Habitat as a determinant of *Pisaster ochraceus* gene expression and microbiome composition

*Keywords: Pisaster ochraceus*, Sea Star Wasting Disease, Microbiome, Differential gene expression, Habitat

Abstract

Sea Star Wasting Disease has led to the mortality of millions of sea stars along the west coast of North America. *Pisaster ochraceus*, a keystone predator in rocky intertidal habitats, is one of many species impacted by this disease. Environmental variables, such as microhabitat have been attributed to determining the susceptibility of *P. ochraceus* to Sea Star Wasting Disease. In this study, we examine the role of habitat type in defining patterns of gene expression and microbiome composition. Through identifying patterns of differential gene expression, we seek to elucidate immune and stress response genes differentially expressed between intertidal and subtidal *P. ochraceus*. In our examination of microbiome composition, we aim to identify microbial taxa associated with a compromised immune system. Our analyses demonstrate similar patterns of gene expression across all *P. ochraceus* individuals, regardless of habitat type. However, genes that differ in their expression indicate differences in immune response, necrotic cell death, and collagen production. Our analyses of the microbiome data indicate differences in microbial composition in different habitats, with conservation of microbiome function across habitat type. This study is a first step in determining the role of habitat type in driving differences in gene expression and microbiome composition in the keystone species, *Pisaster ochraceus*.

Introduction

June 2013, marked the onset of an unprecedented mass mortality of sea stars across a broad range of species, including the rocky intertidal keystone predator *Pisaster ochraceus* (Menge *et al*. 2017; Paine *et al*. 1974; Eisenlord *et al*. 2016). Symptoms preceding mortality include lesions, loss of turgor pressor, and loss of limbs (Hewson *et al*. 2014). The disease associated with this mortality event is Sea Star Wasting Disease. While research has implicated a densovirus in causing sea star wasting, the true causative agent remains unknown (Menge *et al*. 2016).

The impacts of Sea Star Wasting Disease are widespread, however, certain environmental factors influence the susceptibility of sea stars to this disease (Menge *et al*. 2016; Eisenlord *et al*. 2016). Specifically, research shows that temperature, wave exposure, and microhabitat influence the susceptibility of *P. ochraceus* to Sea Star Wasting Disease (Menge *et al*. 2016; Eisenlord *et al*. 2016; Bates *et al*. 2009).

*P. ochraceus* occupies a range of microhabitats within intertidal communities, and is commonly found from the low intertidal zone, to depths of 90m (Lambert *et al*. 2000). The intertidal zone is characterized by high wave action and organisms living in this zone are subjected to long periods of emersion, where they are susceptible to desiccation, high UV irradiation, and rainfall. The subtidal is a more stable environment, with less wave action and no periods of emersion.Additionally, the rocky shore is characterized by strong tidal zonation. For this reason, community composition in the intertidal is distinct from that of the subtidal environment.

Sea stars used in this study varied in their expression of wasting symptoms. Some organisms remained healthy throughout the duration of observation, while others succumbed to the disease. Interestingly, a larger proportion of animals collected from the intertidal developed wasting symptoms as compared to animals collected from a subtidal environment. We motivate that differences in habitat type may result in different stress levels, and overall animal health, potentially influencing patterns of gene expression. Further, differences in community composition may influence the microbiota present in intertidal and subtidal environments.

In this study, we aim to answer two questions: (1) Are there differential patterns of gene expression between intertidal and subtidal *P. ochraceus*? (2) Are there differences in microbial species richness and taxon abundance between intertidal and subtidal *P. ochraceus*? We hypothesize that habitat type will influence gene expression and microbiome composition, resulting in distinct patterns of gene expression and microbial taxon abundance in intertidal and subtidal *P. ochraceus*. Further, we predict that genes associated with immune and stress response will be differentially expressed in intertidal and subtidal *P. ochraceus*. Finally, we expect microbiota associated with pathogens and opportunistic infections to differ in their abundance between intertidal and subtidal *P. ochraceus*. Understanding the role of habitat type in defining host gene expression and microbiome composition will provide further information regarding the dynamics of this enigmatic disease.

Methods

*Field Collection and lab acclimation*

Thirty intertidal *P. ochraceus* individuals were collected from Monterey Harbor in early May, 2016. Eight subtidal individuals were collected from the same location in early June, 2016. Upon arrival in Vermont, sea stars were placed in individual aquaria, maintained at 12°C, and held under constant light conditions. Sea stars were given three days to acclimate to lab conditions, before assessing health and taking samples for the analysis of host and microbiome RNA.

*Time course samples*

Health status was assessed and tissue samples were collected every three days over the course of the two week experiment. Health score for each individual was evaluated using a standardized scale from zero to five (zero being asymptomatic, and five being dead). Tissue samples were taken from the epidermal tissue of each individual and used for the extraction of host RNA and microbial 16s rRNA.

For the purposes of this study, we focused only on the samples collected on day three. We analyzed day three host gene expression for 21 individuals (13 intertidal, and 8 subtidal), and analyzed day three microbiome composition for 37 individuals (29 intertidal, and 8 subtidal). We predict these early stage samples will have the strongest signature of the host environment, and will provide the clearest contrast between intertidal and subtidal *P. ochraceus*.

*Host mRNA and microbiome 16s rRNA library preparation and sequencing*

Total host RNA was extracted from epidermal tissue, and cDNA libraries were prepared with the TruSeq RNA Sample Preparation Kit following the manufacturer’s instructions. Libraries were then pooled and sequenced as 100 bp paired-end reads on four Illumina HiSeq 3000 lanes.

Additional RNA was extracted from epidermal tissue, and reversed transcribed to make cDNA for the 16s rRNA libraries. We then amplified the 16s rRNA gene from the cDNA using primers with overhang adapters. We then prepared libraries using Nextera XT Index Kit following the manufacturer’s instructions. Libraries were then pooled and sequenced on one Illumina MiSeq run to obtain 300bp paired-end reads.

*Differential Gene Expression Analysis*

We assessed the quality of raw RNA-seq data using FASTQC, and cleaned our raw reads using Trimmomatic (Andrews 2010; Bolger *et al*. 2014). After removing adapters and low quality bases from our file, we again evaluated the quality using FASTQC (Andrews 2010). We then used Trinity to assemble a de novo transcriptome using fastq files from four sea stars (both sick and healthy) (Grabherr *et al*. 2011). We functionally annotated our de novo transcriptome by using BLAST+ to search the UniProt and NCBI non redundant (NR) databases for sequence similarity between our assembled contigs and genes with known function (Camacho *et al*. 2009).

Clean reads were mapped to our reference transcriptome, producing sequence alignment files. Count data, for the number of reads that mapped to each contig, were extracted from our sequence alignment files. We then used a subset of these count data, consisting only of day three samples, for differential expression analysis using the package DESEQ2 in R (Love et al. 2014; R Core Team 2016). Further, we conducted a variance stabilizing transformation of our count data and performed a principal components analysis to assess the similarity of our samples, and identify whether or not our samples could be grouped by their habitat type (Love *et al*. 2014).

*Microbiome analyses*

We first joined our paired end reads in Qiime and quality filtered these reads using Qiime’s default parameters (Caporaso *et al*. 2010). We then used open reference picking to identify both characterized and unknown microbial taxa in our sequence data, and compile these taxa into a table of Operational Taxonomic Units (OTUs). Additionally, we aligned our sequence data to the core set of genes in the Greengenes database to test the evolutionary distance between our OTUs and build a phylogenetic tree (DeSantis *et al*. 2006).

The OTU table, phylogenetic tree, and a metadata file containing information on our sampling conditions were used as input for the Phyloseq package in R (McMurdie & Holmes 2013). We then used Phyloseq to assess the microbiome species richness for each *P. ochraceus* individual, and to compare species richness between intertidal and subtidal *P. ochraceus*. Additionally, we used Phyloseq to identify OTUs that differ in their abundance between intertidal and subtidal *P. ochraceus*.

Results

*Differential Gene expression*

Out of a total of 13053 contigs, 9 (0.8%) were significantly downregulated in intertidal *P. ochraceus*, and 58 were upregulated in intertidal *P. ochraceus* (0.52%). This small proportion of differentially expressed genes is supported by the results of our principal components analysis, where there is no clear separation of our samples by habitat type (Figure 1).

The six most significant differentially expressed genes were associated with functions such as necrotic cell death, cell signaling, rRNA, and growth of collagen fibrils (Table 1 and Figure 2).

*Microbiome composition*

Out of a total of 1064 OTUs, 70 (6.6%) were significantly more abundant in intertidal *P. ochraceus*, and 49 (4.6%) were significantly more abundant in subtidal *P. ochraceus*. Of the six OTUs that showed the most significant difference in abundance between habitat types, one was from the family *Alteromonadaceae*, three were from the genus *Pseudomonas*, and two were from the genus *Marinomonas* (Table 2).

Additional significant differences between intertidal and subtidal *P. ochraceus* were found in the abundance of the orders *Sphingomonadales* and *Vibrionales* (Figure 3). *Sphingomonadales* was significantly more abundant in intertidal *P. ochraceus* (p-adjusted < 0.01), and Vibrionales was significantly more abundant in subtidal *P. ochraceus* (p-adjusted < 0.01).

Results from our species richness analysis demonstrate high levels of richness across all individuals, with no significant differences in richness between intertidal and subtidal *P. ochraceus* (Table 3).

Discussion

In this study, we examined the role of habitat type in defining *P. ochraceus* gene expression and microbiome composition. We expected the distinct environmental conditions experienced by intertidal and subtidal *P. ochraceus* would drive differences in patterns of gene expression and microbiome composition, particularly with respect to genes and microbial taxa associated with immune response and stress.

The results from our differential gene expression analysis demonstrate that *P. ochraceus* individuals studied in this experiment exhibit similar patterns of gene expression. While patterns of gene expression of these two groups may differ under field conditions, the common garden of laboratory conditions and the similar genomic background of individuals collected from the same region are likely two reasons we did not see strong patterns of differential gene expression.

Interestingly, genes that play a role in necrotic cell death, growth of collagen fibrils, and rRNA were upregulated in intertidal versus subtidal *P. ochraceus*. The increased expression of genes related to necrotic cell death in intertidal individuals may be a result of more intertidal *P. ochraceus* succumbing to wasting than subtidal *P. ochraceus*. While, the upregulation of a gene that plays a role in the production of collagen fibrils in intertidal individuals may be due to the importance of clinging to substrate for intertidal individuals as compared to subtidal individuals. Sea stars are known to utilize a unique defense mechanism, in which they rapidly stiffen collagen in response to mechanical stimulation (Whittaker *et al*. 2006). This allows for rapid body stiffening, and prevents sea stars (and other echinoderms) from being removed from substrate (Whittaker *et al*. 2006).

The upregulation of an adapter protein associated with the ras signaling pathway in subtidal individuals may be an indicator of a more tightly regulated immune response in subtidal individuals16. Work by Ragab *et al*. (2011) demonstrates the role of the ras pathway in modulating the immune deficiency pathway in Drosophila.

The upregulation of the 5.8s rRNA gene in intertidal *P. ochraceus* can potentially be linked to enhanced RNA virus activity (Johnson *et al*. 2009). RNA viruses, such as the Picornavirus, can hijack host ribosomes, preventing host mRNA translation, and facilitating translation of viral RNA (Johnson *et al*. 2009). These defunct ribosomes may be polyadenylated to signal for their degradation, which would support their appearance in our poly-A selected libraries (Johnson *et al*. 2009). Upregulation in rRNA genes has also been associated with colony collapse disorder in honey bees, further linking the differential expression of these genes with compromised organism health (Johnson *et al*. 2009).

Intertidal *P. ochraceus* had greater abundances of *Alteromonadaceae*,two *Pseudomonas* OTUs, and *Sphingomonadales*. *Alteromonadaceae* is commonly found in marine environments, and is known to degrade complex polysaccharides, such as chitin and agar (Kwak *et al*. 2012). *Pseudomonas* is also commonly found in aquatic environments (Sala-Comorera *et al*. 2016). This genus hosts many species that are known to be opportunistic pathogens in plants, animals, and humans (Sala-Comorera *et al*. 2016). In addition to being an opportunistic pathogen itself, the presence of *Pseudomonas* is often an indicator of other opportunistic pathogens being present (Sala-Comorera *et al*. 2016). *Sphingomonadales* is commonly found in nutrient limited environments, and is known to degrade aromatic compounds (Tang *et al*. 2012).

Subtidal sea stars also had one *Pseudomonas* OTU that was higher in abundance when compared to intertidal sea stars. Additionally, subtidal *P. ochraceus* had a higher abundance of *Vibrionales* and *Oceanospirillales*. *Vibrionales* is a common aquatic bacteria, often associated with animals and plants (Imhoff 2005). Many species of vibrio are associated with pathogens in fish and humans (Imhoff 2005). *Vibrionales* is also associated with indiscriminate infections and wounds (Imhoff 2005). *Oceanospirillales* was found in high abundance following the 2010 Deepwater Horizon oil spill, and is associated with degrading alkanes (Ortmann & Lu *et al*. 2015). *Marinomonas mediterranea*, a species in the order *Oceanospirillales*, is also known to synthesize antibacterial proteins (Lucas-Elío *et al*. 2006; Harayama *et al*. 2004).

The high relative abundance of *Pseudomonas* in intertidal *P. ochraceus*, and *Vibrionales* in subtidal *P. ochraceus* indicate that organisms from both habitat types host microbes associated with opportunistic pathogens. This is not necessarily surprising, as individuals from intertidal and subtidal habitats succumbed to wasting disease. While sea stars from different habitats have different abundances of *Sphingomonadales* and *Oceanospirillales*, these two orders of bacteria overlap in their associated functions. This indicates that while habitat may drive differences in microbiome composition, the functional niches filled by these different OTUs may be the same.

While the limited differences in microbiome composition and gene expression found in this study may be driven by our common garden conditions and lack of genetic differentiation between intertidal and subtidal *P. ochraceus*, they may also be a result of our sampling design. Our analyses included only samples from day three, comparing the gene expression patterns of 21 individuals, and the microbiome composition of 37 individuals. This small sampling size, in addition to a high prevalence of contigs and OTUs with low read counts, may have limited our power to identify existing differences between intertidal and subtidal *P. ochraceus*. Additionally, the differences we did see between habitat types may be an artifact of collection stress rather than a signature of environmental conditions. *P. ochraceus* collected subtidally are easy to remove from their substrate, resulting in a minimal amount of stress to the organism. However, *P. ochraceus* collected from the intertidal needed to be pried from their substrate, possibly resulting in heightened stress in these individuals. Not only may this stress have played a role in altering patterns of gene expression, it may also have compromised the health of our *P. ochraceus* individuals. It is possible that our collection methods are responsible for the differences seen in the health status between intertidal and subtidal *P. ochraceus*, rather than predispositions to disease due to habitat type.

Future analyses of the data analyzed in this study will include a full functional enrichment analysis of differentially expressed genes. Additionally, further annotation of the functional differences in host microbiome will be assessed using the program PICRUSt (Langille *et al*. 2013). Further, we will reanalyze these data including the variable of health status in our experimental design. This will allow us to statistically assess the role of health status in defining patterns of gene expression and microbiome composition, and allow for the identification of a potential interaction effect of health and habitat. Additionally, future studies may include the collection of tissue from sea stars in their natural environment. Getting tissue biopsies in the field, while documenting health status and tidal height, will allow for a more realistic assessment of habitat type as a determinant of *P. ochraceus* gene expression and microbiome composition.

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Author Contributions

M.L. and M.P. designed and planned the experiment. M.L. conducted the experimental procedure and performed the tissue extractions and library preparations. M.P. and S.K. designed the bioinformatics pipeline, and provided guidance in the analysis of host mRNA and microbiome 16s rRNA data. L.A., C.K., and L.C-Q developed the research questions and designed the analytical pipeline for differential gene expression analysis and microbiome composition assessment. L.A. wrote this manuscript.

Tables and Figures:











