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

Introduction: *Pisaster ochraceus* is a keystone predator in rocky intertidal ecosystems, ranging from Alaska to Baja California1,2. *P. ochraceus* feeds on competitively dominant intertidal organisms, facilitating the settlement of competitively subordinate species1,2. The presence of this predation in rocky intertidal habitats supports the diversity of these communities, and maintains ecosystem stability1,2.

June 2013, marked the onset of an unprecedented mass mortality of sea stars across a broad range of species, including *P. ochraceus*3. Symptoms preceding mortality include lesions, loss of turgor pressor, and loss of limbs4. 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 unknown1.

While the impacts of sea star wasting are widespread, certain biological traits and environmental factors are linked to variation in the susceptibility of sea stars to Sea Star Wasting Disease1,3. Research shows that temperature, wave exposure, and microhabitat can all influence the susceptibility of *P. ochraceus* to Sea Star Wasting Disease1,3,5.

*P. ochraceus* occupies a large geographical range, while also inhabiting a range of habitat types within individual intertidal communities. *P. ochraceus* is commonly found from the low intertidal zone, to depths of 90m6. *P. ochraceus* residing in the intertidal zone experience exposure to high wave action, and are subjected to long periods of emersion during low tide. Subtidal *P. ochraceus*, on the other hand, occupy a more stable environment, characterized by 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 subtidal locations. Further, substrate varies with increasing depth. Intertidal sea stars are likely to live on bedrock and boulder substrate, while subtidal individuals may increasingly experience a sandy substrate.

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 and substrate type may influence the microbiota present in intertidal versus 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*.

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, therefore, also predict that genes associated with immune and stress response will be differentially expressed in intertidal and subtidal sea stars. Finally, we expect microbiota associated with immune and stress related functions to differ in their abundance between intertidal and subtidal sea stars. 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 individuals were collected from a subtidal environment in early June, 2016. Upon arrival in Vermont, sea stars were placed in individual aquaria, maintained at 12°C, under constant light conditions. Sea stars were given three days to acclimate to lab conditions, before assessing health and taking samples for host and microbiome RNA.

Time course samples

Health score for each individual was assessed on a standardized scale from zero to five (zero being asymptomatic, and five being dead). A core of epidermal tissue was then collected from each sea star. Tissue samples were used for host RNA and microbial 16s rRNA. Health status and tissue samples were quantified every three days over the course of two weeks.

For the purposes of this paper, we focus only on the samples collected on day 3. We predict these early stage samples will have the strongest signature of the host and microbiome environment, and will provide the strongest contrast between intertidal and subtidal sea stars.

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 on four illumina HiSeq 3000 lanes to obtain 100 bp paired end reads.

Total RNA was extracted from epidermal tissue, and reversed transcribed to make cDNA. 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 paired end 300bp reads.

Differential Gene Expression Analysis

We assessed the quality of raw RNA-seq data using fastqc, and cleaned our raw reads using Trimmomatic. After removing adapters and low quality bases from our file, we again evaluated the quality using fastqc. We then assembled a de novo transcriptome using fastq files from four sea stars (both sick and healthy). 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. These count data were subsetted to include only day 3 samples, and used for differential expression analysis in DESeq2. Further, we conducted a variance stabilizing transformation of our counts data and performed a principal components analysis to assess the similarity of our samples and see whether or not our samples can be grouped by their habitat with respect to patterns of gene expression.

We used BLASTx to search the UniProt database for sequence similarity between significantly differentially expressed genes and proteins with known function. When genes were not associated with any known proteins in the UniProt database, we used BLASTn to search the NCBI non redundant (NR) database to find known genes with sequence similarity.

Microbiome analyses

We first joined our paired end reads in Qiime and quality filtered these reads using Qiime’s default parameters. 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.

The OTU table, phylogenetic tree, and a metadata file containing information on our sampling conditions was used as input for the Phyloseq package in R. We then used Phyloseq to assess the microbiome species richness for each individual sea star, and compare species richness between intertidal and subtidal sea stars. Additionally, we used Phyloseq to identify OTUs that differ in their abundance between intertidal and subtidal sea stars.

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). Further, there is a strikingly small amount of variance (36%) explained by our first two principal components.

The top six significant differentially expressed genes were associated with functions such as necrotic cell death, cell signaling, rRNA, and growth of collagen fibrils (Figure 2 and Figure 3).

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 largest 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* (Figure 4).

Additional significant differences between intertidal and subtidal sea stars were found in the abundance of the orders *Sphingomonadales* and *Vibrionales* (Figure 5). *Sphingomonadales* was significantly more abundant in intertidal sea stars, and *Vibrionales* was significantly more abundant in subtidal sea stars.

Results from our species richness analysis demonstrate high levels of richness across all individuals, with no significant differences in richness between intertidal and subtidal sea stars (Figure 6).

Discussion:

In this study, we examined the role of habitat type in defining P. ochraceus gene expression and microbiome composition. We expected that the distinct environmental conditions (namely in wave exposure, thermal stress, and desiccation stress) experienced by intertidal and subtidal P. ochraceus would drive differences in patterns of gene expression and microbiome composition.

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 in the same region are likely the reasons we did not see strong patterns of differential gene expression.

With respect to genes differentially expressed between intertidal and subtidal individuals, genes that play a role in necrotic cell death, growth of collagen fibrils, and rRNA all were upregulated in intertidal versus subtidal organisms. The increased expression of genes responsible for necrotic cell death in intertidal individuals may be a result of more intertidal sea stars succumbing to wasting than subtidal sea stars. 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 stimulation15. This allows for rapid body stiffening, and prevents sea stars (and other echinoderms) from being removed from substrate15. The upregulations of adapter protein associated with the ras signaling pathway in subtidal individuals may be an indicator of a heightened immune response in subtidal individuals. Work by Ragab et al. (2011) demonstrates the role of the ras pathway in modulating the immune deficiency pathway in Drosophila16. The upregulation of 5.8s rRNA in intertidal sea stars can potentially be linked to enhanced RNA virus activity17. RNA viruses, such as the Picornavirus, have been demonstrated to hijack host ribosomes, preventing host mRNA translation, facilitating translation of viral RNA17. These non-functional ribosomes may be polyadenylated as a signal for their degradation17. This would support their appearance in our poly-A selected libraries. 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 health17.

Results from our microbiome analyses demonstrate that *P. ochraceus* hosts a diverse assemblage of microbial taxa, regardless of host habitat.

Intertidal sea stars 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 agar7. *Pseudomonas* is also commonly found in aquatic environments, in addition to soil samples8,9. This genus hosts many species that are known to be opportunistic pathogens in plants, animals, and humans8,9. In addition to being an opportunistic pathogen, the presence of *Pseudomonas* is often an indicator of other opportunistic pathogens being present8,9. Sphingomonadales is commonly found in nutrient limited environments, is known to degrade aromatic compounds, and may play a role in ocean biogeochemical cycling12.

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 plants13. Many species of vibrio are associated with pathogens in fish and humans13. *Vibrionales* is also associated with indiscriminate infections and wounds13. *Oceanospirillales* was found in high abundance following the 2010 Deepwater Horizon oil spill, and is associated with degrading alkanes14. *Marinomonas mediterranea*, a species in the order Oceanospirillales, is also known to synthesize antibacterial proteins10,11.

The high relative abundance of *Pseudomonas* in intertidal *P. ochraceus*, and *Vibrionales* in subtidal *P. ochraceus* indicate that organisms from both habitat types hosted 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 three orders of bacteria all overlap in their associated functions. This indicates that while habitat may drive differences in microbiome composition, the functional niches filled by these OTUs may be conserved.

While the limited differences in microbiome composition and gene expression between our two groups may be driven by the biology of *P. ochraceus*, it may also be a factor of our sampling design. Our analysis included only samples from day 3, comparing the gene expression patterns of 23 individuals, and the microbiome composition of 37 individuals. This, in addition to a high prevalence of genes and OTUs with low read counts that were included in our analysis may have limited our power to identify existing differenced in gene expression and microbiome composition. Additionally, the differences we did see between our two groups may be a factor of collection stress rather than a signature of their natural environment. *P. ochraceus* collected subtidally are easy to remove from their substrate, resulting in a minimal amount of stress to the organisms. On the other hand, *P. ochraceus* collected from the intertidal needed to be pried from their substrate, which may have resulted in heightened stress in these organisms. Not only may this stress have played a role in altering patterns of gene expression, it may have compromised the health of our *P. ochraceus* individuals. It is possible that collection methods may be responsible for the differences seen in the health status between intertidal and subtidally collected sea stars.

Future analyses of these data will include a full functional enrichment analysis of our gene expression data. Additionally, further annotation of the functional differences in host microbiome will be assessed using the program PICRUST. 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.

As another avenue for identifying the role of habitat in defining gene expression and microbiome composition, 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, would provide a stronger signature of the environment on gene expression and microbiome composition.

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