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Time series analysis of the transcriptional responses of *Biomphalaria glabrata* throughout the course of intramolluscan development of *Schistosoma mansoni* and *Echinostoma paraensei*

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Abstract

Successful colonization of a compatible snail host by a digenetic trematode miracidium initiates a complex, proliferative development program requiring weeks to reach culmination in the form of production of cercariae which, once started, may persist for the remainder of the life span of the infected snail. How are such proliferative and invasive parasites able to circumvent host defenses and establish chronic infections? Using a microarray designed to monitor the internal defense and stress-related responses of the freshwater snail *Biomphalaria glabrata*, we have undertaken a time course study to monitor snail responses following exposure to two different trematode species to which the snail is susceptible: the medically important *Schistosoma mansoni*, exemplifying sporocyst production in its larval development, or *Echinostoma paraensei*, representing an emphasis on rediae production in its larval development. We sampled eight time points (0.5, 1, 2, 4, 8, 16 and 32 days p.i.) that cover the period required for cercariae to be produced. Following exposure to *S. mansoni*, there was a preponderance of up-regulated over down-regulated array features through 2 days p.i. but by 4 days p.i. and thereafter, this pattern was strongly reversed. For *E. paraensei*, there was a preponderance of down-regulated array features over up-regulated features at even 0.5 days p.i., a

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pattern that persists throughout the course of infection except for 1 day p.i., when up-regulated array features slightly outnumbered down-regulated features. Examination of particular array features revealed several that were up-regulated by both parasites early in the course of infection and one, fibrinogen related protein 4 (FREP 4), that remained significantly elevated throughout the course of infection with either parasite, effectively serving as a marker of infection. Many defense-related transcripts were persistently down-regulated, including several fibrinogen-containing lectins and homologs of molecules best known from vertebrate phagocytic cells. Our results are consistent with earlier studies suggesting that both parasites are able to interfere with host defense responses, including a tendency for *E. paraensei* to do so more rapidly and strongly than *S. mansoni*. They further suggest mechanisms for how trematodes are able to establish the chronic infections necessary for their continued success.

Keywords

Schistosoma mansoni; *Echinostoma paraensei*; *Biomphalaria glabrata*; Microarray; Transcript expression; Host-parasite interactions; Immunity; Immunosuppression

1. Introduction

The associations between digenetic trematodes and their molluscan intermediate hosts are specific, complex and long-lasting. The digenean developmental program in molluscs often takes weeks to unfold and the production of cercaria that follows can be sustained for months or even years (Curtis, 2003). The molluscan host is partially or wholly castrated after infection (Sorensen and Minchella, 2001) and not uncommonly dies with an unresolved infection. Such associations pose several questions of fundamental interest for those interested in unraveling the mechanistic bases of host-parasite interactions. Furthermore, many digeneans are of medical or veterinary significance and by knowing more about their associations with molluscs, we may reveal mechanisms whereby their adverse affects can be controlled.

The digeneans *Schistosoma mansoni* and *Echinostoma paraensei* both successfully infect the Neotropical planorbid snail *Biomphalaria glabrata*. Experimental infections of laboratory-reared M-line *B. glabrata*, with either parasite, are successful at least 90% of the time. In the case of *S. mansoni* which exemplifies a “sporocyst only” mode of larval development, a miracidium initiates infection by penetrating the snail, often the head-foot. Within hours the miracidium casts off its miracidial plates and transforms into a mother sporocyst that grows, and by a clonal process of asexual reproduction, begins to produce elongate daughter sporocysts. Depending on the temperature but usually at about 18 days p.i., daughter sporocysts exit the expanded mother sporocyst and migrate to the host’s digestive gland/ovotestis complex where they settle, become amorphous in shape, and begin to produce either additional sporocysts or cercariae, again by a clonal, asexual reproductive process (Pan, 1965). Fully developed cercariae are usually released from daughter sporocysts at about 1 month post-exposure and are then shed from the infected snail’s body. Cercarial production can continue for several months, typically until the infected snail dies.

Echinostoma paraensei exemplifies the “sporocyst-rediae” mode of larval development. A miracidium of this species loses its ciliated plates as it penetrates the snail tegument (Ataev et al., 1997). Once in the snail the parasite, now referred to as a sporocyst, migrates via the snail’s circulatory system to the heart and by 2–3 days p.i. has attached to the ventricular wall. Here the sporocyst rapidly grows and produces mother rediae, one of which, at least in the isolate maintained in our laboratory, is produced before the others, at day 6 p.i.. Mother rediae then colonize other parts of the snail and produce daughter rediae which eventually colonize the host’s digestive gland/ovotestis where they commence production of cercariae. As with *S.*

mansoni, cercariae are first released at about 1 month p.i.. Although the overall length of the pre-patent period is comparable for both parasites, and development culminates with the production of cercariae which can then continue to be produced over many months, their developmental programs are quite distinct.

To establish such long-term infections, digeneans must persistently thwart the host's immune system. Two commonly invoked ideas are that the parasite is either undetected by the host immune response (Yoshino and Bayne, 1983; Hanelt et al., 2008), or the parasite is able to interfere with or suppress the host response in order to establish and maintain infection (Boswell and Bayne, 1986; Loker et al., 1986, 1992). Based on a comprehensive series of in vivo experiments, it was shown that a strain of *B. glabrata* resistant to *S. mansoni* infection, if first infected with *E. paraensei* sporocysts, would lose their resistance to *S. mansoni*, an outcome attributable to the strong interfering capacity of *E. paraensei* larvae (Lie et al., 1977; Lie and Heyneman, 1979). It was concluded that all trematodes studied by Lie and co-workers that developed in *B. glabrata* were able to interfere with this snail's defense responses, and interference was suggested as a mechanism of general significance for understanding the success of trematodes in infecting molluscs (Lie et al., 1982).

Interference by *E. paraensei* has been shown to target hemocytes which are a critical component of the snail defense response against trematode infection (Loker et al., 1989, 1992). *Echinostoma paraensei* sporocysts (Loker et al., 1989) as well as sporocyst excretory/secretory products interfere with phagocytosis of particles (Noda and Loker, 1989) and with hemocyte binding to, and encapsulation of, *E. paraensei* and *S. mansoni* sporocysts (Loker et al., 1992). Hemocytes in the presence of either sporocysts or daughter rediae of *E. paraensei* exhibited abnormal behavior (Adema et al., 1994).

Studies of other snail-trematode systems support the interference hypothesis. Humbert and Coustau (2001) noted that hemocytes from a strain of *B. glabrata* susceptible to *Echinostoma caproni* exhibited diminished adhesiveness and phagocytosis when exposed to parasite secretory/excretory products. Iakovleva et al. (2006) observed reduced rates of phagocytosis of hemocytes of *Littorina littorea* following exposure to the secretory products of echinostome and heterophyid rediae. A number of studies suggest that prior infection of a snail by one trematode can facilitate the snail's colonization by other trematodes (Appleton, 1983; Southgate et al., 1989; Abrous et al., 1998). At the transcriptional level, that *S. mansoni* can also have suppressive effects on host responses has also been shown by Knight et al. (2009) who noted that successful infection caused a down-regulation of defense-related transcripts.

Results from other parasite-invertebrate host model systems exhibiting prolonged parasite development times also suggest that parasite-mediated interference with host defenses occurs. This has been reported with *Plasmodium*-mosquito interactions (Boete et al., 2004), as well as with parasitoid development in arthropods. Parasitoids often possess symbiotic viruses that are responsible for the observed immunosuppression (Davies and Vinson, 1988; Labropoulou et al., 2008; Suderman et al., 2008). Parasite-induced immunosuppression has also been reported in human-trematode interactions (Duvaux-Miret et al., 1992; Cervi et al., 1996), and a number of other parasite infections of vertebrates and invertebrates (Sanad et al., 1991; Guimaraes et al., 1997; Zuniga et al., 2000).

Although effects consistent with trematode-mediated interference have been documented early in the course of infection, additional study is needed to determine how long a state of interference might be maintained by digeneans, given the length and complexity of their developmental patterns in snails. Further documentation of the actual molecular effects of interference on the snail is also needed. Also, although resistance to trematode infection has been associated with various mechanisms such as refractoriness to trematode interference

(Humbert and Coustau, 2001), or expression of high levels of certain immune mediators, (Mitta et al., 2005; Vergote et al., 2005; Bayne, 2009), the immunological mechanisms underlying resistance and the targets of parasite interference remain poorly known.

To help rectify these shortcomings, we report our most recent results using a *B. glabrata* oligo-based microarray focused on transcripts known to be involved in immune and stress responses. We have previously used this array to show that *B. glabrata* is capable of mounting diverse immune responses to different immune challenges, as assessed, 0.5 days p.i. (Adema et al., 2009). A number of immune features were both up- and down-regulated following exposure to either *S. mansoni* or *E. paraensei*, suggesting that neither parasite is able to initiate infection without alerting defense responses from the host. At this single, relatively early time point, a predominance of down-regulated features was noted in snails exposed to *E. paraensei*, whereas snails exposed to *S. mansoni* exhibited a strong predominance of up-regulated features (Adema et al., 2009).

In the present study we monitored the transcriptional profile of susceptible M line *B. glabrata* snails at several time points (0.5, 1, 2, 4, 8, 16 and 32 days) following exposure to *S. mansoni* or *E. paraensei*. We demonstrate that *S. mansoni* infection is associated with an early preponderance of up-regulated transcripts followed by a sharp trend towards a persistent predominance of down-regulated features throughout the course of development. Exposure to *E. paraensei* provoked a predominance of down-regulated features throughout the entire period of development. We also highlight particular immune features that merit additional study based on the transcriptional profiles observed.

2. Materials and methods

2.1. Live material and experimental treatments

M-line *B. glabrata* used in these studies can serve as an intermediate host for both *E. paraensei* and *S. mansoni* (PR-1 strain). Both snails and trematodes were maintained at the University of New Mexico as previously described, and for both trematodes, snails (4–8 mm diameter) were exposed individually to 15–20 miracidia per snail in the wells of a 24-well plate, in artificial spring water (Loker and Hertel, 1987). Size-matched snails were sham exposed to serve as controls. Snails from all groups were kept for 12 h in 24-well plates to remove conditions of the pre-extraction environment as a variable. After this period, whole body extraction of RNA was undertaken on individual snails at the appropriate sampling time (0.5, 1, 2, 4, 8, 16 and 32 days p.i.). For each group, three biological replicates consisting of five snails per group were used.

2.2. *Biomphalaria glabrata* oligonucleotide-based microarray

The oligoarray used in this study has been described previously (Adema et al., 2009). Briefly, it contains 1,152 features that were selected from a set of 4,382 unique sequences identified by cluster analysis of *B. glabrata* expressed sequence tags (ESTs) in GenBank (Blaxter, 2006, <http://www.nematodes.org/NeglectedGenomes/MOLLUSCA/wwwPartiGene.php>). This was combined with open reading frame ESTs (ORESTES) for which a preliminary analysis indicated that their expression was increased after bacterial challenge, suggesting they might play a role in host protection (Lockyer et al., 2007b; Hanelt et al., 2008). In addition, a number of parasite-specific transcripts were included on the array in order to confirm the presence of each trematode infection.

2.3. RNA isolation

After removal of the shell, whole body tissue of individual snails was homogenized in a 1.5 mL tube with a plastic pestle (Kimble/Kontes Glass Co., USA) and RNA was isolated using

500 µL Trizol (Invitrogen) according to the manufacturer's protocols. The RNA samples were treated with TURBO DNA-free® (Ambion) to remove residual DNA. RNA was quantified using a NanoDrop® ND-1000 spectrophotometer using ND-1000 3.3 software (NanoDrop Technologies). RNA integrity was evaluated using an Agilent 2100 Bioanalyzer with a RNA 6000 Nano LabChip® Kit (Agilent Technologies).

2.4. Generation of labeled cDNA probes and microarray hybridization

The protocol used for probe synthesis has been previously reported (Adema et al., 2009). Briefly, cDNAs were generated using 1 µg of *B. glabrata* total RNA in a modified mRNA amplification reaction using template-switching PCR (TS-PCR), according to Petalidis et al. (2003). A universal reference RNA (URR) sample was used for the normalization of gene expression data for all microarray experiments, details of this URR have been published previously (Adema et al., 2009). Total RNA samples were used to generate first strand cDNA; subsequently second-strand cDNA was amplified and labeled with Cy3-dCTP or Cy5-dCTP (GE Healthcare-Amersham) using the BioPrime® DNA Labeling System (Invitrogen). For all experiments, reference cDNA generated from the URR was labeled with Cy3 and cDNA of the experimental groups was labeled with Cy5. Cy3- and Cy5-labeled probes were purified separately using an AutoSeq™ G-50 Dye Terminator Removal Kit (GE Healthcare), and labeling efficiency was determined using a NanoDrop® ND-1000 spectrophotometer. Purified Cy3- and Cy5-labeled cDNAs were pooled and incubated with pre-hybridized arrays under lifterslips (22IX30-2-7059, Erie Scientific Company, USA), one for each of the two array fields on each slide. Hybridization was performed in slide hybridization chambers (Corning) at 45°C for 16–18 h. Three post-hybridization washes with increasing stringency were performed successively, ($2 \times \text{SSC}$, $0.1 \times$ sodium chloride sodium citrate (SSC)/0.1% SDS and $0.1 \times \text{SSC}$) for 5 min each at room temperature with agitation. Only the *B. glabrata* features on the array were considered in the analyses.

2.5. Microarray scanning and analyses

Microarray slides were scanned with a GenePix® 4000B scanner (Axon Instruments) with GenePix® Pro 6.0 (Axon Instruments) software using a modified protocol as previously described (Aragon et al., 2006). Scanning and analysis were carried out as previously reported (Adema et al., 2009). Transcripts expressed at the cut-off for significance of 5% false positive rate (FPR) and $\pm 1.0 \log_2$ in all experimental groups were considered to be differentially expressed. The experimental microarray data were deposited in the MIAME compliant GEO database (<http://www.ncbi.nlm.nih.gov/projects/geo/>) under accession number **GSE18705**.

2.6. Quantitative PCR (qPCR) analysis

Quantitative PCR (qPCR) was used to confirm the results observed using the microarray. Transcripts from each treatment group were selected and analyzed using cDNA generated from 1 µg of tRNA isolated from the same individual samples used to generate the array probes. For each target, three independent reactions were analyzed for each of the individual snails (15) pooled to make the three replicates used in the microarray probe synthesis. Primers used for quantitative reverse transcription (qRT)-PCR analysis were designed using Primer Express 2.0 software (Applied Biosystems). See Table 1 for primer sequences. qPCR analysis was carried out on a Sequence Detection System 7000 (Applied Biosystems) with SYBR Green PCR reagents (Applied Biosystems) using 18S rDNA as an internal reference. The temperature profile was: 1 cycle of 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 58°C for 1 min. Following each qRT-PCR reaction, dissociation curves were analyzed to determine the purity of each product and the specificity of the amplification.

3. Results

3.1. Transcriptional profiles throughout the course of *S. mansoni* development

Infection of M-line *B. glabrata* snails with *S. mansoni* was characterized first by a predominance of up-regulated array features which gave way to a persistent overall pattern of down-regulated features (Fig. 1). There was a steady increase in numbers of up-regulated transcripts early in the course of infection, from 47 (3.6% of the total array features) at 0.5 days p.i., to 86 (7.5% of the total array features) at 1 days p.i., to a maximum of 110 (9.5% of the total array features) at 2 days p.i.. At the latter time point, 20% of the up-regulated features had confirmed immune functions that have been previously described in other studies, 27% had putative immune functions that are inferred based on sequence homology, 39% were transcripts with unknown function, 10% were stress related only, and the remaining 4% had roles in cytoskeleton formation and cellular functioning. At 0.5 and 1 days p.i. there were very few down-regulated transcripts, 20 (1.7% of the total array features) and 19 (1.6% of the total array features), respectively, none with confirmed immune functions. Day 2 p.i. was not only associated with the highest number of up-regulated transcripts, but also represented a marked turning point in the infection because thereafter several transcripts began to be down-regulated. From day 4 until the last time point examined, the number of down-regulated transcripts exceeded the number of up-regulated transcripts. At 4 days p.i., the number of up-regulated transcripts fell to only 32 (2.8% of the total array features), whereas the maximum number, 116 (10% of the total array features), of down-regulated transcripts for any time point was recorded (Fig. 1). Of the latter, 16% had been up-regulated just 2 days prior and a total of 23% had likely immune functions. Importantly, day 2 is when *S. mansoni* target transcripts included on the array were first detected, indicating that the parasite had developed to a point where it comprised enough of the overall probe sample that it could be detected. As expected, given the proliferative nature of the infection, *S. mansoni*-specific transcripts were detected on the array at all subsequent time points.

Among the up-regulated transcripts of immune relevance noted at 2 days p.i. were fibrinogen related proteins (FREPs) 2, 4, 6 and 11, two C1q-like lectins, galectins 4 and 7, as well as superoxide dismutases (Cu-Zn SOD, SOD chaperone and SOD Mn) (Table 1). By 4 days p.i., almost all of these transcripts were no longer up-regulated and, in fact, some were down-regulated (FREP 11, multidrug resistance protein, dual oxidase). FREP 4 was the only immune-related molecule consistently up-regulated throughout the full course of *S. mansoni* development. By 8 days p.i. only three transcripts were up-regulated and although this number increased slightly at 16 and 32 days p.i., none had readily ascribable defense function (Table 2).

This is in contrast to features with a decreased expression profile: from 2 through 32 days p.i., a number of fibrinogen domain-containing lectins were down-regulated including FREPs 1, 5, 7, 10, 11, 12 and fibrinogen related molecule (FReM). In addition, other immune-relevant transcripts such as macrophage mannose receptor, galectin 9, as well as two transcripts resembling epidermal growth factor (EGF) domain-containing proteins were down-regulated relative to controls (Table 2).

3.2. Transcriptional profiles throughout the course of *E. paraensei* infection in M line snails

The entire course of *E. paraensei* infection was characterized by a strong predominance of down-regulated snail transcripts (Fig. 2). Down-regulation was noted at the earliest time point (0.5 days p.i.) whereas, by contrast, *S. mansoni*-induced down-regulation did not occur until after 2 days p.i.. Days 0.5 and 1 had the most up-regulated transcripts, 33 (2.9% of the total array features) and 43 (3.7% of the total array features), respectively. At all time points after 1 day p.i., fewer than 25 total up-regulated transcripts were noted, whereas down-regulated

transcripts consistently exceeded 60 (Fig. 2). On 0.5 days p.i. 32% of the down-regulated transcripts were associated with immune functions, a percentage that dropped to between 15 and 20% from 2 days p.i. until the end of the study.

Similar to the *S. mansoni* infection profile, *E. paraensei*-infected snails up-regulated a number of immune-related transcripts during the initial phase of infection. By 1 day p.i. a number of FREPs (FREP 4, 10, 11 and 13) were up-regulated with FREP 13 displaying a 134-fold increase in expression. Together with FREPs, antimicrobial molecules such as aplysianin-A homolog, lipopolysaccharide (LPS) binding proteins, two C1q-like lectins, Cu-Zn SOD and FReM were also up-regulated. Although many remained up-regulated at 2 days p.i., the total number of up-regulated transcripts sharply decreased from 43 (3.7% of the total array features) on day 1, to 24 (2.1% of the total array features) at 2 days p.i.. In the interval from 1 to 8 days p.i., FREP 13 declined from a 134-fold increase to control levels (Table 3).

The pronounced shift from a pattern of increasing up-regulated transcripts up to 2 days p.i. to one dominated thereafter by down-regulation seen during *S. mansoni* infection was not observed following *E. paraensei* infection. Rather, only a modest up-regulation of transcripts was observed that was over by 1 day p.i.. Furthermore, several defense-related transcripts were down-regulated early in infection, including FREPs 8 and 9, dermatopontin, galectin 4 and 7, lysozymes, serpins, protein kinases and phosphatases. After 4 days p.i. the down-regulated transcript profile changed slightly and emphasized FREPs 5, 10 and 3, and molecules likely involved in phagocyte functions such as macrophage migration inhibition factor, macrophage mannose receptor, alpha-2-macroglobulin receptor and mitogen activated protein (MAP) kinases, and lectins such as plectin-1 (Table 3).

3.4. Comparison of *S. mansoni* and *E. paraensei* infection profiles

As noted in the Venn diagrams and accompanying lists in Fig. 3, for all time points tested, infection with *S. mansoni* or *E. paraensei* elicited very different transcriptional profiles from *B. glabrata*. The early stages of infection had the most up-regulated transcripts shared between the two infections. FREP4 was up-regulated by both infections at all time points and was often recorded as the only commonly up-regulated transcript. The early stages of both infections shared relatively few down-regulated transcripts with only one being common at 0.5 day p.i. and none shared at 1 day p.i.. Starting on day 2 p.i., the number of down-regulated transcripts common to both infections increased. FREP 10 was commonly down-regulated from day 4 p.i. until the end of the study and was the only transcript that exhibited this consistent pattern in both infections. A number of other important immune transcripts including FREP1, fibrinogen related domain (FReD) – FREP2 like protein, cyclic AMP response element binding (CREB) protein, MAP kinase and macrophage mannose receptor were all commonly down-regulated at different stages during both infections.

3.4. qRT-PCR confirmation of microarray results

The fold-changes in transcript expression obtained using microarray analysis were confirmed with select transcripts using qRT-PCR. Transcripts were selected for qRT-PCR analysis to represent the diversity of the expression patterns observed using the microarray. Some transcripts exhibiting a fold increase in expression after challenge (BgLBP/BPI, Spondin, C1q-like lectin, Macrophage migration inhibition factor, and FREP4), some exhibiting a fold decrease in expression (BGC03601 and lysozyme), as well as one transcript that was differentially expressed after infection with *S. mansoni* and *E. paraensei* (Serpins B4), were selected to represent several possible expression patterns observed. In all cases the qRT-PCR analysis confirmed in general the pattern of the microarray results, with the qRT-PCR results exhibiting slightly lower fold changes in expression when compared with the microarray (Fig. 4).

4. Discussion

Infection of a mollusc by the larval stages of digenetic trematodes leads to an intimate, long-term association that fundamentally challenges the physical integrity of the host and severely compromises its biological fitness (Sandland et al., 2007). The initial infective stages (miracidia) are small relative to the host, yet once in the host's body, initiate a complex developmental program that features extensive growth, asexual reproduction and production of additional specialized larval stages, all of which are in intimate contact with host tissues. This process culminates in the production of cercariae that often continues unabated until the host's death. Under favorable temperature regimes it takes about 1 month before cercariae are produced. The massive proliferation of trematode larvae is one that commandeers nutrients and space, and not surprisingly causes widespread internal damage (De Jong-Brink, 1995) and often results in castration of the snail (Crews and Yoshino, 1989). Given the magnitude and duration of the challenge posed by digenean infection, it is of interest to learn how the snail host responds at various times post-exposure, and what this tells us about how digeneans can orchestrate such persistent and profound changes in their hosts.

In this study we observed the transcriptional profiles of *B. glabrata* snails infected with two relatively unrelated trematodes, *S. mansoni* and *E. paraensei*, at seven time points, a sampling regime that emphasizes the early stages of development when infections are first established (0.5 through 4 day p.i.), and that covers later stages of development when trematode larvae are proliferating (8 and 16 days p.i.), or when development culminates with the production of cercariae (32 days p.i.). Each trematode studied represents a prominent digenean family, each family with many species that infect a broad range of gastropod hosts. *Schistosoma mansoni* is a member of the Schistosomatidae which includes over 100 extant species and exemplifies larval development with two or more sporocyst generations. *Echinostoma paraensei*, of the large family Echinostomatidae, exemplifies the mode of larval development featuring a sporocyst generation followed by multiple rediae generations. The results of this study provide a model for what happens in many other trematode-mollusc interactions. Although there are circumstances in which *B. glabrata* can be naturally resistant to, or acquire resistance to, either parasite (Lie et al., 1982; Niemann and Lewis, 1990; Richards et al., 1992), our emphasis in this study is to understand the overall transcriptional response that occurs during successful infection, i.e. when the host is susceptible to infection.

The defense responses of *B. glabrata* to infection with *S. mansoni* have been the subject of considerable study (Bayne, 2009), and one prominent hypothesis has been that *S. mansoni* evades snail immune responses by host mimicry (Yoshino and Bayne, 1983), such that prominent responses to the parasite are not mounted. Another hypothesis, not necessarily in conflict with the concept of mimicry, is that *S. mansoni* is detected in the early stages of the infection but actively interferes with the snail's defense response, thus ensuring its continued success (Lie et al., 1976; Lie and Heyneman, 1977). Our results showed that infection with *S. mansoni* induced a strong up-regulation of a number of defense-related transcripts early in the infection (0.5–2 days p.i.), suggesting the snail can detect the presence of an intruder or the damage it causes. It could be argued that this early phase response is merely in response to tegumental damage resulting from the penetration activities of miracidia but this seems less likely because we previously showed that the snail's response to deliberate wounding of the tegument was relatively modest compared with the response noted following either injection of bacteria or penetration by miracidia (Adema et al., 2009). Furthermore, the responses to injection of bacteria and penetration by *S. mansoni* miracidia were qualitatively and quantitatively different, further implying that *B. glabrata* recognizes the presence of the trematode (Adema et al., 2009).

Among the up-regulated transcripts observed through 2 days p.i. were many immunologically relevant features, including several previously observed by others in similar conditions. Transcripts for the antimicrobial LPS-binding protein were observed to be up-regulated in this study and by Mitta et al. (2005) in isolated hemocytes after challenge with *S. mansoni*. These transcripts exhibited higher expression in hemocytes and albumin glands of snails resistant to *S. mansoni* infection compared with susceptible snails (Guillou et al., 2007a). Antimicrobial molecules routinely appear in other transcriptional studies as being up-regulated in trematode-infected snails (Mitta et al., 2005; Lockyer et al., 2007a). In addition, we and others have found a number of lectins up-regulated including FREPs (Hertel et al., 2005), galectins and sequences that resemble C1q-like lectins (Guillou et al., 2007a). In agreement with our results, studies monitoring *B. glabrata* responses to echinostomes other than *E. paraensei* also noted up-regulation of FREPs, EGF domain containing molecules, antimicrobial molecules such as aplysianin, and adhesion molecules such as dermatopontin (Bouchut et al., 2006a, 2006b, 2007).

In the present study, given that 95% of the snails exposed to *S. mansoni* that were set aside for separate observation eventually shed cercariae, it is clear the early up-regulated response we noted was unsuccessful in fighting off infection. It is possible that the strong early response was directed towards the ciliated plates shed by an *S. mansoni* miracidium as it transforms into a sporocyst. These plates are phagocytosed by snail hemocytes, even in *S. mansoni*-susceptible snails (Pan, 1996). Although vigorous hemocyte activity in the vicinity of a sporocyst could pose obvious dangers to it, it has been proposed that *S. mansoni* sporocysts produce a number of molecules that protect against hemocyte-mediated oxidative damage (Vermeire and Yoshino, 2007). Also, it is possible that the shed ciliated plates serve as a diversion for the sporocyst, which may be able to protect itself against a generalized reactive oxygen and nitrogen response, particularly at this early stage of the infection when the host is responding vigorously.

The initial up-regulation observed early following *S. mansoni* infection is abruptly replaced from 4 days p.i. onward by a sharp shift to a predominance of down-regulated features. This shift coincides with the detection of *S. mansoni*-specific transcripts on the array starting at day 2 p.i.. It is possible that prior to 4 days p.i. *S. mansoni* sporocysts produce factors that down-regulate the snail response but the trend is not yet systemically observable. A localized response may nonetheless enable the parasite to mitigate the snail's defense responses in its immediate vicinity. Thereafter, as the parasite grows, its down-regulatory capabilities would be expected to increase, with the result that the response is systemically manifested. Many of the down-regulated features noted from 4 days p.i. onward have presumptive defense functions. Molecules such as FREPs 1, 5, 7, 10, 11 and 12 (Zhang et al., 2008), as well as molecules associated with phagocytosis mannose receptor (Krarup et al., 2005) and gram-negative binding protein, peptidoglycan recognition protein (Zhang et al., 2007) and production of toxic compounds (dual oxidase, and peroxidase precursor) (Mitta et al., 2005), all exhibited down-regulation after day 4 p.i.. A number of transcripts (FReD, FREP1, 5, 11, 12, macrophage mannose receptor and serpin B4) were all persistently down-regulated after 2 days p.i.. This pattern of consistent down-regulation suggests that these transcripts may be critical for tipping the scales in favor of the snail (if up-regulated) or the parasite (if down-regulated). It continued until our last sampling point, at 32 days p.i., by which time *S. mansoni* infections have achieved patency (Pfluger, 1981). Our study is consistent with the idea that *S. mansoni* infection requires long-term down-regulation of the snail immune response for the infection to establish and reach patency. This hypothesis is congruent with evidence suggesting that *S. mansoni* has the capacity to interfere with *B. glabrata* defense responses (Lie et al., 1976) and that, at least in this model system, the interference capacity of *S. mansoni* appears slower to develop than that of *E. paraensei* (Loker and Adema, 1995).

In contrast with *S. mansoni*, from as early as 0.5 days p.i., infections with *E. paraensei* are characterized by persistent down-regulation of defense-related transcripts, including a number involved in phagocytosis: alpha-2-macroglobulin (Stroschein-Stevenson et al., 2006), macrophage mannose receptor (Krarup et al., 2005), macrophage migration inhibition factor (Onodera et al., 1997), profilin-2 (Pearson et al., 2003), and galectins-4 and-7 (Vasta et al., 1999). These transcripts involved in phagocytosis represent 47% of the confirmed immune-related transcripts down-regulated at this time point, and 18% of all transcripts that were down-regulated, at 0.5 days p.i.. These data suggest that it is a remarkably early and effective reduction in the expression of transcripts relating to cell-based effector mechanisms that allow *E. paraensei* sporocysts to escape rapid attack by host hemocytes. Although some of these molecules remain down-regulated after 12 h, the profile of transcripts displaying decreased expression becomes broader after 1 day p.i. and no longer emphasizes transcripts encoding proteins involved in phagocytosis. The broad functional repertoire of transcripts negatively affected, even at the early stages, by *E. paraensei* infection suggests that *B. glabrata* physiology is profoundly altered by the presence of the parasite.

Echinostoma paraensei infection also resulted in up-regulation of immune transcripts that have been previously associated with infection by this trematode (Hertel et al., 2005). FREP 4 was consistently up-regulated throughout the entire infection reaching a peak of almost a 13-fold increase in expression at day 2 p.i.. Its increased regulation is so consistent, just as with *S. mansoni* infections, that it might be considered as a marker of infection. FREP 4 is known to bind parasite antigens (Zhang et al., 2008), and although further study of its functional relevance is underway, our results imply it is not a molecule that tips the balance in favor of the host and imparts resistance to it. Its presence may still benefit the snail, perhaps by absorbing soluble parasite products including toxins or wastes that would otherwise be detrimental. It is also conceivable that its up-regulation is parasite-mediated, favoring the development of the parasite in a way we do not yet understand. Another FREP, FREP 10, exhibits an initial up-regulation on days 1 and 2 p.i., but is down-regulated thereafter. This type of expression pattern suggests that FREP 10 may be actively down-regulated by the parasite. In addition to these two FREPs, serpins B4 and B6 (Mitta et al., 2005), LPS binding proteins (Guillou et al., 2007a), C1q-like lectins (Guillou et al., 2007a), dermatopontin (Bouchut et al., 2006a) and aplysianin (Bouchut et al., 2007) are all immune-related transcripts that have been demonstrated to be up-regulated after echinostome infection by studies independent of this one.

The transcription profiles evoked by the two parasites were surprisingly different, with relatively few up- or down-regulated transcripts common to both, suggesting each trematode elicits mostly different responses from the snail and potentially employs different mechanisms to interfere with snail defenses. The early stages of infection, which for both parasites showed a common trend towards up-regulated host transcripts, also had the highest number of commonly up-regulated transcripts, a number of which were immune related (FREPs, C1q-like proteins, EGF related proteins and profilin). This suggests the initial response of the snails may be more general, and is then replaced by a more trematode-specific response. For down-regulated transcripts, the highest numbers shared between the two infections appear after day 2 p.i., and include FREPs (FREPs 1, 5 and 10), FReM A, CREB binding protein, FReD – FREP 2-like protein, macrophage mannose receptor and MAP kinase. That infection with either trematode results in down-regulation of these transcripts suggests that they may play important roles in defense, although this awaits further functional characterization.

We have also monitored transcripts associated with stress responses which often accompany, are closely intertwined with, and can influence the outcome of, immune responses including those of snails (De Jong-Brink, 1995; Lockyer et al., 2008). *Schistosoma mansoni* infection induced up-regulation of a number of transcripts that are categorized by gene ontology (GO)

annotation as part of stress-related responses, including the heat-shock proteins 70 and 90 and histones H2 and H3. A number of other features categorized as “stress-related” were also up-regulated including myosin, dynein-associated molecules, a number of transcripts involved in cytoskeletal remodeling, hemocyanin, and transcripts involved in protection against oxidative stress such as dual oxidase (Ha et al., 2009) and Cu-Zn superoxide dismutase (Goodall et al., 2006). Many of these stress-related molecules have implied “secondary” immune functions. Heat shock protein 70 has been repeatedly identified as a molecule up-regulated as a result of parasite infection and in some instances has an immuno-stimulatory role (Oladiran and Belosevic, 2009; Wang et al., 2009). Ittiprasert et al. (2009) found that heat shock protein 70 expression was induced early in susceptible snails following challenge with *S. mansoni* miracidia. Histones have been shown to stimulate an immune response in many phyla including molluscs, via the production of anti-microbial proteins derived from the N-terminal end of the molecule (Li et al., 2007). Hemocyanin, a prominent copper-containing respiratory protein in many invertebrates, has been shown to be involved in invertebrate defense responses as a hemolytic factor dependent on hemocyanin concentration, pH, temperature and the presence of divalent cations (Guo et al., 2009; Zhang et al., 2009). The presence of hemocyanin in *B. glabrata* (Lieb et al., 2006) is particularly enigmatic since the snail is known to rely on iron-containing hemoglobin as its respiratory protein: perhaps the function of hemocyanin is more defense-related in this and other hemoglobin-containing planorbid snails.

Interestingly, infection by *E. paraensei* did not result in such a pronounced stress-like response; only hemocyanin and heat shock proteins 70 and 90 were up-regulated and a number were down-regulated including glutathione-S-transferase (Ouaissi et al., 2002) and transcripts involved in cytoskeletal remodeling such as intermediate filament protein IFB and kinesin-like protein KF-18A (Bhaskar et al., 2007). Given that several of these proteins could be associated with cell or organelle movement, it is possible their down-regulation is a further indication of this parasite’s interference with hemocyte function.

Unlike most studies of trematode–snail interactions which document events occurring within the first few days of infection, at the time that the parasite’s success or failure is typically determined, in this study we have followed the transcriptional profiles of *B. glabrata* throughout the period required for either *S. mansoni* or *E. paraensei* to achieve their full development culminating in the production of cercariae. In so doing, we gain perspective regarding strategies of parasite infectivity and how snails respond to the disastrous consequences of digenean infection. We argue that our results are essentially concordant with those obtained by Lie, Heyneman and colleagues (Lie et al., 1982) in their comprehensive in vivo studies using the same snail and both the same and additional trematode species, in which they documented the “interference effect”. They did not specify a mechanism for interference and we hypothesize that induction of both short- and long-term down-regulation of host immune transcripts is a likely way in which it is achieved. They noted that interference could be mediated by “secondary trematode larvae”, meaning daughter sporocysts in the case of *S. mansoni*, or mother or daughter rediae in the case of echinostomes, and the persistence of down-regulated responses we observed throughout the course of development is again consistent with their observation. Lie et al. (1982) also noted that protection resulting from interference is stronger in the immediate vicinity of the parasite, and we hypothesize there are likely two phases of interference that occur during the course of development of either parasite. The first is a localized suppression of immune cells in the vicinity of the parasite that allows the parasite to establish and begin development. As noted by Guillou et al. (2007b) and Wu et al. (2009), the secretory/excretory products of schistosome and echinostome sporocysts contain a battery of potent factors to counter either host-mediated oxidative stress or phagocytic activities. Early sporocyst development is an especially critical time because the parasite’s biomass is small relative to the host, and our results suggest that the snail can recognize the presence of an intruder and begins to respond. For these reasons, particularly as noted for *S. mansoni*, we

recorded early in the course of infection a number of up-regulated transcripts but relatively few down-regulated transcripts were observable in the whole snail body extractions we used. By contrast, as the parasites grow and become more established, after 2 days p.i. we hypothesize a second and more pronounced systemic phase of interference occurs, one that allows the parasite to begin its proliferative phase of infection that culminates in production of cercaria. This phase is marked by a persistent down-regulation, even in whole body extracts. Although it could be argued that this persistent excess of down-regulated features is simply attributable to a general reduction of overall snail condition, this explanation does not fully explain the patterns observed. If this were the case, as the infections mature and parasite biomass steadily increases it might be expected that the number of down-regulated features would also regularly increase. There is no obvious steady increase in the number of down-regulated transcripts as the infections mature and high numbers of down-regulated transcripts were seen relatively early in the course of infection (e.g. 4 days p.i.). Furthermore, the majority of transcripts on the array did not show diminished expression, even in older infections. Thus it seems unlikely that the persistent preponderance of down-regulated transcripts is due to an overall decline in host condition.

Parasite-induced immunosuppression has been demonstrated in both vertebrate (Cervi et al., 1996) and invertebrate hosts (Loker et al., 1986; Boete et al., 2004) as a strategy to facilitate infection and persistence within a host. It seems particularly likely to occur in associations requiring long-term intimate contact between host and parasite. In addition to digenean infections in snails, the development of malaria parasites in mosquitoes (Boete et al., 2004), acanthocephalan larvae in gammarids (Rigaud and Moret, 2003), or parasitoid wasps in their insect hosts (Shelby and Webb, 1999) also reveal evidence of induction of immunosuppression. Bacteria such as *Wolbachia* (Fytrou et al., 2006) or *Photorhabdus* (Clarke, 2008) can also suppress invertebrate host responses, as can ectoparasites such as the Varroa mite in bees (Yang and Cox-Foster, 2005). Transcriptional profiling studies of parasitoids have noted some defense genes to be down-regulated (Barat-Houari et al., 2006), including some that are persistently down-regulated through the course of parasitoid development (Wertheim et al., 2005). Requiring much further work in the snail-trematode model are more specific studies of the mechanisms of immunosuppression, including whether it is initiated directly by a parasite-produced product or if it is mediated indirectly via host immune regulatory networks. Also requiring further study is the extent to which the snail host's response to other opportunistic pathogens is compromised. Although some studies suggest that interference is selective and the trematode-infected snail can respond normally to nematode infections (Lie et al., 1981), trematode-mediated suppression can also facilitate colonization by opportunistic digeneans (Appleton, 1983; Southgate et al., 1989; Abrous et al., 1998), a reality that helps explain why trematodes act via both direct and indirect means to help prevent the appearance of subsequent trematode infections (Lie, 1973; Sousa, 1993). The extent to which digenean infection predisposes snails to infection with bacteria or viruses or the extent to which the digenean itself might contribute to its host's defense are exciting topics awaiting further study. In the meantime, our study provides new insights into the molecular mechanisms employed by host and parasite in their ongoing struggle, and identifies a number of important transcripts that can now be characterized functionally for their roles in enhancing or suppressing the *B. glabrata* defense response.

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Fig. 1.

Graph showing the total number of up- (black) and down-regulated (white) transcripts in *Biomphalaria glabrata* after infection with *Schistosoma mansoni* for 32 days. Snails infected with *S. mansoni* were collected at 0.5, 1, 2, 4, 8, 16 and 32 days p.i. and analyzed using a *B. glabrata* microarray targeting immune and stress associated transcripts. Each time point represents data collected from 15 individual snails, pooled into three experimental replicates and analyzed using significance analysis of microarrays to determine statistical significance compared with uninfected controls.

Fig. 2.

Graph showing the total number of up- (black) and down-regulated (white) transcripts in *Biomphalaria glabrata* after infection with *Echinostoma paraensei* for 32 days. Snails infected with *E. paraensei* were collected at 0.5, 1, 2, 4, 8, 16 and 32 days p.i. and analyzed using a *B. glabrata* microarray targeting immune and stress associated transcripts. Each time point represents data collected from 15 individual snails, pooled into three experimental replicates and analyzed using significance analysis of microarrays to determine statistical significance compared with uninfected controls.

Fig. 3.

Comparison of the shared up- and down-regulated transcripts over the course of a *Biomphalaria glabrata* 32 day infection with *Schistosoma mansoni* or *Echinostoma paraensei*. Numbers encompassed by only one circle represent the number of up- or down-regulated transcripts in *B. glabrata* unique to each trematode infection (*S. mansoni* in grey, *E. paraensei* in white). Numbers encompassed by both circles represents the number of common transcripts up- or down-regulated by each trematode infection. To the right of each time point is a list of the shared transcripts separated by a line for each time point. FREP; fibrinogen related protein, FRM; fibrinogen related molecule, MAP; mitogen activated protein, CREB; cyclic AMP response element binding, FRD; fibrinogen related domain,

Fig. 4. Confirmation of microarray results using quantitative reverse transcription PCR (qRT-PCR). Transcript expression measured as fold change compared with control is shown for microarray and qRT-PCR analysis methods. Data depicted is from a selected time point for each experimental group. Bg LBP/BPI (2 days p.i.), Spondin (16 days p.i.), BGC03601 (8 days p.i.), Lysozyme (0.5 days p.i.), C1q-like lectin (0.5 days p.i.), Macrophage migration inhibition factor (2 days p.i.), FREP4 (1 days p.i.), Serpin B4 (16 days p.i. (*Schistosoma mansoni*), 1 days p.i. (*Echinostoma paraensei*)).

Table 1

List of primers used to confirm microarray results using quantitative reverse transcription PCR (qRT-PCR).

Primer name	Primer sequence
BgLBP/BPI-F	GTAGAGCGGCAAAGTGTCAC
BgLBP/BPI-R	CAATGCATCGTTTGTACTTGG
Spondin-F	CTGTGGTCAAGCCTGGCTACT
Spondin-R	TCAAAGATATGAAGGATGAAATAACAAG
BGCO3601-F	GGATGATTTAACAATGACAGACATGA
BGCO3601-R	AGACTATGTAAGTGCAAAGTCAATTCT
Lysozyme-F	ATATTATCATTTACTTTCTGCTTTGAATTGTAT
Lysozyme-R	CAAGTTATACTGTCCACGTTTCCA
C1q-like-F	CTGTAATCCTGAAACTGGCTAAAGAA
C1q-like-R	TCCGCGGTTTGACAG
Macrophage mannose receptor F	TCTGAGCCTCGTCCCAAGATTTGT
Macrophage mannose receptor F	TGTCAATCTGGAGGCTACGCCTAT
F4Q3-F	TGATTCGCCGAATGATAATTGT
F4Q3-R	CGTTAAGGTTGACATCAGCACAGT
Serpin B4 F	TTCTACTGAGACGCCGGTCTTTGA
Serpin B4 R	GGACGTTTCACTACGAAGTCCTCT

List of up- and down-regulated defense-associated transcripts after infection with *Schistosoma mansoni* for 32 days. The transcript name is listed, together with the fold change in expression compared with the control for each time point at which significant expression was detected. The function of each transcript as it might pertain to *Biomphalaria glabrata* defense is included together with references.

Table 2

<i>S. mansoni</i>	Time points (days)							Function
	0.5	1	2	4	8	16	32	
Up-regulated								
Clq-like lectin	3.9		2.2					Lectin domain containing molecule that resembles Clq domain. Has been shown to be involved in immune processes in the leech and scallop (Zhang et al., 2009a).
Dual oxidase			3.3					Member of the NADPH oxidase family that is responsible for reactive oxygen species production under various stimuli (Ha et al., 2009).
EGF related protein	4.3	1.4		6.3				Regulates processes of cell proliferation, survival and plays an important role in wound healing and tissue remodeling (Schneider and Wolf, 2009).
FREP 11			2.2					Lectin consisting of a single IgSF domain and fibrinogen domain. Evidence suggests involvement in host defense against parasite infection (Zhang and Loker, 2004b).
FREP 2	1.7	7.7	3.9	1.4				Lectin consisting of a single IgSF domain and fibrinogen domain. Evidence suggests involvement in host defense against parasite infection (Zhang and Loker, 2004b).
FREP 3		2.9	2.4					Lectin consisting of two IgSF domains and a fibrinogen domain. Likely involved in host defense (Zhang and Loker, 2003).
FREP 4	1.7	10.1	2.6	1.5	5	6.9	6.8	Lectin consisting of a single IgSF domain and fibrinogen domain. Evidence suggests involvement in host defense against parasite infection (Zhang et al., 2008c).
FREP 6	1.2	1.9	2.5	3.4				Lectin consisting of a single IgSF domain and fibrinogen domain. Evidence suggests involvement in host defense against parasite infection (Zhang et al., 2008b).
Galectin-4		9	5.7					Binds to beta-galactosides, it has been shown to be pro-inflammatory and have the ability to activate immune cells (Yoshino et al., 2008).
Galectin-7			4.5			4.1	4	Binds to beta-galactosides, it has been shown to be pro-inflammatory and have the ability to activate immune cells (Yoshino et al., 2008).
LPS binding protein	7.6	3.9	2.9					Anti-bacterial protein, increases permeability of bacteria cell walls (Gonzalez et al., 2007).
MAP kinase binding protein		1.9						Regulated MAP kinase signaling to control processes of cell proliferation, differentiation and activation (Sharma et al., 2005).
Multi-drug resistance protein		10.3						Resembles ATP-binding cassette (ABC) drug resistance transporters. These proteins are involved in drug resistance (Sheps et al., 2004).
Profilin-2			3.3					Involved in actin filament positioning and modelling it is an important molecule in phagocytosis (Janssen and Schleicher, 2001)
SOD Copper chaperone		1.8	2.9					Important for the SOD pathway of generating superoxide radicals (Marikovsky et al., 2003)

<i>S. mansoni</i>	Time points (days)							Function
	0.5	1	2	4	8	16	32	
SOD Cu-Zn	6.5		2.5	6.4				Enzyme involved in the production of superoxide radicals, especially from phagocytic/granulocytic cells (Goodall et al., 2004)
SOD Mn		3.5						Anti-oxidant enzyme for detoxifying free radicals inside mitochondria (Jung et al., 2005)
Down-regulated								
Dual oxidase		3.9	1.5					Member of the NADPH oxidase family that is responsible for reactive oxygen species production under various stimuli (Ha et al., 2009).
FReD		3.4	1.9	4.8	11.2	9.2		Fibrinogen domain containing protein (Zhang et al., 2008a).
FReD precursor			1.8	6.5	2.2			Precursor protein for fibrinogen domain containing protein (Zhang et al., 2008a).
FReM A		3.2	1.4					FREP-like molecule possessing two EGF domains which may be important for cell interactions or developmental processes (Zhang et al., 2008a).
FREP 1			2	1.7	1.3	1.6		Lectin consisting of a single IgSF domain and fibrinogen domain. Evidence suggests involvement in host defense against parasite infection (Zhang and Loker, 2003).
FREP 10		3.2	1.4					Lectin consisting of a single IgSF domain and fibrinogen domain. Evidence suggests involvement in host defense against parasite infection (Zhang and Loker, 2003).
FREP 11			1.7	3.4	1.6	8.4		Lectin consisting of a single IgSF domain and fibrinogen domain. Evidence suggests involvement in host defense against parasite infection (Zhang and Loker, 2004b).
FREP 12			1.8	1.5	2.3	2.9		Lectin consisting of a single IgSF domain and fibrinogen domain. Evidence suggests involvement in host defense against parasite infection (Zhang et al., 2008b).
FREP 5			1.8	2.9	7.8	12.4		Lectin consisting of two IgSF domains and a fibrinogen domain. Likely involved in host defense (Zhang and Loker, 2004a).
FREP 7			1.5					Lectin consisting of two IgSF domains and a fibrinogen domain. Likely involved in host defense (Zhang and Loker, 2004a).
Galectin-9				4.5	6.3			Binds to beta-galactosides, it has been shown to be pro-inflammatory and have the ability to activate immune cells (Yoshino et al., 2008)
Gram negative binding protein					3.3			Anti-bacterial protein thought to recognize LPS (Chen et al., 2006).
Macrophage mannose receptor		2.2	1.8	3.5	10.2			Recognize mannan sugar motifs on pathogen surfaces to initiate phagocytosis and activation response in phagocytes (Gruden-Movsesijan and Milosavljevic Lj, 2006)
MAP kinase 2			1.5	1.8		10.1		Involved in the p38 signaling pathway in Drosophila. This pathway is involved in cell responses to inflammatory and environmental stimuli (Zhuang et al., 2006).
Multi-drug resistance protein		3.3	1.5					Resembles ATP-binding cassette (ABC) drug resistance transporters. These proteins are involved in drug resistance (Sheps et al., 2004).
Multiple EGF-like domain protein			1.5					Involved in cell proliferation and survival (Sakamoto et al., 2005).
Peptidoglycan recognition protein-SC2			1.5					Recognition of peptidoglycan. May be important to protect against over-responding to bacterial infection (Bischoff et al., 2006).

<i>S. mansoni</i>	Time points (days)								Function
	0.5	1	2	4	8	16	32		
Peroxidase precursor			1.6	1.4					Enzyme involved in production of reactive oxygen species (Zelck and Von Janowsky, 2004)
Serpin B4					4.6	10.5	12.7		Promotes cell survival (Zou et al., 2009).

GenBank accession numbers for each transcript are available by accessing the GEO database GSE18705 associated with this array.

EGF; epidermal growth factor, FREP; fibrinogen related protein, LPS; lipopolysaccharide, MAP; mitogen activated protein, SOD; superoxide dismutase, FReD; fibrinogen related domain, FReM; fibrinogen related molecule.

Table 3

List of up- and down-regulated defense-associated transcripts after infection with *Echinostoma paraensei* for 32 days. The transcript name is listed, together with the fold change in expression compared with the control for each time point at which significant expression was detected. The function of each transcript as it might pertain to *Biomphalaria glabrata* defense is included together with references.

<i>E. paraensei</i>	Time points (days)							Function
	0.5	1	2	4	8	16	32	
Up-regulated								
Aplysianin-A precursor		3.5						Anti-microbial protein that inhibits growth of gram-positive and negative bacteria and is upregulated after trematode infection (Takamatsu et al., 1995)
Clq-like lectin	2.9	2.4	1.5					Lectin domain containing molecule that resembles C1q domain. Has been shown to be involved in immune processes in the leech and scallop (Zhang et al., 2009a).
Dermatopontin		2.1						Protein involved in cell adhesion and shell matrix formation, possibly important in wound healing (Okamoto and Fujiwara, 2006)
EGF related protein	18.9							Regulates processes of cell proliferation, survival and plays an important role in wound healing and tissue remodeling (Schneider and Wolf, 2009)
FReM B			1.5					FREP-like molecule possessing two EGF domains which may be important for cell interactions or developmental processes (Zhang et al., 2008a).
FREP 10		3.2	6.7					Lectin consisting of a single IgSF domain and fibrinogen domain. Evidence suggests involvement in host defense against parasite infection (Zhang and Loker, 2003).
FREP 11		5.4	2.9					Lectin consisting of a single IgSF domain and fibrinogen domain. Evidence suggests involvement in host defense against parasite infection (Zhang and Loker, 2004b).
FREP 13		134	37.6	6.7				Lectin consisting of two IgSF domains and a fibrinogen domain. Likely involved in host defense (Zhang and Loker, 2003).
FREP 4	3.3	2.4	12.6	6.3	5.7	4.4	9.8	Lectin consisting of a single IgSF domain and fibrinogen domain. Evidence suggests involvement in host defense against parasite infection (Zhang et al., 2008c).
Gram-negative binding protein		1.9						Anti-bacterial protein thought to recognize LPS (Chen et al., 2006).
LBP/BPI		42.1	7.5	4.1	3.8	3	4.7	Involved in response to bacteria, binds LPS (Gonzalez et al., 2007).
Serpin B4		3	3.4					Promotes cell survival (Zou et al., 2009)
Serpin B6		2.6	2.5					Regulates immune response by inhibiting function of proPO-activating proteinase-3 (PAP-3) (Zou and Jiang, 2005)
SMAD 4				3.5	10.4	12.9		Involved in TGF- β signaling pathway to induce proliferation and differentiation of cells (Li et al., 2007b).
SOD Cu-Zn		4.5	3.1		1.6	1.8		Enzyme involved in the production of superoxide radicals, especially from phagocytic/granulocytic cells (Goodall et al., 2004).

<i>E. parvovirus</i>	Time points (days)							Function
	0.5	1	2	4	8	16	32	
SOD Mn	4.5	6.5	1.4					Anti-oxidant enzyme for detoxifying free radicals inside mitochondria (Jung et al., 2005)
Down-regulated								
Alpha-2-macroglobulin receptor	2.2	2.1	1.4					Variety of functions, lipid metabolism, protease degradation, activation of lysosomal enzymes. Important molecule in phagocytic cells (Buresova et al., 2009).
Dermatopontin	2.4							Protein involved in cell adhesion and shell matrix formation, possibly important in wound healing (Okamoto and Fujiwara, 2006)
FRcD						2.9	3.1	Fibrinogen domain containing protein (Zhang et al., 2008a).
FRcM A		2.3	2.2					FRcP-like molecule possessing two EGF domains which may be important for cell interactions or developmental processes (Zhang et al., 2008a).
FRcM B	2.2							FRcP-like molecule possessing two EGF domains which may be important for cell interactions or developmental processes (Zhang et al., 2008a).
FRcP 1						2.8	1.9	Lectin consisting of a single IgSF domain and fibrinogen domain. Evidence suggests involvement in host defense against parasite infection (Zhang and Loker, 2003).
FRcP 10			2.1	1.6	1.5		5.5	Lectin consisting of a single IgSF domain and fibrinogen domain. Evidence suggests involvement in host defense against parasite infection (Zhang and Loker, 2003).
FRcP 3		3.2	16.9	5.3				Lectin consisting of two IgSF domains and a fibrinogen domain. Likely involved in host defense (Zhang and Loker, 2003).
FRcP 5			3.8					Lectin consisting of two IgSF domains and a fibrinogen domain. Likely involved in host defense (Zhang and Loker, 2004a).
FRcP 8	2.6	14.6						Lectin consisting of a single IgSF domain and fibrinogen domain. Evidence suggests involvement in host defense against parasite infection (Zhang and Loker, 2004a).
FRcP 9	2.2							Lectin consisting of a single IgSF domain and fibrinogen domain. Evidence suggests involvement in host defense against parasite infection (Zhang and Loker, 2004a).
Galectin-4	2.4							Binds to beta-galactosides, it has been shown to be pro-inflammatory and have the ability to activate immune cells (Yoshino et al., 2008)
Galectin-7	2.4							Binds to beta-galactosides, it has been shown to be pro-inflammatory and have the ability to activate immune cells (Yoshino et al., 2008)
LPS binding protein	2.2					1.5		Anti-bacterial protein, increases permeability of bacteria cell walls (Gonzalez et al., 2007).
Lysozyme	2.2							Bacteriolytic activity (Zavalova et al., 2006)
Macrophage mannose receptor	3.3	4.3	2.5	2.9	2.7	2.1		Recognize mannan sugar motifs on pathogen surfaces to initiate phagocytosis and activation response in phagocytes (Gruden-Movsesjan and Milosavljevic Lj, 2006)
Macrophage migration inhibition factor		2.7	2.8	3.6				Involved in the inflammatory and immune responses, including processes of phagocytosis, spreading and tumour suppression.

<i>E. parvensei</i>	Time points (days)							Function
	0.5	1	2	4	8	16	32	
MAP kinase 2							1.6	Typically found expressed in many cell types (Nishihira et al., 2003).
MAP kinase binding protein	2.3	1.7	1.4	4				Involved in the p38 signaling pathway in Drosophila. This pathway is involved in cell responses to inflammatory and environmental stimuli (Zhuang et al., 2006).
Multi-drug resistance protein			7.7	5.4	1.6	4.3		Regulated MAP kinase signaling to control processes of cell proliferation, differentiation and activation (Sharma et al., 2005).
								Resembles ATP-binding cassette (ABC) drug resistance transporters. These proteins are involved in drug resistance (Sheps et al., 2004).
Peptidoglycan recognition protein-SC2	2.2							Recognition of peptidoglycan. May be important to protect against over-responding to bacterial infection (Bischoff et al., 2006).
Peroxioredoxin	2.2							Reduce hydrogen peroxide, organic peroxides and peroxynitrite. Involved in reducing oxidative stress, and modulating signaling cascade involving nitric oxide(Knight et al., 2009).
Profilin-2	2.2							Involved in actin filament positioning and modelling it is an important molecule in phagocytosis (Janssen and Schlercher, 2001)
SOD Mn	2.2							Anti-oxidant enzyme for detoxifying free radicals inside mitochondria (Jung et al. 2005)
TGF-beta type 1 receptor-like	10.5	9.5	8.8	3.3	4.9	20.1		Likely involved in binding a TGF-beta-like molecule, possibly induces cell proliferation and differentiation

GenBank accession numbers for each transcript are available by accessing the GEO database GSE18705 associated with this array.

EGF; epidermal growth factor, FReM; fibrinogen related molecule, FREP; fibrinogen related protein, LPS; lipopolysaccharide, SOD; superoxide dismutase, FReD; fibrinogen related domain.