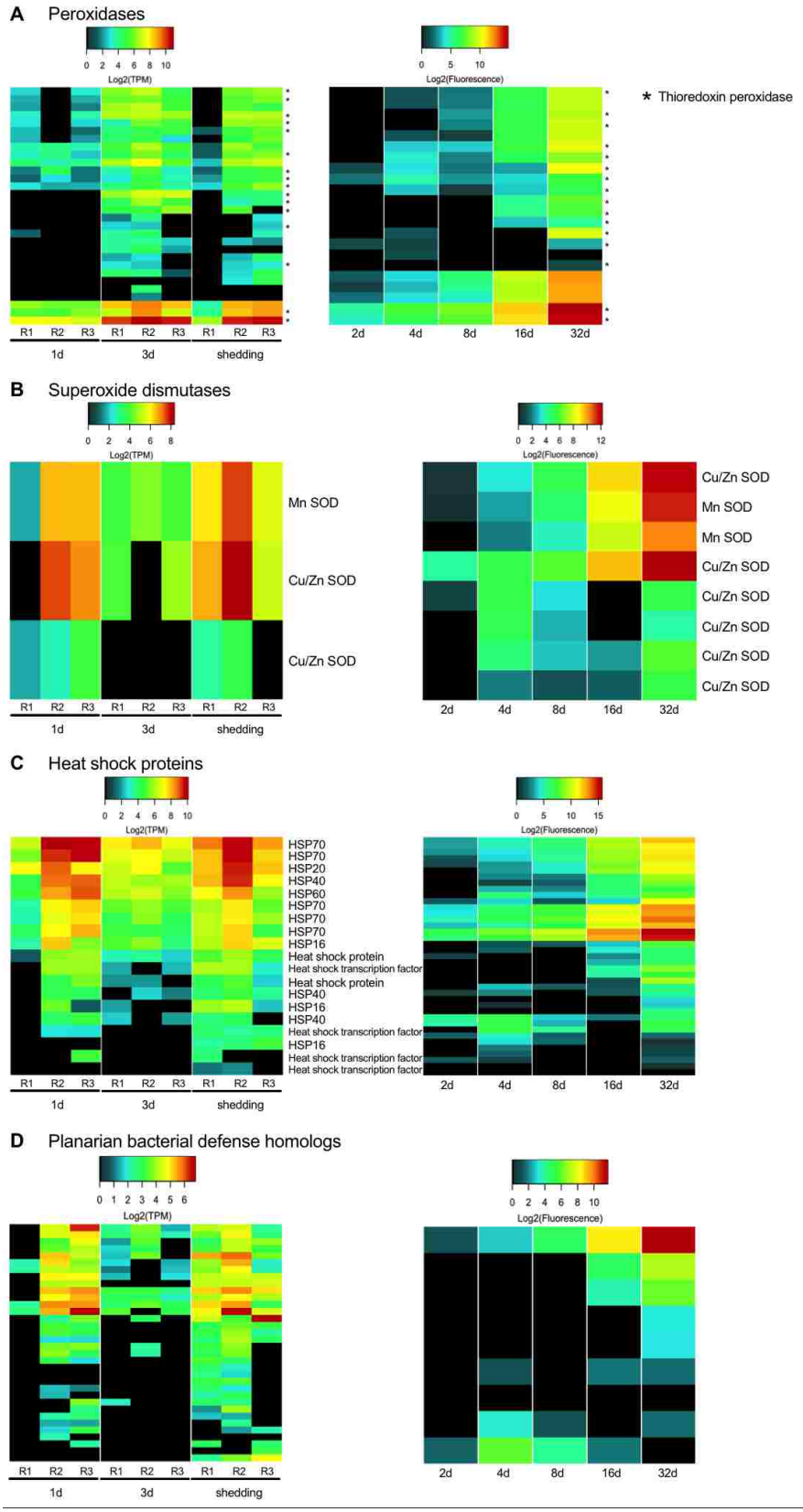
A transcriptional regulatory protein, KRAB-A domain-containing protein and dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit DAD1 homolog that performs post-translational protein glycosylation, were among the most abundantly expressed transcripts across all Illumina samples.

Sporocyst defense and stress responses

It is reasonable to expect that *S. mansoni* intramolluscan stages are under some duress from host immune responses, and we noted that snail Cu,Zn superoxide dismutases (SOD) were upregulated at both 1d and 3di in the highly compatible snail *B. pfeifferi* from which the *S. mansoni* Illumina transcripts discussed here were also obtained (Buddenborg et al. 2017). The H_2O_2 resulting from SOD activity is known to be toxic to *S. mansoni* sporocysts (Hahn et al. 2001; Yoshino et al. 2017) and is a main factor responsible for killing early larval *S. mansoni* in some *B. glabrata* resistant strains (Hahn et al. 2001; Yoshino et al. 2017). Organisms can remove harmful intracellular hydrogen peroxide with catalases, glutathione peroxidases, and peroxiredoxins. Schistosomes lack catalases (Mkoji et al. 1988; Sayed et al. 2006) and have low levels of glutathione peroxidases with limited antioxidant abilities (Mei and LoVerde 1997). It is suggested that peroxiredoxins are the schistosome's main defense against damage from hydrogen peroxide (Kwatia et al. 2000). In our data, thioredoxin peroxidases, peroxiredoxins that scavenge H_2O_2 using thioredoxin (Lu and

Holmgren et al. 2014), are consistently (and highly) expressed throughout all time points in array and Illumina samples (Figure 18A). Thioredoxin peroxidases reduce hydroperoxides with thioredoxin as a hydrogen donor. *S. mansoni* also expresses SOD activity and *S. mansoni*-encoded Mn- and Cu/Zn-type SODs are expressed in every replicate of both Illumina and array-sequenced samples (Figure 18B). Because the abundance of *S. mansoni* SOD transcripts was consistently modest, we suggest that their function is not to mount an anti-snail counter-offensive but rather to take care of the intracellular anti-oxidative needs of the parasite.

Figure 18. Sporocyst defense and stress factors



Cytochrome P450 proteins have been associated with stress responses and in detoxification reactions. *S. mansoni* has but a single cytochrome P450-encoding gene and the associated protein activity has been shown to be essential for survival in both adult worms and eggs, although its underlying function in schistosomes remains unclear (Ziniel et al. 2015). Cytochrome P450 transcripts showed minimal expression at 1d, were absent at 3d, and had modest (~3 Log_2 TPM) in all shedding replicates.

Heat shock proteins (HSPs) are often produced under conditions of stress, but they are also constitutively expressed in actively synthetic cells to serve as chaperones and to facilitate protein folding (Sun and MacRae 2005). *S. mansoni* sHSPs 16 and 20 and HSPs 70 and 90 were all found among proteins released during *in vitro* miracidium to mother sporocyst transformation (Wu et al. 2009). We found sHSPs 16, 20, and 40 and HSPs 60 and 70 but not 90, to be expressed throughout intramolluscan stages with the highest expression seen in HSPs 20, 40, and 70 (Figure 18C). sHSP 20 contributes up to 15% of the soluble proteins of miracidia (Mathieson and Wilson 2010) and is a prominent protein identified in miracidia by LC-MS/MS (Wang et al. 2016). sHSP 40 has been identified as a soluble egg antigen responsible for eliciting immunopathological reactions in the definitive host that result in granuloma formations (Hernandez and Stadecker 1999). Ishida and Jolly (2016) showed that in the absence of HSP 70, cercariae do not orient or penetrate normally, providing additional functional roles for HSP 70 beyond stress responses. We noted that *S. mansoni* HSP 70 was expressed at high levels in cercariae-producing shedding samples.

In addition to defending themselves from attack by host immune components, in long-lived host-parasite associations as represented by *S. mansoni* in *B. pfeifferi*, it might also be reasonable to expect digenean sporocysts to contribute to the stability and maintenance of the host-parasite unit as a whole. Rediae of some digenean species do this in the form of actively attacking newly-colonizing trematode infections (Lim and Heyneman 1972). In complex, natural transmission foci such as the one from which our samples originated, *S. mansoni*-infected *B. pfeifferi* snails are constantly exposed to a variety of viruses,

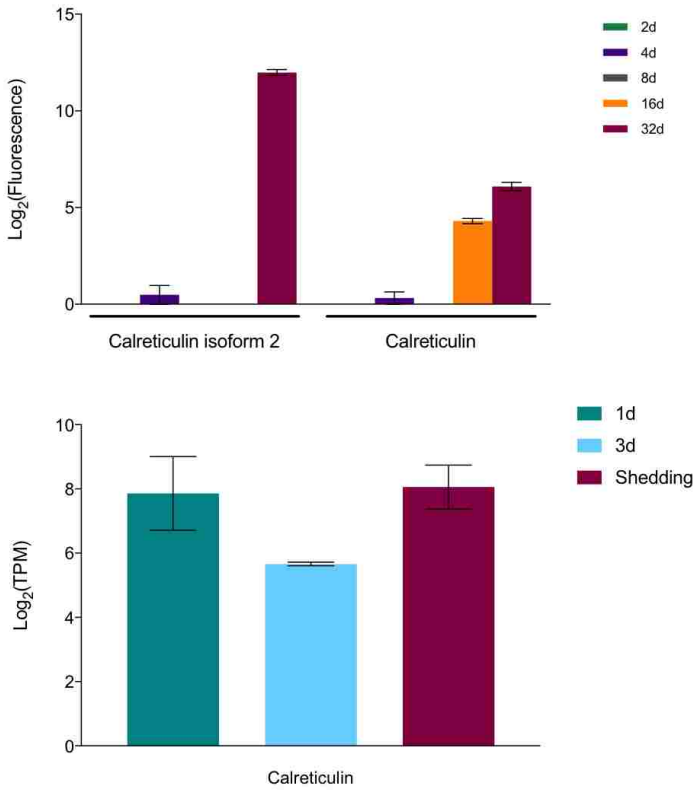
bacteria, and infectious eukaryotes (Buddenborg et al. 2017). Therefore, it seems reasonable that while imposing considerable stresses on its hosts, possibly including immunosuppression, that larval schistosomes might also be expected to contribute to the well-being of the host-parasite unit by expressing transcripts that contribute to repression or elimination of additional parasites.

One way to gain insight into *S. mansoni* sporocyst capabilities in this regard was to review what is known for free-living flatworms, such as the planarian *Dugesia japonica,* that are regularly challenged by pathogenic and non-pathogenic bacteria in their habitats. Planarians are capable of phagocytosing and destroying pathogens like *Staphylococcus aureus* and *Mycobacterium tuberculosis* (Morita 1991; Abnave et al. 2014). Conserved homologs to human genes, such as MORN2 (membrane occupation and recognition nexus-2 protein) are present and known to play a role in LC3-associated phagocytosis (LAP) elimination of bacterial pathogens in human macrophages and in flatworms. Homologs of 34 transcripts of putative flatworm anti-bacterial factors (Abnave et al. 2014) were expressed in *S. mansoni* intramolluscan stages (Figure 18D). Homologs to dual specificity phosphatases were the most prominent group of anti-bacterial factors. They were expressed at all time points, with a general increase in expression with development time. Homologs of MORN2 (membrane occupation and recognition nexus-2 protein) were also present throughout intramolluscan development. MORN2 plays a role elimination of bacterial pathogens in human macrophages as well as in flatworms. MORN2 is present in all replicates in 1d, 3d, and shedding *S. mansoni* samples. Shedding *S. mansoni* samples in both Illumina and microarray contain the most flatworm bacterial defense homologs. How these putative schistosome defense factors may be deployed in sporocysts that lack a gut and that do not engage in phagocytosis as far as we know, remains to be seen.

Among possible *S. mansoni* anti-immune factors we noted to be highly expressed in our samples was calreticulin, previously shown to be present in the excretory/secretory products of *S. mansoni* sporocysts (Figure 19). Because of its calcium-binding capability, Guillou et al. (2007) suggested calreticulin may

interfere with hemocyte spreading and interfere with their ability to initiate encapsulation responses.

Figure 19. Expression of calreticulin in *S. mansoni*



Evidence of amphistome-mediated suppression of *S. mansoni* sporocyst development

As noted in Buddenborg et al. (2017), Illumina replicate 3d-R2 harbored a pre-patent infection of an amphistome species, presumptive *Paramphistomum sukari*, known to be common in *B. pfeifferi* in Kenya, including from the habitat from which these snails were obtained (Laidemitt et al. 2017). The presence of an amphistome infection is of interest because previous studies indicate that amphistomes and schistosomes interact in distinctive ways in the intramolluscan environment, with amphistomes having a permissive effect in enabling development of a schistosome that might not have otherwise developed in a particular snail species (e.g. Southgate et al. 1989). In our context, the effect of the amphistome appears to be the opposite, based both on field results which

suggest that amphistome infections supplant *S. mansoni* infections (Laidemitt, personal communications), and on our transcriptional results. In general, overall *S. mansoni* transcription in the 3d replicate with the amphistome was dampened relative to 3d replicates lacking the amphistome. This dampening took the form of both fewer numbers of *S. mansoni* transcripts expressed, and for those that were expressed, representation at lower copy numbers. It did not appear that specific highly expressed *S. mansoni* transcripts were targeted in any selected way by the presence of the amphistome. In fact, the *S. mansoni* transcriptome in the amphistome-containing replicate most closely resembles that seen for *S. mansoni* in 1d infections (1d-R1), suggestive of an amphistome-imposed delay in development.

Known unknowns and unknown unknowns

Although many of the highly expressed transcripts found in our Illumina samples at 1d, 3d, and shedding time points fell into one (or more) known functional categories, there remained several that either had an annotation and were not in one of our functional categories of interest or had no annotation and remain unknown. One of the advantages of a systematic sequencing approach is the discovery of transcripts for which we have no *a priori* knowledge yet that may play a key role in *S. mansoni* intramolluscan development. Transcripts with unknown characteristics are important to point out and briefly discuss because they may provide brand new insights into molecular functions not yet characterized but that may prove to be important for development and maintenance of infection in the snail host.

The transcripts we highlight in this section function in cytoskeletal maintenance, iron sequestration, oxidation-reduction, and transcription/protein regulation and modification. Cytoskeletal proteins tektin and tubulin beta chain were abundantly expressed in all Illumina *S. mansoni* samples. The tegument of the schistosome changes in shape and size and has to closely interact with its hosts, requiring cytoskeletal molecules to be continually recycled and renewed (Jones et al. 2004). A gene, *nifu*, with homology to nitrogen fixation

genes in bacteria, was highly expressed in all Illumina samples and it likely functions in the *de novo* synthesis of iron-sulfur clusters in mitochondria regulating cellular iron homeostasis (Stehling and Lill 2013). Iron is sequestered by *S. mansoni* from its hosts and is known to be essential to several metabolic processes for adult development and reproduction (Glanfield et al. 2007). Disrupting iron homeostasis has been an area of interest for therapeutics against schistosomes (McManus 2005). Two enzymes involved in the oxidation-reduction process were noted to be highly up-regulated in all samples: NADH-plastoquinone oxidoreductase and NADH ubiquinone oxidoreductase chain 3. A transcript identified as a putative stress associated endoplasmic reticulum protein was also highly expressed in all samples and is linked to the stabilization of membrane proteins during stress and facilitates subsequent glycosylation (Yamaguchi et al. 1999). Lastly, an endothelial differentiation-related factor 1 transcript was constitutively expressed. It is also expressed preferentially in adult male *Schistosoma japonicum* worms (Cheng et al. 2005).

The SmSPO-1 gene, first identified in late stage sporocysts (Ram et al. 1999), is present in all replicates and time points with increasing expression from mother sporocyst to cercariae production. SmSPO-1 is secreted as a lipid bilayer-binding protein that binds to host cell surfaces and induces apoptosis and has been well-characterized in cercariae during skin penetration (Holmfeldt et al. 2007). In addition to penetrating host tissue, intramolluscan stages of *S. mansoni* must also expand into dense host tissue as they grow and must migrate from the head-foot to the digestive gland, all activities for which SmSPO-1 activity may be critical.

We also see several egg CP391B-like and egg CP391S-like transcripts expressed at 1d, 3d, and shedding *S. mansoni* samples. Egg proteins have been reported as differentially expressed in mother sporocyst stages when compared to free-living miracidia (Williams et al. 2007).

With respect to “unknown unknowns,” a *de novo* assembled transcript (TRINITY_DN6450_c0_g1_i1_len=386_path=[654) had no annotation in any

database and a targeted annotation revealed only that the transcript coded for a protein with a distinctive cytoplasmic, transmembrane helix, non-cytoplasmic, and another transmembrane helix. This transcript may be a novel and unique *B. pfeifferi* transmembrane protein.

Because the transcripts mentioned above are abundant in every replicate across all Illumina samples, they represent potential novel targets for eliminating or moderating *S. mansoni* parasite development within *Biomphalaria* snails.

CONCLUDING COMMENTS

As a miriacidium penetrates a snail, it rapidly enters a radically different milieu from what it has previously experienced, and a number of pre-made proteins are released into its new surroundings to effect transition to the mother sporocyst stage adapted for intramolluscan existence (Wu et al. 2009; Wang et al. 2016; Yoshino et al. 2017). Our 1d Illumina samples include many transcripts distinctive from the proteins associated with transformation, indicative of the switch to the needs of existence as sporocysts. As examples, we found representation of different SmVALs, heat shock proteins, protease transcripts (elastases), neural development proteins or neurohormones among 1d Illumina samples than seen as proteins in miracidial transformation products.

From our earliest Illumina samples, it is evident that *S. mansoni* orchestrates a complex transcriptional program within its snail hosts, with a significant percentage of its genetic repertoire (an estimated 66**%** of the *S. mansoni* genome) engaged (see also Verjovski-Almeida who reports that 50-60% genes are expressed in each *S. mansoni* stage). This is particularly evident at the stage of production of cercariae, when large amounts of parasite tissue are present and production and differentiation of the relatively complex cercarial bodies are underway. Also noteworthy is that a core transcriptome required of life in a snail host can be identified which includes transcripts involved in a central glycolysis pathway and the TCA cycle, and for transmembrane transporters for monosaccharides, amino acids, steroids, purines and pyrimidines, indicative of

the dependence of sporocysts on their hosts for key molecular building blocks. Several of these like the amino acid transporters are noted for the first time in schistosome sporocysts. Metabolically, early stage schistosomes focus primarily on acquisition of molecular building blocks and nutrients with a distinct switch from storage to expending these components towards cercariae production in patent stage shedding *S. mansoni*. A role for receptor-mediated endocytosis in sporocyst nutrition should also not be excluded (Wilson 2012). Transcripts for enzymes required for macromolecular synthesis and cell proliferation, the latter a prominent and perpetual part of intramolluscan development and cercarial production, were also part of the core transcriptome. Oxidoreductase activity and cell redox homeostasis are among the most abundant functions across all larval stages of *S. mansoni*.

With respect to particular *S. mansoni* transcripts that may be key to successful intramolluscan development and/or that comprise parts of the "interactome" with snail transcripts, we highlight several findings below. First, by virtue of using both field-derived *B. pfeifferi* and *S. mansoni* from infected children, our Illumina study allows for a broader range of outcomes particularly as measured in the early stages of infection, and the variability that results can provide distinctive insights. For instance, cases where early sporocysts seem to be thriving with respect to read count are accompanied by production of larger quantities of factors associated with infectivity like proteases, fucosyltransferases, SmVALs, and GPCRs. Also, poor transcriptomic productivity for *S. mansoni* sporocysts has been associated with presence of other digenean species, unknown to us to be present at the time of exposure to *S. mansoni*, but that interfere with *S. mansoni* development (Southgate et al. 1989; Laidemitt, personal communication).

One of the surprising things about the genome of *S. mansoni* and of other parasitic helminths is the dearth of genes such as cytochrome P450s involved in degradation of xenobiotics (*S. mansoni* has but one such gene with unknown function), potentially including harmful-snail produced factors as well. The ABC

transporters we have noted to be highly expressed in sporocysts may function to compensate (Kusel et al. 2009).

Of particular interest was the expression of a diverse array of proteases and protease inhibitors at all intramolluscan stages, including some proteases like elastases characteristic of cercariae that were also produced by early sporocyst stages. The up-regulation by the snail host of protease inhibitors during the larval establishment period at 1d and 3d (Buddenborg et al. 2017) seems a likely response to prevent parasite establishment. Also of note was expression of *S. mansoni* protease inhibitors that might inhibit the action of snail trypsin-like proteases up-regulated late in infection (Buddenborg et al. 2017). These protease inhibitors may prevent the activation of the phenoloxidase enzyme laccase, whose activity induces a negative impact on late (7-9 week) *S. mansoni* infections (Le Clec'h et al. 2016).

G-coupled protein receptors (GPCRs) were also well represented in *S. mansoni* intramolluscan stages and are likely to play several important roles in schistosome development. One such GPCR is expressed at 1d and in shedding snails and is known to bind serotonin (El-Shehabi et al. 2009). We noted at the same time points that *B. pfeifferi* down-regulated production of a type 1 serotonin receptor and additionally, at 3d kynurenine 3-monooxygenase which degrades tryptophan and can limit concentrations of serotonin is up-regulated (Buddenborg et al. 2017). It seems reasonable to continue to suspect serotonin of playing a role in parasitic castration. It stimulates egg production when given to castrated snails (Manger et al. 1996). By expressing the appropriate serotonin GPCR, and possibly down-regulating the host receptor, *S. mansoni* sporocysts may limit availability of serotonin to the snail.

Other factors also worthy of additional consideration with respect to parasitic castration are *S. mansoni* neuropeptides Y and F and their receptors which were expressed particularly in shedding snails. In snails, neuropeptide Y has been associated with decreased egg production (de Jong-Brink et al. 2001) and neuropeptide Y receptor was up-regulated at 1 day and in shedding *B. pfeifferi* snails (Buddenborg et al. 2017). Whether these neurotransmitters produced by

S. mansoni might directly affect snail reproduction is not known. Ovipostatins which have a suppressive effect on egg laying in the snail *Lymnaea stagnalis* were found to be up-regulated in shedding *B. pfeifferi* so it is possible their expression may be targeted by *S. mansoni* in some manner as well. We did not see obvious changes in some snail neuroendocrine factors associated with reproduction like calfluxin or schistosomin (Buddenborg et al. 2017). Wang et al. (2017) in a proteomic study of neuropeptides from *B. glabrata*, including snails with 12 day infections with *S. mansoni*, found lower levels of many snail reproductive neuropeptides. The extent to which *S. mansoni* and other digenetic trematodes might effect snail reproduction through interference with their neuroendocrine systems as proposed by de Jong-Brink (2001) remains a topic worthy of more study. As noted by Humphries (2011), it is also possible that castration is more a consequence of depletion of nutrients and alterations of metabolism imposed by metabolically demanding larval schistosomes.

Insights provided by the study of planarians were important to our interpretation of our results in two ways. The first was to confirm in intramolluscan *S. mansoni* samples the common expression of genes associated with maintenance of stem cell-like germinal cells, including fibroblast growth factor receptors (*fgfr*), *vasa*, *argonaute2* (*ago2*), and *nanos-2* (Wang et al. 2013). The second was to examine sporocysts for evidence of homologs of transcripts known to be involved in antibacterial responses in *Dugesia japonica* (Abnave et al. 2014), for which 34 were found. Whether these factors are actually deployed in anti-bacterial or other defense responses remains to be seen. Their presence is somewhat peculiar because sporocysts lack phagocytic activity, unlike the gut cells of planarians. However, perhaps anti-bacterial proteins are deployed along sporocyst membranes, or sporocysts may engage in limited forms of endocytosis (Wilson 2012) that might result in engulfment of bacteria or their products. Possible anti-snail hemocyte factors like calreticulin (Guillou et al. 2007) were also expressed by sporocysts. The possibility that sporocysts contribute to discouraging or preventing growth of third party symbionts that could compromise the snail-schistosome functional unit, especially in light of the need

of schistosme sporocysts to compromise components of host immunity is also a topic worthy of additional study.

One advantage of next gen sequencing is its potential to provide unexpected insights. We were surprised to see two transcripts (MEIG1 and MNS1) associated specifically with prophase I of meiosis and discuss possible interpretations based on previous cytological studies of germinal cell development (Khalil and Cable 1968), observations that might help to explain differences among *S. mansoni* cercariae in genetic content (Grevelding et al. 1999). The expression of recombinases in sporocysts might be consistent with a partial entry into meiosis up to diakinesis, or with mitotic recombination, the latter suggested by Grevelding et al. (1999) to account for genetic differences among cercariae derived from the same miracidium.

Finally, we note that many more highly represented transcripts were found, including those encoding both genes with suspected or unknown functions whose connection with intramolluscan development remain to be elucidated. With ever more complete transcriptional profiles becoming available for schistosomes in their snail hosts, the stage is set for further studies employing the best tools available for gene knockout to address the functional roles of these and the many other transcripts we and others have noted. Of particular interest will be to determine if ingenious use of this information can be made to specifically target and prevent the development of sporocysts and their production of human-infective cercariae, thereby opening a much-needed additional front in the effort to control and eliminate human schistosomiasis.

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CHAPTER FOUR

Transcriptional responses of *Biomphalaria pfeifferi* and *Schistosoma mansoni* Following exposure to niclosamide, with evidence for a synergistic effect on snails following exposure to both stressors

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ABSTRACT

Background

Schistosomiasis is one of the world's most common NTDs. Successful control operations often target snail vectors with the molluscicide niclosamide. Little is known about how niclosamide affects snails, including for *Biomphalaria pfeifferi*, the most important vector for *Schistosoma mansoni* in Africa. We used Illumina technology to explore how field-derived *B. pfeifferi*, either uninfected or harboring cercariae–producing *S. mansoni* sporocysts, respond to a sublethal exposure of niclosamide. This study afforded the opportunity to determine if snails respond differently to biotic or abiotic stressors, and if they reserve unique responses for when presented with both stressors in combination. We also examined how sporocysts respond when their snail host is exposed to niclosamide.

Principal Findings

Cercariae-producing sporocysts within snails exposed to niclosamide express ~68% of the genes in the *S. mansoni* genome, as compared to 66% expressed by intramolluscan stages of *S. mansoni* in snails not exposed to niclosamide. Niclosamide does not disable sporocysts nor does it seem to provoke from them distinctive responses associated with detoxifying a xenobiotic. For *B. pfeifferi*, niclosamide treatment alone increases expression of several features not up-regulated in infected snails including particular cytochrome p450s and heat shock proteins, glutathione-S-transferases, antimicrobial factors like LBP/BPI and protease inhibitors, and also provokes strong down regulation of proteases. Exposure of infected snails to niclosamide resulted in numerous up-regulated responses associated with apoptosis along with down-regulated ribosomal and defense functions, indicative of a distinctive, compromised state not achieved with either stimulus alone.

Conclusions/Significance

This study helps define the transcriptomic responses of an important and under-studied schistosome vector to *S. mansoni* sporocysts, to niclosamide, and

to both in combination. It suggests the response of *S. mansoni* sporocysts to niclosamide is minimal and not reflective of a distinct repertoire of genes to handle xenobiotics while in the snail host. It also offers new insights for how niclosamide affects snails.

INTRODUCTION

Schistosomiasis control remains elusive in many of the world's hyperendemic foci of infection in sub-Saharan Africa, jeopardizing the goals of diminishing schistosomiasis as a public health concern, or of eliminating transmission where possible by 2025 (WHO 2013). Several recent papers have called for the need to adopt more integrated control approaches instead of relying on chemotherapy alone to achieve eventual elimination (Rollinson et al. 2013; Tchuenté et al. 2017), and there has been a resurgence in interest in methods to control the snails that vector human schistosomiasis (King et al. 2015; Lo et al. 2018). Although the practical options available for use in snail control remain limited, molluscicides have been advocated because there are several recorded instances where their use has been associated with successful control (King and Bertsch 2015; Sokolow et al. 2016).

Following the discovery of niclosamide's molluscicidal properties in the 1950s, it has been incorporated into the commercial preparation known as Bayluscide (Andrews et al. 1983) and is the only molluscicide approved for use in schistosomiasis control by the WHO Presticide Evaluation Scheme (WHOPES). Use of niclosamide has enjoyed a modest resurgence and its focal application in snail control is advocated by WHO (WHO 2017). It has been used widely in Egypt and China as a mainstay for control operations, and it is used in both experimental (Kariuki et al. 2013; Xia et al. 2014) and in new control contexts, most notably recently as part of the *S. haematobium* elimination program in Zanzibar (Knopp et al. 2012, 2013).

Although some work on the effects of molluscicides on oxygen consumption and carbohydrate metabolism of snails has been undertaken (Weinbach and Garbus, 1969; El-Gindy and Mohamed, 1976), there have been relatively few

studies employing modern techniques to assess the impacts of molluscicide exposure on schistosome-transmitting snails. Zhao et al. (2015), working with the amphibious snail *Oncomelania hupensis*, the intermediate host for *Schistosoma japonicum*, undertook an Illumina-based *de novo* transcriptome study to show this snail responded to two novel niclosamide-based molluscicides by up-regulating production of two cytochrome p450 (CYPs) genes, and one glutathione-S-transferase. Zhang et al. (2015) examined the effects of three different sublethal concentrations of niclosamide (0.05, 0.10, and 0.15 mg/L for 24 hours) on the transcriptional activity of *Biomphalaria glabrata* as examined using an oligonucleotide microarray and noted up-regulation of several genes associated with biotransformation of xenobiotics (CYPs and glutathione-S-transferase), drug transporters, heat shock proteins (HSP 20, 40 and 70 families) and vesicle trafficking. Down-regulated hemoglobin production was also noted. Niclosamide is able to kill schistosome miracidia and cercariae (Ghandour and Webbe 1975; Tchounwou et al. 1992) and field experiments in China have shown that niclosamide is effective at reducing the number of viable *S. japonicum* cercariae in streams and downstream infection of sentinel mice (Lowe et al. 2005).

With respect to the effects of niclosamide on schistosome-infected snails, or on the schistosome sporocysts within them, there has been remarkably little study. Sturrock (1966) investigated the effects of sublethal concentrations of niclosamide on infections of *S. mansoni* on *Biomphalaria sudanica tanganyicensis* and noted that: 1) snails exposed to molluscicide that survived were still susceptible to infection; 2) snails with prepatent infections were not initially more susceptible to molluscicide but had slightly delayed rate of parasite development and production of cercariae and did eventually exhibit higher mortality as they entered patency; and 3) survivorship of snails exposed during the patent period was less, although it takes some time for the effect to occur. Sturrock (1966) commented that the combined stress of producing cercariae and exposure to molluscicide likely contributed to the higher mortality rate in patent snails. He also noted that doses sufficiently high to kill schistosome sporocysts in

snails were probably above the lethal doses needed to kill the snails themselves.

In this study, building on the microarray results of Zhang et al. (2015) with *B. glabrata*, we sought to obtain a more in-depth view of the transcriptome of molluscicide-exposed snails by using the Illumina platform to examine the responses of *Biomphalaria pfeifferi* to a sublethal dose (0.15 mg/L) of niclosamide. *Biomphalaria pfeifferi* is widely distributed in streams, ponds and impoundments in Africa and is probably responsible for transmitting more cases of *Schistosoma mansoni* than any other *Biomphalaria* species (Frandsen 1979; Brown 1994). In addition, we examined the transcriptional responses to the same dose of molluscicide of *B. pfeifferi* harboring cercariae-producing *S. mansoni* infections. We were able to compare the responses of the above snails to both uninfected and infected *B. pfeifferi* not exposed to molluscicides (see companion studies by Buddenborg et al. 2017, 2018). For both the previous and present studies, we chose to examine the responses of snails recently removed from field habitats and therefore considered to be more representative of what might be expected of snails comprising natural populations actually exposed to molluscicides. The approach taken enables us to ascertain if and how the transcriptional responses of snails already coping with a massive *S. mansoni* infection can be further altered by simultaneous exposure to a toxic xenobiotic. For example, might snail genes up-regulated following exposure to *S. mansoni* trend towards down-regulation if the snail is exposed to niclosamide and required to produce increased quantities of molecules involved in detoxification?

With respect to the sporocysts of *S. mansoni* residing in snails exposed to niclosamide, do they exhibit any tendency to express genes that are not normally expressed during intramolluscan development, and if so, do the ensuing proteins favor survival of the sporocysts or of the stressed snail in which the sporocysts reside? Three possible scenarios for *S. mansoni* transcriptional response to molluscicide exposure can be considered: 1) We see an overall down-regulation of *S. mansoni* transcripts indicating suspension of activity; 2) Cercariae-producing *S. mansoni* sporocysts express unique features that are shut off in response to molluscicide exposure; and 3) Shedding *S. mansoni* stages exposed

to molluscicide show unique transcriptional responses suggestive of a hitherto unseen ability to protect the host-parasite unit in which they reside from a xenobiotic.

METHODS

Biomphalaria pfeifferi used in Illumina sequencing were collected from Kasabong stream in Asembo Village, Nyanza Province, western Kenya (34.42037°E, 0.15869°S) and 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 under natural light to check for shedding of digenetic trematode cercariae (Mutuku et al. 2015). Snails shedding only *S. mansoni* cercariae and non-shedding controls were used in this study. More details of collections and processing can be found in Buddenborg et al. (2017,2018).

The molluscicide niclosamide dissolved in dimethyl sulfoxide (DMSO) was purchased from Sigma. Uninfected and *S. mansoni*-infected *B. pfeifferi* were exposed to a concentration of 0.15 mg/L niclosamide with final DMSO concentrations at 1/1000 (v/v) for 24 hours at 26-28°C with aeration (Zhang et al. 2015). Previous 24 h exposure of *B. glabrata* to varying doses of niclosamide (0.05mg/L, 0.10mg/L, and 0.15mg/L) found that the 0.15mg/L dose produced the most robust transcriptional response, as assessed by microarray analysis (Zhang et al. 2015). All snails exposed to 0.15mg/L niclosamide were alive and responding after the 24 hours dosage period. Therefore, a 0.15mg/L dose was also selected for this study as the sublethal dose administered to *B. pfeifferi*.

RNA extraction, library preparation, sequencing procedures, and sequencing summaries can be found in Buddenborg et al. (2017, 2018). Illumina RNA sequencing reads underwent extensive processing in order to separate host, parasite, and potential symbiont reads. *Biomphalaria pfeifferi* read quantification and differential expression analyses were performed using RSEM (RNA-Seq by expectation maximization) (Li and Dewey 2011) and EBSeq (Leng et al. 2013). Normalized *Schistosoma mansoni* read counts acquired from RSEM were normalized using DESeq's median of ratio method (Love et al. 2014).

RESULTS AND DISCUSSION

Overall *B. pfeifferi* and *S. mansoni* transcriptomic responses to molluscicide exposure

Relative to uninfected and untreated control *B. pfeifferi*, the overall differential gene expression responses were measured for snails i) with shedding *S. mansoni* infections only, ii) exposed for 24 h to a sublethal dose of niclosamide only, or iii) harboring shedding *S. mansoni* infections *and* exposed to niclosamide (Figure 1A). The responses of shedding snails relative to unexposed controls have been discussed extensively by Buddenborg et al. (2017). With respect to molluscicide exposure, this is the first Illumina-based view of the transcriptomics response for any species of planorbid snail, and supplements and extends the view provided by the microarray study for uninfected *B. glabrata* of Zhang et al. (2015). Zhao et al. (2015) undertook an Illumina-based study of the molluscicide-induced transcriptome of *Oncomelania hupensis*, the pomatiopsid snail host of *S. japonicum*. The response of *B. pfeifferi* to simultaneous exposure to schistosome infection and niclosamide exposure is the first glimpse we have for how snails respond transcriptionally to simultaneous exposure to these two relevant stressors.

For each of the three treatments noted, the number of upregulated snail features exceeded the number of down-regulated features. For both up- and down-regulated features, it was remarkable that over half of the transcripts proved to be distinctively represented in the combined treatment group (Figure 1B). Over 4,000 genes were distinctively up-regulated in the snails receiving the combination of stressors. This was the largest number found in any single group of either venn diagram. It was surprising to us that larger numbers of genes were not found in the cells of either venn diagram that represented two or all three of the treatments. It was also evident that although the response of niclosamide-exposed snails had features in common to those evoked by *S. mansoni* exposure, many genes were also uniquely differentially expressed by exposure to just niclosamide. Further inspection of the pattern in expression levels exhibited by genes uniquely expressed in the combined treatment group

revealed that in comparison to genes represented in other cells, they were modest in the degree of their differential expression. The specific nature of the genes responsive to either molluscicide alone, or to molluscicides and *S. mansoni* are discussed further below.

The transcriptomic responses of intramolluscan stages of *S. mansoni*, including those from snails actively shedding cercariae are described by Buddenborg et al. (2018), and are supplemented here by responses of shedding snails exposed to niclosamide (Figure 2A). *S. mansoni* from shedding snails expressed 18,736 transcripts whereas *S. mansoni* from shedding samples exposed to sublethal niclosamide expressed 23,040 transcripts (Figure 2B), with the majority (80.6%) of these shared between the two groups. Most of the remaining transcripts were unique to *S. mansoni* from the niclosamide-exposed samples (19%), with only 0.4% expressed uniquely in the shedding snails. The additional genes expressed only in the presence of niclosamide raises the percentage of the *S. mansoni* genome of 66% shown by Buddenborg et al. (2018) to be expressed in snails to 68%. *S. mansoni* exposed to niclosamide expressed more transcripts, but this response was variable among replicates and in general most (>90%) of these extra transcripts were expressed less than 2 $\log_2$ normalized counts when replicate counts were averaged (Figure 2C). Of the 80.6% of shared *S. mansoni* transcripts, there was little difference in overall expression levels for transcripts from samples with or without niclosamide exposure.

Figure 1. (A) Number of *B. pfeifferi* CDS up- and down-regulated in shedding and shedding plus molluscicide-exposed snails when compared to uninfected snails. (B) Venn diagrams showing shared and unique *B. pfeifferi* CDS between differentially expressed groups.

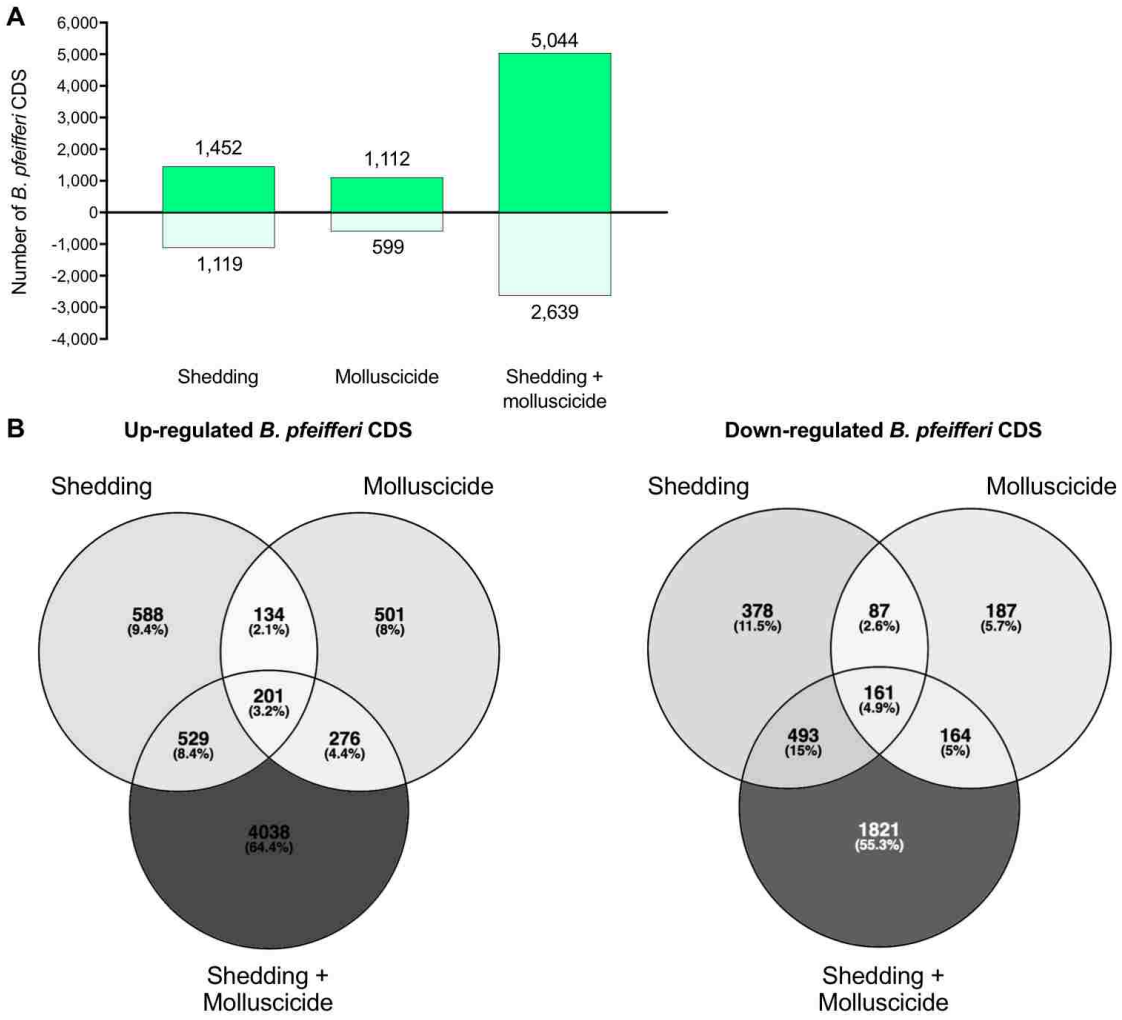
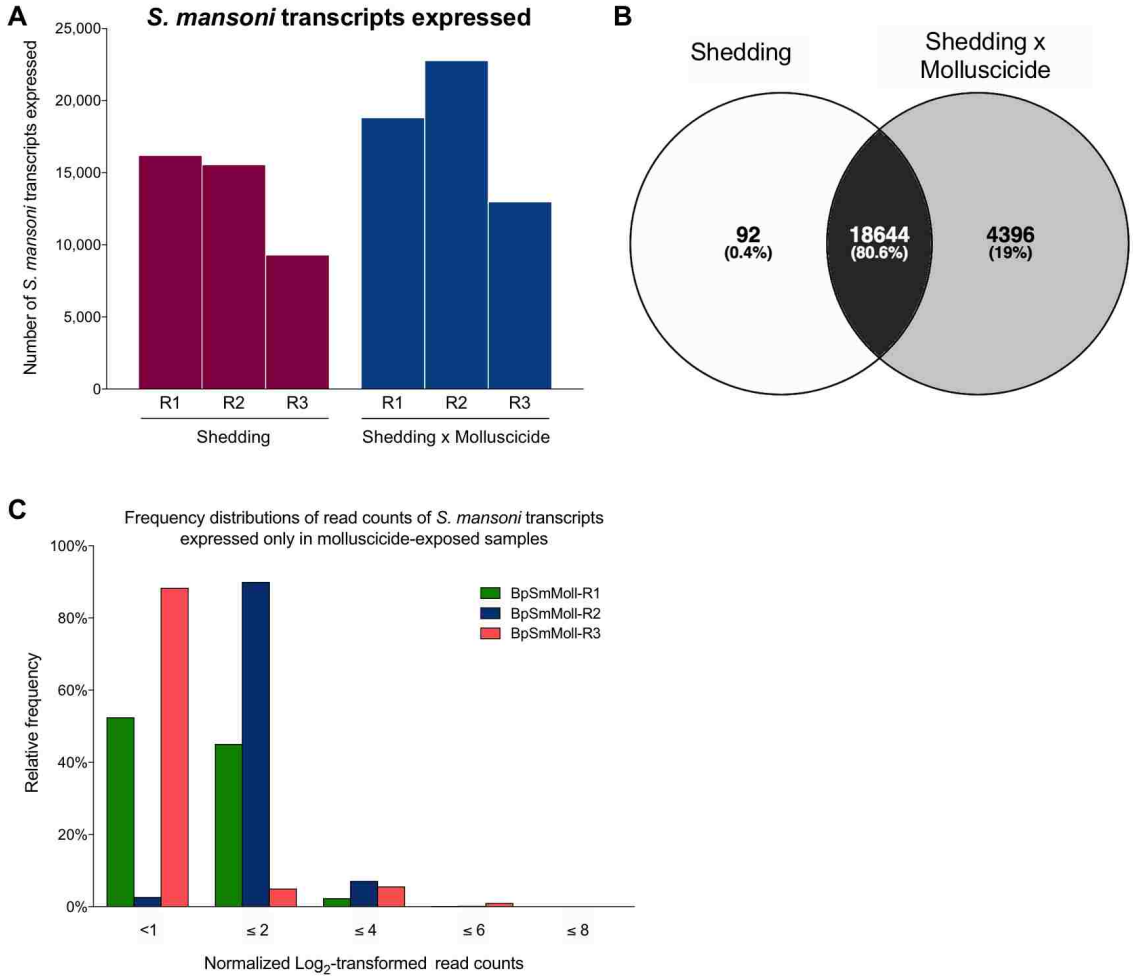


Figure 2. (A) *S. mansoni* transcripts expressed per replicate in shedding and shedding plus molluscicide exposed *S. mansoni.* (B) Venn diagram of shared and unique *S. mansoni* transcripts in tested groups. (C) Frequency distribution of log_2-transformed normalized read counts of *S. mansoni* transcripts uniquely expressed in shedding plus molluscicide replicates.



Specific responses of *S. mansoni* cercariae-producing sporocysts within *B. pfeifferi* exposed for 24 h exposure to sublethal niclosamide treatment:

Sturrock (1966) noted that the lethal dose of niclosamide for intramolluscan schistosomes must be higher than what is needed to kill the host snail. This is likely true since *S. mansoni* sporocysts are protected both by their syncytial tegument (Laumer et al. 2015) and by being embedded in the host snail's tissues. At least with respect to a 24 h exposure to a dose of niclosamide sublethal for *B. pfeifferi*, we observed that sporocysts within such snails produced

more transcripts relative to sporocysts from untreated shedding snails, not fewer (Figure 2). If cercariae-producing sporocysts were strongly and directly affected by niclosamide, we would have expected to see extensive and broad down-regulation or absence of numerous transcripts such as those related to nutrient uptake across the tegument, of elastases indicating a decrease or pause in cercariae production, and of transcripts associated with germ ball development and proliferation. As it was, only 0.4% of transcripts were missing as compared to untreated sporocysts.

Defense and stress responses were sustained in niclosamide-treated sporocysts relative to untreated sporocysts (Figure 3). Peroxiredoxins like glutathione peroxidase and thioredoxin peroxidase, which may be responsible for elimination of potentially lethal hydrogen peroxide produced by the snail (Kwatia et al. 2000; Hahn et al. 2001; Yoshino et al. 2017), were stably maintained. Stable and consistent expression of *S. mansoni* planarian-like bacterial defense homologs, heat shock proteins and SODs are all indicative that protective responses were maintained following exposure to niclosamide. Interestingly, there was no conspicuous evidence of *greater* representation of these transcripts in sporocysts from snails treated with niclosamide. Particularly noteworthy is the lack of an obvious response of the single *S. mansoni* cytochrome p450 gene to niclosamide presence. As noted by Ziniel et al. (2015), the exact function of this gene product in *S. mansoni* is not known, but our results suggest it is not provoked by a xenobiotic like niclosamide. As noted by Ziniel et al. (2015) and by us previously (Buddenborg et al. (2018), parasitic helminths in general lack extensive cytochrome p450 repertoires, quite unlike their hosts. In contrast, *B. pfeifferi* does indeed deploy cytochrome p450 responses upon exposure to molluscicides (see below). Another group of *S. mansoni* transcripts of potential relevance to their response to niclosamide are drug efflux transporters like ABC transporters which are known to be up-regulated in adult schistosomes exposed to praziquantel (Hines-Kay et al. 2012; Kasinathan et al. 2014). We noted several efflux transporters were expressed in cercariae-producing sporocysts of *S. mansoni* (Buddenborg et al. (2018) but did not observe any conspicuous change

in their expression pattern following exposure to niclosamide (Figure 4). A study on the giant liver fluke *Fasciola gigantica* exposed to rhodamine-labeled niclosamide did not reveal substantial changes in ABC transporter activity although the authors did not rule out the potential involvement in these proteins in drug resistance and detoxification (Kumkate, Chunchob, and Janvilisri 2008).

Figure 3. Stress and defense transcripts expressed by *S. mansoni*

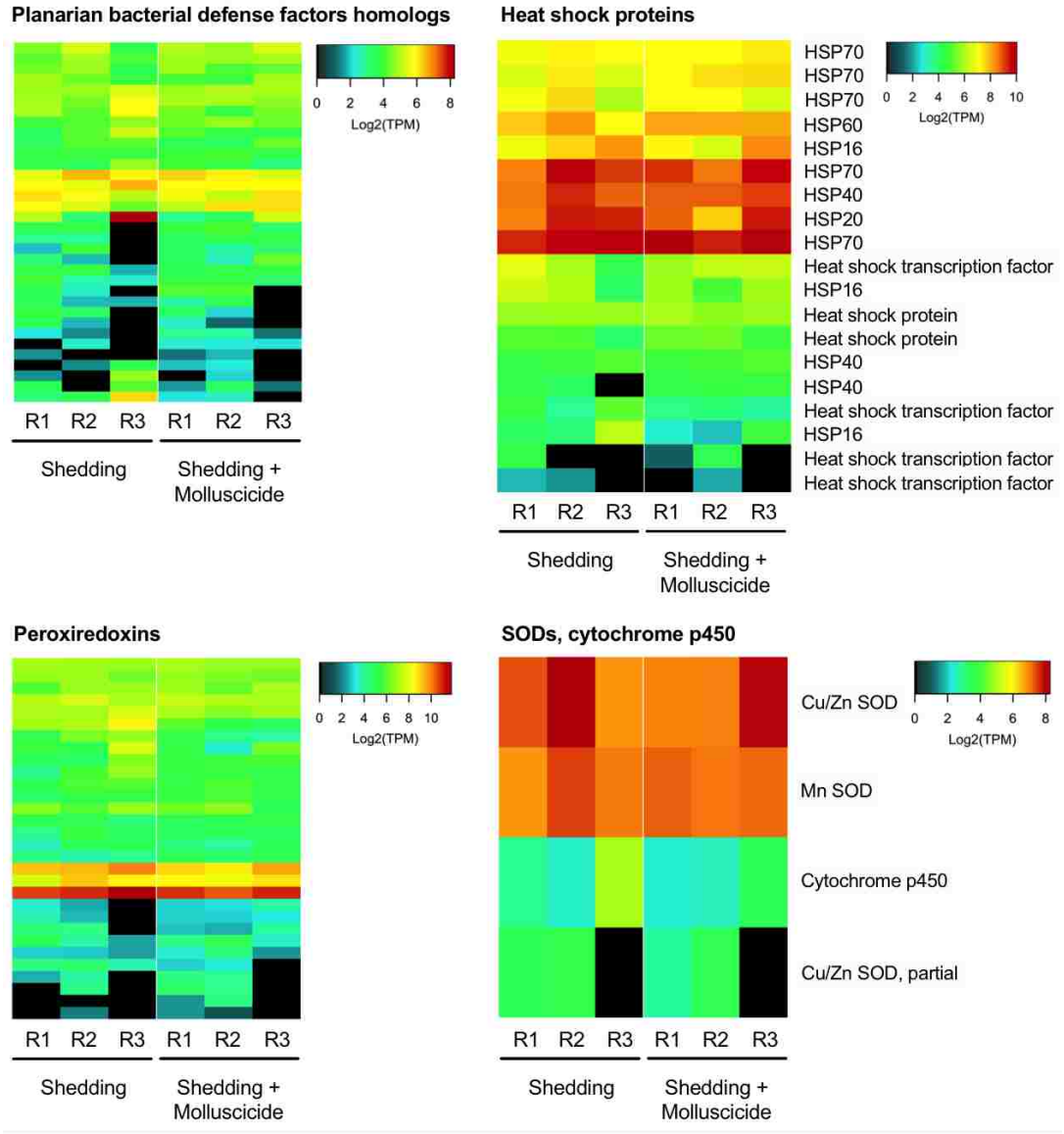
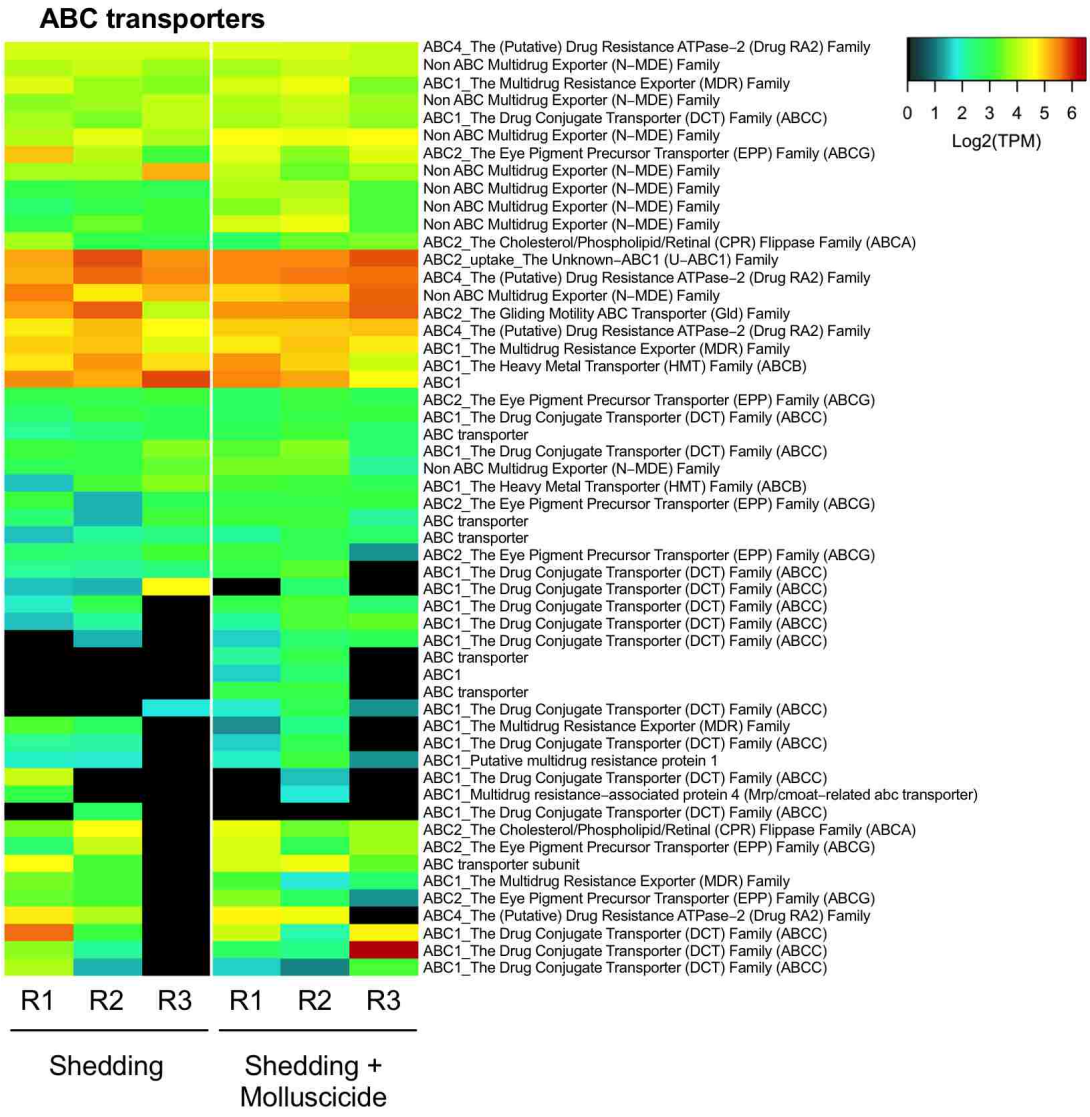


Figure 4. ABC transporters expressed by *S. mansoni* following exposure to 0.15mg/L niclosamide

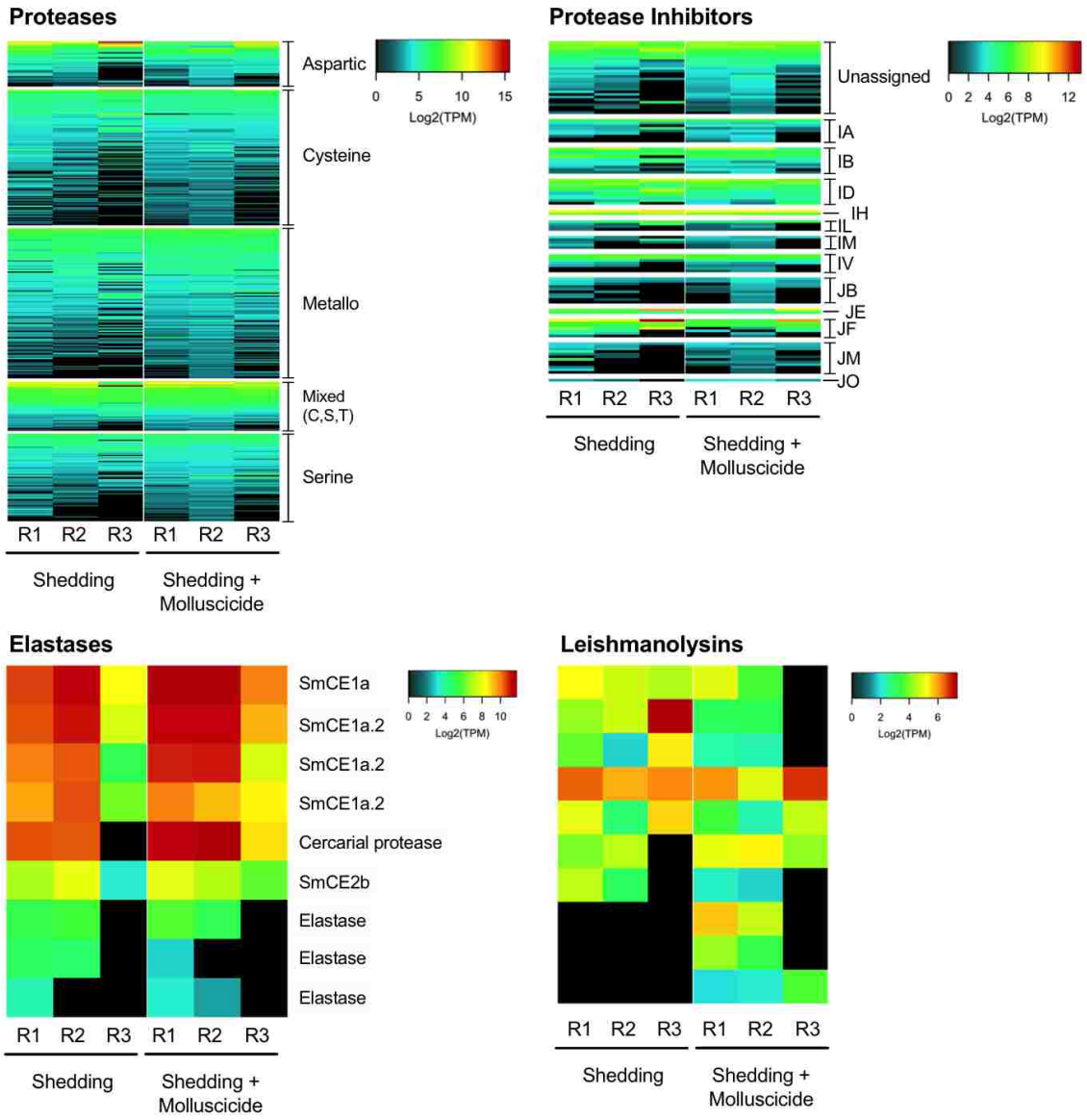


Inspection of the transcripts produced uniquely by niclosamide-exposed sporocysts does not reveal any candidates that would seem to favor resilience to niclosamide. This coupled with the stable expression of known defense or stress response genes noted above leads us to a conclusion that sporocysts have little if any ability to mount protective responses to niclosamide and certainly do not seem to provide anything that would favor enhanced survival of their host snail in

the presence of a chemical that is clearly lethal for the host. It is possible that the parasite is relying on host xenobiotic capabilities to respond to niclosamide.

S. mansoni sporocysts, and the cercariae developing within them, express a diverse array of proteases, including elastases and leishmanolysins (Ingram et al. 2012; Buddenborg et al., chapter 3), with likely functions in disabling snail defenses, dissolution of snail tissues to provide living space, facilitating intra-snail migration of sporocysts and for packaging in cercariae which use them both for exiting the snail host and entering the mammalian definitive host. Protease inhibitors are also produced and likely counteract proteases that the snail expresses late in infection (Le Clec'h et al. 2016; Buddenborg et al., chapter 3). The overall patterns of expression of proteases or protease-inhibitors does not differ substantially between sporocysts in untreated and niclosamide-treated snails (Figure 5).

Figure 5. *Schistosoma mansoni* proteases and protease inhibitor transcripts expressed in shedding and shedding plus molluscicide groups.



When specific proteases like elastases, leishmanolysins, and cathepsins are examined, some changes in expression were noted. Niclosamide-exposed sporocysts exhibited modest increases in expression of cercarial elastases SmCE1a, SmCE1a.2, cercarial protease, and SmCE2b (Figure 5). The SmCE isoforms represent an expanded family of elastases unique to some *Schistosoma* species including *S. mansoni* (Ingram et al. 2012). Transcripts for leishmanolysins which are metalloproteases also known as invadolysins (Curwen et al. 2006), also exhibited a modest increase in representation in niclosamide-

exposed sporocysts as compared to untreated sporocysts (Buddenborg et al., chapter 3).

At least some *S. mansoni* glucose, amino acid, and nucleoside transporter transcripts showed modestly increased representation in molluscicide-exposed sporocysts relative to untreated controls (Figure 6), as did some of the markers for germinal cell proliferation (Wang et al. 2013) such as fibroblast growth factor receptor 2, *vasa,* and *nanos-2* noted in Buddenborg et al. (2018) (Figure 7). Very modest changes in expression were also noted (Figure 8) in neuropeptide hormones or markers of neural development important in flatworm locomotion, feeding, host location, regeneration, and development (Collins et al. 2010; McVeigh et al. 2009). Shedding *S. mansoni* stages exposed to niclosamide had higher transcript levels for cell polarity protein, neuronal differentiation, notch, SOX transcription factor, and septate junction protein (Figure 7) and although modest, these may have important downstream effects on germinal cell proliferation or neurogenesis (Verjovski-Almeida et al. 2003; Parker-Manuel et al. 2011; Lu et al. 2016).

Figure 6. Nutrient transporter expression in shedding and shedding plus molluscicide-exposed *S. mansoni*.

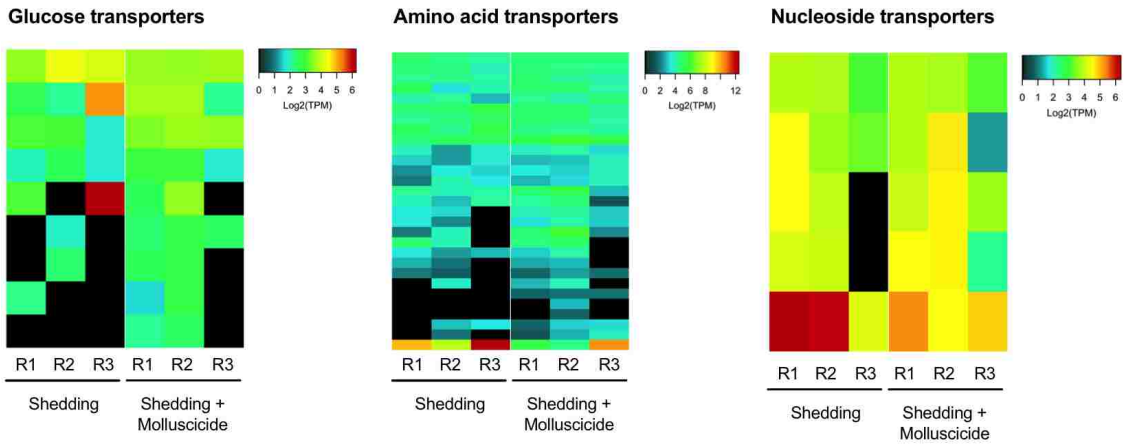


Figure 7. Expression of known germinal cell proliferation transcripts.

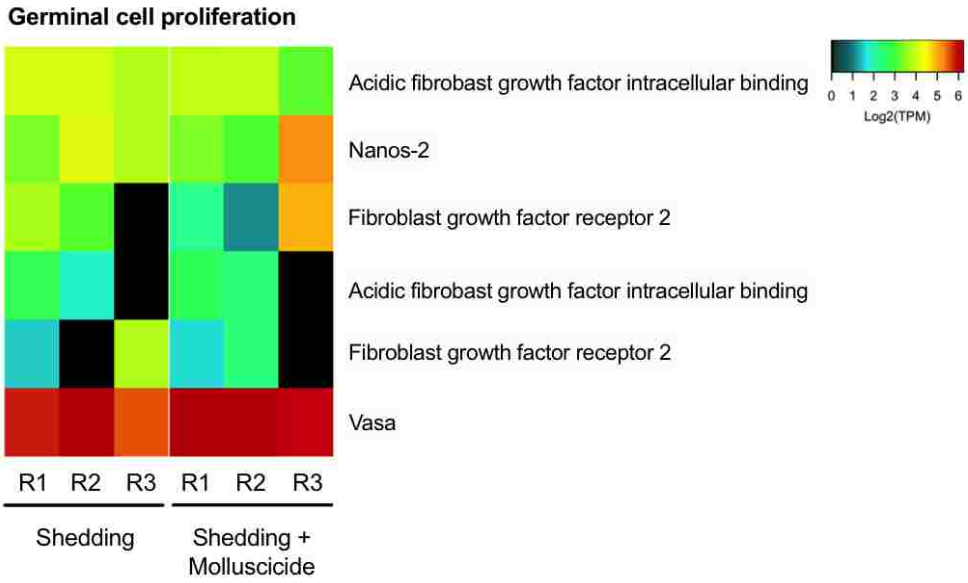
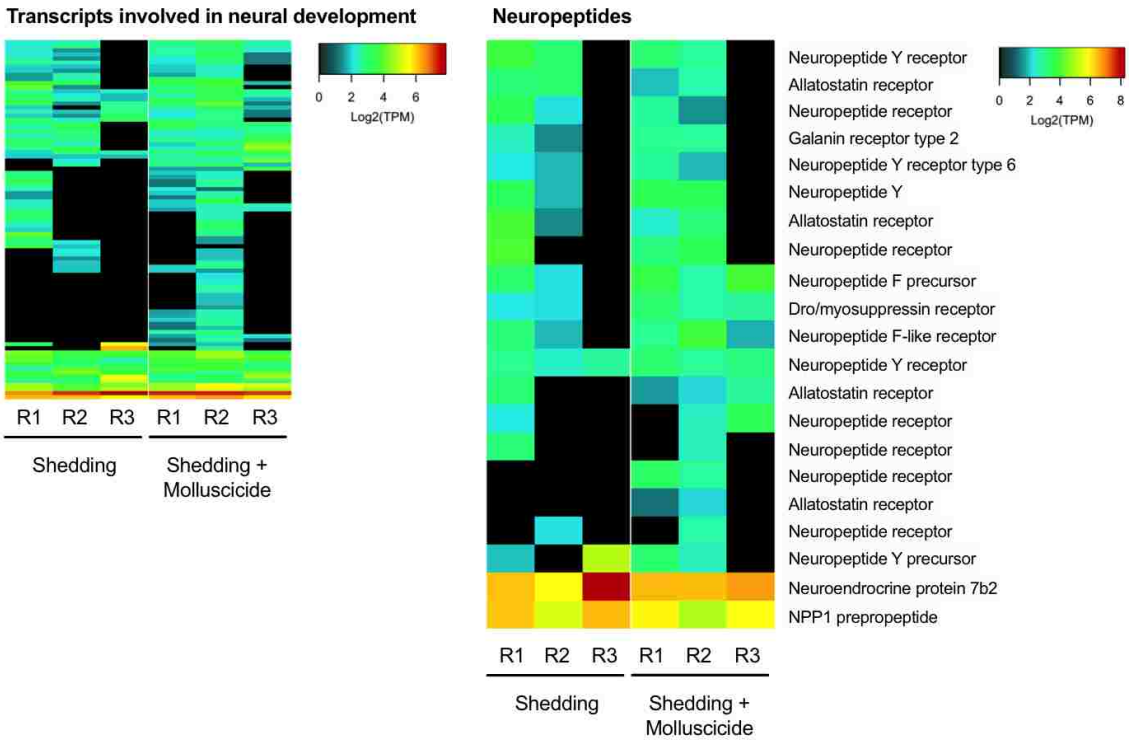


Figure 8. *Schistosoma mansoni* transcripts involved in neural development or encoding neuropeptides



The modest increases in proteases, transporters, germinal cell proliferation factors and neuropeptide or neural development markers in niclosamide-exposed

in vivo sporocysts all serve to further highlight the fact that the 24 h niclosamide exposure we used was certainly not lethal to the sporocysts nor did it seem to significantly curtail their transcriptional production or to invoke transcripts associated either with enhanced efflux or processing of niclosamide or with apoptosis or autolysis of sporocysts. Of course, more extensive exposure of *B. pfeifferi* to niclosamide with attendant loss of the integrity of the snail metabolome would inevitably result in death of *S. mansoni* sporocysts as well.

Shared response of two *Biomphalaria* species to a sublethal dose of niclosamide

Of the 30,647 probe features on the *B. glabrata* microarray used by Zhang et al. (2015), 16,713 (55%) were homologous to a *B. pfeifferi* transcript (Blastn E-value <1e-10, percent identity >75%). Microarray features with homologs to *B. pfeifferi* transcripts and that were differentially expressed in both Zhang et al. (2015) and the present study are shown in Table 1. These features represent a conservative view of genes characteristic of *Biomphalaria*'s response to sublethal niclosamide exposure. The entire differential expression analysis of *B. pfeifferi'*s response to niclosamide showed 895 transcripts up-regulated and 604 down-regulated when compared to uninfected control *B. pfeifferi.*

Table 1. All features shared between *B. glabrata (*Zhang et al., 2015) and *B. pfeifferi* that were significantly differentially expressed after exposed to 0.15mg/L niclosamide.

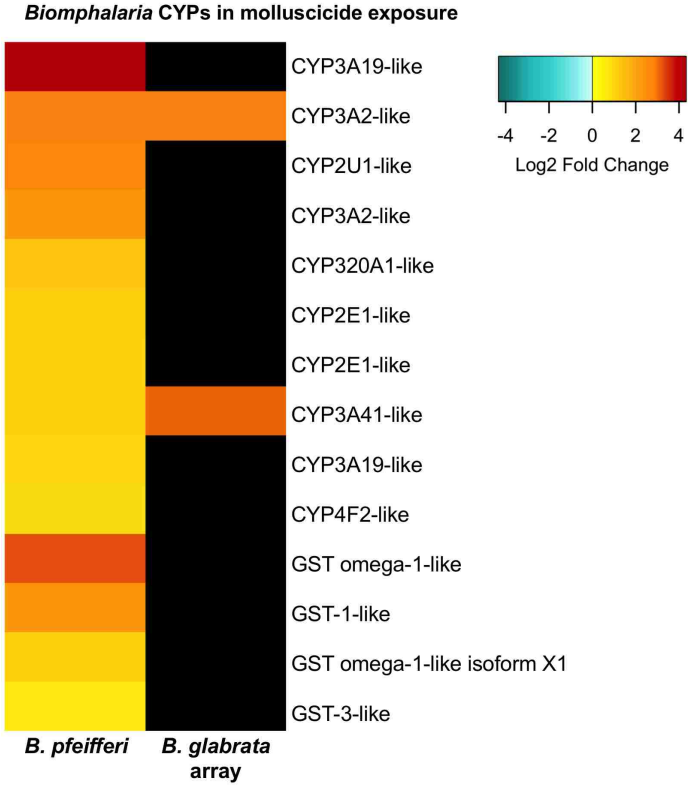
	B. *pfeifferi* Illumina transcript	**Log_2FC**	***B. glabrata* array feature**	**Log_2FC**
ADP-ribosylation factor 3-like	evgTRINITY_DN92963_c1_g2_i1	5.10	c13901	4.73
ADP-ribosylation factor 3-like	evgTRINITY_DN92963_c1_g1_i1	4.45	c13901	4.73
Solute carrier family 28 member 3-like	evgTRINITY_DN88027_c1_g1_i4	6.78	c27272	1.99
Multidrug resistance 1-like	evgTRINITY_BU_DN81217_c7_g4_i1	2.72	contig_14304	1.48
Multidrug resistance 1-like	evgTRINITY_DN90366_c3_g1_i2	5.26	contig_14304	1.48
HSP 12	evglcl|G0WVJSS02FHD9K	2.29	contig_7431	3.79
HSP 12	evglcl|G0WVJSS02JB97J	1.98	contig_7431	3.79
HSP 70	evgTRINITY_GG_25613_c6_g1_i1	1.09	BGC03909	3.64
Solute carrier family 28 member 3-like	evgTRINITY_DN88027_c1_g1_i3	3.79	c27272	1.99
Cytochrome p450	evgTRINITY_BU_DN81631_c8_g1_i1	1.05	c14547_rc	3.10
Cytochrome p450	evgTRINITY_DN93193_c20_g1_i1	2.88	c8814	2.88
Baculoviral IAP repeat-containing 3-like	evgTRINITY_BU_DN78979_c0_g1_i2	1.69	c17676_rc	2.14
Nuclear protein 1-like	evglcl|HJ4YRIA01D0DSV	1.28	contig_4627	2.20
Nuclear protein 1-like	evglcl|HJ4YRIA02HBZUN	1.01	contig_4627	2.20
Growth arrest and DNA damage-inducible alpha-like	evglcl|G0WVJSS02G7JUO	1.85	contig_8438	1.39
Alpha-crystallin B chain	evglcl|HJ4YRIA01ERORD	1.21	contig_2362_rc	1.79
Sequestosome-1-like	evgTRINITY_DN29609_c0_g1_i1	1.00	BGC02302	1.57
Glycogen-binding subunit 76A-like	evgTRINITY_DN70212_c1_g1_i1	0.92	c14016_rc	1.09
Methionine synthase reductase-like	evgTRINITY_DN77579_c0_g1_i1	0.71	c41473	1.00
Glutathione-independent glyoxalase hsp3103	evgTRINITY_DN92822_c15_g1_i1	-1.08	contig_3480	-1.10
Thymidine kinase, cytosolic-like	evgTRINITY_DN90310_c10_g1_i1	-1.29	contig_10981	-1.35
Uncharacterized	evgTRINITY_DN89789_c4_g2_i1	2.39	contig_12514_rc	3.19
Uncharacterized	evglcl|G0WVJSS01A5WAX	3.89	contig_6337_rc	4.16
Uncharacterized	evglcl|G0WVJSS01DEUAY	1.60	contig_3100	2.29
Uncharacterized	evgTRINITY_DN88565_c20_g1_i1	2.00	contig_3944_rc	1.38
Uncharacterized	evgTRINITY_DN22835_c0_g1_i1	1.33	BGC02491	1.02
Uncharacterized	evglcl|G0WVJSS01DKS66	1.36	c43865_rc	1.40
Uncharacterized	evglcl|G0WVJSS02ITT0P	0.82	contig_7634_rc	1.16
Uncharacterized	evgTRINITY_GG_16388_c0_g2_i1	0.86	c13164_rc	1.09
Uncharacterized	evgTRINITY_DN84827_c0_g2_i1	-0.81	c8798_rc	-1.13
Uncharacterized	evgTRINITY_DN93461_c7_g1_i1	-1.49	c1870	-1.80

As a lipophilic xenobiotic, niclosamide would likely be eliminated in animals by increasing its hydrophilicty (phase 1 reaction), conjugating the phase I product with a charged chemical group (phase 2 reaction), and then removing it with the aid of a transmembrane transporter (phase 3 reaction) (Parkinson et al. 2013). A key enzyme superfamily of heme-thiolate proteins responsible for initial phase I detoxification are the cytochrome p450s (CYPs). CYPs are found in all kingdoms of life and most commonly perform monooxygenase reactions adding one oxygen atom to the xenobiotic with the other oxygen atom reduced to water (Parkinson et al. 2013). Zhang et al. (2015) found that 9 of the features that were up-regulated ≥ 2-fold change following exposure to 0.15mg/L of niclosamide were CYPs. The *B. glabrata* genome has about 99 genes encoding heme-

thiolate detoxification enzymes with tissue-specific expression patterns suggesting that CYPs serve specific biological processes (Adema et al., 2017).

CYPs are also up-regulated in *B. pfeifferi* in response to niclosamide exposure, including two in common with *B. glabrata* (Table 1) and 8 more as noted in Figure 9A, underscoring the importance of CYP mixed function oxidases in the snail response to niclosamide. Of the CYPs up-regulated in both snail species, one is a homolog of Cp450 3A2-like found in mouse liver cell microsomes which is responsible for oxidizing steroids, fatty acids, and xenobiotics. The other shared CYP is CYP 3A41-like. It is also microsomal and studies of vertebrate homologs indicate that glucocorticoids may exert control of CYP3A41 gene expression (Sakuma et al. 2004). Modest down-regulation of one CYP in *B. glabrata* (CYP II f2) was also observed (Zhang et al. 2015) and we similarly noted down-regulation of a CYP (1-like isoform X1) in *B. pfeifferi.* This supports the suggestion by Zhang et al. (2015) that different members of the CYPs repertoire are likely to have different functions in *Biomphalaria* snails in response to diverse stimuli, including biotic challenges like *S. mansoni* or abiotic challenges like molluscicides.

Figure 9. *Biomphalaria pfeifferi* CYP and GST transcripts up-regulated in response to niclosamide. Data for *B. glabrata* from Zhang et al. (2015).



Phase 2 in the elimination of xenobiotics would likely include molecules like glutathione transferases that transfer charged chemical species like glutathione to the xenobiotic. Glutathione-S-transferase 7-like (GST) was up-regulated 5-fold following niclosamide exposure in *B. glabrata* (Zhang et al. 2015). In addition to CYPs, GST has also been shown to be up-regulated following niclosamide-based molluscicide exposure in *Oncomelania hupensis* (Zhao et al. 2015). GST was also represented in the *B. pfeifferi* Illumina DE transcripts with up-regulation of GST omega-1-like, and microsomal GST-1 and 3-like (Figure 9).

Transmembrane transporters complement the detoxification and conjugation reactions of phases 1 and 2 by eliminating the xenobiotic or toxin present in an organism (Parkinson et al. 2013). ATP-binding cassette (ABC) transporters, particularly ABC efflux transporters, play an important role in eliminating toxic compounds from cells. For instance, ABCG2, a non-specific, multi-xenobiotic transporter is known to be expressed at high levels in the gills and hemocytes of

Mytilus edulis (Ben Cheikh et al. 2017). One family of ABC efflux transporters, the multidrug resistance proteins (MRPs) act to eliminate drugs and toxic chemicals transporting anionic compounds detoxified in phases 1 and 2. One MRP-1 is expressed 2.8-fold higher than controls in *B. glabrata* (Zhang et al. 2015) and 10 MRP-1 transcripts were up-regulated in *B. pfeifferi* suggesting these transporters are removing toxic waste products produced directly by niclosamide or indirectly through cell death or tissue necrosis (Table 2).

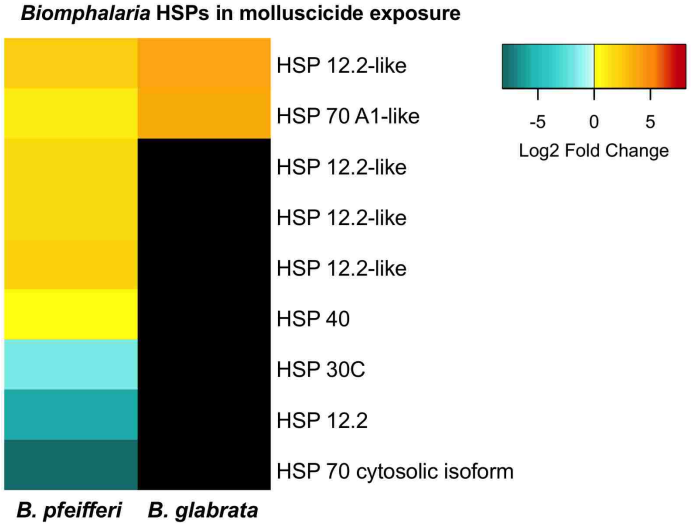
Table 2. *Biomphalaria pfeifferi* multidrug resistant protein 1-like isoforms up-regulated after exposure to sublethal niclosamide.

***B. pfeifferi* Transcript**	**Log_2 FC**
evgTRINITY_GG_18090_c5_g2_i1	7.8
evgTRINITY_BU_DN63065_c0_g1_i1	5.7
evgTRINITY_BU_DN81217_c7_g4_i3	5.7
evgTRINITY_DN90366_c3_g1_i2	5.3
evgTRINITY_DN90366_c3_g1_i1	5.0
evgTRINITY_BU_DN81217_c7_g4_i1	2.7
evgTRINITY_DN1870_c0_g1_i1	2.2
evgTRINITY_DN92909_c10_g1_i1	1.4
evgTRINITY_DN87672_c0_g1_i1	1.3
evgTRINITY_DN84897_c0_g1_i1	1.3

Heat shock proteins show increased expressed to a variety of stressors including elevated temperature, hypoxia, ischemia, heavy metals, radiation, calcium increase, glucose deprivation, various pollutants, drugs, and infections (Feder and Hofmann 1999). Up-regulation of HSPs has been associated with susceptibility of *B. glabrata* to *S. mansoni* (Ittiprasert et al. 2009; Zahoor et al. 2010). HSPs have also been identified in other molluscs as indicative of environmental stress. The disk abalone *Haliotis discus discus* up-regulates HSP 20 when exposed to extreme temperatures, changing salinity, heavy metals, and microbial infection (Wang et al. 2012). The marine bivalve, *Mytilus galloprovincialis* up-regulates HSPs 24.1, 70, 90, and sequestosome-1 following toxic metal exposure (Varotto et al. 2013). *Biomphalaria glabrata* mounts a multifaceted HSP response to niclosamide by up-regulating HSPs 12, 40, and 70

(Zhang et al. 2015) and the selective autophagosome cargo protein sequestosome-1. We also saw up-regulation of these specific HSPs but the more comprehensive sequencing available from the Illumina study revealed mixed responses of isoforms of HSP 12.2 and down-regulation of HSP 30 (Figure 10).

Figure 10. Expression of *B. pfeifferi* HSP transcripts and homologous *B. glabrata* HSP microarray probes in response to 0.15mg/L niclosamide. Data for *B. glabrata* from Zhang et al. (2015).



In response to exposure to *S. mansoni* infection, *B. pfeifferi* shows a more complex transcriptional expression of HSPs, cytochrome p450s, and glutathione-S-transferases than it does to molluscicide with no general up- or down-regulation of any group of these transcripts (Buddenborg et al. 2017). Biotic stressors such as parasites with intimate and prolonged contact with host tissues may induce a more complex stress response with up- and down-regulation of various HSPs in comparison to a general up-regulated of CYPs, glutathione-S-transferases, small and large molecular weight HSPs, and sequestosome noted in the response of several molluscs to abiotic stressors.

Additional responses of *Biomphalaria* to sublethal molluscicide exposure detected with Illumina RNA-Seq

Because of the unbiased sequencing available in Illumina RNA-Seq, we were able to acquire additional information on the transcriptomic responses of *B. pfeifferi* to niclosamide beyond that allowed by the *B. glabrata* microarray study. Transcripts involved in protection from oxidative damage, generalized pathogen defense and innate immunity, protease inhibitors and feeding behavior were all noted.

We observed high expression of several glutathione peroxidase transcripts, presumably associated with enhanced conversion of hydrogen peroxide to water. In cancerous colon cells, niclosamide increased cell death when used with a therapeutic drug through hydrogen peroxide production (Cerles et al. 2017), therefore, it is not inconceivable that niclosamide in snails is directly or indirectly involved in increasing hydrogen peroxide levels although there is thus far no direct evidence for this in exposed snails. Glutathione peroxidase has been shown to increase the general tolerance of cells to oxidative stress resulting from exposure to xenobiotics (Doroshow 1995).

Glutathione reductase, a critical oxidoreductase enzyme that catalyzes the reduction of glutathione disulfide to glutathione, surprisingly was down-regulated. As noted above, glutathione is a key ingredient needed in phase II conjugation mediated by the enzyme glutathione-S-transferase, which is up-regulated in *B. pfeifferi* following molluscicide exposure. An impaired ability to regenerate glutathione because of down-regulated glutathione reductase activity could then impair both the detoxification process and interfere with maintenance of redox balance by allowing hydrogen peroxide to accumulate.

One of the more striking responses of *B. pfeifferi* exposed to niclosamide was the high up-regulation of transcripts for several protease inhibitors including antitrypsin-like and serpins (serine protease inhibitors) and the down-regulation of metallo, cysteine, and serine proteases. In contrast, only one serine protease (chymotrypsin-like elastase family member 1) and a single aminopeptidase N-like transcript were up-regulated. Caspases are cysteine-dependent proteases that

play essential roles in programmed cell death (Kumar et al. 2007) and isoforms of caspase-2 and 3 were down-regulated in niclosamide-exposed *B. pfeifferi*. The down-regulation of protease activity may be part of a compensatory stress response made by the snail to minimize metabolic changes associated with niclosamide exposure that if left unchecked would lead to apoptosis and protein degradation.

Responses typically classified as innate immune responses because they occur following exposure to parasites like *S. man*soni were also noted in *B. pfeifferi* exposed only to niclosamide. One such transcript was homologous to CD109 antigen-like, a thioester-containing protein, which is highly enriched in plasma from both resistant and susceptible strains of *B. glabrata* containing miracidia transforming into mother sporocysts (Wu et al. 2017). We also noted up-regulation of a transcript identified as complement C1q-like protein that we have reported to be up-regulated in early *S. mansoni*-infected *B. pfeifferi* (Buddenborg et al. 2017). Fibrinogen-related proteins (FREPs) 1 and 2 were both up-regulated after niclosamide exposure; FREP2 was also up-regulated in *S. mansoni*-shedding *B. pfeifferi* (Buddenborg et al. 2017). Dermatopontin, a parasite-responsive gene frequently noted in studies of both *B. glabrata* and *B. pfeifferi*, was also up-regulated following niclosamide exposure.

A conspicuous response was the high up-regulation of over 100 diverse transcripts identified as LBP/BPI1 (lipopolysaccharide binding protein/bacterial permeability-increasing protein 1) in *B. pfeifferi* after exposure to niclosamide. LBP/BPI1 is an antimicrobial molecule found in the albumen gland of *B. glabrata* and egg masses (Hathaway et al. 2010). Silencing of LBP/BPI1 expression in *B. glabrata* resulted in significant reduction of egg-laying, and death of eggs attributable to oomycete infections, providing evidence that LBP/BPI is involved in parental immune protection of offspring (Baron et al. 2013).

Transcripts homologous to *B. glabrata* tyrosinases (Tyr) 1, 2, and 3, are also up-regulated in response to niclosamide. In early-stage pre-patent *S. mansoni* infections Tyr-1 is up-regulated, and Tyr-3 is down-regulated in *B. pfeifferi* harboring cercariae-producing sporocysts (Buddenborg et al., chapter 3).

Tyrosinases are involved in melanin synthesis and additionally might mark an early phase in initiation of castration by diverting tyrosine towards the production of melanin instead of dopamine in *S. mansoni*-infected *B. pfeifferi* (Buddenborg et al. 2017). Like LBP/BPI1, tyrosinase has also been isolated from *B. glabrata* egg masses and is presumed to provide an immunoprotective effect for developing embryos by contributing to the melanization of the egg membrane (Bai et al. 1997; Hathaway et al. 2010). The additional considerable effort by the snail to make two egg mass-associated proteins in response to niclosamide is baffling, but might represent a last-ditch attempt to produce offspring before death. Alternatively, perhaps this is best viewed as an example of relatively non-specific innate immune responses that can be invoked by exposure to an unusual stressor, even if it is of an abiotic nature. Another consideration is that it represents a response to the presence of bacteria in the snail that might appear due to impaired hemocyte function or possibly due to failure to contain the gut microbiome in its usual compartment.

Another unexpected response was the high up-regulation of myomodulin-like neuropeptide in niclosamide-treated *B. pfeifferi*. Myomodulins are neurotransmitters involved in regulating feeding behavior by controlling radula protractor muscles used for feeding (Jing et al. 2010) in *Lymnaea stagnalis* (Santama et al. 1994) and *Aplysia californica* (Proekt et al. 2005). Myomodulin is down-regulated in pre-patent *S. mansoni*-infected *B. glabrata* and this was implicated as possibly diminishing feeding efficiency in infected snails (Wang et al. 2017). Down-regulation of a *B. pfeifferi* feeding circuitry peptide was seen in early and patent *S. mansoni* infections (Buddenborg et al. 2017). The up-regulated myomodulin activity noted provides evidence that basic physiological activities such as feeding are altered after niclosamide exposure. The mussel *Mytilus edulis* shows a decreased rate of feeding after exposure to hydrophobic organic chemicals, organochlorine compounds, organophosphate and carbamate pesticides, and pyrethroids (Donkin et al. 1989; Donkin et al. 1997).

With respect to features down-regulated following niclosamide exposure, it would seem transcription and translation efficiency would be hindered as

evidenced by down-regulation of nearly a dozen ribosomal proteins, transcription factors, and mitogen-activated protein kinases (MAPKs). Of transcripts associated with stress responses, HSP 30 and HSP 70 cytosolic isoform were down-regulated along with an HSP 12 isoform. Neuroglobins are members of the hemoglobin superfamily of oxygen carriers, are expressed in the glial cells surrounding neurons and have been found in marine, freshwater, and terrestrial molluscs including the gastropods *Lymnaea stagnalis, Planorbis corneus, Aplysia californica, Helix pomatia* and *Cepaea nemoralis* (Dewilde et al. 2006). Although we did not observe down-regulation of the hemoglobin-encoding gene noted by Zhang et al. (2015) following exposure of *B. glabrata* to niclosamide, down-regulation of neuroglobin in niclosamide-exposed *B. pfeifferi* was observed. This could be associated with reduced availability of oxygen, at least for neural cells.

Significant down-regulation of a Cu-Zn SOD (-9.3 $\log_2$FC) in *B. pfeifferi* indicates that SODs have a more complex response to niclosamide than previously thought from the microarray study by Zhang et al. (2015). High expression of certain alleles of Cu-Zn SOD have been implicated in resistance of *B. glabrata* strain 13-16-R1 to *S. mansoni* (Goodall et al. 2004; Goodall et al. 2006; Blouin et al. 2013) so it is not unlikely that different Cu-Zn SODs show distinctive responses to other stressors like niclosamide. Calmodulins, ubiquitous calcium-dependent signaling proteins responsible for regulating the uptake, transport, and secretion of calcium in gastropod shell formation (Li et al. 2016; Feng et al. 2017), are expressed by *B. glabrata* in response to gram (-) and gram (+) bacteria, yeast (Deleury et al. 2012), and in *B. glabrata* snail plasma containing larval *S. mansoni*. Here, we saw down-regulation of calmodulin in *B. pfeifferi* exposed to the niclosamide, raising the possibility that calmodulin expression is more responsive to biotic challenges. Transcripts related to cell adhesion like spondins that are expressed in *Biomphalaria* hemocytes (Mitta et al. 2005) are also down-regulated.

Responses of *B. pfeifferi* with cercariae-producing *S. mansoni* infections to sublethal niclosamide treatment

As previously noted, snails exposed to the combined effects of the biological stressor *S. mansoni* and the abiotic stressor niclosamide were surprisingly responsive (Figure 1), exhibiting large numbers of uniquely up- and down-regulated features, with many of these only modest in the degree of their differential expression. Among the more notable responses were several features associated with managing cell death in damaged tissues (Table 3). The transmembrane transporter ABCA3 is associated with resistance to xenobiotics and engulfment during apoptosis (Paolini et al. 2016). The enzymes glutaredoxin-2-like and catalase-like are both involved in reduction of hydrogen peroxide that may be released during niclosamide-induced apoptosis. An increase in apoptosis could account for the up-regulation of lysosomal endopeptidases such as cathepsin-L-like. Two mitochondria-associated transcripts that also play a role in gluconeogenesis, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and glycerol-3-phosphate dehydrogenase-like (GPDH) were also up-regulated. GAPDH accumulates in mitochondria during apoptosis and induces pro-apoptotic mitochondrial membrane permeability (Deniaud et al. 2007). Niclosamide has been screened as a potential promoter of mitochondrial fragmentation by disrupting membrane potential, reducing ATP levels, and inducing apoptosis by caspase-3-activation in HeLa cells (Park et al. 2011).

Table 3. Transcripts up-regulated in response to dual stressors (*S. mansoni* infection and sublethal niclosamide exposure) identified for their potential role in responding to programmed cell death. Except where noted, functions were obtained from Entrez Gene at https://www.ncbi.nlm.nih.gov/gene and UniProtKB at www.uniprot.org/uniprot.

Transcript Description	Function
ABCA3 transmembrane transporter	Resistance to xenobiotics and engulfment during apoptosis
Growth arrest-specific protein 2-like	Cell cycle arrest; regulation of cell shape; may act as a cell death substrate for caspases
Glutaredoxin-2-like	Mitochondrial; response to hydrogen peroxide and regulation of apoptosis caused by oxidative stress
Calmodulin 2/4-like, 5, A-like	Can mediate the stress response calcium-dependent signaling that controls a variety of enzymes, ion channels, proteins, kinases, and phosphatases
Heparanase-like	Facilitates cell migration associated with metastasis, wound healing and inflammation
Catalase-like	Reduction of hydrogen peroxide
Caspase 3 and 8-like	TNF binding; endopeptidase activity involved in apoptosis
Tumor necrosis factor (TNF) and receptor	Induces cell death
Cathepsin-L-like	Lysosomal endopeptidase
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Induces pro-apoptotic mitochondrial membrane permeability (Deniaud et al. 2007)

Pattern recognition receptors (PRRs), key elements responsible for the recognition of pathogens, showed mixed responses. Four distinct PRR genes were up-regulated: peptidoglycan-recognition protein SC2-like, ficolin-like, FREP 2, and FREP 10. We have reported the up-regulation of FREP 2 in *S. mansoni*-infected *B. pfeifferi* (Buddenborg et al 2017) but here we see four additional isoforms of FREP 2 up-regulated. Toll-like receptors (TLRs) which are involved in recognizing pathogens and activating conserved innate immune signaling pathways (Kawai and Akira 2010), were conspicuously down-regulated (TLRs 3, 4, 5, 7, and 8). Additional transcripts that function in various aspects of innate immune responses and that were down-regulated are C3 PZP-like alpha-2-macroglobulin domain-containing protein 8, hemolymph trypsin inhibitor B-like, tyrosine-3-monooxygenase, DBH-like monooxygenase 2, and tyramine beta-hydroxylase-like.

As with snails exposed to niclosamide alone, once again a down-regulation of transcripts for ribosomal proteins was noted. Reduction in ribosome production can be considered a stress response because it is a rapid and effective response against misfolded proteins (Guerra-Moreno et al. 2015) but may simply be an indication of a downgrading of general condition. Other down-regulated transcripts show diverse functional activity. Several annexins, intracellular Ca^{2+} and phospholipid binding proteins are down-regulated showing the possible disruption of regulation of membrane organization, trafficking, and the regulation of Ca^{2+} concentrations within cells (Gerke and Moss 2002).

Unlike the general up-regulation of CYPs in *B. pfeifferi* exposed only to niclosamide, *B. pfeifferi* with dual stressors highly down-regulate several CYPs (microsomal CYPs 2J1-like, 2B4-like, 3A29-like, 26A1-like, and mitochondrial CYP12A2-like). Mitochondrial CYP12A2-like is known to metabolize a variety of insecticides and xenobiotics (Guzov et al. 1998). We cannot discount that contribution to the down-regulation of this particular CYP is a result of mitochondrial degradation caused, in part, by niclosamide as noted previously as well as the additional stress of a patent *S. mansoni* infection.

CONCLUDING REMARKS

This study provides a distinctive and detailed view of the nature of the response of field-derived *B. pfeifferi* to relevant stressors likely to be encountered in its environments, including infections with *S. mansoni*, just one of several digenetic trematodes known to commonly infect this snail in Africa (Brown 1994), and exposure to the commonly used molluscicide, niclosamide. It is important to gain additional detailed information regarding the effects of niclosamide on snails, particularly those that harbor schistosome infections. For example, do infected snails succumb more readily to treatment and if so, why? This particular aspect of molluscicide use has not been widely investigated.

In general, exposure to niclosamide alone resulted in the fewest responsive features in *B. pfeifferi* (1,711) followed by infection with *S. mansoni* (2,271) and then by the combination of niclosamide and *S. mansoni* (7,683). Snails in these

three groups all responded in very distinct ways, but in each case with more features up- than down-regulated. Sublethal exposure to a single xenobiotic provoked about 67% as large a transcriptomic response as was noted for snails shedding *S. mansoni* cercariae, snails that had probably been infected with the parasite for at least a month and harbored large numbers of daughter sporocysts. The fact that snails that received the combination of infection and niclosamide responded so much more vigorously with so many distinctive features suggests that they were under greater duress and that their responses in some sense preempted the responses of snails in the other two groups.

Exposure to niclosamide alone provoked up-regulation of several features associated with response to xenobiotics including cytochrome p450s, heat shock proteins, multidrug resistant transporters and glutathione-S-transferases, confirming many of the observations made by Zhang et al (2015) in a microarray study of *B. glabrata* exposed to sublethal doses of niclosamide. Several additional unique aspects of the response to niclosamide were also noted given the increased resolution provided by Illumina sequencing. We note that one of the effects of niclosamide on *B. pfeifferi* may be to contribute to redox imbalance because glutathione is being used by glutathione-S-transferases to conjugate xenobiotics but may not be sufficiently regenerated because of down-regulated activity of glutathione reductase.

Exposure of infected snails to niclosamide was noteworthy in revealing the involvement of several features not found to be responsive to either stressor alone. Although many of the uniquely expressed features did not respond dramatically, the ones that did were indicative of responses associated with apoptosis, reduced protein synthesis, reduced production of some CYPs and thus diminished detoxification ability, and diminished innate immune function. Accordingly, we hypothesize that the combination of stressors was likely overcoming the snail's ability to maintain homeostasis. The snail mounts a considerable transcriptomic response to the presence of cercariae-producing sporocysts (Buddenborg et al. 2017) and it is not hard to imagine that the energy demand placed on infected snails by continual production of cercariae takes an

additional toll. The mortality rate of *B. pfeifferi* infected with *S. mansoni* is significantly higher than that noted for unexposed control snails (Mutuku et al., 2014). The molluscicide-exposed infected snails selected for sequencing were alive when sampled, but the transcriptional profiles suggested they were not thriving. This is broadly in agreement with observations made to indicate that *B. sudanica* with *S. mansoni* infections succumb to sublethal niclosamide treatment at a higher rate than do uninfected controls (Sturrock, 1996). In other words, the combination of stressors used here exposed the limits of what these snails can do to maintain homeostasis.

Even though *S. mansoni* sporocysts within snails exposed to niclosamide expressed more transcripts than in untreated snails, there was little about the response to suggest they possessed any distinctive or large-scale ability to respond to a xenobiotic like niclosamide. Furthermore, the sporocyst response did not appear to be as indicative of a failure to maintain homeostasis as we noted for snails. This is in keeping with the general observation that the lethal dose of niclosamide for sporocysts is probably much higher than for snails (Sturrock, 1966). Although it is clear that both miracidia and cercariae are vulnerable to niclosamide (Ghandour and Webbe 1975; Tchounwou et al. 1992), this may be a reflection of their more aerobic metabolism and that they would be more fully exposed to the action of niclosamide as compared to sporocysts nested within the tissues of an infected snail. Although we did not observe a strong negative effect of molluscicide exposure on the transcriptomics responses of sporocysts, given the relatively unhealthy state of the snail that we detected, it would inevitably follow that the condition of the sporocysts would degenerate.

In conclusion, we noted remarkably distinctive transcriptomics responses for *B. pfeifferi* depending on the nature of the stressor treatment they received, and that the combination of niclosamide and *S. mansoni* infection imposed a level of stress on the snails that resulted in a massive response comprised of many features we had not observed previously. This study contributes to the growing list of molecular participants that may govern the outcomes of the intimate interrelationships between snails and schistosomes, and that may help us

understand how snail host biology might be targeted for disruption by molluscicidal chemicals.

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CONCLUSIONS

Intestinal schistosomiasis, caused by the parasitic helminth *Schistosoma mansoni*, is associated with high morbidity in typically chronic infections that can last decades (Chitsulo et al. 2000). National and international mass drug administration (MDA) programs using praziquantal as preventive chemotherapy have become a mainstay in the control of schistosomiasis. However, although the short-term benefits of these programs are apparent, their maintenance and effectiveness long-term remains uncertain (Olveda et al. 2016). MDA programs are unable to overcome limitations due to rapid reinfection, poor sanitation, lack of access to clean water, low drug coverage, possible zoonotic transmission, and the vast geographical coverage that must be maintained (Olveda et al. 2016; Ross et al. 2017). Schistosomiasis control is further complicated by the possibility of drug resistance and reduced susceptibility after multiple praziquantel treatments (Crellen et al. 2016). It is becoming apparent that an integrated approach to schistosomiasis control that includes targeting specific life cycle stages, including the obligate intermediate host snail, is required for sustainable control (Sokolow et al. 2016; Ross et al. 2017). Thus, we are obliged to better understand the primary snail intermediate host of schistosomiasis in Africa, *Biomphalaria pfeifferi*, to better understand the host-parasite relationship and inform on appropriate control measures. Furthermore, we believe it is necessary to study *B. pfeifferi* as it exists in the wild with natural *S. mansoni* infections so that we can study genetically diverse individuals with natural symbiont populations. This dissertation provides a unique in-depth view of *S. mansoni-B. pfeifferi* molecular associations by looking at transcript expression with and without parasitic infection and also with the addition of the chemical molluscicide, niclosamide.

The first study (Buddenborg et al. 2017), provided a *de novo* transcriptome that was comprehensively mined for immunological, reproductive, defense, and microbiome characteristics of *B. pfeifferi* during natural infections with *S. mansoni* at distinct parasite developmental stages (1d, 3d, and shedding). One of the

most striking discoveries was the extent of transcriptional variability between individuals, suggesting diverse immunological and defense responses of *B. pfeifferi* to *S. mansoni* in this highly compatible host-parasite model. The identification of several potential targets for CRISPR-Cas knock-out could lead to vital applications in the future of snail control. Processes of interest to basic parasitology were also noted including the up-regulation of inhibitory neuropeptides that could lead to parasitic castration. In addition to detecting typical microbiota and eukaryotes one would expect for a natural aquatic habitat, our sequencing approach also detected novel organismal and viral sequences that may be vital to the parasite's success in the snail or aiding the snail in survival during parasitism. The *B. pfeifferi* transcriptome data set represents the most comprehensive and thoroughly analyzed transcriptome for an African *Biomphalaria* snail responsible for >90% of the cases of intestinal schistosomiasis caused by *S. mansoni* in the world.

As a companion to the first study, the third chapter of this dissertation describes the transcriptional responses of *S. mansoni* during intramolluscan development using combined microarray analysis and Illumina RNA-Seq techniques. We provided the first view of the genes transcribed by larval schistosomes in their snail hosts. Larval schistosomes express ~66% of all known *S. mansoni* genes during intramolluscan development with the majority of these constitutively expressed and responsible for nutrient transport, basic metabolic processes, cell division, and protein and nucleic acid synthesis. Using the free-living platyhelminth planarian as a model, we characterized anti-bacterial factors, transcripts involved in neural development, neuropeptides and their receptors, and transcripts essential for germinal cell maintenance. Molecular evidence of the suppressive effects on larval schistosome development due to co-infection with another digeneans species (an amphistome) was reported.

Chapter 4 uncovered the transcriptional changes of *B. pfeifferi* and *S. mansoni* when exposed to a sublethal (0.15mg/L) dosage of the molluscicide, niclosamide. Niclosamide had surprisingly little effect on *S. mansoni* sporocysts with molecular evidence for cercarial production remaining steady or slightly

increasing in individuals. Exposure of uninfected *B. pfeifferi* to niclosamide elicited a strong transcriptional response with expression of transcripts corresponding to chemical detoxification including cytochrome p450s, heat shock proteins, and glutathione-S-transferases. *Biomphalaria pfeifferi* shedding *S. mansoni* and then also exposed to niclosamide showed transcriptional profiles associated with apparent physiological stress, including apoptosis and down-regulation of ribosomal and defense functions. The two stressors combined forced the snail into a distinctive state of decline that was not apparent with either stimulus alone. This information provides novel insight into the molecular impacts of niclosamide.

These studies represent the first in-depth dual transcriptional profiling of the widespread intermediate host *B. pfeifferi* naturally infected with *S. mansoni* from an active transmission focus in western Kenya. In addition to the increased understanding of this host-parasite relationship, it also provides essential biological details necessary for the future of integrated control measures integral for schistosomiasis elimination.

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