



Published in final edited form as:

Dev Comp Immunol. 2016 March ; 56: 25–36. doi:10.1016/j.dci.2015.11.008.

Pathogen-associated molecular patterns activate expression of genes involved in cell proliferation, immunity and detoxification in the amebocyte-producing organ of the snail *Biomphalaria glabrata*

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Abstract

The anterior pericardial wall of the snail *Biomphalaria glabrata* has been identified as a site of hemocyte production, hence has been named the amebocyte-producing organ (APO). A number of studies have shown that exogenous abiotic and biotic substances, including pathogen associated molecular patterns (PAMPs), are able to stimulate APO mitotic activity and/or enlarge its size, implying a role for the APO in innate immunity. The molecular mechanisms underlying such responses have not yet been explored, in part due to the difficulty in obtaining sufficient APO tissue for gene expression studies. By using a modified RNA extraction technique and microarray technology, we investigated transcriptomic responses of APOs dissected from snails at 24 hours post-injection with two bacterial PAMPs, lipopolysaccharide (LPS) and peptidoglycan (PGN), or with fucoidan (FCN), which may mimic fucosyl-rich glycan PAMPs on sporocysts of *Schistosoma mansoni*. Based upon the number of genes differentially expressed, LPS exhibited the strongest activity, relative to saline-injected controls. A concurrent activation of genes involved in cell

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SUPPLEMENTARY DATA

Supplementary Data 1. The details of BlastX result showing fold changes and putative homologous sequence of genes differentially expressed after injected with LPS.

Supplementary Data 2. The details of BlastX result showing fold changes and putative homologous sequence of genes differentially expressed after injected with FCN.

Supplementary Data 3. The details of BlastX result showing fold changes and putative homologous sequence of genes differentially expressed after injected with PGN.

Supplementary Data 4. GO terms of all sequences of up- and down regulated after injection with LPS: biological process, biological function and cellular component.

Supplementary Data 5. GO terms of all sequences of up- and down regulated after injection with FCN: biological process, biological function and cellular component.

Supplementary Data 6. GO terms of all sequences of up- and down regulated after injection with PGN: biological process, biological function and cellular component.

proliferation, immune response and detoxification metabolism was observed. A gene encoding checkpoint 1 kinase, a key regulator of mitosis, was highly expressed after stimulation by LPS. Also, seven different aminoacyl-tRNA synthetases that play an essential role in protein synthesis were found to be highly expressed. In addition to stimulating genes involved in cell proliferation, the injected substances, especially LPS, also induced expression of a number of immune-related genes including arginase, peptidoglycan recognition protein short form, tumor necrosis factor receptor, ficolin, calmodulin, bacterial permeability increasing proteins and E3 ubiquitin-protein ligase. Importantly, significant up-regulation was observed in four GiMAP (GTPase of immunity-associated protein) genes, a result which provides the first evidence suggesting an immune role of GiMAP in protostome animals. Moreover, altered expression of genes encoding cytochrome P450, glutathione-S-transferase, multiple drug resistance protein as well as a large number of genes encoding enzymes associated with degradation and detoxification metabolism was elicited in response to the injected substances.

Keywords

Biomphalaria glabrata; mollusk; amebocyte-producing organ (APO); pathogen associated molecular pattern (PAMP); aminoacyl-tRNA synthetase; GiMAP

1. Introduction

The freshwater snail *Biomphalaria glabrata* is an intermediate host of the digenetic trematode *Schistosoma mansoni*, one of three major etiologic agents of human schistosomiasis, a disease that affects more than 230 million people worldwide (Colley et al., 2014). Recent molecular studies have focused on snail immunity, with an aim to better understand the mechanism of snail defense against schistosome parasites (Adema and Loker, 2015; Knight et al., 2014; Lockyer et al., 2012; Tennessen et al., 2015).

Snail defense cells, called amebocytes or hemocytes, are responsible for the cellular recognition of foreign bodies and for phagocytosis and cytotoxic reactions (Larson et al., 2014; Yoshino and Coustau, 2011; Zahoor et al., 2014). The circulating hemocytes are produced in *B. glabrata* primarily in the amebocyte-producing organ (APO), considered here synonymous with the anterior pericardial wall. This structure contains follicles of hemopoietic cells in a sinus located between the anterior wall of the pericardial sac and the posterior wall of the mantle cavity (Jeong et al., 1983; Lie et al., 1975). In addition to hemopoietic cells and pericardial and mantle epithelia, the APO contains typical cells of snail connective tissue, i.e., fibroblast-like cells, muscle tissue, hemocytes, and large pore cells (Pan, 1958; Sminia, 1972). It has been demonstrated that hemopoietic cells of the APO exhibit increased mitotic activity, and the APO undergoes visible enlargement, following infection with trematode larvae (Lie et al., 1975; Sullivan et al., 1982, 1984) or injection with extracts of larval or adult parasites (Noda, 1992; Sullivan et al., 2004; Sullivan, 2007). Moreover, previous studies have shown that transplanting the APO from schistosome-resistant to schistosome-susceptible snails resulted in increased resistance in the recipients, suggesting a role of the APO in snail resistance to infection (Sullivan and Spence, 1999).

Invertebrates rely on innate immunity for their internal defense against pathogens. To defend against a variety of microbes, one of the strategies that invertebrates employ is expression of pattern recognition receptors (PRR) that recognize relatively invariant microbial ligands called pathogen-associated molecular patterns (PAMPs). Recognition of PAMPs leads to activation of immune signaling pathways, which in turn lead to production of antimicrobial agents (Buchon et al., 2014; Hoffmann, 2003; Zipfel, 2014). Inspired by these observations, investigations have been undertaken to understand the effect of PAMPs on the APO of *B. glabrata*. It has been demonstrated that injected crude lipopolysaccharide (LPS) and crude peptidoglycan (PGN) from *Escherichia coli* (but not purified PGN) show potent mitogenic activity in the APO (Sullivan et al., 2011, 2014). A similar mitotic response occurs to the brown algal polysaccharide fucoidan (FCN), which, although not a pathogen-associated molecule, may mimic fucosylated glycans on sporocysts of *S. mansoni* (Sullivan and Belloir, 2014), and hence is referred to as a PAMP in this study.

Previous work on the APO has been conducted exclusively at the histological or ultrastructural level, and no gene expression studies have been carried out, in part due the difficulty in obtaining sufficient APO tissue for analysis. In the present study, we applied microarray technology to investigate gene expression in the APO of *B. glabrata* following challenge with LPS, FCN or PGN.

2. Materials and Methods

2.1. Snails

Adult Salvador strain (schistosome-resistant) *B. glabrata*, measuring 10–10.5 mm in shell diameter, were used for the experiments. Snails were reared in aerated aquaria containing artificial pond water at 25–27 °C and fed with lettuce as described previously (Sullivan et al., 2011).

2.2. Reagents

Three crude (i.e., not highly purified) PAMPs from commercial sources were utilized: lipopolysaccharide (LPS) from the bacterium *Escherichia coli* O127-B8 (Sigma), peptidoglycan (PGN) from *E. coli* O111-B4 (Invitrogen), and fucoidan (FCN) from the brown alga *Fucus vesiculosus* (Sigma). All three chemicals were dissolved in 1/3 mammalian-strength phosphate buffered saline (PBS) (Kodak, Rochester, NY) at a concentration of 1 mg/ml (LPS and PGN) or 10 mg/ml (FCN).

2.3. Collection, storage and transportation of APO samples

At the University of San Francisco, individual snails were injected with 5 µl of each PAMP described above or with PBS (control) through a hole in the shell and into a hemolymph sinus anterior to the digestive gland on the left side (Sullivan, 1990). At 24 hours post-injection (PI), the time of peak mitotic response (Sullivan et al., 2011), the pericardial sac was removed, and the anterior pericardial wall (or APO) was dissected free (Sullivan, 1990) (Fig. 1). As described previously, the anterior pericardial wall is fused with the lateral pericardial wall, the wall of the pulmonary cavity, saccular kidney, atrium of the heart, and albumin gland (Sullivan and Spence, 1999), and small amounts of tissue from these

adjoining structures unavoidably were included. For each treatment, 80 snails were used and divided into four pooled samples of 20 APOs each. Each sample of 20 APOs was pooled in a 1.5 ml tube containing 1,000 μ l of Trizol (Invitrogen) at 4 °C. Because on a single day only 10 snails were injected, 10 APOs were initially added to the tube, which was then frozen at -80 °C. On a subsequent day, the tube was thawed to 4 °C, and an additional 10 APOs were added. The tube was then capped, sealed with Parafilm, and refrozen at -80 °C. After collection of all samples (20 \times 4 \times 4=320 APOs), the tubes were shipped overnight on dry ice to the University of New Mexico for RNA extraction and microarray analysis. Upon arrival, the still-frozen tubes were stored at -80 °C.

2.4. RNA extraction

A preliminary experiment was performed to determine the best method for RNA extraction, and here we briefly describe our resulting procedure, which is based on a modification of the Trizol manufacturer's instructions (Invitrogen), that yielded a good amount of high quality RNA. All steps were carried out in a sterile hood equipped with a UV light. The hood was sterilized by UV for 30 min before the experiments were conducted. Sample tubes, each containing 20 APOs in 1,000 μ l Trizol, were thawed at 4 °C overnight. On the following morning, 750 μ l of the Trizol supernatant, containing cell lysate, was transferred into a fresh tube. The residual 250 μ l Trizol, with the bulk of the APO tissues, was homogenized by hand using a small plastic homogenizer. After completion of homogenization, the supernatant (750 μ l) was transferred back into the tube containing the APO homogenate, thus reconstituting the original volume. Chloroform (200 μ l) was added to each tube which was then vortexed vigorously for 2 min. The samples were kept on a rotator for 1 hour at room temperature, enabling complete dissociation of nucleoprotein complexes. Next, the samples were centrifuged at 13,000 \times g for 18 min at 4 °C. Following centrifugation, the upper aqueous phase (containing RNA) was transferred into a fresh 1.5 ml tube, taking care to not disturb the interface. Then equal volumes of isopropyl alcohol and 80% ethanol were used for precipitating and washing RNA, respectively. After removing the ethanol supernatant, the tubes were centrifuged briefly and the residual ethanol was removed with a 10- μ l pipette tip. Finally, the samples were air-dried for 2 min. The above procedure was designed to minimize the time required for evaporating ethanol and water, thereby reducing degradation and contamination of RNA samples. Once drying was finished, 15 μ l RNase-Free water (Qiagen) was added to each RNA pellet. The RNA was dissolved by gently passing the solution several times through a pipette tip. The quality and quantity of RNA collected in each tube were measured immediately with the use of an Agilent 2100 Bioanalyzer (RNA 6000 Pico LabChip kit, Agilent) and Thermo Scientific NanoDrop 2000C, respectively. After measurements were completed, the RNA samples were stored at -80 °C and were used for microarray studies within one week, to avoid decay of the purified RNA. All sample tubes were processed at the same time.

2.5. Microarray experiment

A two-color microarray-based gene expression system was used for quantitative analysis of differential gene expression (Version 6.6, Agilent Technologies). To provide a positive hybridization signal at as many elements of the array as possible, the universal reference RNA (URR) consisted of 80% from the PBS-injected group (control) and 20% from the

three experimental groups (LPS, FCN and PGN). The 12 samples in the three experimental groups contributed equally.

Microarray—A 60-nt oligonucleotide *B. glabrata* microarray with 30,647 features on a glass slide, each with duplication, was used. This array has been successfully applied to reveal molecular mechanisms underlying toxicological effects of niclosamide, the active ingredient of a widely used molluscicide, on the snail *B. glabrata* (Zhang et al., 2015). Details of microarray design are available at Gene Expression Omnibus (GEO) at the Platform Accession Number GPL20716 (www.ncbi.nlm.nih.gov/geo/). The microarray results from this study were submitted to GEO at Accession Number GSE71607.

Spike-in and Labeling—A total of 200 ng RNA was used as the starting sample. Spike A and B mix were added into the RNA samples of the experimental groups and URR, respectively. Spike A contained the cyanine dye Cy-3 whereas Spike B contained Cy-5. Complementary RNA (cRNA) amplification and labeling reactions with Cy-3 and Cy-5 were conducted at 42 °C for 2 hours, according to the protocol of Agilent. Unless otherwise mentioned, all reagents were purchased from Agilent. The labeled cRNA was purified using the RNeasy Mini Kit (Qiagen). The cRNA was quantified to determine the concentration and specific-label activity using a NanoDrop spectrophotometer.

Hybridization—300 ng of the purified Cy-3 and Cy-5 labeled cRNA were added to 10× hybridization buffer and incubated at 60 °C for 30 min to fragment RNA. 40 µl of the hybridization samples were added to a gasket chamber on a microscope slide. After all eight chambers were filled with the hybridization sample, the microarray slide was clamped in place. The assembled chamber was placed in a hybridization oven at 65 °C for 17 hours (rotating at 10×g).

Wash—After hybridization, the slides were washed carefully in Agilent's wash buffer I for 1 min at room temperature, followed by wash buffer II for 1 min (37 °C). After treatment with a stabilization buffer to protect against ozone deterioration, the dry slides were scanned with Agilent's Microarray G2505C scanner and extracted and normalized using Agilent's Feature Extraction software by the University of California San Francisco's Viral Diagnostics and Discovery Center.

2.6. Microarray data analysis

Microarray data analysis was conducted using GeneSpring GX (version 12.6.1). For quality control, the program called "Filter probesets on data" was used. Under this model, all control probes were removed. Genes were retained for further analyses when they were detected as expressed in at least 75% of all replicates from at least one treatment (experimental or control). The features that were not positive, not significant, not above background noise, not uniform, not saturated or that were population outliers were not analyzed further.

After filtration, the remaining features were further analyzed by a volcano plot (http://genespring-support.com/files/gs_12_6/GeneSpring-manual.pdf). A *t*-test using an unpaired unequal variance model under asymptotic *P*-value computation was applied. For the multiple

testing corrections, the Benjamin Hochberg FDR was employed. Corrected P -value cut-off and fold change cut-off were set at 0.05 and 2, respectively.

2.7. BlastX search for features that passed criteria

The features that passed the criteria of ≥ 2 fold change and $P < 0.05$ were selected for subsequent analyses. Based on the probe codes that showed differential expression, all the corresponding target sequences used to design the probes were selected for BlastX search and gene ontology (GO) analysis. Blast2GO (v.2.8.0) (www.blast2go.com) was used because it was able to perform both BlastX search and GO analysis. Three GO term categories, biological process, molecular function and cellular component (all at the level 3), were generated under the program.

3. Results

3.1. Quality of RNA generated from the APO

To profile transcriptomic changes using microarrays, a sufficient amount of high quality RNA obviously is a prerequisite. Meeting this requirement was a challenge, due to the small size of the APO (Fig. 1) and the length of time required to inject and dissect all 320 snails, approximately 3.5 months. By using the protocol developed in preliminary trials, we were able to harvest RNA at concentrations ranging from 350 ng/ μ l to 950 ng/ μ l. Thus, a total of 5,000 ng to 14,000 ng total RNA per 20-APO sample was obtained (equivalent to 250–700 ng per APO). More importantly, we confirmed that all RNA samples were of excellent quality (Fig. 2). No obvious differences in quality were seen among samples that were collected at different time points over the course of the experiment. For example, RNA from APOs collected at the start did not differ in terms of quality and quantity from those collected over three months later.

3.2. Pattern of transcriptomic response to the three PAMPs

Of 30,647 unique probe features presented in the array, 19,229 remained for subsequent analyses after stringent filtration for quality control was applied. As indicated by the high number of genes that were differentially expressed, all three PAMPs were able to alter gene expression, with LPS exhibiting the strongest activity. Specifically, LPS injections caused a change in gene expression in a total of 151 genes, with 106 up-regulated and 45 down-regulated. In contrast, FCN and PGN showed relatively less activity in altering gene expression, with 54 and 41 genes differentially expressed following exposure to FCN and PGN, respectively (Fig. 3).

3.3. Genes activated by LPS, FCN, or PGN

With respect to genes activated by LPS, the transcripts that passed the cut-off criteria (≥ 2 fold change and $P < 0.05$) were further analyzed by BlastX search, in which homologs of transcripts were identified (Supplementary data 1). Table 1 shows differential expression of genes encoding proteins that are activated and have known or putative functions. Highly expressed (≥ 5 fold) genes included arginase, checkpoint kinase, aminoacyl-tRNA synthetases (ARS), GTPase of immunity-associated protein (GiMAP), cytochrome P450 (CYP), peptidoglycan recognition protein short form (PGRP-SA) and tumor necrosis factor

receptor (TNFR) genes. In addition, genes for a number of enzymes that included kynurenine 3-monooxygenase, amine oxidase, threo-3-hydroxyaspartate-ammonia-lyase, cytosolic non-specific dipeptidase-like isoform, cutA, nucleoredoxin were also highly expressed. Notably, seven members of ARS, including tryptophan-, tyrosine-, asparagine-, arginine-, methionine-, lysine- and bifunctional glutamate-proline-tRNA synthetases, and four GiMAPs were repeatedly over-expressed.

With regard to down-regulation, several genes encoding proteins that are involved in lipid metabolism, such as proprotein convertase subtilisin kexin type 9, apolipoprotein, and long-chain-fatty acid ligase, were down-regulated in response LPS.

As described above, the total number of genes differentially expressed in response to either FCN or PGN was significantly lower than that observed in LPS-treated snails (Table 2, Supplementary data 2 and 3). In the snails exposed to FCN, the number of genes down-regulated is higher than that of up-regulated genes, a pattern which is in contrast to that observed in snails challenged with PGN or LPS. Many genes up-regulated in response to LPS were also up-regulated after challenge with PGN (Table 1 and 2). Interestingly, up-regulation of bacterial permeability increasing protein gene (BPI), also called lipopolysaccharide-binding protein (LBP), occurred only in PGN-injected snails.

3.4. Comparison of transcriptome profiles elicited by the three PAMPs

Among all up-regulated genes, none were induced by all three PAMPs. However, LPS and PGN shared 21 genes, the highest number found in all three comparisons (i.e., LPS-PGN, LPS-FCN and PGN-FCN). Among them were kynurenine 3-monooxygenase, tryptophan tRNA synthetase, threo-3-hydroxyaspartate ammonia lyase, cytosolic non-specific dipeptidase, cutA, bis(5-adenosyl)-triphosphatase, E3 ubiquitin-protein ligase XIAP isoform, and thymidine phosphorylase.

A similar pattern was observed in down-regulated genes. Only two genes, c34347_rc (apolipoprotein-like) and c3542 (aldehyde dehydrogenase) were down-regulated following exposure to all three PAMPs. Although a small number of genes were down-regulated by PGN, the proportion of down-regulated genes shared with the LPS group (8/14) was greater than that shared by the FCN group with the LPS group (8/42) (Fig. 4).

3.5. Gene ontology analysis

Among the large number of genes whose expression was altered by LPS, some of the same genes were also affected by FCN or PGN. GO analysis is presented here only on the genes differentially expressed in response to LPS. However, GO analysis data for all three PAMPs are provided in Supplementary data 4, 5, and 6. BlastX search revealed that approximately half (52%) of the genes differentially expressed in response to LPS have no GenBank match, and thus no homologs have been identified for those sequences. Among up-regulated genes with homologs in other organisms, GO analyses revealed that the GO term biological process of the majority of genes relates to cellular metabolism, which mainly included organic substance (17%), primary (15%), single-organism (14%), cellular (14%), and nitrogen compound (14%) metabolic processes. With regard to GO term molecular function, proteins that play roles in ligand binding represented the majority of proteins with known

functions in GenBank. These molecules included ion (16%), protein (16%), organic cyclic compound (12%), heterocyclic compound (12%) and carbohydrate derivative (12%) binding proteins. With respect to genes that were down-regulated by LPS, the distributions and proportions were very much similar to up-regulated genes (Fig. 5).

4. Discussion

4.1. Expression pattern of genes elicited by PAMPs

Except for studies involving hemocytes (Lockyer et al., 2012; Zahoor et al., 2014), studies of gene expression in snails have involved whole-body extraction of nucleic acid (Adema et al., 2010; Hanington et al., 2010, 2012; Lockyer et al., 2007, 2008; Zhang et al., 2015). The amount and quality of RNA we obtained from the APO shows the feasibility of conducting transcriptomic studies on individual snail organs. Although the present microarray contained a large number of features (31K features) (Zhang et al., 2015) compared to previous studies (i.e., arrays with 1.1K features (Adema et al., 2010) and 5K features (Lockyer et al., 2012)), it is possible additional transcriptional information remains to be captured. Genome-wide transcriptome analysis certainly provides an alternative, powerful tool for understanding gene expression in response to PAMPs for future studies. Specifically, a large scale molecular screen using next generation sequencing platforms could be applied to the APO to provide additional perspective on the developmental and molecular responses of the snail hemopoietic organ.

The present study demonstrates that the three PAMPs we employed alter gene expression in the snail APO, to varying degrees. Our findings can be compared with observations made in previous histological studies, providing the first associated molecular data to help interpret these previous results and to guide future analysis. LPS had the strongest effect on gene expression in the APO whereas PGN and FCN produced significantly less response. In previous histological studies, although LPS had slightly higher potency in inducing the mitotic division, the mitogenic activity of the three PAMPs was comparable, ranging between approximately 600 to 900 mitotic figures/APO at 24 hours PI, all significantly higher than in saline-injected controls (Sullivan et al., 2011, 2014; Sullivan and Belloir, 2014). This implies that LPS may play a broader role in activating additional responses beyond those involved in hemopoiesis (Table 1). Another interesting finding is that the patterns of the transcriptomic responses to LPS and PGN in our study have several features in common. As noted, both LPS and PGN are PAMPs that are derived from bacteria, whereas FCN is extracted from a brown alga, and is hypothesized to mimic fucosylated glycan PAMPs from *S. mansoni*. Consequently, a higher concordance among responses to bacterial PAMPs is not surprising. Moreover, some of the overlap in gene expression between LPS and PGN may be a result of the presence of LPS in crude PGN (Sullivan and Belloir, 2014).

4.2. Cell division and proliferation

The present study revealed that checkpoint kinase 1 (Chk1) was the second most highly up-regulated gene after challenge with LPS. Chk1 is a serine/threonine-specific kinase, a key regulator of mitosis. In mammalian genomes, two subtypes, Chk1 and Chk2, have been

identified (Patil et al., 2013). Although the number of *Chk* genes in the snail genome is not yet known, our results suggest that Chk1 is involved in cell proliferation in the APO. The reason for the lack of upregulation of Chk1 in APOs from snails injected with PGN of FCN, also mitogenic in previous histological studies, is not known. A potential useful application of our results is that Chk1 may serve as a genetic marker to identify other locations in the snail that produce hemocytes, as the APO may not be the sole site of hematopoiesis.

An increased rate of cell division requires active protein synthesis. We observed elevated expression of seven ARS genes, although it is unlikely that probes for all possible ARS transcripts were included in the array. ARSs are ubiquitous enzymes responsible for charging amino acids to their cognate tRNAs, providing the substrates for global protein synthesis. Simultaneous activation of seven ARSs clearly indicates high levels of protein synthesis in cells of the APO, suggesting their role in proliferation and differentiation of hemocyte precursors in the APO. Among the ARSs, two versions of tryptophan tRNA synthetase (contig_13013 and contig_6234) showed significantly high expression in LPS-injected snails, with 37- and 31-fold up-regulation, respectively. Intriguingly, these two transcripts were also highly up-regulated in PGN-challenged snails (both showing 7-fold up-regulation), as mentioned above. A concurrently high expression of the two tryptophan tRNA synthetases, but not other ARSs, implies an additional role for this enzyme. Tryptophan tRNA synthetase possesses unusual properties in eukaryotic systems. Up-regulation of tryptophan tRNA synthetase by interferon gamma was found in a number of cell types, including several human cell culture lines (Kisselev, 1993). A previous study demonstrated high expression of tryptophan-tRNA synthetase in the salivary gland of *Drosophila*, but the investigators suggested that this up-regulation did not necessarily indicate a requirement for synthesis of proteins enriched in tryptophan, and that instead this enzyme may be involved in noncanonical functions, such as immune response and control of cell growth (Seshaiah and Andrew, 1999).

4.3. Immune responses

In addition to stimulating hemocyte precursors to proliferate, presumably increasing the number of defense cells, PAMPs, particularly LPS, also elicited increased expression of a number of potential immune-related genes that include arginase, PGRP-SA, TNFR, ficolin, E3 ubiquitin ligase, BPI and GiMAPs. Among these, it is worth noting that arginase and GiMAP expression have not previously been reported in studies of protostome invertebrate immunity.

The most highly expressed gene uncovered in the study was an arginase gene. Macrophage arginase plays an important role in vertebrate immune responses and wound healing (Dzik, 2014; Munder, 2009). The molluscan hemocyte has been proposed as the invertebrate morphological and functional counterpart of the vertebrate macrophage (McKerrow et al., 1985). In mammals, there are two types of macrophages, M1 and M2. Biochemically, the two types of macrophages are characterized by expression of two enzymes, nitric oxide (NO) synthase and arginase (Rath et al., 2014). M1 macrophages express NO synthase, which metabolizes arginine to NO and citrulline. M2 macrophages are characterized by expression of arginase, which hydrolyzes arginine to ornithine and urea. Arginase and NO

synthase compete for the same substrate, L-arginine. Therefore, over-expression of arginase could interfere with NO synthase activity, subsequently diminishing formation of NO, an important immune effector. It has been demonstrated that the balance of expression between the enzymes is critical to regulate immune responses (Rath et al., 2014; Dzik, 2014).

The other striking finding is that four versions of GiMAP were upregulated concurrently. GiMAP is also called IAN (immune associated nucleotide binding protein). GiMAPs are ancient in origin, and have been lost in some animal lineages yet undergone expansions in others (Weiss et al., 2013). GiAMPs have been found to play an important role in plant defense and in regulating T-cell development and in maintaining T-cell homeostasis for normal immune function in mammals (Nitta and Takahama, 2007; Reuber and Ausubel, 1996). Recently, it has been demonstrated that GiMAPs appeared as the most prominent immune factors in the coral *Acropora millepora*, a basal metazoan, after challenge with the bacterial cell wall-derived muramyl dipeptide and polyinosinic:polycytidylic acid (Weiss et al., 2013). Still, except for mammals, a clear immune role for GiMAPs in the metazoa has not been documented. Our study provides evidence suggesting that GiMAPs play a role in immunity in mollusks.

This study also uncovered a novel fibrinogen (FBG)-related gene, namely ficolin. A large number of studies have shown that ficolins play an important role in innate immunity in vertebrates and invertebrates (Endo et al., 2015). BlastX search revealed that the snail ficolin (contig_7199) was more closely related to ficolins found in other animals than to fibrinogen-related proteins (FREPs) or fibrinogen-related molecules (FREM) described in *B. glabrata* (Zhang et al., 2004, 2008). In addition to containing a C-terminal FBG domain, FREPs and FREMs have an N-terminal IgSF (immunoglobulin superfamily) or EGF (epidermal growth factor) domain(s), respectively. However, snail ficolin does not appear to contain IgSF or EGF domains, and thus its structure and function may be different from that of FREPs or FREMs (Hanington and Zhang, 2011). A potential reason for not finding ficolin expression in the past is that our work on FREPs or FREMs focused on snail-trematode interactions. Possibly, snail ficolins may be immune molecules specifically responding to microbes rather than metazoan parasites. With respect to FBG-containing proteins, we also noted down-regulation of FREP12 in response to FCN, albeit at the threshold level (2 fold). To date, the function of FREP12 is unknown, although the full-length sequence has been characterized (Zhang et al., 2003). Nevertheless, revealing an additional molecule that contains a conserved FBG domain enhances our understanding of the evolution, diversity and function of FBG-containing molecules in snails.

PGRP-SA that was upregulated after exposure of snails to LPS was identical to the snail PGRP-SA reported previously (Zhang et al., 2007). Recently it was reported that microbes elicited elevated expression of PGRP-SA in *B. glabrata* (Deleury et al., 2012), an observation consistent with our study. In *Drosophila*, PGRP-SA is an extracellular component of the Toll-like receptor (TLR) pathway and plays a vital role in antimicrobial responses (Hoffmann, 2003). With regard to TLR and IMD (immune deficiency) pathways, two evolutionarily conserved immune pathways, a number of relevant components, including gram-negative bacterial binding protein (GNBP), PGRP-LA, NF- κ B/Relish and NF- κ B/Rel, were characterized (Zhang et al., 2007; Zhang and Coultas, 2011) and included

in the array. However, we did not detect altered expression of these components in response to PAMPs, implying no obvious involvement of the TLR or IMD pathways. As PGRPs have both PGN recognition capability and amidase activity, which can degrade PGN (Zaidman-Rémy et al., 2006), it is possible that PGRP-SA functions as an antibacterial amidase, rather than a recognition molecule for activating the TLR signaling pathway.

TNFR is involved in the Jun-N-terminal Kinase signaling pathway (JNK). The JNK pathway is a mitogen-activated protein (MAP) kinase pathway involved in the regulation of numerous physiological processes during development and in response to various stresses. This signaling pathway is highly conserved in *Drosophila*, in which the TNF ligand Eiger triggers cell death through its TNF receptor Wengen (Igaki and Miura, 2014). In mammals, LPS-induced TNF- α factor (LITAF) mediates inflammatory cytokine expression (Tang et al., 2006). In the mosquito *Anopheles gambiae*, it has been shown that LITAF is an important regulator of hemocyte differentiation and plays a key role in the mosquito late-phase immune response against *Plasmodium* parasites (Smith et al., 2015). In addition, it has been reported that TNFR is activated by bacteria or PAMPs in mollusks (Yang et al., 2011) and shrimp (Wang et al., 2012).

Recent studies have revealed a role of ubiquitylation in the regulation of immune responses in mammals (Park et al., 2014), plants (Duplan and Rivas, 2014) and insects (Severo et al., 2013). Differential expression of ubiquitin-related proteins has been described in tick cells upon infection with *Anaplasma marginale* (de la Fuente et al., 2007). Still, little is known about how ubiquitin relates specifically to vector immunology. The inducible activation of NF- κ B by a wide range of immuno-receptors such as the TLR, TNFR, and T cell and B cell antigen receptors requires the ubiquitin-triggered proteasomal degradation of I κ B α to promote the nuclear translocation and transcriptional activity of NF- κ B dimers (Skaug et al., 2009; Vandenabeele and Bertrand, 2012). We found increased expression of E3 ubiquitin-protein ligases in snails injected with LPS and PGN, suggesting their possible role in snail immunity.

A prior study reported that calmodulin, a calcium-binding messenger protein expressed in all eukaryotic cells, was upregulated in *B. glabrata* in response to three kinds of microbes, *Escherichia coli*, *Bacillus cereus* and *Saccharomyces cerevisiae* (Deleury et al., 2012). In addition, proteomics analysis also revealed that *Schistosoma mansoni* was able to induce expression of calmodulin in the cardiac and pericardial tissues of *B. tenagophila*, a species closely related to *B. glabrata* (Jannotti-Passos et al., 2008). Our study agrees with the previous findings in showing calmodulin upregulation, though the purpose of this response, immune-related or otherwise, requires clarification.

Surprisingly, we found up-regulation of the bacterial permeability increasing protein (BPI) gene in response to PGN, but not in response to LPS which is bound by BPI. BPI has been found to play a defense role in *B. glabrata* (Adema et al., 2010; Baron et al., 2013; Deleury et al., 2012; Mitta et al., 2005).

In general, exposure to abiotic and biotic substances may lead to stress responses in animals. However, we did not observe obvious stress responses in the APO, since genes encoding

heat shock proteins (HSP), the hallmark of stress response, did not appear expressed differentially. Using the same array, we found expression of six HSP genes was highly up-regulated in the whole body of *B. glabrata* in response to sublethal doses of niclosamide, the active ingredient of the molluscicide Bayluscide (Zhang et al., 2015). Therefore, it is suggested that the pattern of gene expression revealed by the present study is more indicative of immune responses, rather than stress responses, to PAMPs.

4.4. Degradation and detoxification metabolism

Exposure to xenobiotics typically induces activation of biotransformation pathways (Klaassen, 2013). Indeed, we observed differential expression of a number of genes associated with degradation or detoxification, including CYP (c29331_rc), GST (contig_6441, contig_2097), and multidrug resistance protein (c27924_rc) in snails injected with PAMPs. Not unexpectedly, a larger number of genes associated with biotransformation activities was activated by exposure to niclosamide (Zhang et al., 2015). For example, 10 CYP genes were expressed differentially in response to niclosamide, with 9 up-regulated and 1 down-regulated (Zhang et al., 2015). In contrast, only one CYP gene (c29331_rc) was up-regulated by LPS, and this gene does not correspond to any of the 10 CYP genes differentially expressed in response to niclosamide. This finding further supports our notion regarding the diversity of CYPs in snails in terms of their coding sequence and biological function (Zhang et al., 2015).

With respect to GST, two GST genes (i.e., contig_6441 and contig_2097) exhibited differential expression. Up-regulation of contig_6441 was observed in FCN- (6 fold) and LPS- (5 fold) challenged snails. Down-regulation appeared only in the contig_2097, with 6-fold and 3-fold in PGN- and FCN-injected snails, respectively (Table 1, 2). Thus, FCN exposure had opposite effects on expression of the two GST genes. Neither responsive GST noted here is the same as the GST gene (BGC02292) upregulated in response to niclosamide (Zhang et al., 2015). It seems that expression of *B. glabrata* GSTs in response to xenobiotics is complex, suggesting differences in structure and function of GSTs. In addition to their well-known ability to conjugate xenobiotics to glutathione during phase II detoxification reactions (Klaassen, 2013), GSTs are also capable of binding nonsubstrate ligands, and thereby can regulate intracellular signaling (Laborde, 2010).

A gene encoding the enzyme aldehyde dehydrogenase (ALDH) was down-regulated in all three PAMP-challenged snails. ALDH belongs to a group of enzymes that catalyze the oxidation of aldehydes, and it commonly functions in phase I detoxification in insects such as *Drosophila* (Leal and Barbancho, 1992) and the bumble bee (Xu et al., 2013). In humans, ALDHs are also important in oxidizing reactive aldehydes derived from lipid peroxidation and thereby can help maintain cellular homeostasis (Koppaka et al., 2012). In addition, it has been shown that ALDHs protect ocular surface tissues from reactive oxygen species (Chen et al., 2009).

In this study, we also observed that a gene encoding Cu/Zn superoxide dismutase (SOD) (contig_1415_rc), the enzyme that catalyzes dismutation of superoxide anion to H₂O₂, was down-regulated by FCN, but not by LPS or PGN. LPS has been found to up-regulate expression of Cu/Zn SOD in mice (Marikovsky et al., 2003) although we did not see a

similar effect of LPS in the APO of *B. glabrata*. Previous studies have demonstrated that allelic variation at the Cu/Zn SOD locus plays an important role in susceptibility of *B. glabrata* to schistosome infection (Bender et al., 2007; Blouin et al., 2013; Goodall et al., 2006). Therefore, we compared our SOD nucleotide sequence (contig_1415_rc, 1364 bp) to the complete cDNA of Cu/Zn SOD1-B allele (GenBank accession number DQ239578; 3076bp) (Goodall et al., 2006). No significant similarity between the two sequences was found, suggesting multiple members of the Cu/Zn SOD gene family with diverse biological functions exist in the snail genome.

4.5. Additional proteins

As in the case with aldehyde dehydrogenase, apolipoprotein expression was downregulated by all three PAMPs. Apolipoproteins are proteins that bind lipids to form lipoproteins. Insect apolipoprotein-III has been implicated in both detoxification of LPS and as a pattern recognition receptor in immune responses (Whitten et al., 2004), and has been shown to mediate anti-plasmodial epithelial responses in *Anopheles gambiae* (G3) mosquitoes (Gupta et al., 2010). Similarly, long chain fatty acid ligase and proprotein convertase subtilisin were down-regulated in response to LPS. These three proteins are involved in lipid metabolism, implying that LPS, a microbial lipid, may suppress expression of genes involved with lipid metabolism.

Expression of kynurenine 3-monooxygenase was highly induced by LPS (37-fold) or PGN (17-fold) in treated snails. This enzyme belongs to the family of oxidoreductases, and is involved in tryptophan metabolism. Human kynurenine 3-monooxygenase has been a target for treatment of neurodegenerative disease (Amaral et al., 2013), but little is known about its function in invertebrates. Tryptophan, which typically comprises less than 1.5% of amino acids in proteins (Henderson et al., 1962) acts as a precursor for several biomolecules in animals, including serotonin, melatonin, and niacin. Thus, our data not only indicate some commonalities in responses to LPS and PGN, as discussed above, but also suggest that both PAMPs activate tryptophan metabolism (recall also upregulated tryptophan tRNAs synthetases). The biological role of increased tryptophan metabolism in snails remains to be elucidated.

Finally, two enzymes, amine oxidase and threo-3-hydroxyaspartate ammonia-lyase, were found highly expressed in response to LPS. Amine oxidases are enzymes that catalyze the oxidation of a wide range of biogenic amines including many neurotransmitters, histamine and xenobiotic amines. They also function in controlling cell growth through degradation of diamines and polyamines (Finney et al., 2014; Høgdall, et al., 1998). The significance of the altered expression of these and other proteins listed in Tables 1 and 2 is unclear, because little is known about their biological roles in snails.

4.6. Cellular source of genes showing altered expression

Unlike a mammalian lymph node, the APO is not a discrete organ separated from surrounding structures by a connective tissue capsule, and in addition to follicles of developing hemocytes, it consists of epithelial and connective tissue cells, as well as small numbers of cells from adjacent organs. Therefore, it is possible that some of the altered gene

expression we observed is due to changes occurring in cells other than the developing hemocytes. However, histological studies have never revealed obvious morphological or proliferative changes in non-hemopoietic cells in response to challenge with nonself, nor do transplants of these other types of tissues from schistosome-resistant snails alter susceptibility of recipient snails to infection with *S. mansoni* (Sullivan and Spence, 1999). Consequently, the transcriptomic responses reported here are almost certainly due to altered gene expression in the developing hemocytes. At what stage of development this altered gene expression occurs, i.e., whether in the immature hemocytoblast (Jeong et al., 1983) or mature hemocyte about to be released into the hemolymph, is not known.

Conclusions

From the above results, we conclude the following. First, the first molecular study on the snail APO presented here shows the feasibility of such studies on the APO and on other snail tissues and organs. Second, LPS serves as a potent PAMP as it induced the highest number of differentially expressed genes. Third, LPS activation of checkpoint kinase 1 and aminoacyl-tRNA synthetases provides a molecular basis for the high mitotic activity observed in previous histological studies. Fourth, involvement of a complex immune response in the APO is inferred from up-regulation of genes with putative immune functions. Fifth, biotransformation activity in the APO was enhanced by challenge with xenobiotics, as shown by differential expression of a number of detoxification-associated molecules such as CYP, GST, and multidrug resistance protein. Finally, this study has uncovered a potential novel immunological role for arginase and GiMAP in snails. Further investigation of the functions of differentially expressed genes in the APO in response to challenge with nonself should improve our understanding of the immunological role, if any, of this enigmatic structure beyond that of a site of hemocyte proliferation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH/NIAID grants AI097967 and AI101438 and the Fletcher Jones Foundation.

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Highlights

- The first transcriptome study in the APO provides molecular evidence supporting histological observations of high mitotic activity in the APO following challenge with PAMPs.
- PAMPs elicit immune responses in the APO by up-regulating expression of immune genes and increasing proliferation of defense cells (hemocytes).
- Compared to FCN and PGN, LPS is a more potent PAMP in activating gene expression.
- Role of arginase and GiMAP in snail immunity is suggested.
- Biotransformation activity is enhanced in the APO following exposure to PAMPs.

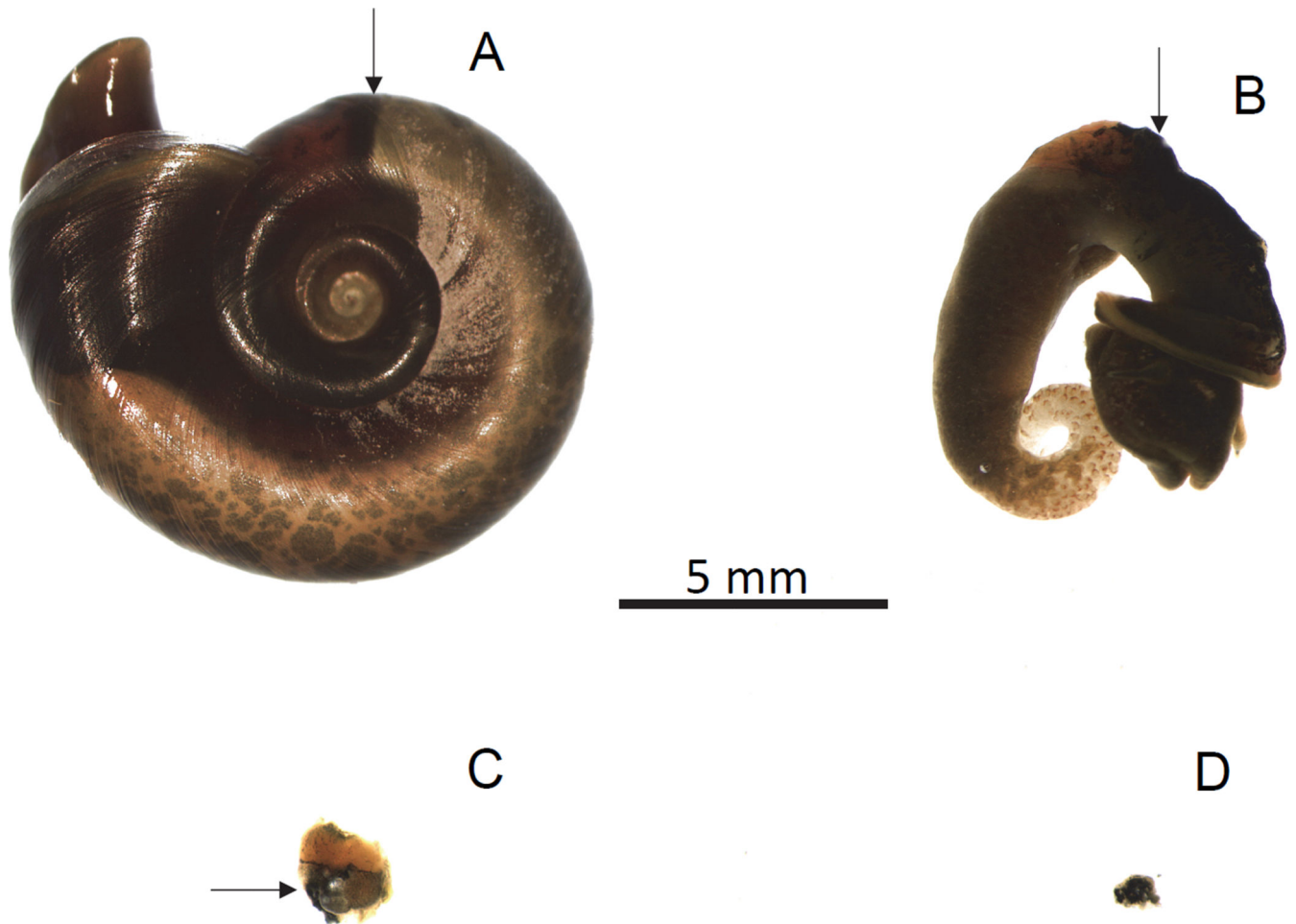


Figure 1.

Dissection of APO showing its relative size. **A)** 10.4 mm snail on dry surface. **B)** Same snail as in A, dissected from its shell and submerged in 0.03% NaCl. **C)** Anterior pericardial wall, inside view, still attached to portion of kidney above, mantle on left and exterior pericardial wall underneath. **D)** Anterior pericardial wall. Arrows show location of the anterior pericardial wall.

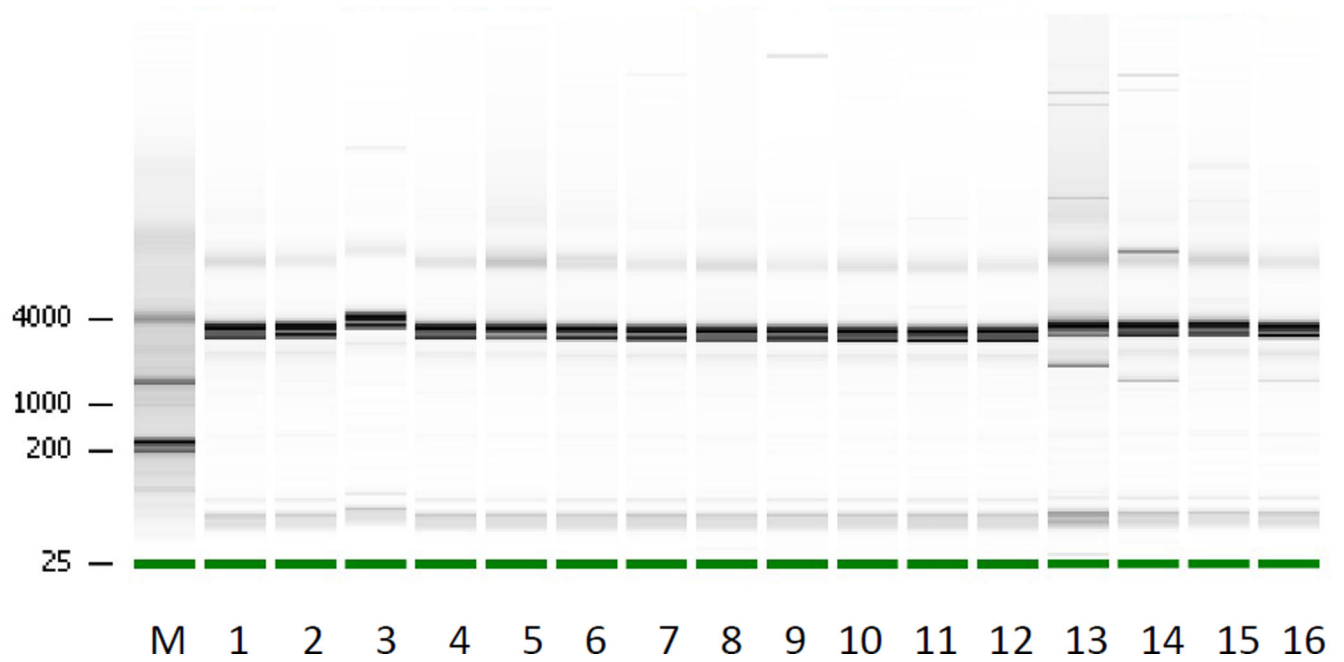


Figure 2.

High quality RNA samples generated from APOs. Gel image produced by Agilent 2100 Bioanalyzer shows quality of RNA generated for microarray analysis. Lane 1–4, 5–8, 8–12 and 13–16 show RNA samples from snails injected with PBS (control), LPS, FCN and PGN, respectively. A260/A280 ratio for all samples determined by Thermo Scientific NanoDrop 2000C is 1.9–2.1.

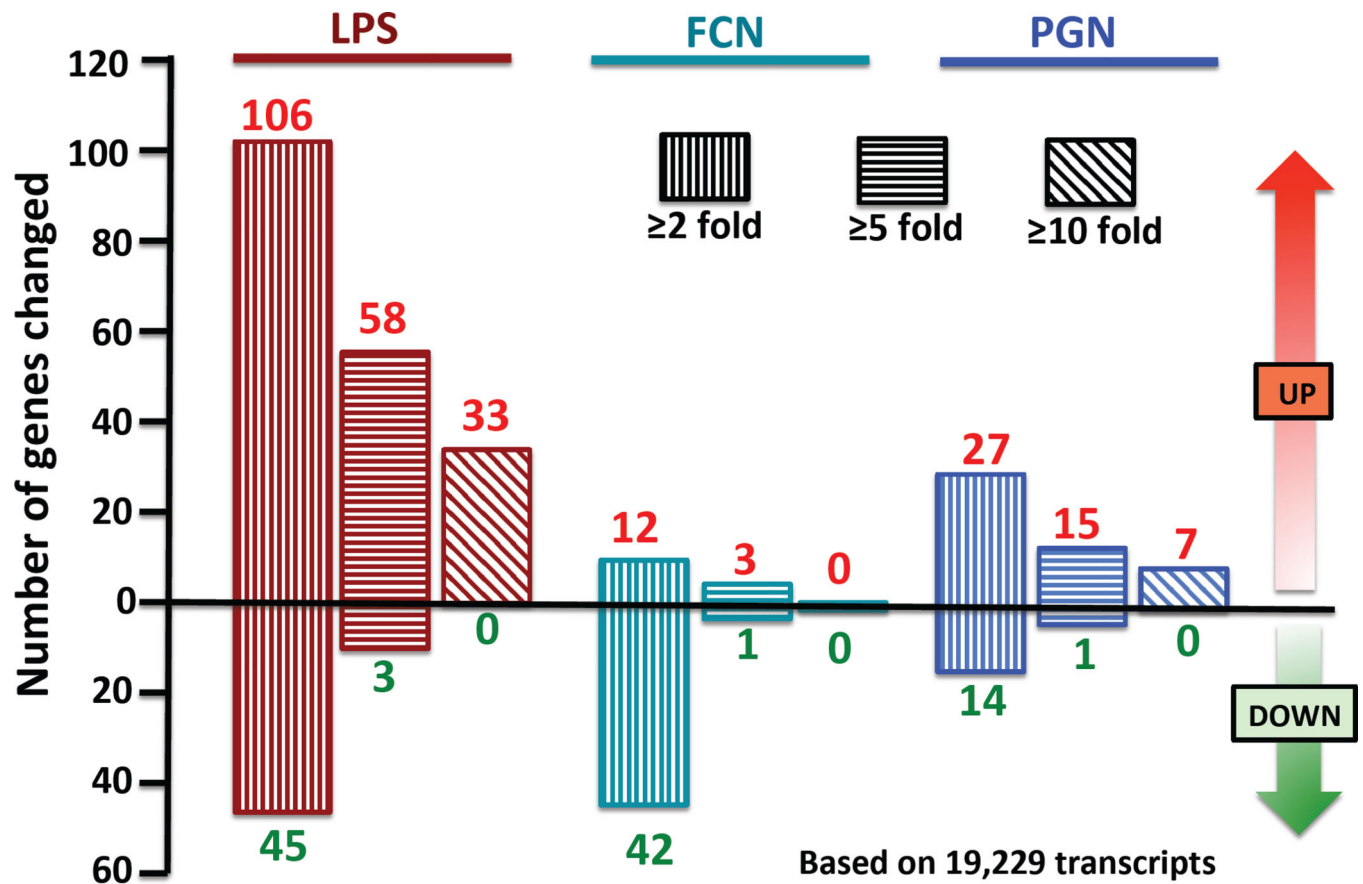


Figure 3.

The number of genes that are expressed differentially in APOs of snails that were injected by the three PAMPs.

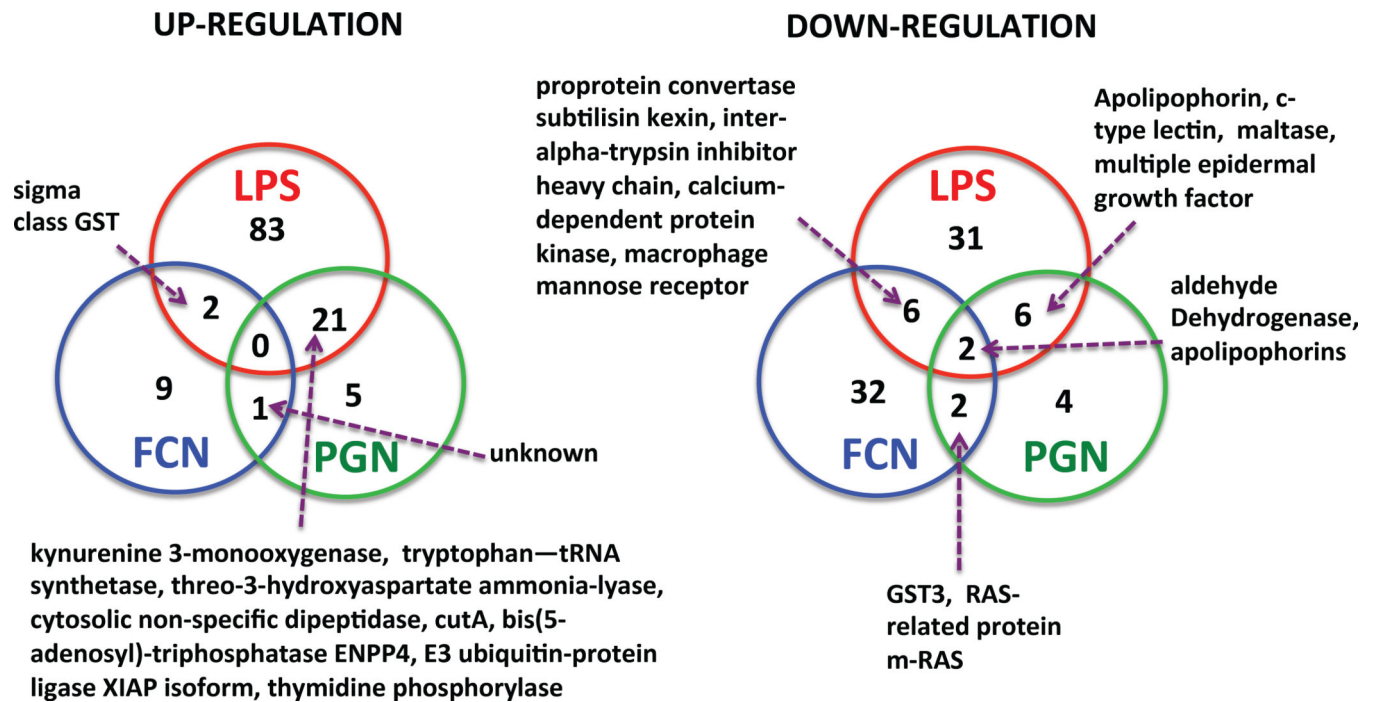


Figure 4.

Venn diagram showing an overlap of genes of either up- or down-regulated by the three PAMPs. The gene products that have homologs in other organisms are shown.

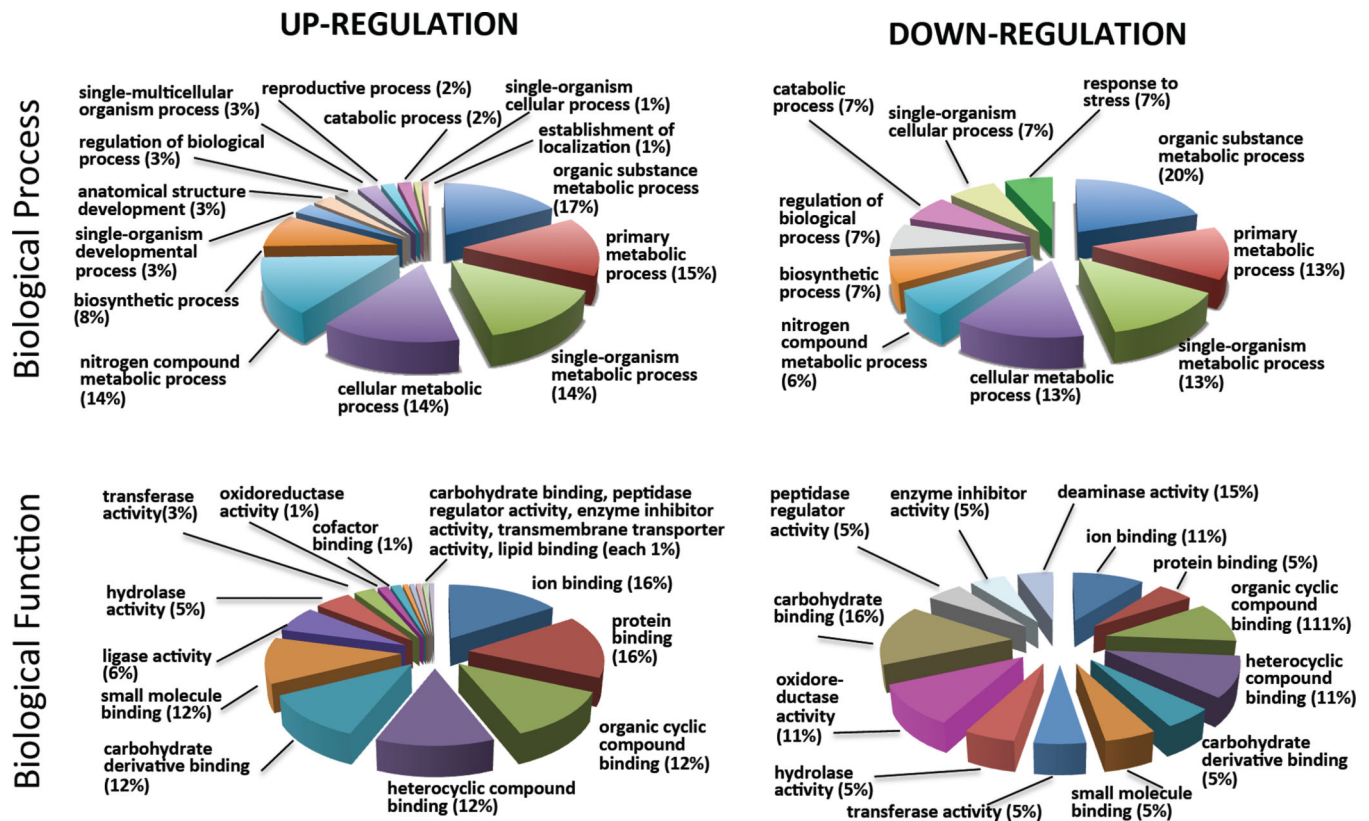


Figure 5.
Distributions of GO terms for genes elicited by LPS.

Table 1

Differential expression of genes in snails after exposed to LPS

| Up-Regulation | | | | |
|----------------------|-------------|----------|--|----------|
| Probe | Fold change | P value | Sequence Description | E value |
| contig_3728 | 75 | 8.37E-05 | arginase type I | 3.36E-74 |
| BGC00022 | 41 | 2.82E-04 | checkpoint kinase 1 | 6.04E-14 |
| contig_15126_rc | 37 | 1.62E-04 | kynurenine 3-monooxygenase-like | 5.95E-33 |
| contig_13013 | 37 | 1.98E-04 | tryptophan-tRNA synthetase, cytoplasmic-like isoform | 1.12E-80 |
| c9579 | 37 | 1.01E-05 | amine oxidase | 3.23E-85 |
| contig_6234 | 31 | 2.81E-05 | tryptophan-tRNA synthetase, cytoplasmic | 1.43E-63 |
| lrc25226_rc | 23 | 2.47E-05 | cytochrome p450 2u1 | 1.42E-19 |
| c42297_rc | 17 | 4.53E-04 | tyrosyl-tRNA synthetase | 5.38E-58 |
| contig_14397_rc | 17 | 1.32E-04 | threo-3-hydroxyaspartate ammonia-lyase | 1.61E-18 |
| BGC04403 | 15 | 3.97E-04 | cytosolic non-specific dipeptidase-like isoform | 2.45E-86 |
| c29331_rc | 15 | 2.44E-05 | cytochrome p450 2c3-like isoform | 7.16E-31 |
| c12695_rc | 14 | 7.69E-05 | threo-3-hydroxyaspartate ammonia-lyase | 5.15E-08 |
| c39491 | 13 | 2.60E-05 | GTPase IMAP family member 3-like | 2.37E-07 |
| c21101 | 13 | 6.08E-04 | cytosolic non-specific dipeptidase-like isoform | 3.00E-20 |
| contig_7508 | 11 | 1.09E-04 | GTPase IMAP family member 2 | 2.52E-39 |
| contig_4488 | 10 | 6.51E-06 | cytosolic non-specific dipeptidase-like isoform | 2.42E-67 |
| contig_12676 | 8 | 1.12E-04 | asparagine-tRNA synthetase, cytoplasmic | 4.49E-49 |
| contig_1916_rc | 8 | 2.25E-04 | peptidoglycan recognition protein short form | 2.77E-82 |
| contig_2994 | 8 | 1.84E-04 | GTPase IMAP | 4.84E-27 |
| contig_4543 | 7 | 1.88E-07 | cutA-like | 1.81E-51 |
| c11140_rc | 7 | 5.60E-05 | tumor necrosis factor receptor superfamily member | 1.70E-04 |
| contig_12935 | 7 | 2.71E-04 | nucleoredoxin-like | 1.00E-38 |
| contig_7199 | 6 | 6.07E-05 | ficolin-like | 1.50E-34 |
| c19028_rc | 6 | 2.71E-04 | ectonucleotide pyrophosphatase/phosphodiesterase family member | 1.69E-23 |
| contig_6441 | 5 | 2.71E-04 | sigma class glutathione-s-transferase 2 | 7.95E-44 |
| contig_7273 | 5 | 4.29E-06 | arginyl-tRNA synthetase, cytoplasmic | 4.22E-49 |
| c32044 | 5 | 4.72E-04 | spermine oxidase-like | 9.37E-13 |
| contig_12053_rc | 4 | 1.60E-04 | methionine-tRNA synthetase, cytoplasmic-like isoform | |
| c13927 | 4 | 6.98E-06 | bis(5-adenosyl)-triphosphatase ENPP4 | 1.02E-30 |
| c31446 | 4 | 6.00E-05 | E3 ubiquitin-protein ligase XIAP isoform | 1.32E-05 |

| | | | | |
|------------------------|---|----------|--|----------|
| c35643_rc | 3 | 3.35E-04 | methionine-tRNA synthetase, cytoplasmic-like isoform | 4.10E-13 |
| c20384 | 3 | 6.25E-05 | bifunctional glutamate proline-tRNA synthetase | 1.11E-14 |
| BGC03187 | 3 | 1.61E-04 | calmodulin 2 | 2.25E-49 |
| BGC02291 | 3 | 1.70E-04 | cystatin-a1-like isoform | 1.28E-30 |
| contig_7605 | 3 | 2.96E-06 | thymidine phosphorylase | 7.19E-21 |
| contig_5672_rc | 3 | 5.00E-06 | asparagine-tRNA synthetase, cytoplasmic-like | 2.79E-85 |
| c1620 | 3 | 2.998227 | GTPase IAMP family 2 | 2.67E-18 |
| c27924_rc | 3 | 2.16E-04 | multidrug resistance-associated protein 4-like | 2.83E-38 |
| c27924_rc | 3 | 3.00E-04 | lysine-tRNA synthetase-like isoform | 4.33E-98 |
| contig_1925 | 2 | 5.61E-04 | Zgc:153317 protein | 2.61E-29 |
| BGC00117 | 2 | 4.98E-04 | alpha-protein kinase vwka-like | 6.57E-11 |
| contig_6430 | 2 | 3.86E-04 | myelin regulatory factor-like | 9.97E-44 |
| contig_11753 | 2 | 5.00E-05 | chitinase-3-like protein 1-like | 2.50E-13 |
| Down-Regulation | | | | |
| contig_5268 | 7 | 3.67E-04 | selectin 1 | 5.36E-06 |
| contig_4393 | 7 | 1.40E-04 | proprotein convertase subtilisin kexin type 9 | 7.27E-69 |
| contig_6450 | 4 | 2.36E-04 | inter-alpha-trypsin inhibitor heavy chain H3 | 1.25E-10 |
| c32965_rc | 4 | 4.51E-04 | inter-alpha-trypsin inhibitor heavy chain H3 | 1.44E-23 |
| c17612 | 3 | 1.56E-04 | apolipoporphins-like | 1.68E-13 |
| c34347_rc | 3 | 2.14E-78 | apolipoporphins-like | 6.70E-25 |
| c10746_rc | 3 | 1.41E-04 | long-chain-fatty-acid--ligase 5 | 2.14E-78 |
| c16806_rc | 3 | 5.01E-04 | C-type lectin domain family 3 member a | 6.99E-14 |
| contig_3275_rc | 3 | 2.63E-04 | ATP-dependent RNA helicase ddx58-like | 1.66E-82 |
| c87_rc | 3 | 1.47E-04 | C-type lectin domain family 11 | 1.27E-17 |
| contig_3975 | 3 | 1.43E-04 | peptidase inhibitor 16 | 2.19E-13 |
| c3542 | 3 | 9.93E-05 | aldehyde dehydrogenase | 8.79E-61 |
| c18878_rc | 3 | 4.22E-04 | maltase-intestinal | 3.75E-19 |
| c31505 | 3 | 1.06E-04 | long-chain-fatty-acid-ligase 1 | 6.99E-24 |
| c7502_rc | 2 | 1.44E-04 | proton myo-inositol co-transporter-like isoform | 6.66E-24 |
| contig_13623 | 2 | 9.63E-05 | multiple epidermal growth factor-like domains 10 | 1.39E-19 |
| c14865 | 2 | 4.99E-04 | calcium-dependent protein kinase 5 | 1.81E-24 |
| Bcscontig_0033 | 2 | 8.45E-07 | phosphoglycerate kinase | 3.19E-60 |
| BGC04266 | 2 | 1.93E-04 | macrophage mannose receptor 1 | 2.37E-23 |
| c10235 | 2 | 5.05E-04 | CDK5 regulatory subunit-associated protein 1 | 2.24E-20 |

| | | | | |
|-----------|---|----------|--|----------|
| c19138_rc | 2 | 1.05E-04 | general transcription factor IIH subunit 3-like | 2.17E-93 |
|-----------|---|----------|--|----------|

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Table 2

Differential expression of genes in snails after exposed to FCN or PGN

| FCN: Up-Regulation | | | | |
|-----------------------------|---|----------|--|-----------|
| contig_6441 | 6 | 4.21E-04 | sigma class glutathione-s-transferase 2 | 7.95E-44 |
| c25173_rc | 5 | 8.90E-06 | transcription factor sp8 | 2.41E-06 |
| contig_2190_rc | 4 | 1.80E-06 | kruppel-like factor | 9.37E-47 |
| c3148_rc | 3 | 3.54E-04 | fibrillin-1 | 3.69E-17 |
| c10946 | 2 | 3.01E-06 | arginine-tRNA synthetase, cytoplasmic | 1.04E-106 |
| FCN: Down-Regulation | | | | |
| contig_4393 | 5 | 4.17E-04 | proprotein convertase subtilisin kexin type | 7.27E-69 |
| BGC04266 | 4 | 3.47E-04 | macrophage mannose receptor 1 | 2.37E-23 |
| BGC04703 | 3 | 1.57E-04 | kunitz protease inhibitor | 1.14E-50 |
| c454_ctg_0562 | 3 | 8.15E-05 | serine carboxypeptidase CPVL-like | 3.52E-106 |
| contig_6105 | 3 | 1.66E-04 | SPTR prohormone /whitnin | 2.14E-42 |
| c3542 | 3 | 5.20E-05 | aldehyde dehydrogenase | 8.79E-61 |
| c34347_rc | 3 | 8.90E-06 | apolipoporphins-like | 6.70E-25 |
| contig_1415_rc | 3 | 8.90E-06 | superoxide dismutase | 2.62E-38 |
| c42991 | 3 | 2.59E-04 | peptidyl-prolyl CIS-trans isomerase c | 3.50E-09 |
| lrc21214 | 3 | 2.59E-04 | carbonyl reductase | 7.66E-35 |
| contig_2097 | 3 | 8.72E-06 | glutathione-s-transferase 3-like | 9.03E-49 |
| contig_11028 | 3 | 4.35E-04 | cell growth-regulating nucleolar isoform | 9.56E-42 |
| contig_4240_rc | 3 | 3.35E-07 | p21-activated protein kinase-interacting protein 1 | 1.84E-11 |
| FREP12_1 | 2 | 7.60E-05 | fibrinogen-related protein 12 | 1.86E-23 |
| c10976 | 2 | 1.43E-04 | surfeit locus protein 2 | 1.15E-37 |
| 4.53E-05 | 2 | 1.57E-05 | cytidine deaminase | 7.99E-57 |
| c6857_rc | 2 | 8.84E-05 | RNA exonuclease 4 | 1.92E-18 |
| contig_15610 | 2 | 4.53E-05 | nucleolar protein 12 | 4.80E-47 |
| c21994_rc | 2 | 4.47E-04 | dentin sialophospho isoform | 2.17E-17 |
| contig_14398 | 2 | 4.63E-05 | protein-lysine methyltransferase | 1.96E-36 |
| c454_ctg_0683 | 2 | 1.35E-04 | actin-binding protein | 3.48E-17 |
| c4544 | 2 | 1.35E-04 | RNA exonuclease 4 | 5.16E-114 |
| c14865 | 2 | 2.92E-04 | calcium-dependent protein kinase 5 | 1.81E-25 |
| c14865 | 2 | 7.22E-05 | annexin a11 | 1.81E-25 |
| c40718_rc | 2 | 7.74E-05 | kazrin | 3.42E-18 |
| contig_9514 | 2 | 4.44E-04 | transmembrane protein 223 | 3.42E-18 |
| contig_6450 | 2 | 1.97E-04 | inter-alpha-trypsin inhibitor heavy chain h3 | 1.25E-10 |

| | | | | |
|-----------------------------|----|----------|---|----------|
| contig_5169 | 2 | 1.13E-06 | bifunctional polynucleotide phosphatase kinase | 2.57E-31 |
| PGN: Up-Regulation | | | | |
| contig_15126_rc | 17 | 3.94E-05 | kynurenine 3-monooxygenase | 5.95E-33 |
| contig_14397_rc | 15 | 1.33E-04 | threo-3-hydroxyaspartate-ammonia-lyase | 1.61E-18 |
| c12695_rc | 14 | 7.81E-05 | threo-3-hydroxyaspartate ammonia-lyase | 5.15E-08 |
| contig_4488 | 8 | 1.63E-06 | cytosolic non-specific dipeptidase | 2.42E-67 |
| BGC00933 | 7 | 2.59E-06 | bactericidal permeability-increasing protein | 5.41E-05 |
| contig_13013 | 7 | 2.11E-06 | Tryptophan-tRNA synthetase cytoplasmic | 1.12E-80 |
| contig_6234 | 7 | 1.32E-05 | tryptophan-tRNA synthetase,cytoplasmic | 1.43E-63 |
| contig_4543 | 3 | 3.11E-06 | cutA-like precursor | 1.81E-51 |
| c31446 | 3 | 4.64E-05 | E3 ubiquitin-protein ligase XIAP isoform | 1.32E-05 |
| c13927 | 3 | 1.44E-05 | bis(5-adenosyl)-triphosphatase ENPP4 | 1.02E-30 |
| contig_7605 | 2 | 1.54E-04 | thymidine phosphorylase | 1.02E-30 |
| PGN: Down-Regulation | | | | |
| contig_2097 | 6 | 6.37E-05 | glutathione-s-transferase 3 | 9.03E-49 |
| c34347_rc | 4 | 8.96E-06 | apolipoporphins-like | 6.70E-25 |
| contig_13623 | 3 | 4.37E-05 | multiple epidermal growth factor-like domains 10 | 1.39E-19 |
| c17612 | 3 | 1.68E-13 | apolipoporphins | 1.68E-13 |
| c87_rc | 3 | 1.56E-04 | C-type lectin domain family 11 member a | 2.68E-15 |
| c18878_rc | 3 | 3.05E-06 | maltase-intestinal | 3.75E-19 |
| c3542 | 3 | 1.89E-04 | aldehyde dehydrogenase | 8.79E-61 |
| c9967_rc | 2 | 1.77E-04 | RAS-related protein M-RAS | 1.56E-27 |
| c37416 | 2 | 1.61E-04 | NF-kappa-B inhibitor-interacting RAS-like protein 1 | 2.21E-39 |
| contig_3537 | 2 | 2.11E-04 | collagen alpha-1 chain | 1.43E-54 |