TITLE : **Pulling apart the host response to sea star wasting disease**

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Introduction

Echinoderms provide a powerful system to examine invertebrate immune function from both an organismal and evolutionary perspective. With a taxonomic position at the base of the deuterostomes, echinoderms are more similar to chordates and vertebrates than other invertebrate lineages (Blair and Hedges 2005). This is evident in part through their complex immune system with specialized phagocytic cells, signalling molecules that circulate in coelomic fluid, and a melanization response (Franco et al. 2011; Smith et al. 2010; Smith and Soderhall 1991). Laboratory based studies have established the existence of echinoderm recognition proteins (Bulgakov et al. 2013; Gowda et al. 2013), cytokine signaling molecules (Beck et al. 1996), and effector mechanisms, such as antimicrobial peptides (Li et al. 2010). Conserved immune response pathways include the complement system, Toll pathway, and cell adhesion regulation (Hibino et al. 2006; Smith et al. 2010; Dheilly et al. 2013). While the discovery of these major pathways highlights broad similarities across the phylogenetic tree, echinoderms also have regions of unusually high diversification as well as unique combinations of innate and adaptive immune capabilities. For example, it has been reported that the sea urchin *Strongylocentrotus purpuratus* possess over two hundred Toll Like Receptor (TLR) genes as well as genes that suggest overlap with vertebrate immunity (Hibino et al., 2006).

Infectious diseases of marine echinoderms are on the rise (Burge et al. 2014), providing an opportunity to investigate the varied immune responses of these animals to multiple pathogens. This rise in disease epidemics further underscores the need for a thorough understanding of the echinoderm immune system. Sea star wasting disease (SSWD), a newly emerging infectious disease affecting echinoderms, has caused severe population declines in multiple sea star species along the North American Pacific coast since summer 2013 (MARINe 2013). Early symptoms of disease include abnormally twisted arms, a deflated appearance, and white lesions on the aboral body wall; this rapidly progresses to tissue degradation, structure loss, arm loss, and death (Eckert et al. 2000). Many basic details of SSWD are still unknown, including the causative pathogen, the mode of disease spread, and conditions influencing its severity (MARINe 2013). As with any keystone species, loss of asteroid populations to disease outbreaks has the potential to shift community composition of intertidal and subtidal ecosystems (Paine 1974; Duggins 1983).

In this study, we characterize the sea star immune response in the sunflower star, *Pycnopodia helianthoides,* by injecting healthy individuals with homogenate from stars with signs of wasting disease and examining gene expression patterns of control and treated stars. The *P. helianthoides* transcriptome generated here is the first immune-related transcriptome in the class Asteroidea. Physiological responses to SSWD included activation of the the Toll and complement pathways, alterations in nervous and connective tissues, and Wnt signaling pathway disruption. This transcriptome not only provides an important resource for future evolutionary and organismal studies but provides the first evidence of how sea stars mount a response to this devastating disease. Understanding the response to this rapidly-progressing disease will certainly provide insight into dynamics of host-pathogen interactions in marine systems. Furthermore this detailed exploration of the sea star response to disease can enhance our understanding of the evolution of immune response.

Methods

Experimental Design

Pycnopodia helianthoides (radius 160.5 +/- 30.9 mm) were collected from four sites in Washington State: Langley (LA) (48.038, -122.404); Dabob Bay (DB) (47.813, -122.820); Port Hadlock Marina (PH) (48.030, -122.745); Friday Harbor (FH) (48.545, -123.012). Sites had no reports of SSWD infection at time of collection. An effort was made to collect animals within a similar size range to reduce variation based on body mass but otherwise selection was random. Experiments were conducted at Marrowstone USGS Laboratory, WA. *P. helianthoides* were transported to the lab on the day of collection in coolers, wrapped in cloth soaked in seawater from their respective sites.

Upon arrival, animals were examined for signs of disease or trauma and then transferred to individual, 37.8 L aquariums with separate flow-through, sand-filtered, and UV-treated seawater kept at ~8.5°C. The acclimation period for each star ranged from 3 days to 2.5 months, depending on time of collection. During this period, animals were examined for signs of disease and fed live manila clams (*Ruditapes philippinarum*), every 3-4 days. Water flow was checked daily.

An infection challenge was conducted with tissue homogenate prepared from the tube feet, dermal tissue, and coelomic fluid of three *P. helianthoides* with clinical signs of SSWD. The tissue was ground with a mortar and pestle, then placed in a Stomacher with 10 mL seawater, and centrifuged at 1000 x *g* for 5 min at 4°C. Organisms injected with the homogenate boiled for 7 minutes are considered the control group. The treated group were injected with homogenate that was syringe-filtered through a 0.22 µm polyethylsulfone filter. Specifically, one animal from each site was inoculated on 6 April 2014 with either boiled (controls) or filtered (treated) inocula (0.5 mL) by injection into the coelomic cavity, under the dermis on the aboral side of the arm, and at the arm base. Animals were checked twice daily and any physical or behavioral changes recorded. All animals were sacrificed 9 days post injection once all three treatment animals showed small lesions. Stars were sampled while lesions were small and they still had turgor so the coelomic fluid could be extracted from an intact animal. Coelomic fluid was collected from the arm using a syringe. The samples were centrifuged for 5 minutes at 1200 rpm at 4°C to separate the coelomocytes. The fluid was then removed and the pellet containing the coelomocytes flash frozen in liquid nitrogen and stored at -80C.

High Throughput Sequencing and Assembly

Total RNA was extracted using Tri-Reagent per manufacturer's instruction. Potential DNA carry-over was removed from extracted RNA using the Turbo DNA-free treatment according to the manufacturer's instructions (Ambion). RNA quality, library preparation, and sequencing was performed by the Cornell University Institute for Biotechnology. RNA quality was assessed using an Advanced Analytical Fragment Analyzer. Libraries were prepared using the Illumina TruSeq RNA Sample Preparation kit according to the manufacturer's protocol (including bar-coding for multiplexing), except our sample concentration was between 99-1503 ngs per sample. Samples were multiplexed where six samples were run in one lane for Illumina Hiseq 100 bp paired end sequencing.

Quality trimming of resulting sequencing reads was performed using CLC Genomics Workbench v. 7.0 (CLC Bio, Germany) with the following parameters: quality limit = 0.05, number of ambiguous nucleotides < 2 on ends, reads shorter than 20 bp were removed, and Illumina PCR primers were removed. De novo assembly was performed with Genomics Workbench 7.0 (CLC Bio, Germany) on quality trimmed sequences with the following parameters; automatic bubble size, automatic word size, auto-detect paired distances, perform scaffolding, and minimum contig size of 500 bp. In order to remove bacterial sequences from the transcriptome data, consensus sequences were compared to the NCBI nt database using the BLASTn algorithm. Consensus sequences with significant matches (evalue = 0) to bacterial sequences (n=1102) were removed and not considered in subsequent analyses.

Transcriptome Characterization

In order to characterize relative completeness of the transcriptome, it was compared to complete transcriptomes available for the bat star *Patira miniata* (http://echinobase.org) and purple sea urchin *Strongylocentrotus purpuratus* (http://spbase.org, Cameron et al. 2009). Specifically, gene sets of *P. minata* (29,805) and *S. purpuratus* (31,159) were used as query with blastn comparison to our *P. helianthoides* transcriptome. *P. helianthoides* transcriptomic sequences were subsequently annotated by comparing contiguous sequences to the UniProtKB/Swiss-Prot database. Comparisons were made using the BLASTx algorithm with a 1.0E-5 e-value threshold. Genes were classified according to Swiss-Prot Gene Ontology (GO) associations, as well as respective parent categories (GO Slim).

Differential Expression Analysis

Differential expression of contigs was calculated using a negative binomial GLM in the R package DESeq2 (Anders and Huber 2010). Read counts were determined by aligning reads with CLC v7.0 with the following parameters: mismatch cost = 2, insertion cost = 3, deletion cost = 3, length fraction = 0.8, similarity fraction = 0.8, maximum number of hits for a read = 10. The read counts were first normalized using the size factors method and fit to a negative binomial distribution. Significantly differential contig expression (Benjamini-Hochberg adjusted $p<0.05$) between control and treated animals was determined using the Wald test for significance of GLM terms.

Enrichment Analysis

Enriched GO terms associated with differentially expressed genes were identified using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v. 6.7 (Huang et al. 2009). Specifically, UniProt accession numbers for differentially expressed genes were uploaded as a gene list, while UniProt accession numbers for all annotated contigs were used as a background. Significantly enriched GO terms were identified as those with Benjamini-Hochberg adjusted $p<0.05$.

Results

Inoculation Experiment

During the acclimation period, no signs of disease were observed. All treated *P. helianthoides* developed signs of SSWD including curling and lesions. Lesions were noted at 8 to 9 days post injection in all three treatment animals. There was some individual variation in the onset and duration of clinical sign but signs were consistent between animals. Control animals did not show any clinical signs.

Transcriptome

This sequencing effort results in a combined $2.9x10^8$ paired end reads among all six libraries (three control individuals and three treated individuals. Sequencing reads are available in NCBI SRA Accession # (*). After quality trimming, reads were assembled into 29476 consensus sequences with an N50 value of 1757 bp (SUPPLEMENTAL FILE). Using *Patria miniata* and *Stronglycentrotus purpuratus* transcriptomes as for references we found that 52% and and 26% of the respective transcriptomes had match to the *Pycnopodia helianthoides* transcriptome using an 1.0E-5 e-value threshold. Comparisons of the *Pycnopodia helianthoides* transcriptome sequences to the UniProtKB/Swiss-Prot database resulted in annotation of 10513 contigs (SUPPLEMENTAL FILE).

Differentially Expressed Genes

Of those contigs identified as differentially expressed (n=3773), 1629 were less expressed and 2103 were more expressed in treated individuals compared to control individuals (Figure 1). A total of 1183

differentially expressed contigs (31.7%) were annotated based on comparison to Uniprot/SwissProt database.

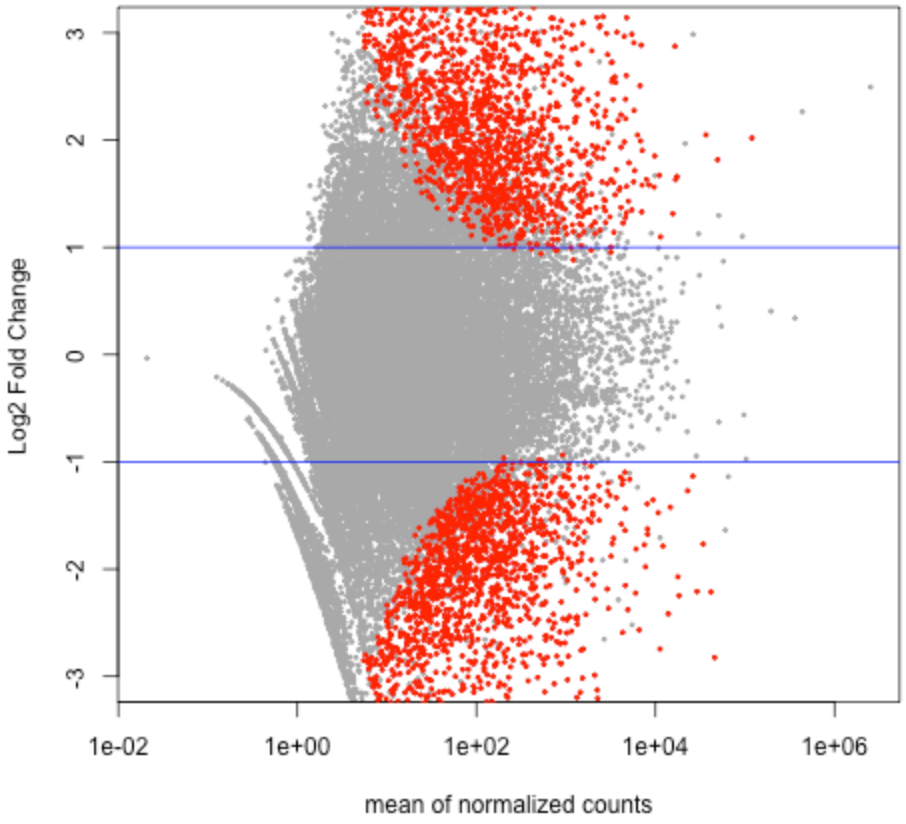

Figure 1. Volcano plot showing Log2 fold change between treated and control organisms. Positive values represent contigs expressed at higher level in treated. Red circles indicate those contigs determined to be differentially expressed (n=3773).

Seventeen BP_FAT gene ontology (GO) terms were significantly enriched based on the subset of annotated differentially expressed genes. These terms fell into three broad categories: immune response, regulation of cytokine production, and biological adhesion. Immune response was the largest of these categories, with thirteen associated enriched GO terms. **Table S1** lists all significantly enriched GO terms. Regulation of cytokine biosynthetic process (GO:0042035) and activation of immune response (GO:0002253) were the two most enriched GO terms with fold enrichments of 5.881791 and 5.614436 respectively (**Figure 2**).

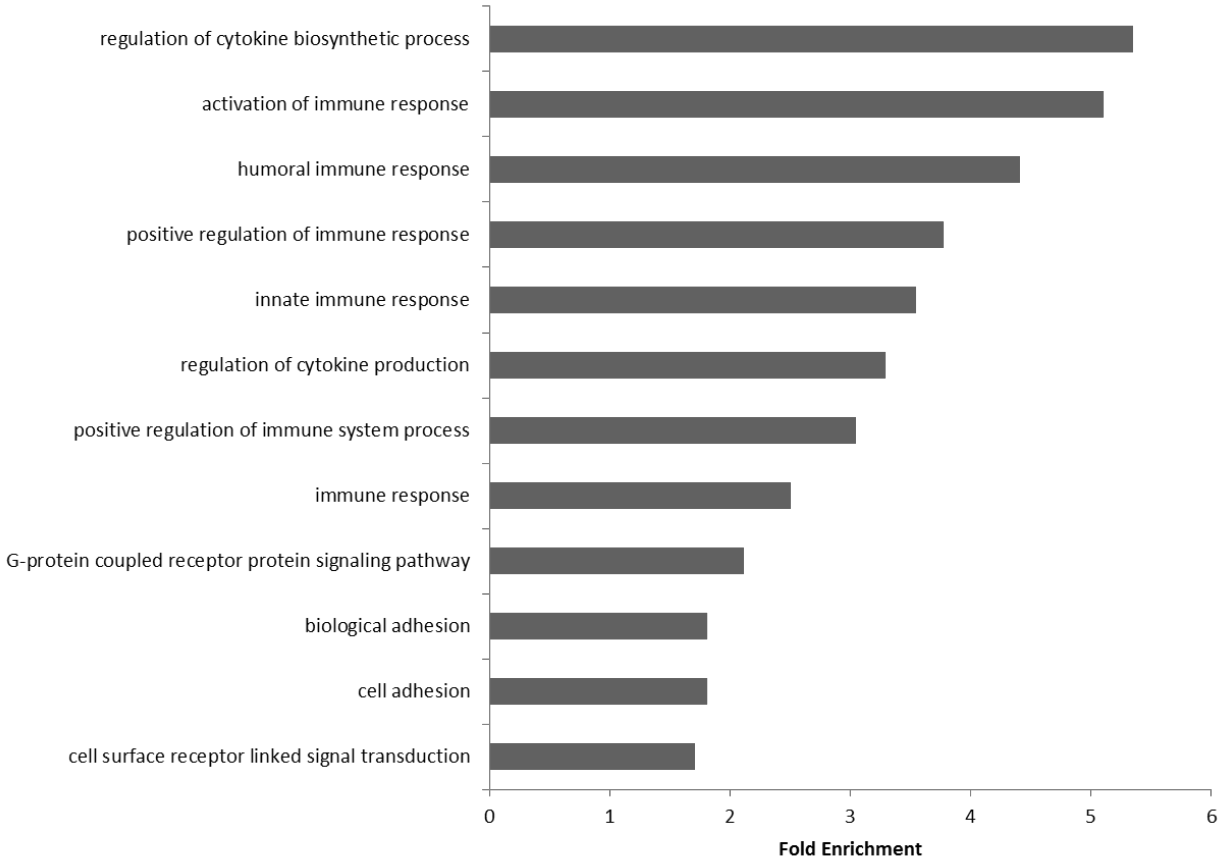

Figure 2 Fold Enrichment of all significantly enriched (padj<.05) biological processes.

In order to visualize individual variability among sea stars additional focus was place in pathways that were associated with enriched biological processes, differentially expressed genes, and pathways of interest. Specifically, we generated heat maps for genes associated with immune response (**Figure 3**), nervous system growth and organization (**Figure 4**), clotting (**Figure 5**), WNT-signaling (**Figure 6**) and WTF (**Figure 7**). Fold change information and p-values for all contigs is found in **Table S1**.

For reference, I'm changing HK_CF2 = Control_1, HK_CF35= Control_2, HK_CF70=Control_3, V_CF71=Treated_1, V_CF34=Treated_2, V_CF26=Treated_3

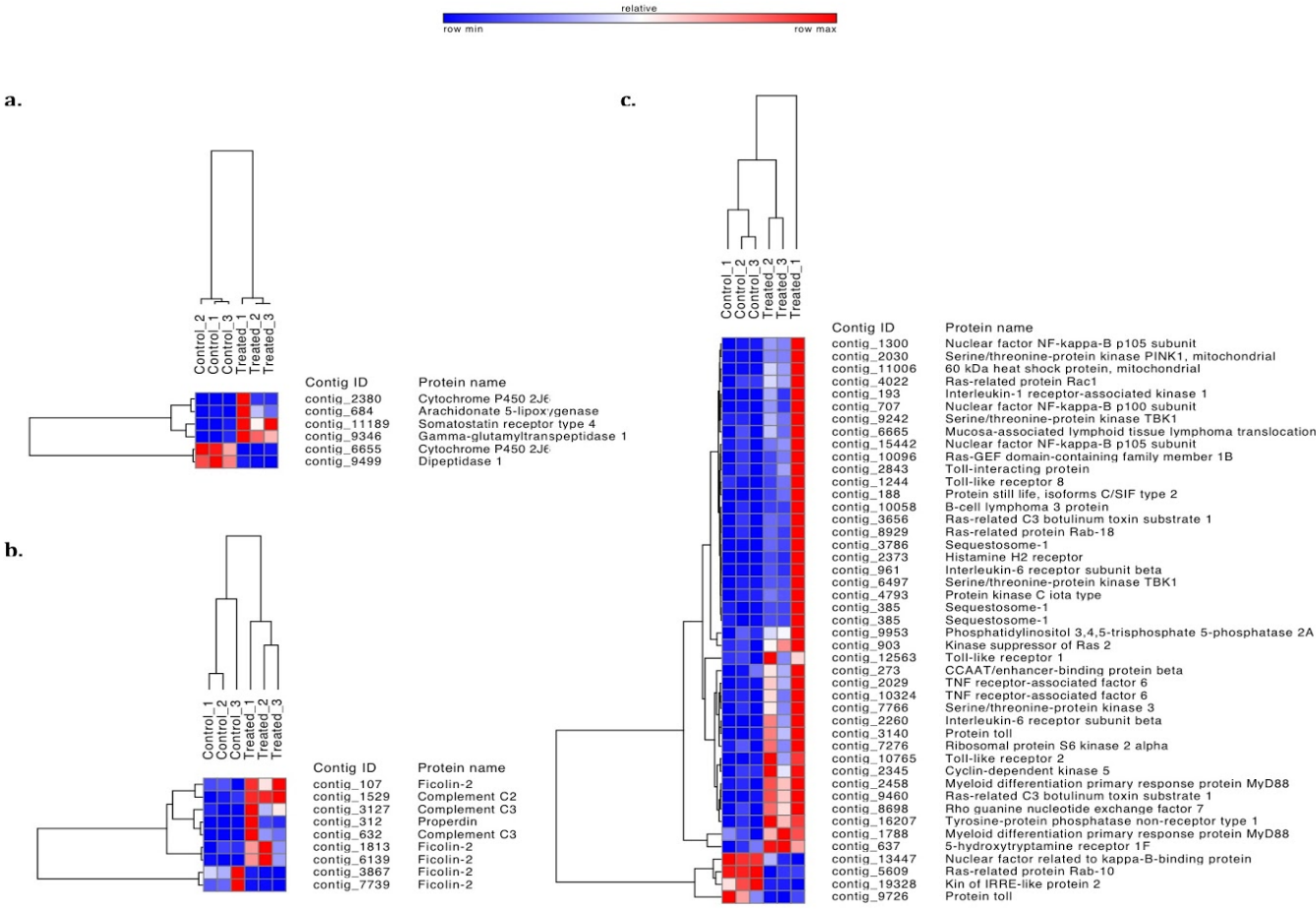

Figure 2. Heatmaps of immune-related differentially expressed transcripts between control and treated seastars. Heatmaps are subdivided by related pathway a) Arachidonic acid metabolism b) Complement cascade c) Toll-mediated pathways. Increased expression is shown in red and decreased expression is shown in blue.

~~**FIGURE 3 ID 1 = Friday Harbor seastars** (HK_CF2 = Control_1; V_CF71=Treated_1) **- ID 2 = Port Hadlock seastars** (HK_CF35= Control_2, V_CF34=Treated_2) **- ID 3 = Port Hadlock seastarts** (HK_CF70=Control_3, V_CF26=Treated_3)~~

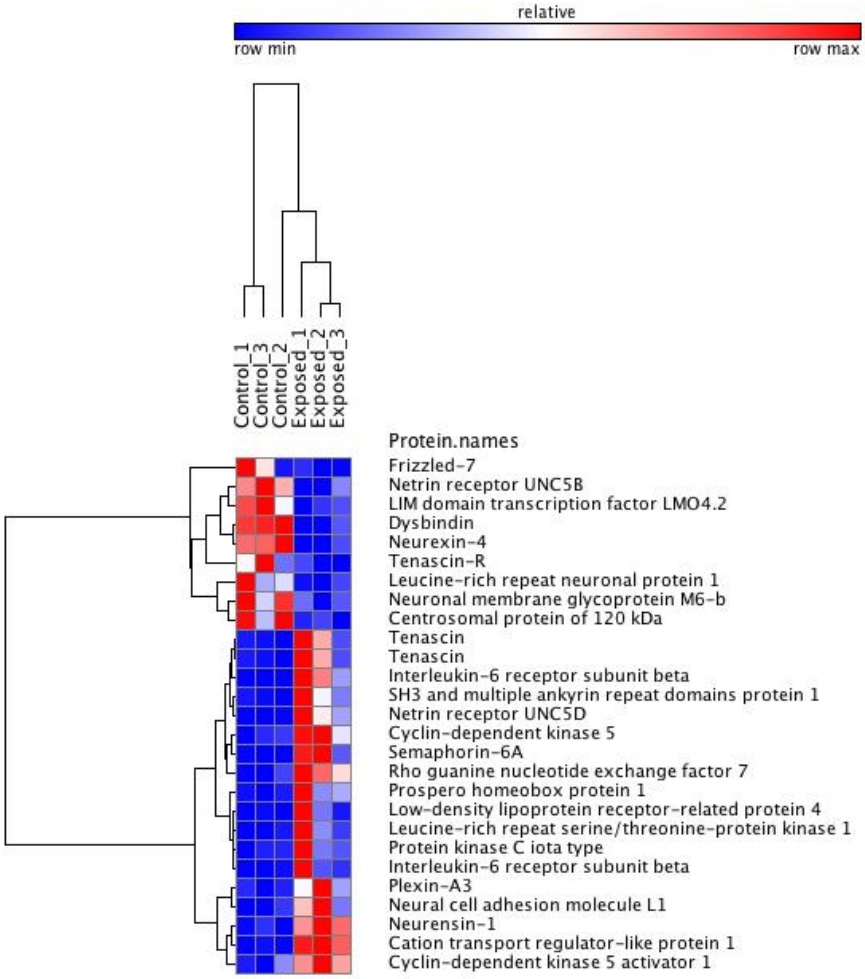

FIGURE 4 *Neural- Growth /organization*

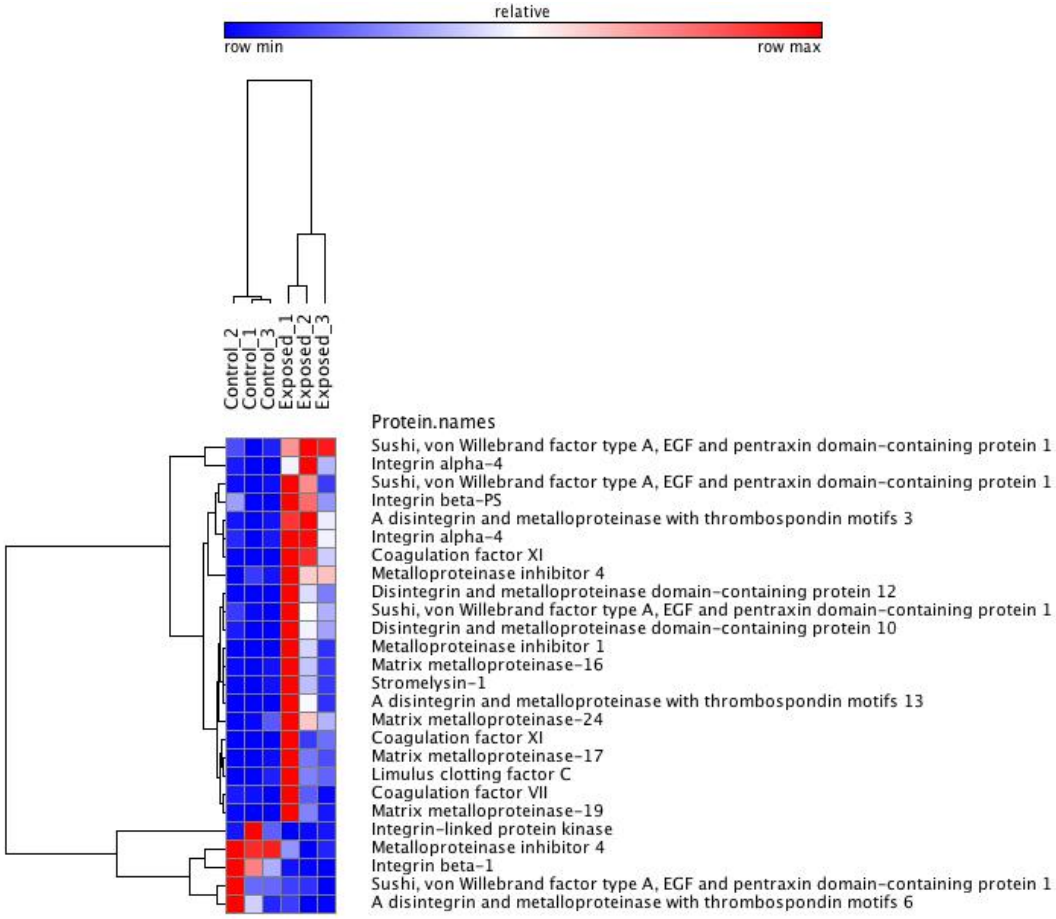

FIGURE 5. *Clotting*

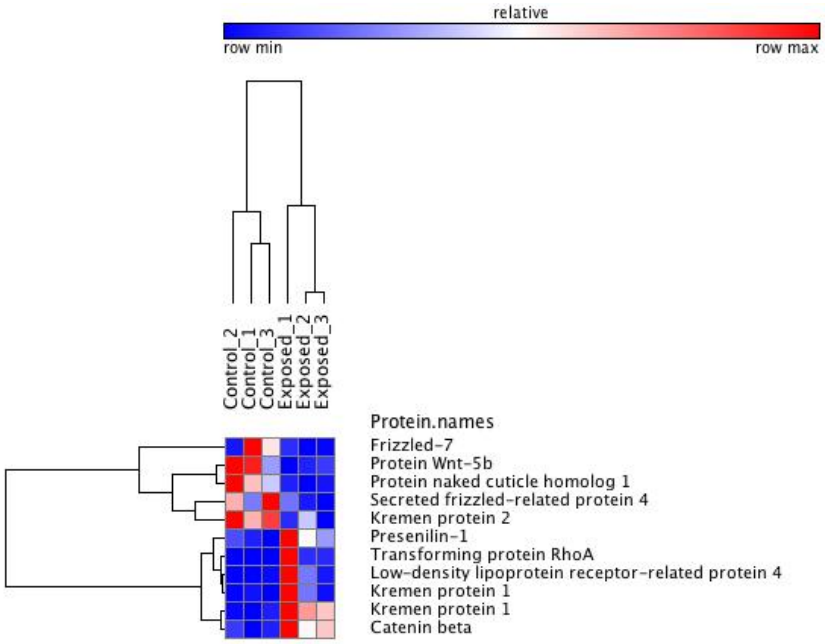

FIGURE 6*. Wnt*

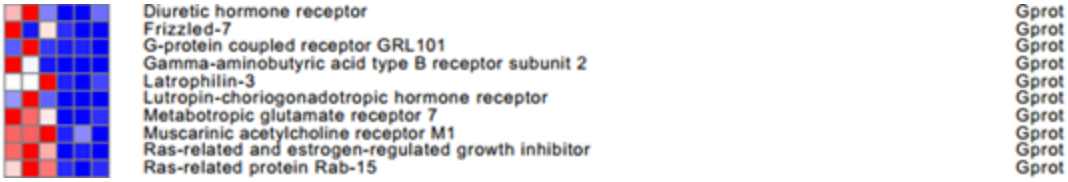

Figure 7

Discussion

Here we provide the first transcriptome description of *P. helianthoides* in relation to (or "following"?) pathogen exposure. Our transcriptome consisted of 29476 consensus sequences, 10413 of which were annotated using SwissProt databases. The abundance of available annotations for this transcriptome makes it an excellent resource for studying a variety of relevant genes and pathways in *P. helianthoids*. Furthermore, comparison of the *P. helianthoides* transcriptome presented here with other Echinoid transcriptomes (*Patria miniata* and *Stronglycentrotus purpuratus*) suggests that our transcriptome is fairly complete. [**Sentence here about the relevance of this completeness.]** Finally, differential expression analyses revealed 3773 genes which were differentially expressed between treatment and control organisms. Specifically, the comparison of gene expression patterns between treatment and control organisms ~~was in an effort to gain~~ provides a better understanding of the immune response of *P. helianthoides* to SSWD. As might be predicted from the dramatic mortality events that have been reported from Canada to …. (CITE), SSWD poses an existential threat to many seastar populations. **[This could continue straight into the next paragraph]**

The results of our examination of the *P. helianthoides* transcriptome indicate that *P. helianthoides* mounts a strong immune response when infected with homogenate from sea stars with signs of SSWD. This response is characterized by the increased expression of genes associated with known immune pathways such as the Toll pathway, complement cascade, melanization, and arachidonic acid metabolism. In addition to understanding the immune response of these stars to SSWD, we identified a number of differences in gene expression between treated and control seastars that may contribute to the observed clinical signs of SSWD. These include contigs involved in G-coupled protein processes, WNT signaling, cell adhesion, and neural processes. A close examination of these genes enhances knowledge of host-pathogen dynamics in sea stars.

Toll Pathway

The differential expression and enrichment of multiple genes involved in the Toll-like receptor-mediated pathway of the infection treatment sea stars suggests that this pathway plays an important role in *P. helianthoides* immunity. Toll-like receptors (TLRs) in both vertebrates and invertebrates recognize patterns to discriminate between self and non-self, inducing a response to bacterial and viral pathogens (Aderem and Ulevitch, 2000; Finberg et al. 2007; Zambon et al. 2005; Valanne et al. 2011). The

sea urchin genome contains 222 TLRs (Rast et al. 2006). It is not surprising, therefore, that we find differentially expressed TLRs in the sea star transcriptome (TLR1,TLR2,TLR8). The differential expression of TLR8 is especially interesting as this receptor has been linked to viral recognition (Akira et al. 2006). Following recognition, TLRs act through a network of signaling molecules to activate different immune and inflammatory cascades (Valanne et al. 2011). One potential pathway following TLR recognition in the treated sea stars is activation of Rac1, which initiates a pathway to activate NF-kB (Cuadrado et al. 2014), a transcription factor that triggers multiple immune effectors (Janeway et al. 2001). This response has been seen in immune-challenged Manila clams (Moreira et al. 2012). Additional enriched genes could act through an alternate pathway of NF-kB activation: TOLLIP and Myd88 could be activated by TLR1 and TLR2, in turn activating IRAK1 (Takeda et al. 2004; Muzio et al. 1997). Evidence of the cascade from Myd88 to IRAK1 to TRAF6 has been found in both invertebrate and vertebrate systems (Wang et al. 2011), including Myd88 in both sea urchins and sea cucumbers and TRAF6 in sea cucumbers (Rauta et al. 2014). Downstream signaling ~~in~~ linked to the Myd88 pathway includes a suite of closely interacting genes that belong to enriched processes in the treatment sea stars: PINK1, Traf6, Sqstm-1, HSP60 and MALT1 (Sun et al. 2004, Bitto et al 2014, Muroi et al. 2008; Coscia et al. 2011; Into et al. 2010; Cohen-Sfady et al. 2005). Further downstream in the TLR-mediated pathways, the enriched gene BLC3 ~~is~~ could be involved in negatively regulating NF-kB (Wang et al. 2009). NF-kB, NF-kB1 subunits, and NF-rKB are themselves enriched genes in treatment sea stars. The signaling cascade initiated by TLRs leads to the production of inflammatory cytokines including IL-6, which is differentially expressed in treatment sea stars and has been found in past research on immune-challenged sea stars (Beck & Habicht 1996).

We also identified other enriched genes that have been linked with the TLR-mediated pathway including RASGEF1B (Andrade et al. 2010), TBK1, which activates IRF3 instead of NF-kB (Shin et al. 2013), and Cebp-b, another pro-inflammatory transcription factor (Rojo et al. 2007; Grohmann et al. 2010). The histamine H2 receptor (Hrh2) is also differentially expressed and has been linked to the regulation of IL-10 and IL-12 regulation in mammals (Elenkov et al. 1998). Differentially expressed genes in enriched processes appear in multiple branches of the TLR-mediated pathway, serving as evidence that, as in other echinoderms, Toll pathways play an important role in *P. helianthoides* immunity.

Complement Cascades

The presence of multiple differentially expressed components of the complement cascade suggests that sea stars utilize this pathway, in addition to Toll-like receptors, to combat infection by SSWD. The complement cascade oposonizes pathogens leading to inactivation and phagocytosis by immune cells and also damages pathogens directly through the formation of the membrane attack complex (Janeway 2001). Although a previous study into the sea star coelomocyte proteome did not show homologous complement proteins, this study identifies four complement cascade proteins that are differentially expressed: Ficolin-2 (FCN2; contig_7739, contig_3867, contig_107, contig_1813, contig_6139), Complement C3 (C3; contig_3127, contig_632), Properdin (CFP; contig_312), and Complement C2 (C2; contig_1529) (Franco et al 2011). For all of four protein annotations, at least one corresponding contig was more highly expressed in infected stars than in control samples. However, contigs annotated as FCN2 had opposing log fold changes, suggesting either gene duplication and differential function or alternative splicing.

The complement cascade has been well documented in Echinoderms previously and contains C3, which functions in opsonization and phagocytosis, and factor B/C2, which increases C3 production (Smith et al. 1999). Here we document the presence of two additional complement cascade proteins, FCN2 and CFP. Both FCN2 and CFP induce the complement cascade: Ficolin through association with mannan-binding lectin-associated serine proteases (Matsushita et al 2000), and CFP by binding to apoptotic cells resulting in complement activation (Kemper et al, 2008). Our results suggest that the complement

cascade is an important part of sea star response to wasting disease, and furthermore, that echinoderms may have a more complex complement cascade system than previously thought.

Melanization

The melanin synthesis cascade in invertebrates serves roles in wound healing and immune response by creating chemical/physical barriers and encapsulating pathogens for phagocytosis (Cerenius & Söderhäll 2004). One of the contigs with the largest log2 fold change was annotated as a quinone oxidoreductase (CRYZ). Similar oxidoreductases (NQO1) in humans have been described as increasing melanin synthesis by increasing tyrosinase catalytic activity (Yamaguchi et al, 2010). Various aspects of the melanin synthesis cascade have been documented in a number of Echinoderms (Canicatti and D'Ancona, 1998). Previous experiments using sheep erythrocytes as an immune challenge in the sea cucumber *Holothuria polii* resulted in both clearance of the antigen and the production of 'brown masses' which were positive for Schmorl's, Lillie's, and Hueck's reactions, indicating the presence of melanin (Canicatti and D'Ancona, 1998). Furthermore, various urchins, sea stars, and sea cucumbers are characterized by low levels of phenoloxidase, an important part of the melanin synthesis cascade (Smith and Soderhall, 1991). Our results serve as the first genetic evidence of melanin synthesis in an echnioderm and suggest that melanin production may play an important role in the healing of lesions on infected sea stars.

~~*Clotting response and ECM remodeling*~~

~~Focal adhesion complexes are essential for binding cell surfaces to the extracellular matrix (ECM) and have roles in ECM remodeling and signal transduction. Integrins are a large family of transmembrane receptors in the focal adhesion complex that interact with a variety of ligand binding factors (Plow et al, 2000).They provide structure by linking the ECM to the actin cytoskeleton, contribute to growth factor receptor signaling transduction, and regulate the extracellular affinity of receptors to ECM proteins, leading to the focal adhesion "inside-out" signaling theory (Widmaier et al, 2012). The focal adhesion complex also has a role in initiation of the clotting response through the large EGF- and pentraxin-domain containing protein vWF, and can be affected by ECM connective tissue remodeling through metalloproteinase and disintegrin proteolysis (Page-McCaw et al, 2007).~~

~~In the treated *P. helianthoides*, we report differential expression in 3 integrin contigs, annotating to integrin beta-PS, CD antigen CD29 and CD antigen CD49d. Additionally, there are changes in 12 integrin-binding contigs, including collagenase 3, stromylesin-1, the disintegrins ADAM-10, ADAM-TS3, ADAM12, ADAM-TS6, ADAM-TS13, the metalloproteinases MMP-16, MMP-17, MMP19, MMP24, and the clotting factors vWF, FVII, and FXI. Log2 fold change for the clotting factors, MMPs, and ADAM disintegrins are all increased in the treated samples, while three out of the four contigs for protein vWF (cleaved by the anticlotting ADAM superfamily) also increase expression. Three out of the four annotated integrins also have increased differential expression. Matrix metalloproteinases degrade components of the ECM such as fibronectinin and laminin and are shown to be essential in connective tissue remodeling in the sea cucumber *Holothuria glaberrima* (Quinones 2004). These metalloproteinases may have a role in connective tissue mutability and lead to some of the signs of SSWD.~~

~~*G protein coupled receptors*~~

~~Guanosine nucleotide-binding proteins (G proteins) are signal transducers attached to the plasma membrane of the cell, which act as molecular switches, communicating signals from a variety of stimuli within and outside the cell (Neves *et al*., 2002). G protein coupled receptors (GPCRs) are the largest family of cell-surface molecules involved with the transmission of signals (Dorsam & Gutkin, 2007). While the family is large, enriched GPCRs were exclusively down in the treatment samples and show suppression of particular signalling cascades in contrast to healthy samples. GPCRs involved with adenylate cyclase~~

activity such as Luteinizing hormone receptor (contig_8658), Metabotropic glutamate receptor 7 (contig_2786), Muscarinic acetylcholine receptor M (contig_7895), and Diuretic hormone receptor (contig_11919) were down in the treatment condition. This enrichment is important as adenylate cyclase has been shown to cause muscle relaxation in sea stars (Elphick & Melarange, 2001), which parallels one physiological clinical sign of SSWD - loss of structural turgor (Eckert *et al.*, 2000). GPCRs involved with neural processes such as GABA-B receptor 2 (contig_29418), calcium-independent alpha-latrotoxin receptor 3 (contig_8096), and GRL101 (contig_15446) were also down in treatment samples. These among others were found in the sea urchin, *Strongylocentrotus purpuratus*, as well as *Saccoglossus kowalevskii,* the acorn worm, genomes and were linked to neural processes (Burke *et al., 2006; Krishna et al.*, 2013). Additional proteins, such as Ras-related and estrogen-regulated growth inhibitor (contig_8892) and Ras-related protein Rab-15 (contig_1757) both bind GTP for activation of G protein were also down in treatment samples. GTP activates G proteins of subsequent signaling pathways within the cell (Dorsam & Gutkin, 2007) and therefor without these processes being activated the subsequent signaling cascades cannot occur.

Insights into Sea Star Wasting Disease Pathology

Observable signs of SSWD also include a deflated appearance, twisting arms, lesions, and in advanced cases arm autotomy and death (Eckert et al. 2000). These suggest a role of nervous system and connective tissues in the disease pathology. The nervous system is the primary control of adhesion and connective tissues, including the mutable collagenous tissues (MCT), which are key in maintaining sea star structure (Sugni et al 2014). Our results yielded several differentially expressed genes that suggest a disruption of neural function, possibly having downstream effects to connective tissue function. Norepinephrine transporter, responsible for the uptake of the stress hormone norepinephrine into synaptic terminals, had strongly opposite expression between two contigs. BCL-2-like protein 1 (BCLx) was over-expressed. While it has a role in apoptosis (Adams & Cory 1998, Boise et al 1993), BCLx also regulates synaptic activity (Li et al 2013). Also, gamma-aminobutyric acid B receptor 2 ($GABA_BR2$), a neuroinhibitor that helps fine-tune neural function (Bettler et al 2004) was under-expressed. There is also evidence for neurogenesis: nerve guide and growth protein transcripts such as Blocks Notch protein (Botch, Chi et al 2012), neurensin (Araki & Taketani 2009), and netrin receptor UNC5D (Katow 2008) were highly expressed in treatment stars.

Neural changes in tandem with or mediating focal adhesion complexes are likely affecting MCT through manipulation of matrix metalloproteinases. The neurotransmitter acetylcholine is known to be a stiffening modulator of MCT in urchins (Ribeiro et al 2012, Hidaka & Takahashi 2009), and its degrading enzyme, acetylcholinesterase, is under-expressed in the treatment stars. While this suggests a greater stiffening response, which is opposite to what is observed, MCT response to neurotransmitters is often varied between echinoderm classes (Sugni et al 2014) or could be an attempt by the star to maintain structure. Matrix metalloproteinases such as collagenase and stromelysin degrade components MCT and soften it (Ribeiro et al 2012, Quinones et al 2002), while their antagonists Tissue Inhibitors of Metalloproteinases (TIMPs) stiffen it (Sugni et al 2014, Tipper et al 2003). Both collagenase-3 and stromelysin were over-expressed, which is consistent with the “melted” appearance of diseased stars. TIMPs had mixed expressions. Interestingly, collagen alpha-1(XIV) chain, a component of ECM/MCT, was over-expressed. However, we expected to observe some opposing expression in our transcriptome as some DEGs may represent disease signs while others could be the sea stars’ attempt to resist further development of the disease.

Insights into Sea Star Wasting Disease Pathology

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and connective tissues, including the mutable collagenous tissues (MCT), which ~~are key in maintaining~~ help maintain sea star structure (Sugni et al 2014). Therefore the signs of SSWD suggest a role ~~of~~ for the nervous system and connective tissues in the disease pathology.

Our results yielded differentially expressed genes that suggest a disruption of neural function, possibly having downstream effects to connective tissue function. Norepinephrine transporter, which is responsible for the uptake of the stress hormone norepinephrine into synaptic terminals, had opposite expression between two contigs (contig_1368, contig_6750). BCL-2-like protein 1 (BCLx, contig_3774) was over-expressed in treatment stars and has a role in apoptosis (Adams & Cory 1998, Boise et al 1993), but BCLx also regulates synaptic activity (Li et al 2013). There is also evidence for neurogenesis, as nerve guide and growth protein transcripts such as Blocks Notch protein (Botch, Chi et al 2012, contig_7409), neurensin (Araki & Taketani 2009, contig_18669), and netrin receptor UNC5D (Katow 2008, contig_4949) were all highly expressed in the treatment stars.
Enriched G-protein coupled receptors (GPCRs) were exclusively down in the treatment samples and show suppression of particular signaling cascades in contrast to healthy samples. [Sentence on what G-proteins CAN do - list a few things. Otherwise it jumps in too quickly]. Gamma-aminobutyric acid B receptor 2 ($GABA_BR2$, contig_29418), a neuroinhibitor that helps fine-tune neural function (Bettler et al 2004), was under-expressed. Other GPCRs involved with neural processes such as calcium-independent alpha-latrotoxin receptor 3 (contig_8096), and GRL101 (contig_15446) were also down in the treatment samples. These among others were found in the *S. purpuratus* (sea urchin) and *Saccoglossus kowalevskii* (acorn worm) genomes and were linked to neural processes (Burke et al., 2006; Krishna et al., 2013). Additionally, Ras-related and estrogen-regulated growth inhibitor (contig_8892) and Ras-related protein Rab-15 (contig_1757), both of which bind GTP for activation of G protein, were also down in treatment samples. GTP activates G proteins of subsequent signaling pathways within the cell (Dorsam & Gutkin, 2007) ~~and therefore~~ Without ~~these processes being activated~~ the activation of these processes, ~~the~~ subsequent signaling cascades cannot occur.
The neurotransmitter acetylcholine is known to be a stiffening modulator of MCT in urchins (Ribeiro et al 2012, Hidaka & Takahashi 2009). Its degrading enzyme, acetylcholinesterase (contig_16508, contig_17140, contig_24134), is down in the treatment stars while the GPCR Muscarinic acetylcholine receptor M (contig_7895) is down in the treatment condition, indicating a reduction of acetylcholine-mediated MCT stiffening. While this suggests greater stiffening response, which is opposite to what is observed, the treatment stars may be over-expressing this as an attempt to maintain turgor.
GPCRs involved with adenylate cyclase activity such as Luteinizing hormone receptor (contig_8658), Metabotropic glutamate receptor 7 (contig_2786), and Diuretic hormone receptor (contig_11919) were down in the treatment condition. This enrichment is important, as adenylate cyclase has been shown to cause muscle relaxation in sea stars by possibly mediating the nitric oxide signaling pathway (Elphick & Melarange, 2001).
Matrix metalloproteinases (MMPs) and the clotting factor vWF have a physiological role in extracellular matrix (ECM) remodeling and the clotting response involved in wound healing, respectively cleaving or binding the focal adhesion complex. They are essential for connecting cell surfaces to the ECM, for providing tissue structure by linking the ECM to the actin cytoskeleton, and for initiation of the clotting response (Widmaier et al, 2012). In the treated P. helianthoides, we report changes in the clotting factors vWF (contig_1077, contig_12224, contig_163, contig_5796), FVII (contig_3408), and FXI (contig_1871, contig_20104). Three of the four contigs for protein vWF (cleaved by the anticlotting ADAM superfamily) and both clotting factors FVII and FXI show increased expression in the treated ~~conditions~~ stars. Additionally, there is increased expression in the protein integrins, annotating to integrin beta-PS (contig_1522), CD antigen CD29 and CD antigen CD49d (contig_13337, contig_2142).

ECM connective tissue remodeling occurs through matrix metalloproteinases (MMPs) and disintegrin proteolysis (Page-McCaw et al, 2007). MMPs, such as collagenase and stromelysin, degrade components of the ECM and are shown to be essential in tissue remodeling in the sea cucumber *Holothuria glaberrima*, indicating a key role in mutable connective tissue (MCT) changes that may lead to some of the signs exhibited in SSWD[CC3] [k4] (Ribeiro et al 2012, Quinones et al 2002, Quinones et al 2004). In the treated stars, both collagenase-3 (contig_235, contig_296, contig_475) and stromelysin (contig_360) were over-expressed. Additionally, 9 other proteases change expression with treatment, disintegrins ADAM-10 (contig_1865) and
ADAM12 (contig_1112), the ADAMTS proteins ADAM-TS3, (contig_1128), ADAM-TS6 (contig_6569), and ADAM-TS13 (contig_7590), and the membrane-associated metalloproteinases MMP-3 (contig_360), MMP-13 (contig_235, contig_296, contig_475), MMP-16 (contig_202), MMP-17 (contig_1508), MMP19 (contig_6080), and MMP24 (contig_61). Differential expression for the disintegrins and MMPs are all increased in the treated samples except for ADAM-TS6, indicating a massive MCT response. This physiological response is likely linked to the physical "melted" appearance of diseased stars. The antagonists Tissue Inhibitors of Metalloproteinases (TIMPs) have been shown to stiffen MCT (Sugni et al 2014, Tipper et al 2003) and had mixed expression changes between treated and control (contig_1066, contig_2994, contig_4458, contig_27280). Interestingly, collagen alpha-1(XIV) chain (contig_2464), a component of the ECM, was over-expressed, possibly due to the sea stars' attempt to reconstruct tissues affected by immense changes in tissue organization[CC5] .

This analysis of differentially expressed genes and enriched pathways in the sea star transcriptome shows that, similar to urchins, sea stars have a complex immune response involving thousands of genes. *Pycnopodia helianthoides* exhibiting clinical signs of SSWD had differential expression in the toll pathway, complement cascades, melanization, clotting response, ECM remodeling, G protein coupled receptors, and neural involvement, highlighting that the experimental treatment caused a significant host response. While further studies are needed to differentiate the gene expression changes following infection with SSWD from other pathogens, this insight into the *P. helianthoides* transcriptome lays the foundations for better characterization of sea star immune responses. Comparisons with the few other echinoderm transcriptomes can provide insight into the evolution of immunity-related genes in deuterostomes.

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Fake Appendix

Table 4 List of contigs involved in clotting/ MCT

Contig	ID	Annotation	log 2 FC	padj
Phel_clc_contig_1871, Phel_clc_contig_20104	Q91Y47	Coagulation factor XI (FXI)	7.75 5.37	0.00 0.00
Phel_clc_contig_1128	O15072	ADAM-TS 3	3.77	0.00
Phel_clc_contig_6569	Q9UKP5	ADAM-TS6	-2.33	0.02
Phel_clc_contig_7590	Q76LX8	ADAM-TS 13 (vWF-cleaving protease)	7.96	0.00
Phel_clc_contig_3408	P98139	Coagulation factor VII	2.68	0.02
Phel_clc_contig_1865	Q8JIY1	ADAM 10	2.77	0.00
Phel_clc_contig_1112	Q61824	ADAM 12	4.17	0.00
Phel_clc_contig_163, Phel_clc_contig_12224, Phel_clc_contig_1077, Phel_clc_contig_5796	A2AVA0	Sushi, von Willebrand factor	2.20 4.20 1.61 -1.91	0.00 0.00 0.00 0.03
Phel_clc_contig_1522	P11584	Integrin beta-PS	1.74	0.05
Phel_clc_contig_2142, Phel_clc_contig_13337	Q00651	CD antigen CD49d	3.07 3.63	0.00 0.00
Phel_clc_contig_9206	B0FYY4	CD antigen CD29	-1.40	0.01
Phel_clc_contig_6080	Q9JHI0	MMP-19	4.22	0.00
Phel_clc_contig_61	Q9R0S2	MMP-24	2.24	0.00
Phel_clc_contig_360	P28863	Stromelysin-1	3.97	0.00
Phel_clc_contig_1508	Q9ULZ9	MMP-17	4.08	0.00
Phel_clc_contig_202	Q9WTR0	MMP-16	3.43	0.00

Phel_clc_contig_25693	P57044	Integrin-linked protein kinase (ILK)	0.00	-3.37

Table 5 List of contigs involved in the wnt signaling pathway

Contig	SPID	Annotation	log2 FC	padj
Phel_clc_contig_1252	Q5REY6	Transforming protein RhoA	6.22	2.52E-11
Phel_clc_contig_25	Q9QYP1	Low-density lipoprotein receptor-related protein 4	4.48	1.79E-06
Phel_clc_contig_9741	Q90Y90	Kremen protein 1	3.52	4.98E-04
Phel_clc_contig_3608	Q99N43	Kremen protein 1	2.64	2.61E-10
Phel_clc_contig_2240	Q4JIM4	Presenilin-1	1.8	1.07E-03
Phel_clc_contig_7024	P35223	Catenin beta	1.47	4.02E-04
Phel_clc_contig_9770	Q8NCW0	Kremen protein 2	-1.42	6.13E-03
Phel_clc_contig_9571	Q6FHJ7	Secreted frizzled-related protein 4	-1.93	3.01E-03
Phel_clc_contig_12417	Q5BL72	Frizzled-7	-2.26	1.61E-03
Phel_clc_contig_13198	P33945	Protein Wnt-5b	-2.92	1.35E-05
Phel_clc_contig_24757	Q2TJA6	Protein naked cuticle homolog 1 (Naked-1)	-3.62	6.70E-05

Table 6: List of selected neurological/MCT contigs

Contig	SPID	Annotation	log2 FC	padj
Phel_clc_contig_296	O77656	Collagenase 3 / Matrix Metalloproteinase 13	10.84	1.01E-39
Phel_clc_contig_475	O77656	Collagenase 3 / Matrix Metalloproteinase 13	7.82	5.07E-16
Phel_clc_contig_235	O77656	Collagenase 3 / Matrix Metalloproteinase 13	3.39	0.000411
Phel_clc_contig_29418	Q80T41	Gamma-aminobutyric acid type B receptor subunit 2	-3.56	0.0168

Phel_clc_contig_1368	P23975	Sodium-dependent noradrenaline transporter / Norepinephrine transporter	4.64	1.51E-13
Phel_clc_contig_6750	P23975	Sodium-dependent noradrenaline transporter / Norepinephrine transporter	-4.76	2.08E-21
Phel_clc_contig_7409	B3STU3	Botch (Blocks Notch protein)	4.25	7.63E-20
Phel_clc_contig_3774	Q07817	Bcl-2-like protein 1	4.31	6.23E-08
Phel_clc_contig_18669	P97799	Neurensin-1	3.85	4.40E-06
Phel_clc_contig_4949	Q8K1S2	Netrin Receptor UNC5D	6.05	4.27E-15
Phel_clc_contig_1127	Q8JGT4	Netrin Receptor UNC5B	-1.19	0.0262
Phel_clc_contig_360	P28863	Stromelysin-1	3.97	5.91E-05
Phel_clc_contig_1066	P35624	Tissue Inhibitor of Metalloproteinases 1	6.58	8.50E-16
Phel_clc_contig_4458	Q9JHB3	Tissue Inhibitor of Metalloproteinases 4	3.27	0.000347
Phel_clc_contig_2994	P81556	Tissue Inhibitor of Metalloproteinases 4	-1.81	0.00149
Phel_clc_contig_27280	P26652	Tissue Inhibitor of Metalloproteinases 3	-4.17	2.33E-05
Phel_clc_contig_24134	Q86GC8	Acetylcholinesterase	-2.34	0.0446
Phel_clc_contig_16508	P21836	Acetylcholinesterase	-1.94	0.0411
Phel_clc_contig_17140	Q29499	Acetylcholinesterase	-1.6	0.0214

Table 7: List of contigs involved in G-proteins