



Associations between transcriptional changes and protein phenotypes provide insights into immune regulation in corals



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ABSTRACT

Disease outbreaks in marine ecosystems have driven worldwide declines of numerous taxa, including corals. Some corals, such as *Orbicella faveolata*, are particularly susceptible to disease. To explore the mechanisms contributing to susceptibility, colonies of *O. faveolata* were exposed to immune challenge with lipopolysaccharides. RNA sequencing and protein activity assays were used to characterize the response of corals to immune challenge. Differential expression analyses identified 17 immune-related transcripts that varied in expression post-immune challenge. Network analyses revealed several groups of transcripts correlated to immune protein activity. Several transcripts, which were annotated as positive regulators of immunity were included in these groups, and some were downregulated following immune challenge. Correlations between expression of these transcripts and protein activity results further supported the role of these transcripts in positive regulation of immunity. The observed pattern of gene expression and protein activity may elucidate the processes contributing to the disease susceptibility of species like *O. faveolata*.

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1. Introduction

Marine disease outbreaks have become one of the most serious threats to marine ecosystems (Burge et al., 2014; Harvell et al., 1999, 2004; Ward and Lafferty, 2004). Increases in marine disease have affected a diversity of taxa including: turtles, corals, marine mammals, mollusks, and urchins (Ward and Lafferty, 2004). Scleractinian corals form the basis of coral reefs (Bozec et al., 2013; Graham and Nash, 2013), which are some of the most diverse ecosystems on the planet (Bellwood and Hughes, 2001; Bellwood et al., 2006; Odum and Odum, 1955; Renema et al., 2008; Roberts, 1995; Sebens, 1994). However, coral reefs are currently in decline due to losses in coral coverage as a result of increasing disease prevalence (Daszak et al., 2001; Harvell et al., 1999; Sutherland et al., 2004).

Some corals, such as the major reef building Caribbean coral *Orbicella faveolata*, are particularly susceptible to disease (Sutherland et al., 2004; Weil, 2004; Weil and Rogers, 2011).

O. faveolata is affected by as many as eight different diseases, including single pathogen bacterial diseases such as white plague type II, as well as microbial consortium diseases such as yellow band, black band, red band and dark spot syndrome, and diseases of unknown cause such as tumor growth syndrome (Weil, 2004; Weil and Rogers, 2011). Outbreaks of these diseases, coupled with other stressors, have driven massive declines in populations of this species across the Caribbean (Bruckner and Hill, 2009; Nugues, 2002; Weil et al., 2009). Our understanding of *O. faveolata* susceptibility is limited due to a lack of knowledge in coral immunity.

Components of each of the three main processes of invertebrate immunity (i.e. pathogen recognition, signaling pathways, and effector responses) have been documented in corals and are essential to the host's defense against disease (Palmer and Traylor-Knowles, 2012). Pattern recognition receptors (PRRs) are essential for pathogen recognition in invertebrate systems. These molecules recognize and bind to potential microbial pathogens, triggering molecular pathways and inducing immune responses through signaling pathways (Akira and Takeda, 2004; Akira et al., 2006; Janeway and Medzhitov, 2002; Kawai and Akira, 2011; Kumar et al., 2011; Takeuchi and Akira, 2010). Signaling pathways consist of a number of intermediate molecules which promote the necessary changes in gene expression and protein activity to generate an

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effective defense response against potential pathogen(s) (Aderem and Ulevitch, 2000; Akira and Takeda, 2004; Akira et al., 2006; Arthur and Ley, 2013; Newton and Dixit, 2012; O'Shea and Plenge, 2012). This chain of immune events is recognized as the “effector responses”. Effector responses include, but are not limited to, the production of antimicrobial compounds and the activation of phagocytic cells (Aderem and Ulevitch, 2000; Medzhitov, 2007; Underhill and Ozinsky, 2002).

Representative segments of all three immune processes have been described in corals and other cnidarians. A number of different immune receptors have been identified, including Toll-like receptors (TLRs) (Burge et al., 2014; Franzenburg et al., 2012; Miller et al., 2007; Poole and Weis, 2014; Shinzato et al., 2011; Wolenski et al., 2011), and various types of lectins (Hayes et al., 2010; Kvennefors et al., 2010; Schwarz et al., 2007; Wood-Charlson and Weis, 2009). Unfortunately, there is a paucity of data supporting their functional role in the recognition of pathogens in coral systems (Burge et al., 2013; Libro et al., 2013). In addition to signaling pathways, such as the melanin synthesis (Mydlarz et al., 2008; Palmer et al., 2008) and complement pathways (Brown et al., 2013; Miller et al., 2007), corals and other cnidarians, have been found to produce a number of immune effector responses such as the antioxidants catalase (Dash and Phillips, 2012; Merle et al., 2007; Palmer et al., 2011), peroxidase (Mydlarz et al., 2009; Mydlarz and Harvell, 2007; Palmer et al., 2011), and superoxide dismutase (Couch et al., 2008; Dash et al., 2007; Richier et al., 2003), as well as antimicrobial compounds (Jensen et al., 1996; Vidal-Dupiol et al., 2011a, 2011b).

Advancements of next generation technology have led to rapid increases in our understanding of coral genomics and transcriptomics (Barshis et al., 2013, 2014; Miller et al., 2011; Palumbi et al., 2014; Pinzon et al., 2015; Shinzato et al., 2011). Many studies have examined modulation of coral immunity in response to a variety of stressors, including immune challenge (Anderson et al., 2016; Burge et al., 2013; Libro et al., 2013; Libro and Vollmer, 2016; Pinzon et al., 2015; Weiss et al., 2013; Wright et al., 2015). Additionally, many studies have described and documented changes in immune proteins associated with the coral response to disease (Couch et al., 2008; Mann et al., 2014; Mydlarz and Harvell, 2007; Mydlarz and Palmer, 2011; Vidal-Dupiol et al., 2011a, 2011b). However, few studies have used both a genomic and protein-based approach to study coral immune response, resulting in limited understanding of the connections between changes in gene expression and phenotypic response.

In order to better understand the genomic mechanisms underlying the coral immune response, and observed phenotypic differences in this non-model system, this study experimentally exposed colonies of *Orbicella faveolata* to bacterial pathogen-associated molecular patterns (PAMPs) and examined associated changes in both protein activity and gene expression. By leveraging existing knowledge and well-developed protein activity assays, gene expression and protein activity data were correlated to one another using novel analytical techniques in order to better understand the connections between gene expression and proteins. Using these techniques we show that complementing gene expression with protein activity data can provide new insight regarding the response of corals to immune challenge and provide a more holistic image of coral response to immune challenge.

2. Methods

2.1. Sample collection

Coral fragments were collected in July of 2012 from five randomly selected *Orbicella faveolata* colonies from on Media Luna

reef (17° 56.096 N; 67° 02.911 W) near La Parguera, Puerto Rico. Six small fragments (5 × 5 cm) were chipped off randomly from each colony with a hammer and chisel for a total of 30 fragments. Upon collection, the fragments were placed in labeled zip-lock bags and transported in ambient seawater to an indoor running saltwater facility at the Dept. of Marine Sciences (University of Puerto Rico – Mayagüez in Isla Magueyes). At the facility, three fragments from each colony were randomly assigned to one of the two treatment groups (control and PAMP exposure).

Five fragments from the same treatment were placed in one of six large plastic containers. Each container was aerated using an electric air pump and supplied with continuous flow of seawater. To control for temperature, the water was initially contained in a 500 gallon barrel where the temperature was maintained at 26 °C using electric heaters and chillers when needed. Overhead lamps were used to maintain a 12 h light/dark cycle. Fragments were maintained in these conditions for seven days prior to experimentation to allow for acclimatization and healing from fragmentation.

2.2. Experimental design

Following the acclimatization period, continuous water flow and aeration were ceased and water levels in each of the large containers reduced to 3L. A piece of PVC pipe (6.35 cm high and 5.08 cm wide) was placed around each coral fragment, making a temporary microenvironment. Using a micropipette, 1 mL of 7.57 mg/mL lipopolysaccharides (LPS), a pathogen-associated molecular pattern (PAMP), from *Escherichia coli* 0127:B8 (Sigma-Aldrich L3129-100MG) was added just above the surface of each treatment fragment. Final concentration of LPS in the container was 10 µg/mL spread over the five fragments on each PAMP exposure container. Control fragments received 1 mL of sterile seawater used in preparation of the LPS solution.

Exposure conditions were maintained for 30 min to ensure the LPS was taken into the coral, after which the aeration was resumed. Then the fragments were allowed in continuous flow for an additional 4 h before being removed and frozen in liquid nitrogen. All samples were shipped on dry ice to the University of Texas at Arlington, where they were divided in two, leaving a small (~1 cm²) piece for RNA extraction and the rest for protein extraction. Samples were then stored at –80 °C until tissues were collected.

2.3. Protein extraction

Proteins were extracted over ice using a Paansche airbrush (Chicago, IL, USA) with coral extraction buffer (50 mmol tris buffer, pH 7.8, with 0.05 mmol dithiothreitol). Tissues were then homogenized using a Power Gen 125 tissue homogenizer with a medium saw tooth generator (Fisher Scientific, Pittsburgh, PA, USA) for 60 s on ice. Samples were then left on ice for 10 min. From the resulting extract, 1 mL was reserved for melanin analysis. The remaining volume was centrifuged for 5 min at 4 °C and 3500 RPM in an Eppendorf centrifuge 5810R. The resulting supernatant, or coral extract, was split into two ~2 mL aliquots mL which were frozen in liquid nitrogen and stored at –80 °C (Mydlarz and Palmer, 2011).

Total protein in each sample was determined using the Red660 protein assay (G Biosciences, St. Louis, MO) standardized to BSA. These concentrations were used to standardize all biochemical assays conducted on the samples. All colorimetric assays were run in duplicate on 96 well plates using a Synergy two multi-detection microplate reader and Gen5 software (Biotek Instruments, Winooski, VT, USA).

2.4. Prophenoloxidase cascade assays (PPO and MEL)

Prophenoloxidase was measured by diluting 20 μ L of coral extracts in 20 μ L of 50 mM phosphate buffer, pH 7.0. Samples were incubated with 25 μ L trypsin (0.1 mg/mL) for 30 min at room temperature. After incubation, 30 μ L of 10 mM Dopamine was added to each well. Absorbance of the product was then read every minute for 20 min at 490 nm. PPO activities were calculated as the change of absorbance (final-initial) per mg protein per minute at the steepest point of the 20 min curve (Mydlarz and Palmer, 2011).

The portion of extract reserved for melanin analysis was lyophilized in a tared two mL microcentrifuge tube overnight. Following removal of the water contact, the total dry tissue weight in the 1 mL aliquot was determined. Then, a spatula full of glass beads (~200 μ L in volume) was added to each tube and vortexed for 10 s. Lastly, 400 μ L of 10 M NaOH was added and the tubes were vortexed for 20 s. Tubes were incubated for 48 h at room temperature in the dark. After incubation, the tubes were centrifuged for 10 min at room temperature at 1000 RPM. The supernatant (40 μ L) was transferred to a 1/2 well UV plate (Costar, Corning Life Sciences, Lowell, MA, USA). Absorbance was recorded at 410 and 490 nm and a standard curve of melanin dissolved in 10 M NaOH was used to convert absorbance to mg melanin (Mydlarz and Palmer, 2011). The results are presented as μ g of melanin per mg of tissue.

2.5. Antioxidant assays

Activity of three antioxidants was assessed: catalase (CAT), peroxidase (POX), and superoxide dismutase (SOD). Catalase activity was measured by adding 5 μ L sample and 45 μ L 50 mM phosphate buffer, pH 7.0 to a UV transparent microtiter plate (Costar, Corning Life Sciences, Lowell, MA, USA). Subsequently, 75 μ L of 25 mM hydrogen peroxide was added to each well to initiate the reaction. Absorbance at 240 nm was determined every 30 s for 15 min. Scavenged H₂O₂ was calculated by subtracting the final absorbance from the initial during the linear portion of the curve. A standard curve of hydrogen peroxide was used to determine the concentration (mM). Results were standardized by mg of protein (Mydlarz and Palmer, 2011).

To measure peroxidase, 20 μ L of sample were diluted in 10 mM phosphate buffer, pH 6.0. Then 50 μ L of 25 mM guaiacol (Sigma-Aldrich) in 10 mM phosphate buffer, pH 6.0 were added to each well. The reaction was initiated with 20 μ L of 20 mM hydrogen peroxide and optical density was measured every minute for 15 min at 470 nm. Results were calculated as change in absorbance normalized to mg of protein per minute, (Mydlarz and Harvell, 2007).

The SOD Assay Determination Kit-WST (Fluka, Switzerland) was used to measure superoxide dismutase activity (Mydlarz and Palmer, 2011). The procedure followed the manufacturer's protocol, briefly, 10 μ L of coral extract was incubated with 10 μ L deionized water, 200 μ L WST-1 and 20 μ L xanthine oxidase for 20 min at 37 °C. Percent inhibition of absorbance was then measured at 450 nm by comparing the absorbance of the samples to that of the control wells. Activity is reported as superoxide dismutase activity standardized by mg protein (Mydlarz and Palmer, 2011).

2.6. Antibacterial activity

Antibacterial activity of each sample was assessed against *Vibrio alginolyticus* using a strain isolated from *O. faveolata* (Strain provided by K. Ritchie, Mote Marine Laboratory, GenBank #X744690). This bacterial species has been proposed as the putative causative agent of Yellow Band Disease (Cervino et al., 2004, 2008). Bacteria were grown overnight in salt amended (2.5% NaCl) Luria Broth

(EMD Chemicals, Gibbstown, NJ) and diluted to a final optical density of 0.2 at 600 nm prior to use in the assay. Sample wells consisted of 140 μ L of each sample and 60 μ L of diluted bacteria. Plates were then incubated in the spectrophotometer for 6 h at 29 °C, with reads at 600 nm recorded every 10 min. Growth rate of the bacteria was determined as the change in absorbance during the logarithmic phase of growth (Pinzon et al., 2014).

2.7. RNA extractions and sequencing

Total RNA was extracted from a small fragment (~3–5 μ g) of tissue and skeleton using the RNAqueous Kit with DNase step kit (Life Technologies AM1914). Fragments were initially ground with 800 μ L of Lysis Buffer in a 2 mL microcentrifuge tube for 2 min. The tube was then centrifuged on an AccuSpin Micro (Fisher Scientific) and 700 μ L of the supernatant were removed and placed in a new tube with 700 μ L of 64% Ethanol solution from the kit. The resulting solution was passed through the kit's column and washed once with the wash one solution and twice with the wash two solution. The column was spun dry for 2 min and the resulting RNA was eluted with 50 μ L elution solution. Possible DNA contamination was removed from samples by mixing 25 μ L of the final extract with 1.5 μ L DNase solution and 2.95 μ L of Master Mix and incubating at 37 °C for 1 h. This solution was then incubated at room temperature for 2 min with 2.95 μ L inactivation reagent. The final extract (~30 μ L) was transferred to a new tube and stored at –80 °C.

2.8. cDNA library prep and sequencing

Following extraction the three replicates from each colony within a treatment were pooled for RNA library sequencing, due to budgetary constraints (n = 5 per treatment). The quality of the combined extract was assessed using an Agilent BioAnalyzer 2100 at the University of Texas at Arlington Genomics Core facility. Samples with quality values (RIN number) greater than 8 were processed for cDNA library creation using an Illumina TruSeq RNA with Poly-A selection libraries kit (Illumina). Combined samples were sent to the University of Texas Southwestern Medical Center Genomics Core facility for library construction and sequencing. All 10 libraries were sequenced on a single lane.

2.9. Transcriptome assembly and RNA-seq analysis

RNA-seq libraries were sorted and assessed for quality of reads. Adaptors and low quality reads were removed using Trimmomatic v. 3 (Bolger et al., 2014). Non-host sequences were filtered out using methods described in Pinzon et al., 2015. All alignments were performed using BLAT (parameters: 90% identity and e-value < 0.000001). Duplicate hits were removed and coral-only sequences were retrieved for further analysis using cdbfasta/cdbbank (<http://sourceforge.net/projects/cdbfasta/>).

Differential expression analyses were conducted using Cufflinks software package (v. 2.2.1; (Trapnell et al., 2013)). Read counts for each transcript were obtained by aligning coral only reads from each sample to the *O. faveolata* reference transcriptome (Pinzon et al., 2015) using default parameters in the Cufflinks package (Trapnell et al., 2013). Normalized expression values were then generated in the Cuffdiff package with the default parameters (Trapnell et al., 2013). These normalized expression values were then compared between treatment groups using default parameters of the Cuffdiff package to estimate average log₂fold change of each transcript. These log₂fold change values were used to identify significantly differentially expressed transcripts (adjusted p < 0.05) across treatments.

2.10. Transcriptome annotation and gene ontology (GO) analyses

Following transcriptome assembly, transcriptomic sequences were annotated against the UniProtKB/Swiss-Prot and TrEMBL databases (blastx algorithm, $1.0E-5$ e-value threshold). Enriched GO terms associated with the differentially expressed transcripts were identified using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v. 6.7. Analyses were performed using the Biological Processes domain of annotations. A list of UniProt accession numbers for all annotated transcripts in the transcriptome was used as a background. GO terms with a Benjamini-Hochberg adjusted p -value lower than 0.05 were considered significantly enriched.

2.11. Weighted co-expression network analysis

Gene expression data was analyzed using the Weighted Co-Expression Network Analysis (WGCNA) R package (Langfelder and Horvath, 2008) to identify modules of co-expression between transcripts and proteins for all samples combined. Normalized read counts for all differentially expressed transcripts were \log_2 transformed. These values were used to create a signed, consensus network using automatic network construction methods (parameters: soft power = 20, minimum module size = 8, deep split = 2, merged cut height = 0.4, minimum KE for module assignment = 0.2, verbose = 3, cutHeight = 0.999). Eigengene values (average expression of all transcripts in a module) of each module were correlated to results from the five biochemical assays using a Pearson correlation. Correlations were conducted on two sets of samples: all of the samples ($n = 10$) and samples from the PAMP exposure only ($n = 5$). Modules with significant correlations (p -value ≤ 0.05) were selected for further analyses. Gene ontology (GO) enrichment for each significant module was conducted in DAVID using SwissProt IDs (spIDs) for all annotated transcripts in a given module, with spIDs of all significantly differentially expressed transcripts used as a background.

2.12. Statistical analysis: gene expression to phenotype correlations

To account for potential intracolony variation this experiment was run with three replicates per colony. Proteins were extracted and assayed for each replicate. However, as mentioned above, RNA extracts from each of the three replicates from each colony within a treatment were pooled for RNA library sequencing (resulting in $n = 5$). In order to compare gene expression with protein data, protein data was averaged for each colony and treatment group, resulting in a sample size of 5 for this analysis.

All transcripts belonging to modules with significant correlations to one or more measures of immunity were assessed for correlations to protein activity from one of the assays performed in this study, herein referred to as 'immune phenotypes'. While there were no statistical differences between the controls and corals exposed to PAMPs within the biochemical assays, we used this data to examine the possible correlation between gene expression and the activity of immune proteins. \log_2 transformed normalized (FPKM) read counts from cufflinks for each transcript of interest were correlated to values for each of the immune assays using a Pearson correlation. Correlations were calculated using the same two sets of samples used for module to trait correlations (all samples i.e. controls and PAMP exposure, $n = 10$, or the PAMP exposed subset of samples, $n = 5$). Two separate sample sets were used in order to identify transcripts, such as effector molecules, which are correlated to immune protein activity under all conditions, as well as transcripts, such as receptors, which would only be correlated to immune protein activity when in the presence of an elicitor such as

bacterial PAMPs.

3. Results

3.1. Transcriptome assembly

Sequencing of all ten samples (5 control, 5 PAMP treated) resulted in a total of 120,020,664 paired end reads. Sequencing reads are available for download on NCBI (SRA Accession # SRP044398). Final alignment of quality assessed and trimmed reads to the *O. faveolata* reference transcriptome resulted in consensus 31,958 contigs with an N50 value of 1928 base pairs. Comparison of these consensus sequences to the UniProtKB/Swiss-Prot database yielded annotations for 10,638 (~33%) transcripts.

3.2. Differential expression and gene ontology analysis

Of the 31,947 expressed transcripts, 371 were differentially expressed, 111 downregulated and 260 upregulated in the PAMP-exposed compared to control samples. Only 40% (149) of these genes could be annotated against UniProt and TrEMBL protein databases (Supplementary Table 1). Gene ontology analyses of these 149 annotated differentially expressed transcripts found no enriched biological processes. However, from this set of transcripts, we identified 17 involved in immune processes (Fig. 1, Table 1). These immune-related transcripts could be categorized as one of three processes of immunity: recognition, signaling, and effector response.

3.2.1. Immune recognition

Seven putative immune recognition transcripts were differentially expressed upon exposure to immune challenge: galaxin, an apolipoprotein, a transcript containing a leucine-rich repeat (LRR) domain, and four mucin-like proteins, Galaxin, which may have a dual function as a recognition molecule (Heath-Heckman et al., 2014), decreased in expression 1.5 \log_2 fold in samples exposed to PAMPs. The homolog of the insect pattern recognition molecule apolipoprotein (Whitten et al., 2004; Zdybicka-Barabas and Cytryńska, 2013) increased in expression by 1.4 \log_2 fold. Additionally, a protein containing a LRR-domain, which is characteristic of immune receptors such as TLRs (Buchanan and Gay, 1996), decreased in expression by 1.5 \log_2 fold in samples exposed to bacterial PAMPs. Finally, we documented four mucin-like proteins that were significantly differentially in our treated samples. Mucin domains are common to immune receptors in the TIM family (T cell, immunoglobulin, mucin) (Anderson et al., 2007; Kuchroo et al., 2003; Zhang et al., 2012). Expression of three of these mucin-domain containing proteins was increased (1.8, 3.3, 5.0 \log_2 fold increases); however expression of the fourth was decreased 1.9 \log_2 fold in immune challenged samples.

3.2.2. Signaling processes

Four transcripts involved in either downstream TLR signaling or regulation of NF- κ B were differentially expressed. Two transcripts associated with TLR signaling, ATP-binding cassette sub-family A member one (Yvan-Charvet et al., 2010) and deleted in malignant brain tumor one (DMBT1; (Rosentiel et al., 2007)), were upregulated with one and 1.3 \log_2 fold change respectively. In contrast, expression of γ -taxilin, a negative regulator of TLR signaling (Yu et al., 2006), decreased (γ -taxilin had no detectable expression in treatment samples). Finally, a transcript involved in activity of NF- κ B, γ -secretase subunit PEN-2 (Poellinger and Lendahl, 2008), increased 1.7 \log_2 fold in samples exposed to bacterial PAMPs.

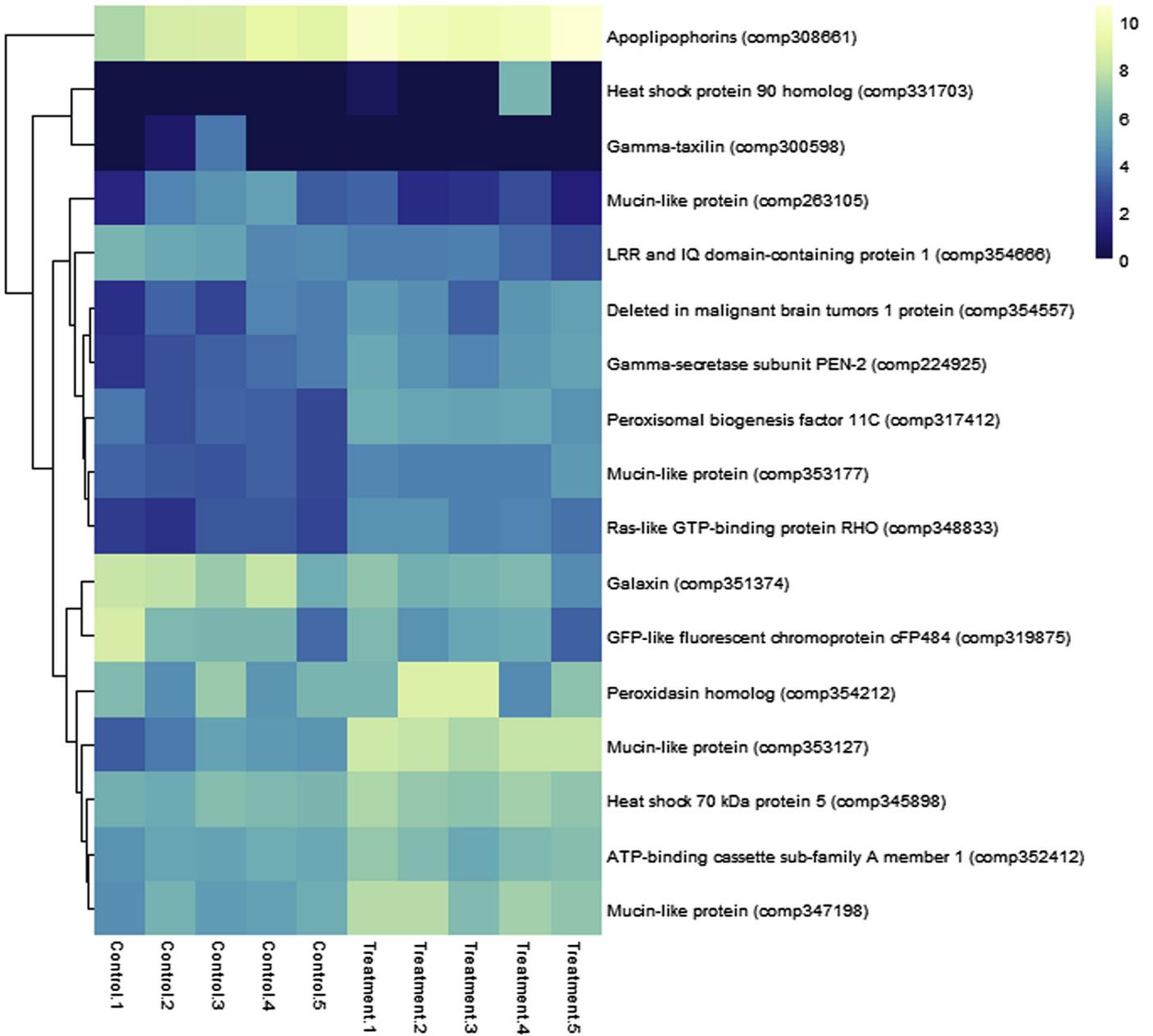


Fig. 1. Heatmap of expression of the 17 differentially expressed transcripts involved in immunity, clustered based on expression values.

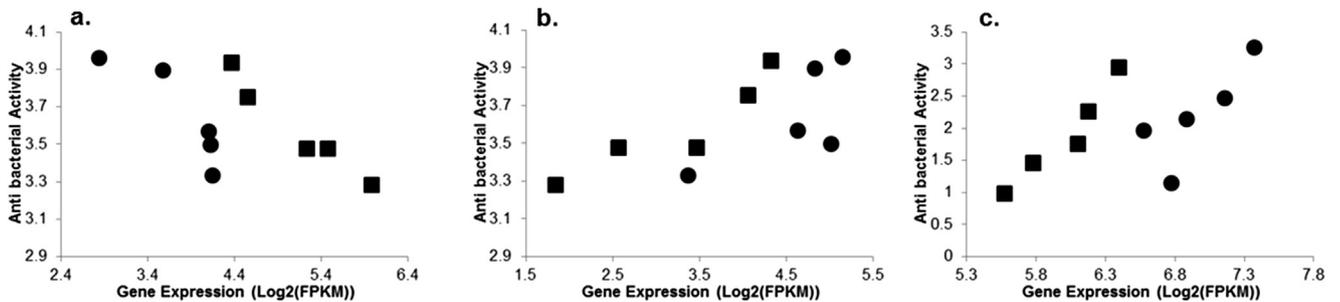


Fig. 2. Summary of significant gene to immune-related protein activity Pearson correlations for all samples (n = 10). a) correlations between antibacterial activity (Doubling time) and expression of LRR and IQ domain-containing protein 1 ($r = -0.688$, $p = 0.028$); b) correlations between antibacterial activity and expression of DMBT1 ($r = 0.711$, $p = 0.021$); c) correlations between PPO activity and expression of heat shock 70 kDa protein 5 ($r = 0.643$, $p = 0.045$). Squares represent control samples, circles represent LPS-treated samples.

Table 1
Immune transcript blast annotation results.

Locus	spID	Protein name	Percent match	eValue	Bit score
comp308661	Q9U943	Apolipoporphins	29.06	7.00E–37	155
comp352412	P41233	ATP-binding cassette sub-family A member 1	48.06	0	1702
comp354557	Q9UGM3	Deleted in malignant brain tumors 1 protein	39.96	7.00E–95	348
comp351374	D9IQ16	Galaxin	36.58	4.00E–35	142
comp224925	Q8JHF0	Gamma-secretase subunit PEN-2	63.41	1.00E–31	119
comp300598	Q9NUQ3	Gamma-taxilin	30.95	4.00E–22	100
comp319875	Q9U6Y3	GFP-like fluorescent chromoprotein cFP484	62.86	3.00E–76	237
comp345898	Q90593	Heat shock 70 kDa protein 5	84.73	0	961
comp331703	P41887	Heat Shock Protein 90 Homolog	73.72	6.00E–64	213
comp354666	Q96JM4	LRR and IQ domain-containing protein 1	29.39	4.00E–80	300
comp263105	B3EWM9	Mucin-like Protein	49.71	1.00E–41	167
comp347198	B3EWY9	Mucin-like Protein	37.48	9.00E–128	432
comp353127	B3EWY9	Mucin-like Protein	34.02	1.00E–51	203
comp353177	B3EWZ6	Mucin-like Protein	38.89	5.00E–34	147
comp354212	Q3UQ28	Peroxidase homolog	35.81	4.00E–91	321
comp317412	Q96HA9	Peroxisomal membrane protein 11C	39.24	2.00E–46	169
comp348833	P01122	Ras-like GTP-binding protein RHO	73.77	2.00E–19	92.8

List of blast results for annotated, differentially expressed immune genes. Transcripts were annotated against the Uniprot/SwissProt and TrEMBL databases using default parameters with a minimum e-value cutoff of $1E-5$. Listed in alphabetical order by protein name.

3.2.3. Effector processes

Six transcripts identified as immune effectors were differentially expressed. Two heat shock transcripts (Hsps), *Hsp70* and *Hsp90*, which play roles in immune effector responses (Asea et al., 2002; Kaufman, 1990; Mayor et al., 2007), increased in expression (*Hsp70* increased 1 log₂fold; *Hsp90* expression was not measurable in control samples). A transcript involved in phagocytosis, Ras-like GTP-binding protein Rho (Caron and Hall, 1998), increased 1.8 log₂fold in corals following exposure to bacterial PAMPs. Additionally, 3 transcripts which may function in antioxidant responses were differentially expressed following exposure to bacterial PAMPs: a GFP-like fluorescent chromoprotein (Palmer et al., 2009), Peroxisomal biogenesis factor 11C (PEX11C; (Aruoma, 1998; Schrader et al., 1998)), and peroxidase (Nelson et al., 1994). Expression of the GFP-like transcript decreased 1.6 log₂fold, while PEX11C and peroxidase increased 1.7 and 2.1 log₂fold respectively.

3.3. WGCNA network construction

WGCNA network construction of 371 transcripts resulted in eight modules ranging in size from 10 to 57 transcripts. Additionally, a ninth module contained 1 gene which could not be grouped in any other module. This module was excluded from further analyses. Seven of these modules were significantly correlated with an immune phenotype in the control or PAMP exposed subsets of samples. In the control subset, bacterial doubling time was positively correlated with Module 7 ($r = 0.94$, $p = 0.01$). Also in the control subset, melanin was positively correlated with Module 4 ($r = 0.93$, $p = 0.02$) (Supplementary Fig. 1). In the PAMP exposed subset, Modules 4 ($r = -0.92$, $p = 0.03$) and 8 ($r = -0.99$, $p = 1e^{-04}$) were negatively correlated with melanin. Prophenoloxidase activity was positively correlated to both Module 5 ($r = 0.93$, $p = 0.02$) and 6 ($r = 0.89$, $p = 0.04$). Peroxidase was positively correlated to Module 5 ($r = 0.95$, $p = 0.008$) and negatively correlated to Module 2 ($r = -0.91$, $p = 0.03$) (Supplementary Fig. 2).

While none of the seven modules significantly correlated to any immune phenotype showed significant gene ontology enrichment, a number of immune-related transcripts were found in these modules (Supplementary Table 2). For example: Module 4 included putative immune recognition molecule galaxin. Module 7 included the receptor, apolipoporphin, signaling molecules DMBT1 and γ -secretase subunit PEN-2, and the effector *Hsp70*.

3.4. Expression to protein correlations

Analysis of protein data alone revealed no significant differences between control and PAMP-treated samples (Table 2). However, of the 247 transcripts included in the seven significant modules, 91 (36.8%) were significantly correlated to one or more immune phenotypes in the entire set (35 transcripts; $n = 10$) or in the PAMP treatment (76 transcripts; $n = 5$). Of these 91 transcripts, 45 were assigned annotations with sufficient confidence (e-value < $1.0E^{-5}$; transcripts annotated as uncharacterized/hypothetical/predicted proteins excluded; Table 3). Correlations of relevant immune and stress transcripts with immune phenotypes are summarized in Figs. 2 and 3.

4. Discussion

Climate change and other anthropogenic stressors have been affecting natural communities at what appears an unprecedented scale. One of the most prominent impacts of climate change on natural communities has been drastic increases in the prevalence of disease or disease-like symptoms, causing unprecedented declines in many taxa including corals (Aronson and Precht, 2001; Bellwood et al., 2004; Daszak et al., 2001; Harvell et al., 1999; Hughes et al., 2003; Schutte et al., 2010). Despite this continuing and growing issue, our knowledge of the cellular and molecular mechanisms used by corals to resist and battle infections is still limited. Progress has been made in identifying elements of immunity on both phenotypic and genetic scales (Brown et al., 2013; Miller et al., 2007; Mydlarz and Harvell, 2007; Mydlarz et al., 2008, 2010; Mydlarz and Palmer, 2011; Palmer et al., 2009; Poole and Weis, 2014). Still, current knowledge is fragmented and few studies have investigated pathways of immunity or the relationship between changes in gene expression and immune protein activity (Vidal-Dupiol et al., 2014). Here we provide a comprehensive description of the response of the Caribbean coral *Orbicella faveolata* to an immune challenge with PAMPs. Our analyses include both transcriptional and biochemical data that document changes in gene expression and protein activity associated with the corals' immune response (Fig. 4).

4.1. Transcriptional expression patterns

In this study we documented significant changes in 17 potential

Table 2
Protein assay results.

	Units	Control	Treatment
Prophenoloxidase	ΔAbs 490 nm/mg protein/min	1.88 ± 0.34	2.20 ± 0.35
Melanin	μg melanin/mg tissue	3.04 ± 0.60	3.56 ± 0.12
Catalase	mM H ₂ O ₂ scavenged/mg protein/min	1130.39 ± 123.76	1230.49 ± 207.52
Peroxidase	ΔAbs 470 nm/mg protein/min	7.36 ± 0.80	8.34 ± 1.08
Superoxide dismutase	% Inhibition/mg protein	15278.17 ± 1450.81	16589.71 ± 1580.37
Antibacterial activity	Bacterial growth rate	3.58 ± 0.12	3.65 ± 0.12

Results of the protein activity assays for all five phenotypic measures of immunity. No assays showed significant differences ($p < 0.05$) between control and treatment groups.

immune transcripts. This group of seventeen transcripts was representative of all three major processes of invertebrate immunity: recognition, signaling, and effector responses. Seven transcripts from this group were potential immune recognition transcripts, four of which increased post-PAMP exposure and three of which decreased. Interestingly, three transcripts identified as potential immune recognition molecules all decreased following exposure to bacterial PAMPs: galaxin, a mucin-like protein, and a LRR-domain containing protein. Galaxin has previously been described as a protein component of the coral exoskeleton (Watanabe et al., 2003), however other studies have also suggested this molecule may function in the recognition of symbionts in squid-*Vibrio* associations (Heath-Heckman et al., 2014). In this study, expression of galaxin decreased significantly following exposure to PAMPs, suggesting a more complex or novel role for this protein in coral immunity.

A gene known as TIM-3 (T cell, immunoglobulin, mucin-3) with a mucin domain, both positively and negatively regulates expression of immune cytokines (Zhang et al., 2012). While three of the four documented mucin-like transcripts increased in expression following exposure to PAMPs, a fourth transcript decreased significantly. It is possible that the function of mucin domain-containing proteins in corals is divided between splice variants or gene duplicates as observed in our results, thus explaining the conflicting patterns of expression of mucin-like protein transcripts seen here. A transcript containing a LRR domain was downregulated in immune-challenged corals. LRR domains are key components of many immune recognition molecules (Bell et al., 2003; Inohara et al., 2005; Martinon and Tschopp, 2005). In many Cnidarians, such as *Psudeodiploria strigosa*, TLR-like receptors are believed to be constructed of two separate transcripts, one containing the LRR domain, and the second containing a TIR domain (Ocampo et al., 2015). In the Cnidarian, *Hydra*, the gene HyLRR-2 encodes a protein that binds flagellin and elicits an immune response in the presence of other immune receptor genes (Bosch et al., 2009). Finally, other immune recognition molecules which contain LRR, such as NOD-like receptors, have been described in *Orbicella faveolata* (Anderson et al., 2016). The downregulation of the galaxin and LRR recognition transcripts is counter-intuitive based on the function inferred from their annotations.

In addition to documenting changes in several potential immune recognition transcripts, we also identified four putative signaling molecules which were differentially expressed following exposure to bacterial PAMPs. One of these, γ -taxilin, which negatively regulates TLR4 signaling by inhibiting activating transcription factor four activity (Yu et al., 2006), was downregulated following immune challenge likely promoting immune response. Upregulated immune signaling transcripts included ATP-binding cassette sub-family A member 1 (ABCA1), which negatively regulates cytokine production by macrophages and resultant TLR4 signaling (Yvan-Charvet et al., 2010). This pattern of expression is contrary to expected patterns following immune stimulation by bacterial PAMPs as it likely results in immune suppression.

Finally, six transcripts involved in effector responses were differentially expressed. These included two heat shock proteins (70 and 90). Both of these proteins, which act as molecular chaperones (Feder and Hofmann, 1999), assist in coral response to stress (Barshis et al., 2013; Black et al., 1995; Császár et al., 2009; DeSalvo et al., 2008). Expression of both *Hsp70* and *Hsp90*, increased following exposure to bacterial PAMPs. Expression of several other effector transcripts including two transcripts potentially involved in antioxidant response, PEX11C (Aruoma, 1998; Schrader et al., 1998) and peroxidasin (Nelson et al., 1994), and the phagocytosis associated transcript Rho (Caron and Hall, 1998), also increased following exposure to bacterial PAMPs. However, expression of a third transcript, green fluorescent protein (GFP), decreased following exposure to bacterial PAMPs. Even though GFP's have been shown to decrease during heat stress (DeSalvo et al., 2008; Dove et al., 2006; Roth and Deheyn, 2013; Smith-Keune and Dove, 2008), their possible function as antioxidant suggest that they should increase as part of an appropriate immune response (Mydlarz and Palmer, 2011; Palmer et al., 2009).

4.2. WGCNA analysis

Analysis of all differentially expressed transcripts with WGCNA software yielded eight modules of co-expressed transcripts. Each module should consist of transcripts with similar biological function and be relatable to specific downstream responses, i.e. protein activity. While lack of annotations limited characterization of many of these modules, several patterns could be ascertained. Module 3, containing 35 annotated transcripts, included six transcripts associated with actin activity. Module 7, with 22 annotated transcripts, contained transcripts associated with lipid processing (four in total). Finally, Module 6 contained 12 annotated transcripts, four of which were associated with apoptotic processes. Seven of these modules were significantly correlated at least one immunity phenotype (i.e. protein activity). Module 5 correlated to both prophenoloxidase and peroxidase activity, indicating that they contain transcripts that either regulate or are regulated by these immune proteins. This link provides further support to previous findings that the production of peroxidase (Cerenius and Soderhall, 2004) and other antioxidants (Mydlarz and Palmer, 2011) is associated with the melanin synthesis cascade.

4.3. Correlations between transcript expression and immune phenotypes

To further explore the roles of specific transcripts in the immune response of corals, we correlated the expression of individual transcripts to immune-related protein activity to add new depth to studies of gene expression. While correlation does not necessary equal causation, our approach attempts to isolate, or filter, the transcripts associated with a general stress response from those more directly related to the biochemical immune response. Using this approach, we identified six immune receptor transcripts,

Table 3
Correlations between putative immune transcripts and immune protein activity.

Transcript ID	Protein name	Protein activity assay	Treatment	r	p
comp343799	2-acylglycerol O-acyltransferase 1	Prophenoloxidase	PAMP	−0.988	0.002
		Peroxidase	PAMP	−0.913	0.030
comp333208	2-acylglycerol O-acyltransferase 2-A	Melanin	PAMP	0.925	0.024
comp351374	39S ribosomal protein L41-A, mitochondrial	Catalase	PAMP	0.881	0.048
comp346081	3-hydroxypropionyl-coenzyme A dehydratase	Antibacterial Activity	PAMP	0.931	0.021
comp345898	78 kDa glucose-regulated protein (<i>Hsp70</i>)	Prophenoloxidase	All	0.643	0.045
		Melanin	PAMP	−0.985	0.002
comp226382	Arrestin-domain containing protein 4	Antibacterial Activity	All	0.672	0.033
comp341607	Bifunctional ATP-dependent dihydroxyacetone kinase/FAD-AMP lyase (cyclizing)	Prophenoloxidase	PAMP	0.931	0.021
		Peroxidase	PAMP	0.971	0.006
comp317101	Calumenin	Superoxide Dismutase	All	0.638	0.047
		Prophenoloxidase	PAMP	0.956	0.011
comp339880	Carbonic anhydrase 2	Antibacterial Activity	PAMP	0.980	0.003
comp347148	Carbonic anhydrase 2	Antibacterial Activity	PAMP	0.970	0.006
comp340734	CTD small phosphatase-like protein	Antibacterial Activity	All	0.655	0.040
comp349474	Cyclic AMP-dependent transcription factor ATF-5	Catalase	PAMP	0.910	0.032
comp340439	Cysteine string protein	Antibacterial Activity	PAMP	0.958	0.010
comp349461	Cytochrome C oxidase subunit NDUFA4	Antibacterial Activity	All	0.641	0.048
comp345160	Cytochrome P450 3A24	Prophenoloxidase	PAMP	−0.959	0.010
		Peroxidase	PAMP	−0.989	0.001
comp354557	Deleted in malignant brain tumors one protein	Antibacterial Activity	All	−0.700	0.024
comp347593	EGF-like repeat and discoidin I-like domain-containing protein 3	Peroxidase	PAMP	−0.969	0.006
comp343301	Estradiol 17-beta-dehydrogenase 8	Prophenoloxidase	All	−0.656	0.039
comp351548	Ferric-chelate reductase 1	Superoxide dismutase	PAMP	0.907	0.034
comp332277	Flavin Reductase	Prophenoloxidase	All	0.702	0.024
comp353272	Formate acetyltransferase 2	Doubling Time	All	0.652	0.041
comp351374	Galaxin	Prophenoloxidase	PAMP	0.956	0.011
		Peroxidase	PAMP	0.948	0.014
comp319875	GFP-like fluorescent chromoprotein cFP484	Prophenoloxidase	PAMP	0.922	0.026
		Peroxidase	PAMP	0.921	0.027
comp315290	High mobility group protein B3	Antibacterial Activity	All	0.640	0.046
comp354666	Leucine-rich repeat and IQ domain-containing protein 1	Antibacterial Activity	All	0.662	0.037
comp353127	MAM and LDL-receptor class A domain-containing protein 2	Melanin	PAMP	−0.882	0.048
comp263105	Mucin-like protein	Prophenoloxidase	PAMP	0.960	0.010
		Peroxidase	PAMP	0.907	0.034
comp347198	Mucin-like protein	Catalase	PAMP	0.948	0.014
comp345211	Multidrug resistance-associated protein 1	Melanin	PAMP	−0.880	0.049
comp354720	NADH-ubiquinone oxidoreductase chain 4	Prophenoloxidase	PAMP	0.910	0.032
		Peroxidase	PAMP	0.931	0.021
comp317412	Peroxisomal biogenesis factor 11C	Prophenoloxidase	PAMP	0.968	0.007
		Peroxidase	PAMP	0.937	0.019
comp343191	Probable UMP/CMP kinase 1	Prophenoloxidase	All	0.733	0.016
		Superoxide Dismutase	All	0.671	0.034
comp331913	Protein zinc induced facilitator-like 1	Prophenoloxidase	PAMP	−0.923	0.025
		Peroxidase	PAMP	−0.943	0.016
comp337513	RNA-binding protein Musashi homolog 1	Antibacterial Activity	PAMP	0.914	0.030
comp337740	Sodium-coupled monocarboxylate transporter 1	Melanin	PAMP	−0.952	0.013
comp333933	Succinate dehydrogenase [ubiquinone] cytochrome b small subunit B, mitochondrial	Antibacterial Activity	PAMP	0.943	0.016
comp352754	Tetratricopeptide repeat protein 28	Peroxidase	PAMP	−0.934	0.020
comp354523	Tetratricopeptide repeat protein 28	Prophenoloxidase	PAMP	−0.934	0.020
		Peroxidase	PAMP	−0.918	0.028
comp328348	Tetratricopeptide repeat protein 28	Peroxidase	PAMP	−0.955	0.011
comp353371	Tubulin beta chain	Superoxide Dismutase	PAMP	0.881	0.049
comp343899	Tyrosine-protein kinase STK	Antibacterial Activity	All	0.641	0.046
comp327632	Ubiquinone/menaquinone biosynthesis C-methyltransferase UbiE	Prophenoloxidase	All	0.637	0.047
		Melanin	PAMP	−0.928	0.023
comp350393	Uncharacterized skeletal organic matrix protein 2	Peroxidase	PAMP	−0.895	0.040
comp325048	Unconventional myosin-IXb	Melanin	PAMP	−0.949	0.014
comp345834	Valine-tRNA ligase	Prophenoloxidase	PAMP	0.934	0.020

Summary of transcripts with significant ($p < 0.05$) Pearson correlations to one or more measures of immune protein activity. Annotations were determined via blastx against the UniProtKB/Swiss-Prot database (1.0E-5 e-value threshold). Listed in alphabetical order by protein name.

galaxin, LRR and IQ domain-containing transcript, and three mucin-like transcripts, as well as one signaling transcript (DMBT1) that were correlated to an immune phenotype. Galaxin was positively correlated to prophenoloxidase and peroxidase activity, even though it was downregulated after PAMP treatment. This suggests that the transcript may in fact be serving as a recognition molecule and activator of immunity. LRR and IQ-domain-containing protein expression was negatively correlated to antibacterial activity. Based on these correlation results, this protein may in fact be involved in

negative regulation of antibacterial activity, thus explaining its downregulation following immune challenge. Furthermore, two mucin-like transcripts were both upregulated and significant correlated to one or more immune phenotypes (comp263105 to prophenoloxidase and peroxidase and comp347198 to catalase) in samples exposed to PAMP. Each transcript was correlated to a different measure of immunity, suggesting that either duplication or alternative splicing of a mucin, or similar immune receptor genes, may have led to division of labor which each unique

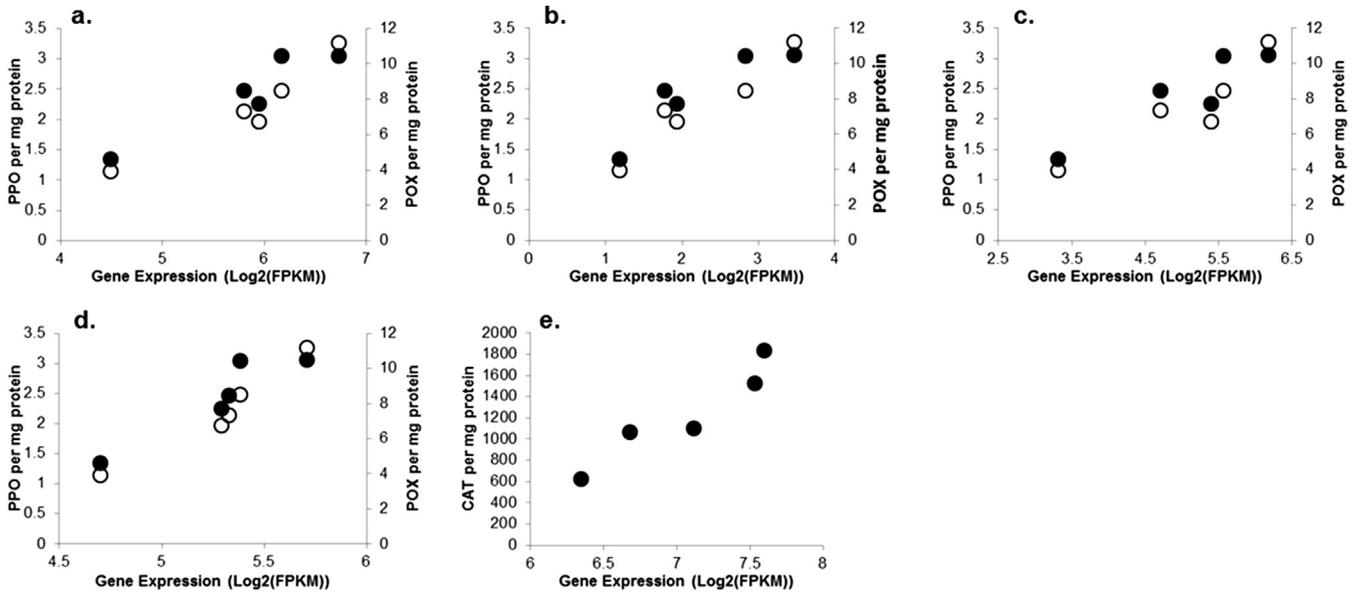


Fig. 3. Summary of significant transcript to phenotype correlations for the LPS subset of samples (n = 5). a) correlations between galaxin expression and activity of prophenoloxidase (PPO; r = 0.956, p = 0.011) and peroxidase (POX; r = 0.948, p = 0.014); b) correlations between mucin-like protein (comp263105) expression and activity of PPO (r = 0.960, p = 0.010) and POX (r = 0.907, p = 0.034); c) correlations between GFP-like fluorescent chromoprotein cFP484 expression and activity of PPO (r = 0.922, p = 0.026) and POX (r = 0.921, p = 0.027); d) correlations between peroxisomal biogenesis factor 11C expression and activity of PPO (r = 0.98, p < 0.01) and POX (r = 0.937, p = 0.019); e) correlations between mucin-like protein (comp347198) expression and activity of catalase (CAT; r = 0.948; p = 0.014). For graphs a–d: open circles correspond to POX values, closed circles correspond to PPO values.

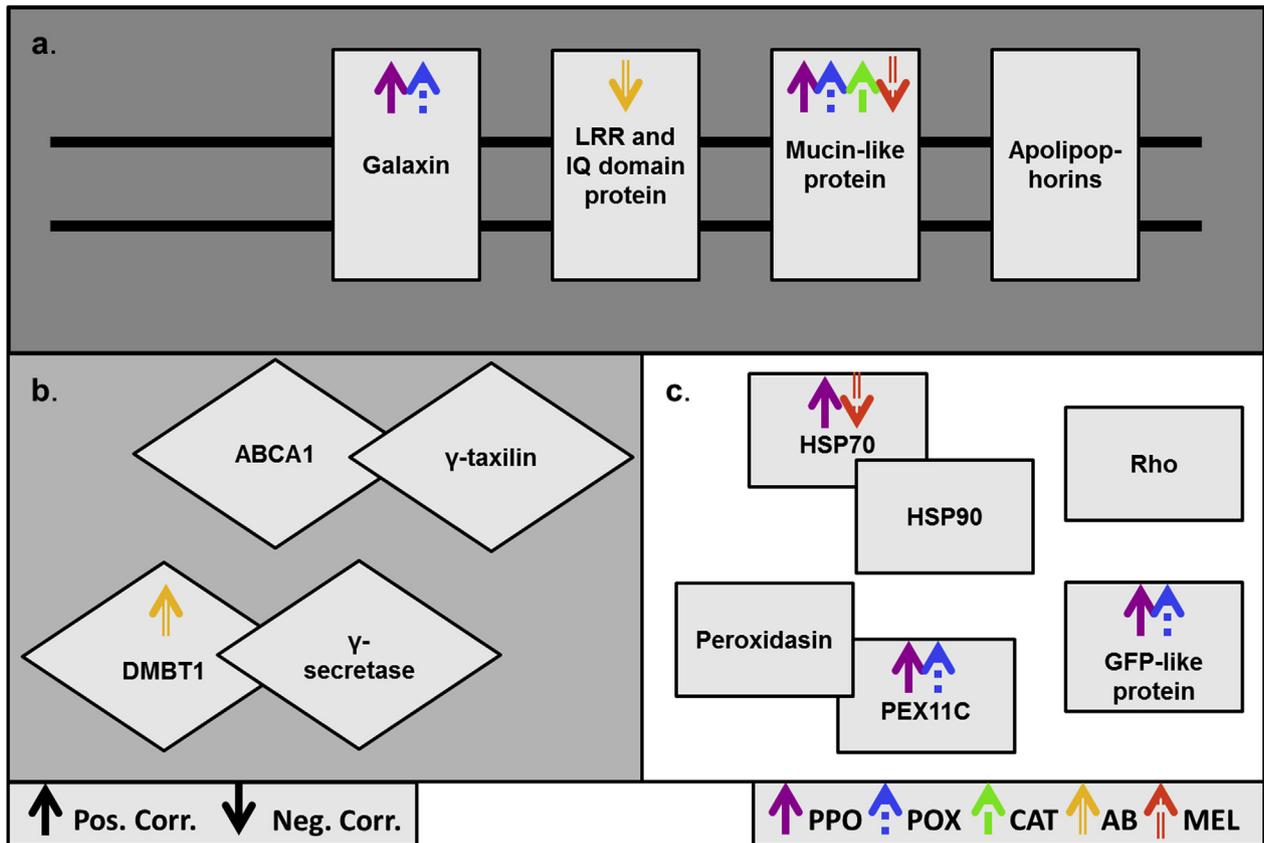


Fig. 4. Summary of the significantly differentially expressed transcripts involved in immunity divided by immune component (a. receptors, b. signaling molecules, c. effector molecules). Arrows indicate correlation between transcript expression and immune protein activity. Directionality of arrows corresponds to the direction of correlation (up = positive correlation, down = negative correlation).

transcript activating a separate arm of immunity. Finally, the DMBT1 signaling transcript was positively correlated to antibacterial activity when all samples were analyzed. This is indicative of its proposed role in agglutination and inactivation of bacterial pathogens (Bikker et al., 2002).

Our results indicated a number of transcripts related to effector responses were correlated to at least one measure of immune-related protein activities. This is consistent with the nature of the biochemical assays, which measure activity of effector responses. In total we found three immune effector transcripts which were correlated to one or more measures of immunity: *Hsp70*, GFP, and PEX11C. Expression of *Hsp70* was positively correlated to prophenoloxidase activity, supporting previous findings that *Hsp70* expression increases following immune challenge in corals (Brown et al., 2013). While there is no known direct link between prophenoloxidase activity and expression of *Hsp70*, it is possible similar upstream pathways regulate these processes.

PEX11C and GFP, two effector transcripts associated with antioxidant activity, were positively correlated to both prophenoloxidase and peroxidase activity in the PAMP treatment. PEX11 proteins control the proliferation of peroxisomes, which are major sites of hydrogen peroxide degradation. Peroxidases also contribute to the degradation of hydrogen peroxide in other parts of the cell (Aruoma, 1998). Co-regulation of peroxidase proteins, PEX11, and other antioxidant transcripts would explain this correlation. Coral fluorescent proteins, such as GFP, have antioxidant properties and contribute to immune functions in corals (Palmer et al., 2009). The results of correlation analyses provide strong evidence that both of these transcripts contribute to peroxidase activity in samples. Furthermore, production of multiple antioxidants is correlated to prophenoloxidase activity in corals (Mydlarz and Palmer, 2011), thus explaining the correlation between expression of PEX11 and GFP to prophenoloxidase in addition to peroxidase activity during exposure to PAMPs. Importantly, while the results of our correlation analyses suggest that GFP expression is positively correlated to immune activity, this transcript was downregulated during immune challenge.

4.4. Global patterns of immune regulation and conclusions

While many transcripts, such as *Hsp70* and DMBT1, were regulated in the expected pattern to confer resistance and immunity to a pathogen, there were other transcripts that displayed counter-intuitive patterns of regulation. Several of these transcripts were not only annotated as potential immune genes, but also were significantly correlated to immune phenotypes. While it is possible that observed downregulation of these transcripts is an artifact of the time point selected for sampling, it is also possible that these genes are undergoing dysfunctional regulation (referred to as “dysregulation”). “Dysregulation” of transcripts may significantly affect the response of *O. faveolata* to disease and may partially explain previously documented susceptibility of the species to disease (Bruckner and Hill, 2009; Sutherland et al., 2004; Weil, 2004; Weil and Rogers, 2011). Similar phenomena have been noted in other systems, where “dysregulation” of immune transcripts and consequential immune compromise result from transcriptomic mutations (Hodge et al., 1996; Kobasa et al., 2007; Welker et al., 2007). Our findings here suggest that similar patterns may exist in corals, thus potentially explaining disease susceptibility in certain coral species (Raymundo et al., 2005; Sutherland et al., 2004; Ward et al., 2006; Weil et al., 2009). Further studies examining immune response at multiple time points in corals will be necessary to fully understand the extent and importance of potential immune transcript “dysregulation” in *O. faveolata*.

In conclusion, by using novel approaches to compare changes in immune-related proteins activity to those in transcript expression, we were able to identify transcripts involved in the three major aspects of coral immune response: recognition, signaling, and effector responses. Our findings extend beyond transcriptional description by correlating expression of individual transcripts to biochemical measures of immune proteins activity. Further use of similar methods will serve to expand our knowledge of invertebrate immunity in general, and specifically regarding the mechanisms controlling host responses to infection in corals. These data provide a foundational resource that will lead to even greater understanding of important questions of invertebrate immunity.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.dci.2016.04.017>.

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