Published in final edited form as:
Mol Immunol. 2010 January ; 47(4): 849. doi:10.1016/j.molimm.2009.10.019.

Differential transcriptomic responses of *Biomphalaria glabrata* (Gastropoda, Mollusca) to bacteria and metazoan parasites, *Schistosoma mansoni* and *Echinostoma paraensei* (Digenea, Platyhelminthes)

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Abstract

A 70-mer oligonucleotide-based microarray (1152 features) that emphasizes stress and immune responses factors was constructed to study transcriptomic responses of the snail *Biomphalaria glabrata* to different immune challenges. In addition to sequences with relevant putative ID and Gene Ontology (GO) annotation, the array features non-immune factors and unknown *B. glabrata* ESTs for functional gene discovery. The transcription profiles of *B. glabrata* (3 biological replicates, each a pool of 5 snails) were recorded at 12 hours post wounding, exposure to Gram negative or Gram positive bacteria (*Escherichia coli* and *Micrococcus luteus*, respectively), or infection with compatible trematode parasites (*S. mansoni* or *E. paraensei*, 20 miracidia/snail), relative to controls, using universal reference RNA. The data were subjected to Significance Analysis for Microarrays (SAM), with a false positive rate (FPR) ≤10%. Wounding yielded a modest differential expression profile (27 up/21 down) with affected features mostly dissimilar from other treatments. Partially overlapping, yet distinct expression profiles were recorded from snails challenged with *E. coli* (83 up/20 down) or *M. luteus* (120 up/42 down), mostly showing up-regulation of defense and stress-related features. Significantly altered expression of selected immune features indicates that *B. glabrata* detects and responds differently to compatible trematodes. *Echinostoma paraensei* infection was associated mostly with down regulation of many (immune-) transcripts (42 up/68 down), whereas *S. mansoni* exposure yielded a preponderance of up-regulated features (140 up/23 down), with only few known immune genes affected. These observations may reflect the divergent strategies developed by trematodes during their evolution as specialized pathogens of snails to negate host

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defense responses. Clearly, the immune defenses of *B. glabrata* distinguish and respond differently to various immune challenges.

Keywords

oligonucleotide microarray; immune challenges; comparative immunology; lophotrochozoan

Introduction

The planorbid snail *Biomphalaria glabrata* is an important intermediate host for the larval stages of the digenetic trematode *Schistosoma mansoni*, a parasite that infects nearly 100 million people in Africa and the neotropics (Morgan et al., 2001). In addition to *S. mansoni*, *B. glabrata* can be infected by pathogens including other trematode parasites such as *Echinostoma paraensei* (Loker et al., 1987), bacteria (Bean-Knudsen et al., 1988), and likely viruses (Rondelaud and Barthe, 1992). Commonly, *B. glabrata* counters pathogens with an effective immune response that involves soluble components and cell-mediated cytotoxicity (Adema et al., 1997; Bayne, 2009; Hahn et al., 2001). In case of infection by *S. mansoni* or *E. paraensei*, however, the snail immune response may fail to clear the infection, due in part to trematode-mediated avoidance or inhibition of snail defense mechanisms (Coustau and Yoshino, 1994; Douglas et al., 1993; Lie and Heyneman, 1977; Loker et al., 1986; Loker and Hertel, 1987; Noda and Loker, 1989a,b; Roger et al., 2008).

Current understanding of the immunity of *B. glabrata* and other molluscs is incomplete (Bayne, 2009), yet a number of recent studies have identified molecules that may be relevant to their defense (Bouchut et al., 2007; Bouchut et al., 2006a; Bouchut et al., 2006b; Guillou et al., 2007; Hanelt et al., 2008; Knight et al., 2009; Lockyer et al., 2007a; Lockyer et al., 2007b; Mitta et al., 2005; Raghavan et al., 2003; Stout et al., 2009; Vergote et al., 2005). Little is known about the specific functions of many of these molecules, but we can nonetheless gain valuable information about the roles of these molecules by assessing the snail response as a whole, documenting transcriptional trends displayed in response to specific stimuli. Of particular interest is to determine the extent to which snails, as representative lophotrochozoans, can mount responses that are tailored to specific groups of pathogens, as suggested by previous comparative analysis of ESTs profiles of *B. glabrata* exposed to bacteria or parasites (Mitta et al., 2005; Hanelt et al., 2008). A comprehensive microarray-based approach can reveal whether exposure to infection elicits a “one size fits all” type of defense response, or whether snails mount different kinds of responses depending on the stimulus. This is particularly of interest when the infectious agents such as digenetic trematodes, that have an intimate evolutionary association with snails, initiate complex developmental programs that result in long-term infections that are overtly deleterious to their snail hosts.

This study employed an oligo-based microarray to survey transcriptional responses of *B. glabrata* to wounding, exposure to bacteria (Gram negative *Escherichia coli* or Gram positive *Micrococcus luteus*) and to digenetic trematodes (*S. mansoni*, and *E. paraensei*). The design of the oligo-based array was targeted in the sense that it emphasizes features involved in immune or stress-related responses. The study of transcriptomic responses of snails to parasitism and other environmental stimuli is currently in its infancy, only a single other report featuring a *B. glabrata* cDNA microarray, different from the one employed here, has been published (Lockyer et al., 2008). Studies of other invertebrate host-parasite associations indicate the great potential for this approach to reveal immunological mechanisms critical to host defense or parasite survival (Abraham et al., 2004; Dimopoulos et al., 2002; Srinivasan et al., 2004; Xu et al., 2005; Baton et al., 2009). This paper aims to present and validate the oligo-based array, and to exemplify its use by comparative analysis of the responses of *B.*

glabrata at a relatively early time point (12 hours) following the immune challenges noted above. The results indicate that *B. glabrata* snails mount different defense responses depending on the nature of the biological stimulus.

Materials and Methods

Live material and experimental treatments

The M line strain of the snail *Biomphalaria glabrata* used in these studies serves as intermediate host for both *Echinostoma paraensei* and *Schistosoma mansoni* (PR-1 strain). Both snails and trematodes were maintained at the University of New Mexico as previously described (Stibbs et al., 1979; Loker and Hertel, 1987). Seven groups of snails were used in this study. The first group consisted of un-manipulated snails (10–12mm shell diameter) that served as controls for snails of similar size in groups two through four. Snails in the second group were stab-wounded with a 27G hypodermic needle. Groups three and four were exposed to *Escherichia coli* or *Micrococcus luteus*, respectively, by injection in the headfoot with 50 μL of bacterial culture in LB medium (OD_{600} of 1.0 = 8×10^8 cells/ml) using a G27 hypodermic needle (Hanelt et al., 2008). These bacteria were selected because they are common in nature, their genomes have been characterized and they are frequently used as model infectious organisms in invertebrates (Hetru and Bulet, 1997). Groups five and six consisted of snails (4–8 mm) that were exposed to *E. paraensei* or *S. mansoni*, respectively. For both trematodes, snails were exposed individually to 15–20 miracidia per snail in the wells of a 24-well plate, in artificial spring water (ASW) (Loker and Hertel, 1987) for 12 hours. Size-matched snails (group seven) were sham exposed as controls. Snails from all groups were kept for 12 hours in 24-well plates before RNA was extracted from whole bodies of individual snails. For each group, three biological replicates consisting of pools of 5 snails were used.

Design and generation of a *B. glabrata* oligonucleotide-based microarray

The *B. glabrata* 70-mer oligoarray was designed at the Center for Evolutionary and Theoretical Immunology (CETI), University of New Mexico (UNM) and contains 1152 features. Target sequences were selected from the set of 4382 unique sequences identified by cluster analysis of *B. glabrata* ESTs in GeneBank (Blaxter, 2006, http://www.nematodes.org/NeglectedGenomes/MOLLUSCA/wwwPartiGene.php), combined with ORESTES many of which were recorded uniquely from *B. glabrata* after bacterial challenge (Hanelt et al., 2008; Lockyer et al., 2007b). The use as selection criteria of Gene Ontology terms related to immunity, stress response, phagocytosis, encapsulation, defense, lectin, lysosome, oxidant, radical, adhesion, apoptosis, cytoskeleton, kinase, and signal transduction yielded 557 targets for features on the array. An additional 502 features on the array represent novel sequences (i.e. no similarity to entries in GenBank databases) with unknown functions were incorporated with the goal of identifying new candidate factors important to the response of *B. glabrata*. Detection of coding regions using ESTscan v2.1 (Iseli et al., 1999) was used to infer 5'−3' directionality for unknown ESTs. The array also includes nuclear rDNA sequences and genes of the mitochondrial genome of *B. glabrata*. An additional 37 features represent mitochondrial and rDNA genes of *S. mansoni* and *E. paraensei*, as well as transcripts expressed by intramolluscan larvae of these parasites.

Within the derived population of ESTs, unique 70-mer oligos were selected with the bioinformatics tools Yoda (Nordberg, 2005) and OligoArray v2.1 (Rouillard et al., 2003). Inclusion of 10 alien sequences (SpotReport® Alien® cDNA Array Validation System, Stratagene) facilitates normalization of relative signals for different probes. Sense 70 mer-oligonucleotides were obtained from Integrated DNA Technologies (IDT) and printing and quality control testing were performed at the Hollings Marine Laboratory Genomics Core Facility (HML-GCF)/MUSC in Charleston, SC. The GenePix Array List (GAL) file and details

of the features on the array (feature ID, complete cDNA sequence, oligo sequence, BLAST similarity) are provided in supplementary table 1 and supplementary table 2. Note that many features were assigned putative identities based on BLAST results, pending validation by full experimental characterization.

For spotting on the microarray, the oligonucleotides (100μM in deionized water) were diluted into 50% water and 50% Epoxide Spotting Buffer (ESB, Integrated DNA Technologies) to a final concentration of 40 μM. Twenty microliters of each oligonucleotide was transferred to 384 well microarray plates (Genetix). Eight landing lights (consisting of 5 μM Cy5 labeled oligo, 5 μM Cy3 labeled oligo, 40 μM of unlabeled oligo (GCF3), and ESB) were printed on every subarray for orientation. A total of 300 Epoxide-coated glass slides (Corning Life Sciences) were spotted with two complete arrays, using the QArray Max (Genetix). The slides were dried for one hour at 80% humidity and overnight at 60% humidity. The testing of six slides across the printing batch revealed uniform shape and consistent presence of spotted features.

RNA isolation

After removal of the shell, whole body tissues of individual snails was homogenized in a 1.5 mL tube with a plastic pestle (Kimble/Kontes Glass Co.) in 500 μL Trizol (Invitrogen). The homogenate was extracted with 100 μL of chloroform and centrifuged (12,000 g, 15 min, 4° C). The aqueous phase was transferred to a new tube and the RNA was precipitated with 0.5 mL isopropanol, pelleted (12,000 g, 10 min, 4°C), washed (75% ethanol), air dried, and dissolved in 50 μL deionized water. The RNA samples were treated with TURBO DNA-FREE® (Ambion) to remove residual DNA, quantified spectrophotometrically, and evaluated using an Agilent 2100 Bioanalyzer.

Universal Reference RNA (URR)

A universal reference RNA (URR) sample was used for the normalization of gene expression data for all microarray experiments (Novoradovskaya et al., 2004). This URR was a mixture of RNA from control (non-infected) *B. glabrata* (95%), 1.25% from *S. mansoni* -infected *B. glabrata*, 1.25% from *E. paraensei*-infected *B. glabrata*, 1.25% from *E. coli*-exposed *B. glabrata* and 1.25% of *M. luteus*-exposed *B. glabrata*, in order to generate a positive signal for as many array features as possible.

Generation of labeled cDNA probes and microarray hybridization

cDNAs were generated from 1 μg of *B. glabrata* total RNA using a modified mRNA amplification reaction with template-switching PCR (Petalidis et al., 2003). RNA was mixed with 7 pg of each SpotReport Alien mRNA 1–10 (Stratagene), 20 pMol 3' SMART CDS primer IIA (5′-AAGCAGTGGTATCAACGCAGAGTACT$_{30}$VN-3′) and 20 pMol template switching primer [5′-d(AAGCAGTGGTATCAACGCAGAGTACGC)r(GGG)-3′] in a 12 μL volume, incubated at 72°C for 5 minutes, and placed on ice. Two μL of 10x ArrayScript™ buffer, 4 μL of 10mM dNTPs, 1 μL of RNase inhibitor (Applied Biosystems), 1 μL of ArrayScript™ Reverse transcriptase (Ambion) were added to generate first strand cDNA at 42°C for 2 hrs. Ten μL of the first-strand cDNA reaction was combined with 62 μL dH_2O, 10 μL of 10x PCR buffer II, 10 μL of 25 mM $MgCl_2$, 2 μL of 10 mM dNTPs, 4 μL of 10 μM 5' PCR primer (5'-AAGCAGTGGTATCAACGCAGAGT-3') and 2 μL of AmpliTaq® (40 U/ μL) (Applied Biosystems) for amplification of second-strand cDNA. Amplification conditions were 95°C for 1 min for one cycle, 95°C for 5s, 68°C for 6 mins for 15 cycles. Double stranded cDNA was purified (QIAquick® PCR Purification Kit, Qiagen) and quantified spectrophotometrically. For labeling (BioPrime® DNA Labeling System, Invitrogen) with Cy3-dCTP or Cy5-dCTP (GE Healthcare-Amersham), 200 ng of ds cDNA in 21 μL dH_2O was combined with 20 μL of 2.5x random primer reaction buffer, incubated at 95°C for 5 min and

placed on ice for addition of 5 μL low-C dNTP mix (5mM dATP, 5mM dGTP, 5mM dTTP, 2mM dCTP), 2 μL Cy3 or Cy5 dCTP (1 mM) and 1 μL Klenow enzyme (40U/ μL). The labeling reaction (37°C, 2 hrs) was stopped by adding 5 μL Stop Buffer. Labeled probes were purified separately (AutoSeq™ G-50 Dye Terminator Removal Kit, GE Healthcare). After spectrophotometric determination of labeling efficiency, the cDNA probes (Cy5-labeled expereimental, Cy3-labeled URR) were pooled, ethanol precipitated, resuspended in 43 μL hybridization solution (40% formamide, 5x SSC, 5x Denhardt's solution, 1mM sodium phosphate, 50 mM Tris (pH 7.4) and 0.1% SDS) and incubated at 95°C for 5 min and 50°C for 5 min. Arrays were pre-hybridized for 12 hours at 42°C in a rotating hybridization oven. Labeled probes were added to the microarrays under lifterslips (22IX30-2-7059, Erie Scientific Company), 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 hours. Three post hybridization washes were performed, (2x SSC, 0.1x SSC/0.1% SDS and 0.1x SSC, respectively) for 5 min each, at room temperature with agitation. Only the *B. glabrata* features on the array were considered in the analyses.

Microarray scanning and analyses

Microarray slides were recorded with a GenePix® 4000B scanner (Axon Instruments) with GenePix® Pro 6.0 (Axon Instruments) software using a modified protocol (Aragon et al., 2006). A preloaded *B. glabrata* grid was used to align and identify array spots. Alignment diameter limits ranged from 50% and 200%. Nearest negative control spots were selected for background subtraction. Using Acuity v4.0 (Axon Instruments), arrays were normalized using a ratio of medians. Signals were further normalized using the relative signal strength of SpotReport mRNA Alien sequence numbers 9 and 10. Microarray analysis was performed with raw expression data for each element, and signals expressed as a ratio of the experimental group (Cy5) to the URR (Cy3). The oligo-based array platform was validated by probing independent arrays with cDNA derived from each of the three biological replicate samples of unmanipulated *B. glabrata* (pools of 5 snails). The plotting of pair wise comparisons of the signals (ratio versus URR) recorded from all features for each biological replicate yielded graphs with signals falling closely along a 45° angle line, thus confirming functionality and reproducibility of the experimental methods (Fig. 1). Expression ratios obtained for features relative to size-matched control (unmanipulated) samples were then analyzed with Significance Analysis for Microarrays (SAM; Tusher et al., 2001) which utilized repeated permutations of the data to determine if the expressions of any genes are significantly related to the response. Transcripts expressed at the cutoff for significance of 10% false positive rate (FPR) and ± 1.0 $\log_2$ in all experimental groups were considered to be differentially expressed. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE16596 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE16596).

Quantitative PCR analysis

Quantitative PCR was used to validate the micro array results, employing cDNA templates that were generated from the same RNA samples as used to generate the array probes. For each target, three reactions were done for each of three biological replicate templates. Primers were designed with Primer Express 2.0 software (Applied Biosystems; Table 2) and used to perform qPCR reactions (SYBR Green PCR, Applied Biosystems) on a Sequence Detection System 7000 (Applied Biosystems), using *18S* rDNA as an internal reference. The temperature profile was: 1 cycle of 95°C for 10 minutes; 40 cycles of 95°C for 15 seconds; 58°C for 1 minute. Dissociation curves were generated to check specificity of each amplification reaction.

Results

Comparison of wounding to bacterial and trematode challenge

Snails were wounded to ascertain the response to integumental breach, without deliberate introduction of pathogens, relative to un-manipulated control snails. The wounding treatment also served as a control for the two groups of snails exposed to bacteria using the same means of breaching the tegument. Wounding alone evoked less transcriptional response than exposure to either bacteria or trematodes (Fig. 2 and Fig. 3), resulting in an increase in expression for 27 transcripts (48% unknowns), and a decrease for 21 (57% unknowns). Of the transcripts with increased expression levels, 20 were also up-regulated following bacterial and trematode challenges (Fig. 3a). Specifically, 17 were up-regulated following exposure with *E. coli*, 19 with *M. luteus*, one with *E. paraensei*, and two with *S. mansoni*. None of the up-regulated transcripts occurred in all treatment groups. Transcripts involved in cell proliferation, including CDC7-related kinase, SMAD 4 and TGF-beta receptor 1, were common between bacterial injection and wounding (Table 3). Sixteen of the 21 transcripts with reduced expression were unique to the wounding treatment (Fig. 3b). The remaining five (all unknowns) were all down-regulated following injection with *M. luteus*, whereas two were shared with *E. coli* injection, none with *E. paraensei* infection, and one with *S. mansoni* infection.

Profile of transcripts with increased expression after Gram positive or Gram negative bacterial challenge

The number of differentially expressed features in response to challenge with bacteria or trematode parasites is shown in Fig. 2 and Fig. 4. The proportion of unknown features amounted to 65% and 64% of the up-regulated transcripts and 65% and 62% of down regulated features for *E. coli and M. luteus*, respectively. Among the 52 transcripts that were elevated in response to both Gram negative and Gram positive bacteria are several factors that likely play a role in antibacterial immunity. These included MAP kinase 2, lipopolysaccharide binding protein/ bacterial permeability increasing protein (LBP/BPI), lysozyme and serpins B4 and B6 (Table 3).

Escherichia coli increased expression of another 31 *B. glabrata* transcripts, four of which responded similarly to *S. mansoni*, and another that was up-regulated following infections with either trematode species. These latter five transcripts have unknown functions. Among the remaining 26 transcripts are several with immune functions: JNK interacting protein, SMAD 4, C1q-like protein (a lectin), LPS binding protein, and Cu-Zn superoxide dismutase (SOD) (Table 3).

Among the 68 features that yielded increased signals in response to *M. luteus*, but not to *E. coli*, were immune-relevant sequences such as fibrinogen-related protein (FREP) 7, NFκB subunit p105 (transcription factor), a TGF receptor homolog, multi-drug resistance-associated protein, and two SH3 domain binding proteins. FREP 4 and another nine features were also responsive to trematode infection (Table 3).

Transcripts with decreased expression following bacterial challenge

Fewer features were down-regulated following bacterial exposure compared to those with elevated expression levels (Fig. 2). The assembly of down-regulated transcripts with immune relevance suggest that bacteria do not appreciably suppress snail immunity (Fig. 4b). Transcripts encoding for immune proteins, peptidoglycan recognition protein (PGRP) SC2, multi-drug resistance protein 2, ELP1 IKK complex-associated protein and alkaline phosphatase were decreased in the presence of *M. luteus*. No known immune-relevant transcripts exhibited decreased expression after injection with *E. coli*. (Table 3).

Transcriptional responses of *B. glabrata* 12 h post exposure to S. mansoni or E. paraensei

Comparison of the response profiles of *B. glabrata* to the two trematode species disclosed remarkable differences. *S. mansoni* infection resulted in more features being up- than down-regulated (140 and 23, respectively), whereas *E. paraensei* yielded the opposite pattern (42 up/68 down). Of the up-regulated transcripts; 57% and 11% were unknown sequences for *E. paraensei* and *S. mansoni*, respectively. Of the down-regulated transcripts 29% and 43% were unknown sequences for *E. paraensei* and *S. mansoni* respectively (Fig 2). *Schistosoma mansoni* induced increased expression of more transcripts than any of the treatments tested. The 89 up-regulated transcripts unique to *S. mansoni* exposure included 7 factors with immune function: FREP 2, FREP 6, FREP 11, LPS binding protein, superoxide dismutase 1 copper chaperone, Cu-Zn SOD, and dual oxidase. Both parasite infections led to increased expression of transcripts resembling C1q TNF-related protein and C1q like protein 4 (Paidassi et al., 2008;Zhang et al., 2008a) (Table 3). All three of the *B. glabrata* transcripts uniquely up-regulated in the response to *E. paraensei* are of unknown function (Table 3).

None of the features uniquely down-regulated in the presence of *S. mansoni* have known immune relevance. However, the 66 transcripts uniquely down-regulated by *E. paraensei* infection include FREP 8, FReM, alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein (LRP/Alpha 2 MR), lysozyme, macrophage migration inhibition factor (MIF), MAP kinase binding protein Mp1, peptidoglycan recognition protein (PGRP SC2), SH_3 containing growth factor receptor-bound protein 2 (GRB2)-like protein, and Cu-Zn SOD, that are all likely to have defense functions. No down-regulated transcripts were common between the two trematode infections. The transcriptional profile of *B. glabrata* at 12 hours post exposure, likely a composite of immune responses of the host and immuno-modulation efforts of the parasite is clearly particular to the species of infecting trematode.

Functional interpretation of challenge-specific transcript expression profiles

To facilitate overall interpretation of the different transcription profiles, immune-related features on the array were arbitrarily divided into the following categories: signal transduction, lectin, antimicrobial, cell adhesion, transcription, apoptosis, muscle, lysozyme, immune response, and proliferation/development. Also delineated were features related to structure and function of the cytoskeleton. Stress-related features were divided by general stress response and detoxification.

Considering these functional categories (Fig. 5), the wounding challenge caused up-regulation of cytoskeletal and proliferation/developmental transcripts in *B. glabrata*. The two antibacterial responses shared common features but the reaction to *E. coli* was distinct in consisting of increased expression of transcripts involved in signal transduction, anti-microbial and general immune response, in particular involving lectins. The transcriptomic response to *M. luteus* featured up-regulation of transcripts involved in signal transduction, but also down-regulation of transcription factors and anti-microbial factors (alkaline phosphatase and peptidoglycan recognition protein; PGRP). At 12 h post infection, the response of *B. glabrata* to *E. paraensei* was characterized largely by down-regulation of transcripts encoding lectins, anti-microbial factors, and features involved in signal transduction, cell proliferation, and general immune response. In contrast, *S. mansoni* infection led to down-regulation of some cytoskeletal and immune response transcripts, but was largely associated with a profile of up-regulation that included few known immune features except for several lectins.

Each of the immune challenges also affected expression levels of so-called “unknown” *B. glabrata* transcripts. Because no functional context exists, however, the implication of differential regulation of these unknowns cannot be interpreted at this time. These unknowns are obvious candidates for additional study.

Confirmation of differential expression by qPCR

Differential expression as detected by the microarray approach was confirmed for a set of selected transcripts using qPCR (Fig. 6). Values for fold change in expression measured by qPCR analysis tended to be greater in magnitude when compared to the values obtained from the array. The qPCR results did correlate closely to the trends observed in transcript expression for all of the array experiments, however.

Discussion

This study provides the first microarray-based comparison of the defense responses of *B. glabrata* at 12 hours post exposure to a variety of immune challenges: wounding, Gram negative and Gram positive bacteria, and two different digenetic trematode species, *S. mansoni* and *E. paraensei*. The distinct responses that were generated to the different insults suggest that considerable complexity and discriminatory ability are associated with the immune response of *B. glabrata*.

The oligo-based microarray used in this study was designed with a focus on transcripts with relevance to defense and stress response. Our microarray results, validated by qPCR in this study as well as by transcriptome level responses for selected genes reported elsewhere e.g for FREPs, FReM (Zhang et al., 2008b), Cu-Zn SOD and Mn SOD (Guillou et al., 2007; Jung et al., 2005), are consistent with a functional role of homologs of known immune genes in responses of *B. glabrata* to challenge, and identify unknowns as candidate immune factors.

The wound response in *B. glabrata*, especially when compared to the reaction profiles generated in response to infectious agents, is relatively modest and distinct (Fig. 1), as it incorporates few known immune genes. This pattern, recorded from a lophotrochozoan, resembles that reported from the ecdysozoans *Anopheles gambiae* (Dimopoulos et al., 2002) and *Drosophila melanogaster* (Galko and Krasnow, 2004;Stramer et al., 2008). Larval *D. melanogaster* exhibit increased expression of many transcription factors and hematopoietic transcripts following injury (Pearson et al., 2009), consistent with production of an increased number of circulating hemocytes after wounding. However, other than melanization responses likely involved in repair (Stramer et al., 2008), wounded *Drosophila* larvae do not up-regulate a transcription profile indicative of an extensive immune response. Melanization responses play a much less conspicuous role in defense or repair in molluscs such as *B. glabrata* (Bahgat et al., 2002;Luna-Gonzalez et al., 2003), so it is not surprising that we did not observe up-regulated features involved in melanization cascades. In *B. glabrata*, transcripts resembling TGF-beta receptor 1 and SMAD 4 were both up-regulated after wounding. Given that homologs of these transcripts are important for fibroblast proliferation and function in vertebrates (Clark et al., 1997), we hypothesize that their increased expression after wounding in *B. glabrata* indicates activation of cell proliferation for tissue repair.

Invertebrates routinely contend with bacterial challenges and their immune cells swiftly engage in effective phagocytosis and nodulation responses accompanied by oxygen and nitrogen radical production to eliminate a majority of bacterial challenges (Bayne and Fryer, 1994; Hillyer et al., 2003; Molina-Cruz et al., 2008; Noda and Loker, 1989b; van der Knaap et al., 1981). Ecdysozoans like *Drosophila* (Ferrandon et al., 2007), and *Anopheles gambiae* (Dong et al., 2006; Heard et al., 2005) are capable of distinguishing between different types of bacterial pathogens. Our study demonstrated that *B. glabrata* also differentiates between Gram positive and Gram negative bacteria. Several features were in common between the snail's responses to *E. coli* and *M. luteus*: for example, both included increased expression of classical anti-bacterial transcripts such as lysozyme, and LBP/BPI (Beamer et al., 1998; Gonzalez et al., 2007; Li et al., 2008a; Li et al., 2008b). It was evident however, that each bacteria provoked distinct subsets of features. A transcript that was up-regulated in response to *E. coli* was for

Cu-Zn SOD 1, an enzyme involved in the regulation of production of oxygen radicals during the respiratory burst, an important process for killing of bacteria. This enzyme is a component of snail responses to *Echinostoma caproni* (Guillou et al., 2007). *Biomphalaria glabrata* is able to resolve *E. coli* infections quickly (Matricon-Gondran and Letocart, 1999), so the array results provide a better understanding of components involved in this successful response.

The response of *B. glabrata* to *M. luteus* encompassed both more uniquely up-regulated and down-regulated features. Notable among the down-regulated transcripts is a homolog of peptidoglycan recognition protein SC2 (PGRP SC2), a scavenger receptor that sequesters soluble peptidoglycan. High levels of circulating PGRP SC2 reduce the response to Gram positive bacteria by limiting the amount of free peptidoglycan available to bind directly to cell-surface PGRPs, an event that activates immune cells in the fruit fly (Charroux et al., 2009). The down-regulation of this transcript in snails suggests that there should be an enhanced capacity to respond to peptidoglycan, potentially increasing the efficiency of bacterial clearance.

Among the transcripts that were up-regulated in response to *M. luteus* were FREP 4 and FREP 7, these are lectins from *B. glabrata* that contain fibrinogen-related domains (FREDs). Such lectins have been recovered in surprising diversity from several invertebrates (Adema et al., 1997; Christophides et al., 2002; Dong and Dimopoulos, 2009; Hibino et al., 2006; Stout et al., 2009), and were identified as components of responses against bacteria and parasites of the immune systems of both invertebrates and vertebrates (Dong and Dimopoulos, 2009; Zhang and Loker, 2004, Azumi et al., 2003; Frederiksen et al., 2005). Our results suggest a role for FREPs in response to Gram positive but not Gram negative bacteria at 12 hours post exposure. FREP 3 and 7 were shown to bind both Gram positive and negative antigens (Zhang et al., 2008c), so they likely act as pattern recognition molecules for bacteria, as noted for other FRED-containing lectins in other invertebrates.

In addition to common pathogens such as viruses, bacteria and fungi, molluscs like *B. glabrata* must also contend with a major group of specialized pathogens, the digenetic trematodes. Almost all known digeneans have an obligatory dependence on molluscs, usually snails, for their larval development. Digenean infections typically are highly specific with respect to the molluscan host, are long-term and carry pronounced detrimental consequences, including parasitic castration (Crews and Yoshino, 1989). *Biomphalaria glabrata* can host multiple species of digeneans and study of these different parasite/host interactions is particularly relevant given that digeneans in their adult stages can cause significant disease in humans (Orihel and Ash, 1995). Some strains of *B. glabrata* are resistant to *S. mansoni* (Lie et al., 1979), and this remains an important topic for exploration (Bayne, 2009), as it may lead to novel methods of interrupting transmission of human schistosomaisis. Trematodes actively undertake efforts to avoid immune elimination by host defenses (Lie et al., 1981; Loker and Adema, 1994). Accordingly, gene expression profiles recorded from trematode infected snails will reflect an amalgam of anti-pathogen defense efforts mounted by the snail and of parasite-directed modifications of expression of host genes that may function in defense or other aspects of homeostasis.

In the case of *S. mansoni* infection, a number of defense-related lectin transcripts of *B. glabrata* were upregulated, including transcripts encoding for FREPs 2, 4 and 6 (Adema et al., 1997), galectin-4 (Yoshino et al., 2008), and two transcripts encoding lectins with sequence similarities to complement C1q-like lectins (Païdassi et al., 2008; Zhang et al., 2009). Also up-regulated were sequences encoding histones H2A, H2AV, and H3.3. Classically, histones play a role in chromatin remodeling during mitosis (Georgatos et al., 2009), however, in molluscs they have potential anti-microbial properties (Li et al., 2007). Interestingly, after *S. mansoni* infection histone H3.3 had the highest increase (43 fold) of any of the array features. Theories

regarding successful *S. mansoni* infection in *B. glabrata* have centered on the concept of the parasite either acquiring host molecules or of producing host-mimicking molecules (Chacon et al., 2002) such that it evades detection by the snail's immune response and 'flies under the radar' of the snail defenses (Loker and Adema 1995; Hanelt et al., 2008). On the contrary, at least at 12 h post-infection, this study suggests that the snail recognizes and responds to this parasite. A few known immune relevant features were up-regulated but since M line snails eventually succumb to *S. mansoni* infection, the immune response must in some way be inadequate or actively modulated by the parasite, it is also possible that immune compatibility is achieved later during this *S. mansoni-B. glabrata* interaction.

The cDNA array-based transcriptome study of Lockyer et al., (2008) showed that hemocytes from resistant *B. glabrata* transcribe many genes at 24 hours post exposure to *S. mansoni*, while few genes were differentially expressed in susceptible snails. We observed a number of upregulated transcripts in M line *B. glabrata*, which are also susceptible to *S. mansoni*. These apparently divergent observations likely stem from different experimental designs. Lockyer et al., (2008), directly comparing RNA expressed in hemocytes from susceptible versus resistant strains of *B. glabrata*. This highly valid approach is less likely to reveal transcripts that are similarly up- or down-regulated in both experimental groups: such genes will be identified from a comparison of the transcription profile of a susceptible *B. glabrata* infected with *S. mansoni* versus that of untreated control snails, as performed in this study. Additional differences may stem from the source of the cDNA probe (RNA from hemocytes versus whole body), bonafide changes in the parasite host interactions between time points, or a combination of these issues. Still, some transcripts were found up-regulated in this study and associated with resistant snails in their study (Lockyer et al., 2008), suggesting that heat shock protein 70, titin and multi-drug resistance associated protein do indeed play an important role in the internal defense system of *B. glabrata*.

In contrast to *S. mansoni*, infection with *E. paraensei* resulted in a predominance of down-regulated features, many of which have immune functions such as macrophage migration inhibition factor (MIF) (Nishihira, 2000), peroxiredoxin-4 (Diet et al., 2007; Knight et al., 2009), cAMP-responsive element modulator (CREM) binding protein (Matt, 2002) and Cu-Zn SOD1 (Marikovsky et al., 2003). Furthermore, FREP 8 and a transcript encoding a distinct category of fibrinogen related molecule (FReM) with an unusual N-terminal domain were also down-regulated. Previously, Zhang et al. (2008b) also observed decreased expression of FReM after *E. paraensei* infection. This strong trend of down-regulation of transcripts involved in host defense lends support to the hypothesis that *E. paraensei* actively interferes with the snail immune response (Lie et al., 1981; Loker et al., 1986; Loker et al., 1992).

Many of the features up-regulated by *S. mansoni*-infected snails were also expressed at elevated levels in response to *E. paraensei*. This included FREP 4, a plasma lectin that in our experience is the most consistently up-regulated feature accompanying trematode infection in *B. glabrata*, and that binds and precipitates secretory/excretory products from *E. paraensei* larvae (Adema et al., 1997). FREP 4 production increases dramatically upon *E. paraensei* infection, with maximum amounts of protein detected at 4–8 days post infection, followed by a decrease thereafter. These protein expression levels correlate with FREP 4 expression observed in this study. Infection of *B. glabrata* with *E. paraensei* also provokes an increase in the number of circulating hemocytes (Noda and Loker, 1989a). We observed an increase in expression of a transcript encoding a homolog of epidermal growth factor, the latter being a pivotal growth factor in vertebrates (Pastore et al., 2008). Given that many transcripts are up-regulated following exposure to *E. paraensei, B. glabrata* does detect this trematode and attempts to mount a response, but at least at 12 hours, *E. paraensei* is more capable than *S. mansoni* of down-regulating array features. This accords broadly with the conclusion of Lie et al., (1982), that the immunosuppressive/interference of *E. paraensei* is stronger than that of *S.*

mansoni. Strikingly, there was no overlap in the features that were significantly down-regulated by *B. glabrata* in response to these two trematode species (Fig 3b).

Several recent studies revealed in invertebrates the capacity to recognize specific pathogens, to produce diversified recognition molecules, and even the potential for mounting responses of increased efficacy after prior exposure (Zhang and Loker, 2003; 2004; Kurtz and Armitage, 2006; Baton et al., 2009; Dong and Dimopoulos, 2009). This study demonstrates that juvenile and adult *B. glabrata* too mount different immune responses to different pathogens. Particularly with respect to *E. paraensei*, a parasite specifically adapted to colonize *B. glabrata*, there is evidence consistent with a strategy of parasite-mediated immuno-suppression. With a functional oligo-based microarray platform in hand, further studies are in progress to better define the immune responsiveness of *B. glabrata* to either *S. mansoni* or *E. paraensei*. These approaches will enable us to better understand how trematodes, including species of medical significance such as *S. mansoni*, continue to thrive in their natural environments, and provide valuable perspective on the immune capabilities of a prominent phylum of lophotrochozoan invertebrates, the Mollusca.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Dr. Mark Blaxter provided us access to his cluster analysis of *B. glabrata* ESTs in aid of selecting features for the array. This study was supported in part by NIH grant number 1P20RR18754 from the Institute Development Award (IDeA) Program of the National Center for Research Resources, NIH AI024340 (ESL), NIH AI062363 (CMA) and an NSERC PDF fellowship (PCH).

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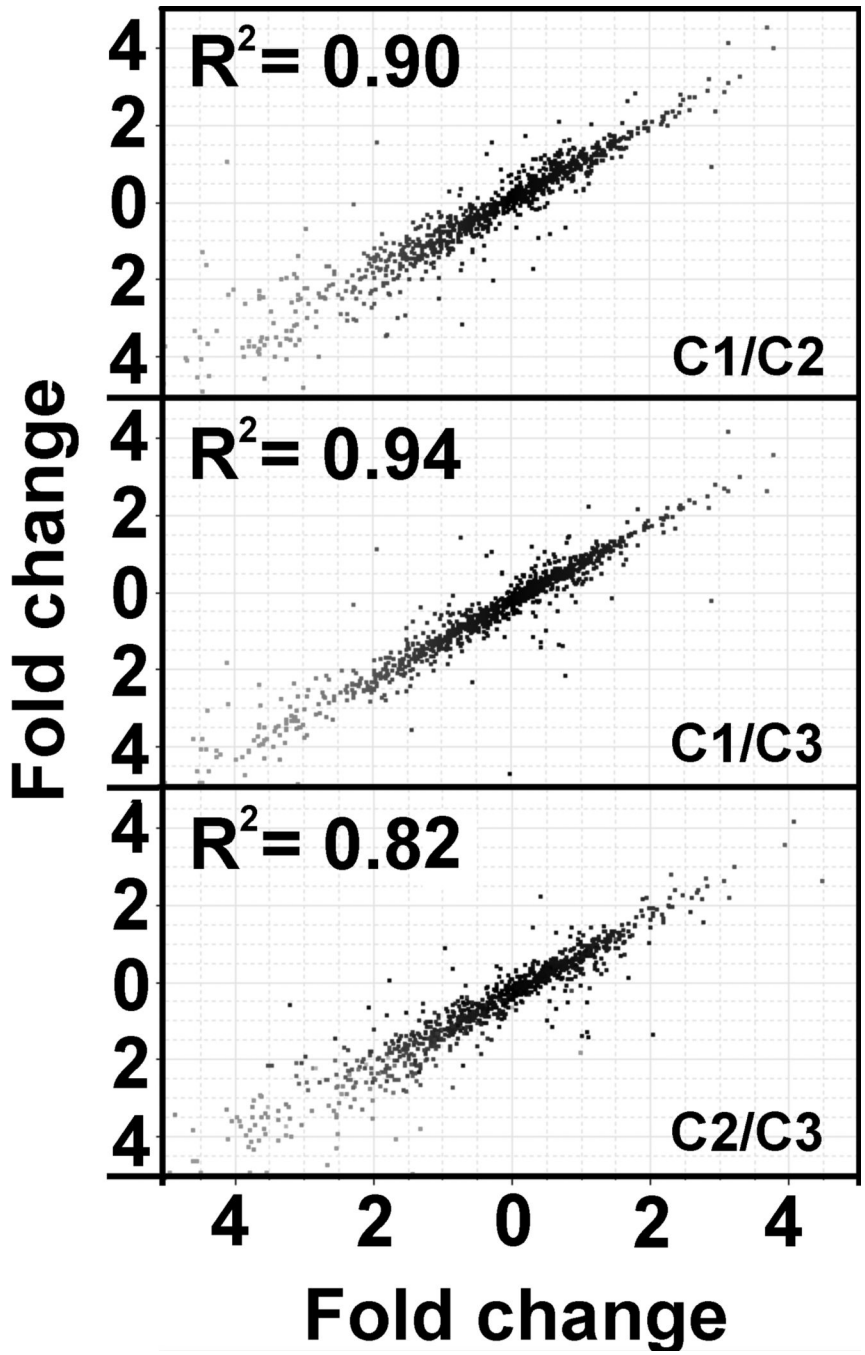

Figure 1. Reproducibility of the *B. glabrata* oligo-based microarray

Three different arrays were hybridized with cDNA probes derived from RNA of three independent pools of untreated control *B. glabrata* (10–12mm shell diameter, n=5/pool). Pairwise comparisons showed that the signals obtained for features of the array (expressed as $\log_2$ ratio of experimental versus universal RNA reference) grouped closely along a 45° angle line (R^2 values ranging from 0.82 to 0.94). This demonstrates that biological replicate samples yield highly similar signals between array hybridizations. C1, C2, C3 designate the cDNA probes produced with different biological replicates.

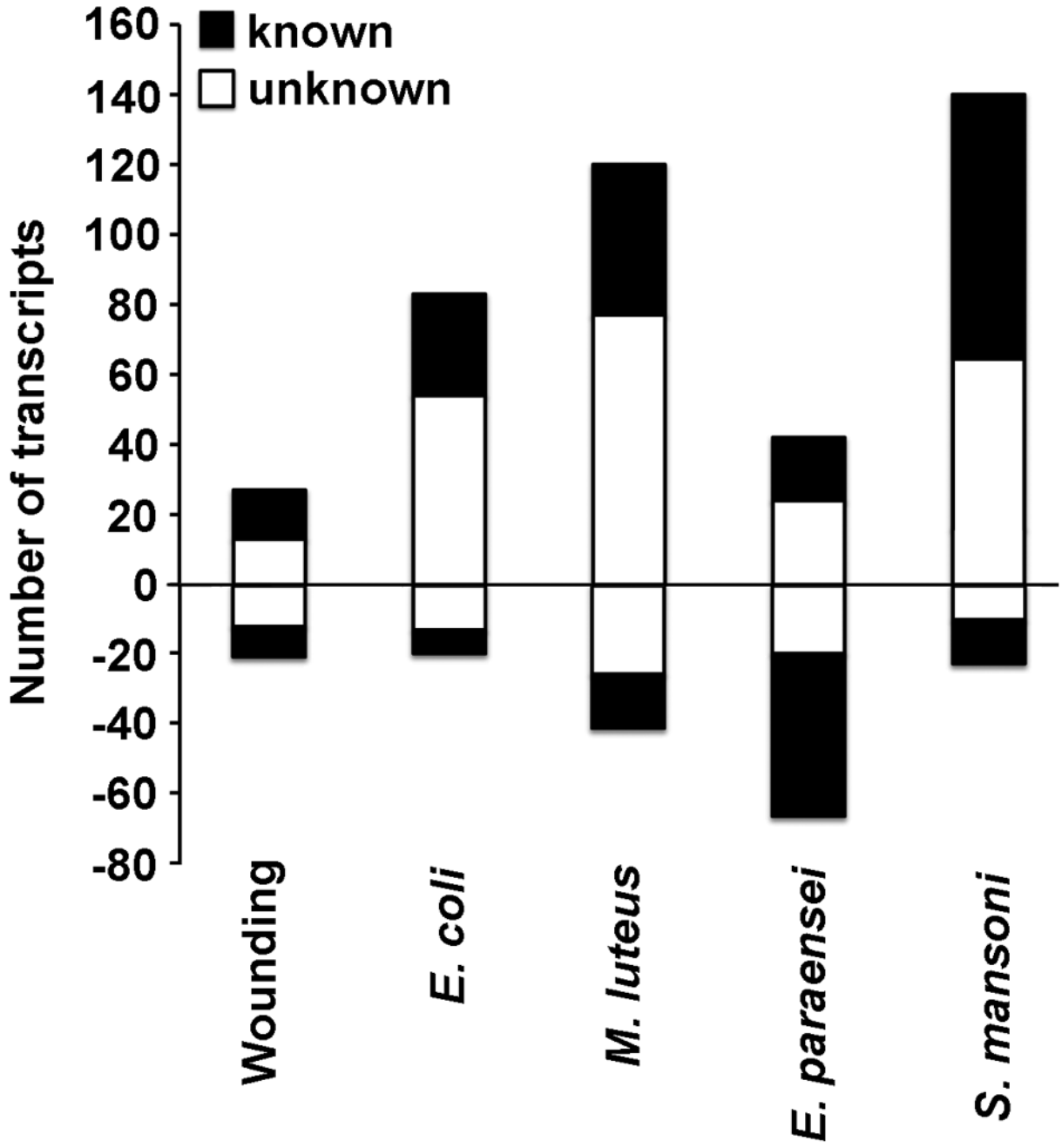

Figure 2. Transcriptional responses of *B. glabrata* to experimental challenges

The number of features that were up- or down-regulated at 12 hours after challenge is shown for each experimental treatment. False Positive Rate ≤ 10%. Features with a (putative) ID are numbered in black bars, white bars indicate unknown (novel) sequences.

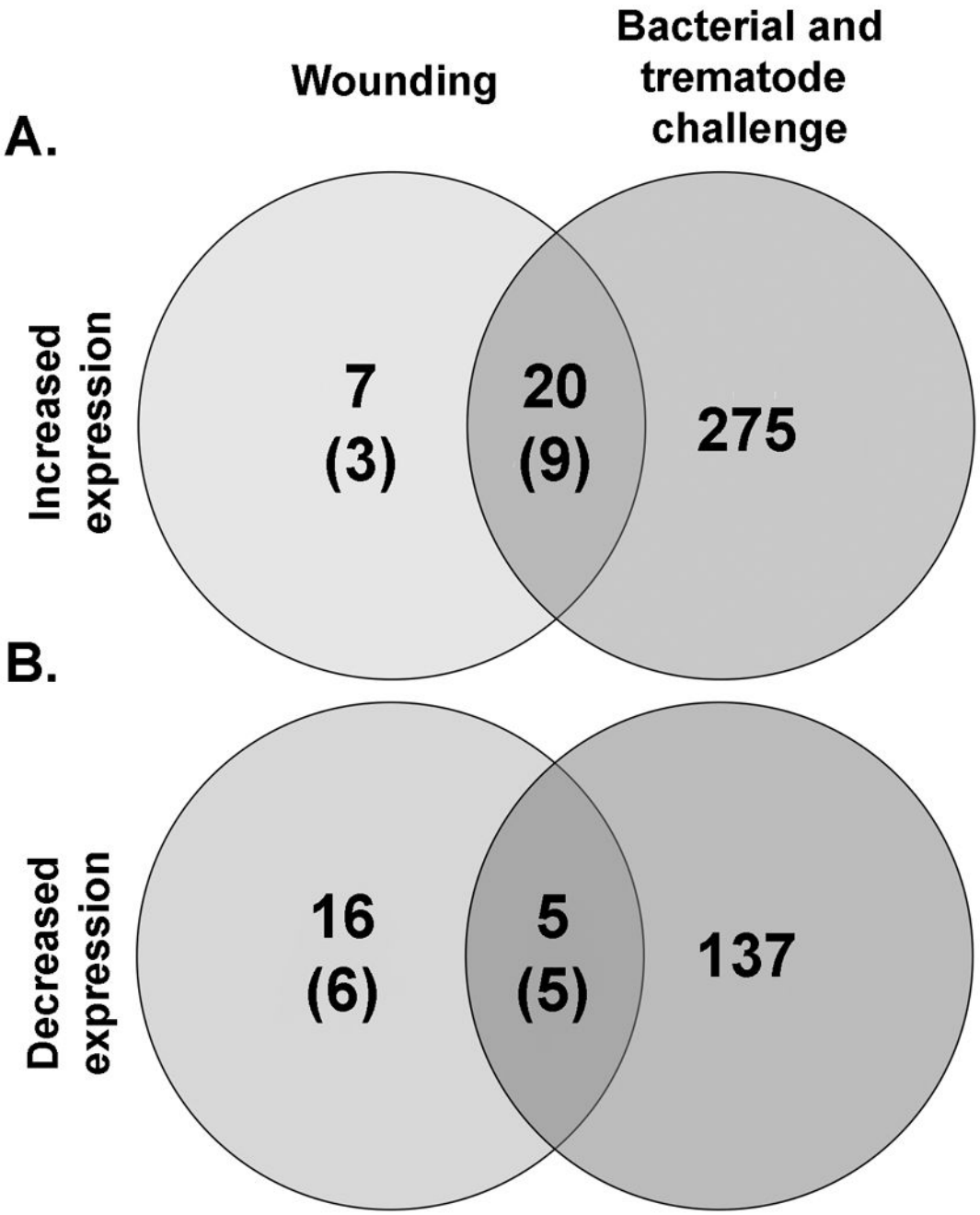

Figure 3. Response of *B. glabrata* to wounding compared to other challenges

Transcripts significantly increased (a) or decreased (b) at 12 hours post wounding were modest in number and encompassed different sequences in relation to the features affected by challenge with bacteria and trematodes (shown combined). For wounding, the total number of differentially expressed features is shown with the number features with unknown function in brackets. False Positive Rate ≤ 10%.

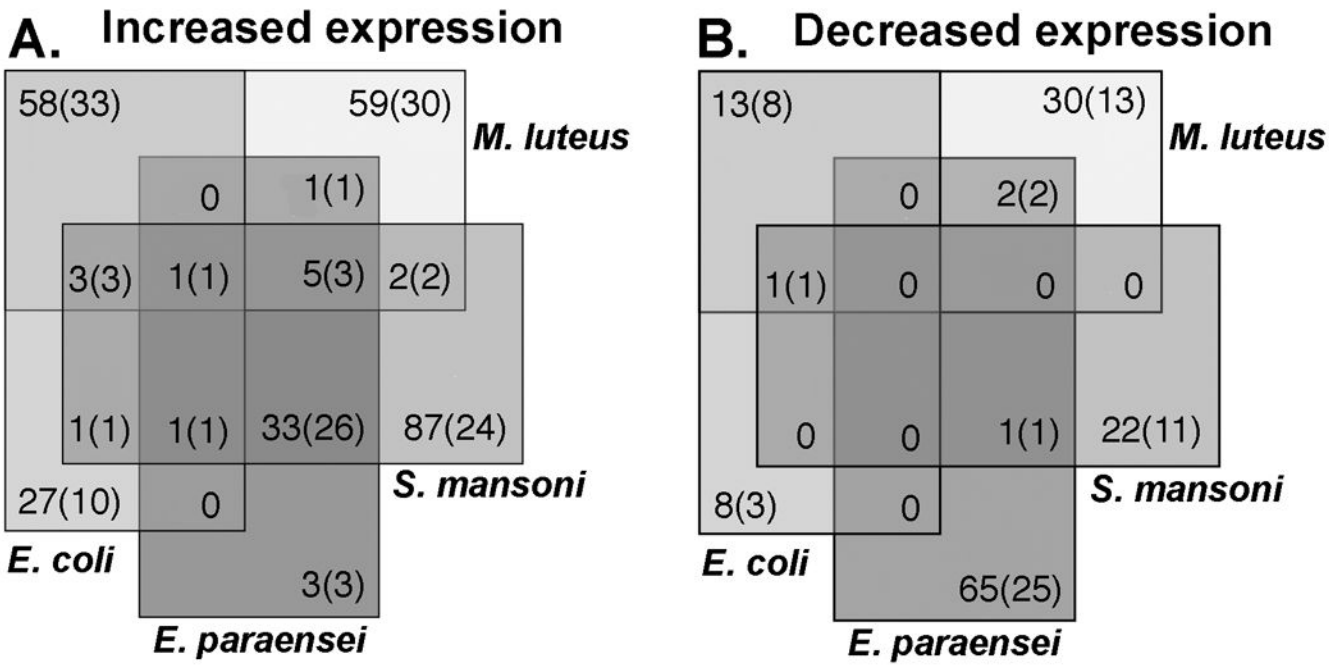

Figure 4. Comparison of transcription profiles of *B. glabrata* after challenge with bacteria or digenetic trematodes

These Venn diagrams show the number of shared and unique features that were up-regulated (a) or down-regulated (b) at 12 hour post exposure to *E.coli* (Gram negative), *M. luteus* (Gram positive), *E. paraensei* and *S. mansoni*. Each challenge yielded a distinct transcriptome. Note the absence of overlap in features with decreased expression in response to *E. paraensei* and *S. mansoni*. The numbers in the Venn diagram represent total number of differentially expressed features with the number of features with unknown function shown in brackets. False Positive Rate ≤ 10%.

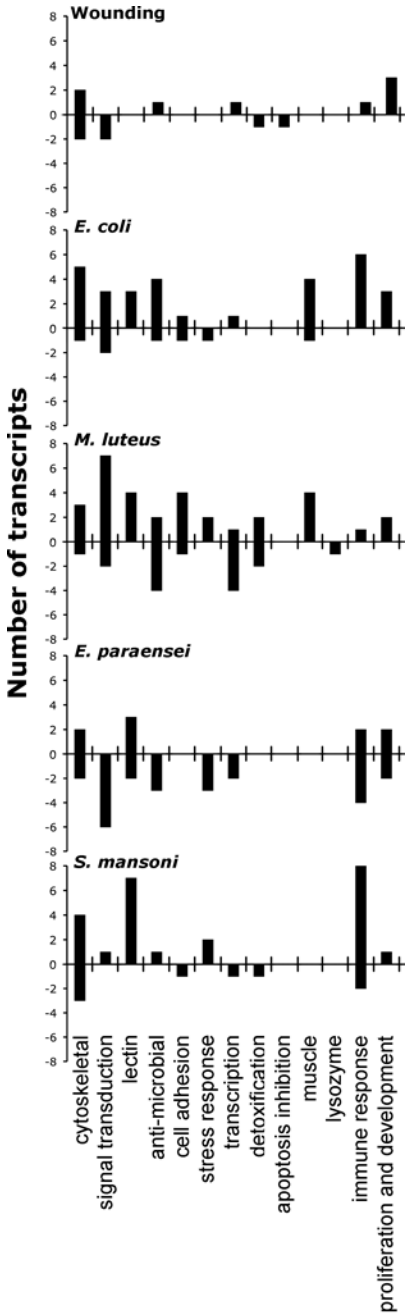

Figure 5. Impact of different challenges on categories of immune or stress response features

The number of differentially expressed features that was assigned to an immune or stress response category (based on putative ID and GO annotation) is presented for each separate experimental treatment. False Positive Rate ≤ 10%. Bacterial challenge evoked transcriptional responses with mostly increased expression. The responses of *B. glabrata* to the compatible digenean parasites *E. paraensei* and *S. mansoni* include many down-regulated features, likely reflecting less effective immune responses.

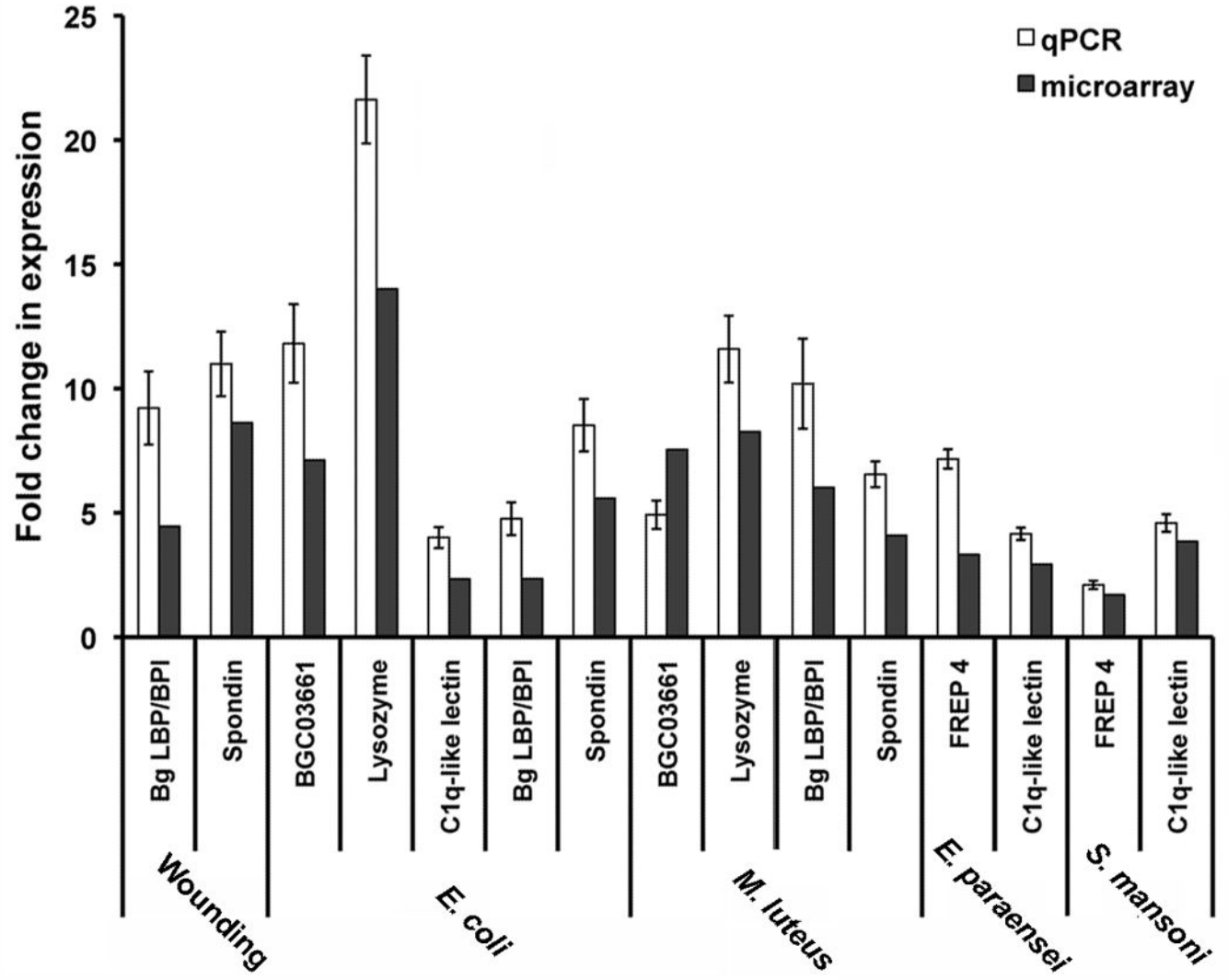

Figure 6. Differential expression validated by reverse transcripton quantitative PCR

RT-qPCR was applied as independent means to validate differential expression as detected by the microarray approach. Primers were designed for selected features and qPCR was performed with the same templates that were used to generate the cDNA probes for the microarray experiments. While the results tended to indicate greater fold change in expression, RT-qPCR results did correlate closely to the trends observed in transcript expression for all the array treatments.

Table 1

General description of features on the array.

Category	Number	Comments
Biomphalaria (with putative ID)	557	Genes, ESTs, ORESTES (clusters, singletons)
Biomphalaria (unkown, novel)	502	Genes, ESTs, ORESTES (clusters, singletons)
Biomphalaria (other)	25	rDNA genes, mitochondrial genes
Echinostoma paraensei	12	ESTs, rDNA genes, mitochondrial genes
Schistosoma mansoni	26	ESTs, rDNA genes, mitochondrial genes
SpotReport Aliens	20	10 sense, 10 antisense
Negative controls	10	Plant origin
Total:	1152	

Table 2

Primers used for RT-qPCR.
Indicated are the names and sequences (5'–3') of the transcript-specific primers.

Primer name	Primer sequence
BgLBP/BPI-F	GTAGAGCGGCAAAGTGTCCAC
BgLBP/BPI-R	CAATGCATCGTTTTGTTACTTGG
Spondin-F	CTGTGGTCAAGCCTGGCTACT
Spondin-R	TCAAAGATATGAAGGATGAAATAAACAAG
BGCO3661-F	GGATGATTTAACAATGACAGACATGA
BGCO3661-R	AGACTATGTAAGTGCAAAGTCAATTCT
Lysozyme-F	ATATTATCATTTACTTTCTGCTTTGAATTGTAT
Lysozyme-R	CAAGTTATACTGTCCACGTTTCCA
C1q-like-F	CTGTAATCCTGAAACTGGCTAAAGAA
C1q-like-R	TCCGCGGTTTGCACG
F4Q3-F	TGATTCGCCGAATGATAATTGT
F4Q3-R	CGTTAAGGTTGACATCAGCACAGT

Table 3

Differentially expressed (putative) immune-relevant features,.
Listed for each experimental treatment are the affected transcripts (name, feature code from GAL file), fold change (negative = down-regulated, positive = up-regulated), and putative function.

	Fold Change	Putative Function
WOUNDING		
Bg LBP/BPI, BGC03345	4.458	Lipopolysaccharide-binding protein/Bactericidal permeability-increasing protein Anti-bacterial protein, binds LPS (Gonzalez et al., 2007).
Peptidoglycan-recognition protein-(PGRP) SC2, P073H12	−4.829	Recognition of peptidoglycan. May protect against over-responding to bacterial infection (Bischoff et al., 2006).
SMAD4, BGC04177	6.812	Transcription factor, Involved in TGF-B signaling pathway to induce proliferation and differentiation of cells (Brummel et al., 1999).
TGF-beta type I receptor (TGFR-1), BGC04521	2.667	Likely involved in binding a TGF-beta-like molecule, possibly induces cell proliferation and differentiation (Dworkin and Gibson, 2006).
***E. coli* INJECTION**		
Bg LBP/BPI, BGC03345	2.351	Lipopolysaccharide-binding protein/Bactericidal permeability-increasing protein Anti-bacterial protein, binds LPS (Gonzalez et al., 2007).
CDC7-related kinase, BGC01771	9.339	Required for the initiation and progression of cell cycle (Masai et al., 1999).
C1q-like protein 4, BGC00252	2.331	Contains C1q-like domain with lectin properties. Related sequence responds to bacteria in molluscs (Zhang et al., 2008a).
JNK-interacting protein 3, BGC02448	2.141	Involved in signal transduction after TLR 4 activation in mammals. Presumed to also interact with Toll in Drosophila (Matsuguchi et al., 2003).
Low density lipoprotein (LDL) receptor, BGC04358	2.098	Potentially involved in the recognition of oxidized lipoproteins. Cholesterol metabolism (Hazen, 2008).
LPS binding protein-like protein, BGC03011	2.708	LBP/BPI (Lipopolysaccharide-binding protein/ Bactericidal permeability-increasing protein) Anti-bacterial protein, binds LPS (sequence differs from BGC03345) (Gonzalez et al., 2007).
Lysozyme, BGC00466	2.013	Bacteriolytic activity (Zavalova et al., 2006).
MAP kinase 2 (MPK2), BGC04690	3.677	Involved in the p38 signaling pathway in Drosophila. This pathway is involved in cell responses to inflammatory and environmental stimuli (Zhuang et al., 2006).
Pulmonary surfactant-associated protein DSP-D, BGC03371	4.092	C-type lectin domain, activities include binding and agglutination of pathogens (Haagsman et al., 2008).
Serpin B4, P075G03	2.294	Promotes cell survival (Zou et al., 2009)
Serpin B6, BGC01370	2.009	Regulates immune response by inhibiting function of proPO-

	Fold Change	Putative Function
		activating proteinase-3 (Zou and Jiang, 2005)
SMAD4, BGC04177	4.607	Involved in TGF-B signaling pathway to induce proliferation and differentiation of cells (Brummel et al., 1999).
Cu-Zn Superoxide dismutase 1 (SOD1), BGC03242	2.201	Detoxification/production of superoxide radicals, especially from phagocytic/granulocytic cells (Marikovsky et al., 2003)
***M. luteus* INJECTION**		
Bg LBP/BPI, BGC03345	2.025	Lipopolysaccharide-binding protein/Bactericidal permeability-increasing protein Anti-bacterial protein, binds LPS (Gonzalez et al., 2007).
Bg M-line FREP 4, BgMFREP4_1	2.348	Lectin consisting of a single IgSF domain and fibrinogen domain. Evidence suggests involvement in host defense against parasite infection (Adema et al., 1997).
Bg M-line FREP 7, BgFibro7_1	2.056	Lectin consisting of two IgSF domains and a fibrinogen domain. Likely involved in host defense (Zhang and Loker, 2004).
Low density lipoprotein (LDL) receptor, BGC04358	2.855	Potentially involved in the recognition of oxidized lipoproteins. Cholesterol metabolism (Hazen, 2008).
Lysozyme, BGC00466	2.262	Bacteriolytic activity (Zavalova et al., 2006).
MAP kinase 2 (MPK2), BGC04690	4.450	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-associated protein, P077E11	2.302	Resembles ATP-binding cassette (ABC) drug resistance transporters. These proteins are involved in drug resistance (Sheps et al., 2004).
NF-kappa-B p105 subunit, 139917075	2.481	Transcription factor of NF-kappa-B/Toll pathway, regulation inflammatory response (Chang et al., 2009).
Pulmonary surfactant-associated protein DSP-D, BGC03371	3.323	C-type lectin domain, activities include binding and agglutination of pathogens (Haagsman et al., 2008).
Serpin B4, P075G03	2.471	Promotes cell survival (Zou et al., 2009)
Serpin B6, BGC01370	2.772	Regulates immune response by inhibiting function of proPO-activating proteinase-3 (Zou and Jiang, 2005)
SH3 domain-binding protein 5, BGC02605	2.361	Binds to SH_3 domains of intracellular proteins. May regulate signaling by SH_3 binding (Kang et al., 2000).
TGF-beta type I receptor (TGFR-1), BGC04521	2.450	Likely involved in binding a TGF-beta-like molecule, possibly induces cell proliferation and differentiation (Dworkin and Gibson, 2006).
Alkaline phosphatase, BGC04218	−4.613	Lysosomal enzyme associated with granulocytic hemocytes of invertebrates (Xing et al., 2002).
ELP1 IKK complex-associated protein A, P077G11	−4.577	Binds to NF-kappa-B inducing kinase and IKK which regulates 3

	Fold Change	Putative Function
		other kinases involved in inflammation (Strnad and Burke, 2007).
Peptidoglycan-recognition protein-(PGRP) SC2, P073H12	−4.829	Recognition of peptidoglycan. May protect against over-responding to bacterial infection (Bischoff et al., 2006).
Multi-drug resistance protein 2, BGC01951	−2.563	Resembles ATP-binding cassette (ABC) drug resistance transporters. These proteins are involved in drug resistance (Sheps et al., 2004).
***S. mansoni* INFECTION**		
Agrin precursor, BGC02479	10.283	Important ECM protein involved in immunological synapse formation which is critical for immune cell activation (Zhang et al., 2006).
Bg M-line FREP 2, BgMFREP2_1	9.142	Lectin consisting of a single IgSF domain and fibrinogen domain. Host response to parasite infection (Adema et al., 1997).
Bg M-line FREP 4, BgMFREP4_1	1.698	Lectin consisting of a single IgSF domain and fibrinogen domain. Host response to parasite infection (Adema et al., 1997).
Bg M-line FREP 6, BgMFREP6	1.981	Lectin consisting of a single IgSF domain and fibrinogen domain. Host response to parasite infection (Adema et al., 1997).
C1q TNF-related protein 3, BGC00252	3.951	Induces the proliferation and migration of endothelial cells as well as the up-regulated expression of pro-inflammatory molecules (Paidassi et al., 2008).
C1q-like protein 4, BGC002524	3.851	Contains C1q-like domain with lectin properties. Related sequence responds to bacteria in molluscs (Zhang et al., 2008a).
Epidermal growth factor (EGF)-related protein, BGC04033	4.312	Regulates processes of cell proliferation, survival and plays an important role in wound healing and tissue remodeling (Schneider and Wolf, 2009)
Galectin-4, BGC00934	10.574	Binds beta-galactosides, shown to be pro-inflammatory and have the ability to activate immune cells (Yang et al., 2008; Yoshino et al., 2008).
Glutathione S-transferase (GST), BGC02292	9.251	Heat shock response and drug resistance, also has been shown to be produced by parasites to alter host immune cell activation and development (Ouaissi et al., 2002).
Histone H2A, BGC00456	4.591	Host defense response through producing novel antimicrobial peptides (AMPs) from its N-terminus in vertebrates and invertebrates (Georgatos et al., 2009).
Histone H2AV H2A.F/Z, BGC02328	5.741	Host defense response through producing novel antimicrobial peptides (AMPs) from its N-terminus in vertebrates and invertebrates (Georgatos et al., 2009)
Histone H3.3, BGC02445	43.017	Host defense response through producing novel antimicrobial peptides (AMPs) from its N-terminus in vertebrates and

	Fold Change	Putative Function
		invertebrates (Georgatos et al., 2009)
Lipopolysaccharide-binding prot LBP, BGC02348	7.658	LBP/BPI (Lipopolysaccharide-binding protein/ Bactericidal permeability-increasing protein) Anti-bacterial protein, binds LPS (sequence differs from BGC03345, BGC03011) (Gonzalez et al., 2007).
Cu-Zn Superoxide dismutase 1 (SOD1), BGC03242	6.495	Enzyme involved in the detoxification/production of superoxide radicals, especially from phagocytic/granulocytic cells (Marikovsky et al., 2003)
***E. paraensei* INFECTION**		
Bg M-line FREP 4, BgMFREP4_1	3.324	Lectin consisting of a single IgSF domain and fibrinogen domain. Host response to parasite infection (Adema et al., 1997)..
C1q TNF-related protein 3, BGC00252	1.896	Induces the proliferation and migration of endothelial cells as well as the upregulated expression of pro-inflammatory molecules (Paidassi et al., 2008).
C1q-like protein 4, BGC002524	2.924	contains C1q-like domain with lectin properties. Related sequence responds to bacteria in molluscs (Zhang et al., 2008a).
Epidermal growth factor (EGF)-related protein, BGC04033	18.866	Regulates processes of cell proliferation, survival and plays an important role in wound healing and tissue remodeling (Schneider and Wolf, 2009).
Ras-related protein Rab-6.1, BGC01836	1.829	Involved in organelle targetting, important for phagolysosome formation after initial phagocytic event (Kinchen and Ravichandran, 2008).
Serine/threonine-protein kinase Polo-like, P077E04	2.122	Important for entry of the cell into M phase mitosis (Archambault and Glover, 2009).
Bg M-line FREP 8, BgMFREP8	−2.553	Lectin consisting of a single IgSF domain and fibrinogen domain. Evidence suggests involvement in host defense against parasite infection (Zhang et al., 2008b).
CREB-binding protein, BGC02298	−1.753	Important transcription factor involved in the acute and cronic inflammatory response after triggering the NF-kappa-B or AP-1 pathways (Matt, 2002).
Bg FREM, P079H03	−2.203	Fibrinogen-related domain (FReD) containing sequece wit two EGF domains which may be important for cell interactions or developmental processes (Zhang et al., 2008b).
Glutathione S-transferase (GST), BGC02292 OR BGC02321 OR P071F12	−2.411	Important in the processes of heat shock response and drug resistance, also has been shown to be produced by parasites to alter host immune cell activation and development (Ouaissi et al., 2002).
Heat shock 70 kDa protein (HSP70) BGC02350	−2.166	Enhances cell survival by preventing apoptotic signaling. Can inhibit inflammatory response if intracellular, enhance inflammatory response if extracellular (Giffard et al., 2008).

	Fold Change	Putative Function
Histone H3.3, BGC02445	−2.211	Reported to participate in host defense response through producing novel antimicrobial peptides (AMPs) from its N-terminus in vertebrates and invertebrates (Georgatos et al., 2009).
LRP/Alpha-2-macroglobulin receptor, BGC04652	−2.193	Variety of functions, lipid metabolism, protease degredation, activation of lysosomal enzymes. Important molecule in phagocytic cells (Correia Soeiro et al., 2001).
Macrophage migration inhibit factor (MIF), BGC02909	−1.888	Involved in the inflammatory and immune responses, including phagocytosis, cell spreading and tumor suppression (Nishihira, 2000).
Mitogen-activated protein kinase-binding Mp1, BGC02379	−2.300	Regulated MAP kinase signaling to control processes of cell proliferation, differentiation and activation (Sharma et al., 2005).
Peptidoglycan-recognition protein-(PGRP) SC2, P073H12	−2.227	Recognition of peptidoglycan. May protect against over-responding to bacterial infection (Zhang et al., 2007).
Peroxiredoxin-4, BGC03555	−2.233	Reduces hydrogen peroxide, organic peroxides and peroxynitrite. Involved in reducing oxidative stress, and modulating signaling cascades involving nitric oxide (Knight et al., 2009).
Cu-Zn Superoxide dismutase 1 (SOD1), BGC03242	−1.234	Enzyme involved in the detoxification/production of superoxide radicals, especially from phagocytic/granulocytic cells (Marikovsky et al., 2003).