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Variation in global transcriptomic response to *Perkinsus marinus* infection among eastern oyster families highlights potential mechanisms of disease resistance

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ABSTRACT

Dermo disease, caused by the protozoan parasite *Perkinsus marinus*, negatively impacts wild and cultured Eastern oyster populations, yet our knowledge of the mechanistic bases for parasite pathogenicity and the Eastern oyster's response to it is limited. To better understand host responses to the parasite and identify molecular mechanisms underlying disease-resistance phenotypes, we experimentally challenged two families exhibiting divergent Dermo-resistance phenotypes with the parasite, generated global expression profiles using RNAseq and identified differentially expressed transcripts between control and challenged oysters from each family at multiple time points post-parasite injection. The susceptible and resistant families exhibited strikingly different transcriptomic responses to the parasite over a 28-day time period. The resistant family exhibited a strong, focused, early response to *P. marinus* infection, where many significantly upregulated transcripts were associated with the biological processes "regulation of proteolysis" and "oxidation-reduction process." *P. marinus* virulence factors are mainly comprised of proteases that facilitate parasite invasion and weaken host humoral defenses, thus host upregulation of transcripts associated with negative regulation of proteolysis is consistent with a Dermo-resistant phenotype. In contrast, the susceptible family mounted a very weak, disorganized, initial response to the parasite. Few transcripts were differentially expressed between control and injected oysters, and no functional enrichment was detected among them. At the final 28 d time point 2450 differentially expressed transcripts were identified and were associated with either "G-protein coupled receptor activity" (upregulated) or "microtubule-based process" (downregulated). A handful of protease inhibitors were differentially expressed between control and injected susceptible oysters, but this function was not enriched in the susceptible data set. The differential expression patterns observed in this study provide valuable insight into the functional basis of Dermo resistance and suggest that the timing of expression is just as important as the transcripts being expressed.

1. Introduction

Disease significantly limits fish and shellfish production worldwide and there are few efficient, long-term management strategies to protect stocks exposed to naturally-occurring pathogens in open environments [1]. Dermo disease, a chronic condition of the Eastern oyster, *Crassostrea virginica*, was first detected in the Gulf of Mexico in the 1940s and by the 1980's had spread to the Chesapeake and Delaware Bays [2,3]. Oysters are exposed to the disease through suspension feeding and infections initiate along the digestive epithelia and pallial tissues [4,5]. Superficially, disease progression is characterized by reduced condition index, slowed growth, loss of reproductive output, atrophy/lysis of host tissues, and usually leads to death after oysters reach reproductive maturity [6]. While the effects of Dermo on cultured and wild *C. virginica* populations vary over space and time, populations spanning nearly the entire geographic range of the species are impacted and epizootics resulting in greater than 50% mortality are common [7,8].

Substantial economic losses to Eastern oyster fisheries and the aquaculture industry from Dermo disease spurred considerable research into the virulence and transmission of its causative agent and the host's response to it [9]. Infection with *Perkinsus marinus*, a protozoan parasite with a complex life cycle, causes Dermo disease. The meront or trophozoite stage of the parasite proliferates within oyster tissues [4] and attracts immune cells called hemocytes to the site of infection by promoting host cell motility [10]. In accordance with typical bivalve cellular defense mechanisms, hemocytes promptly internalize the parasitic

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cells; however, whether the phagocytic process is fully realized and effectively reduces infection is unclear [11,12]. Reports suggest that *P. marinus* meronts evade degradation by lysosomal enzymes and reactive oxygen species (ROS), actively divide within hemocytes, and spread throughout host tissues by inducing apoptosis or causing parasite-laden hemocytes to burst [13–15]. Furthermore, *P. marinus* secretes extracellular products (ECPs), primarily consisting of serine proteases, immediately after entering the host [16,17]. Serine proteases may degrade the extracellular matrix to facilitate parasite invasion [18]. *P. marinus* ECPs have also been shown to weaken the oyster's humoral immune defenses by reducing lysosomal activity, ROS production, cell motility, and hemagglutination [19].

The immune-related gene repertoire has been catalogued for several bivalve species using comparative genomics and/or transcriptomics. Based on homology searches against model species, consistently represented stress response and defense associated genes in bivalves include pattern recognition receptors (PRRs; e.g. toll-like receptors, scavenger receptors, lectins, fibroginen-related proteins, peptidoglycan recognition proteins, and retinoic acid inducible genes), transcription factors from the NF-kβ and interferon regulatory factor families, signal transducers such as G-protein-coupled receptors and Ras GTPases, select components of the complement system (e.g. C1q and complement C3), sequences coding for cytokines (e.g. tumor necrosis factors), antioxidant defense proteins (superoxide dismutase, thioredoxin, and peroxiredoxin), and a complex apoptosis machinery [20–23]. Many of these gene families are highly expanded in all bivalve genomes surveyed to date, likely reflecting adaptation to highly variable and stressful environments [21,24].

A relatively small number of bivalve studies have attempted to characterize transcriptomic responses to specific pathogens and the majority are focused on interactions between the host and bacteria [25–27] or viruses [28,29]. Only a handful have investigated the effect of *Perkinsus* parasites on host gene expression. In the Manila clam [30], found that hemocyte gene expression in response to *Perkinsus olseni* exposure (quantified with a 15K microarray) changed over a 31-day period and changes corresponded to the stage of infection. The largest number of genes responding to the parasite were detected at 5 d post-exposure and the number of upregulated and downregulated genes was roughly equal. Early in the infection cycle many genes associated with innate immune function (response to stimulus, signal transduction, and cytoskeletal organization) were downregulated; however, genes involved in pathogen recognition, the antimicrobial response, and serine protease inhibition were upregulated. By day 31, when the pathogen became established and was causing severe disease, genes involved in lipid and carbohydrate metabolism (which *Perkinsus* species sequester from their hosts to facilitate survival and proliferation) were significantly upregulated.

Two prior studies investigating the Eastern oyster transcriptomic response to *P. marinus* also detected a modest number of annotated, differentially expressed genes between control and infected samples. For example, a disease challenge experiment coupled with the suppression-subtractive hybridization technique identified 105 transcripts involved in the molecular response to *P. marinus* at 45 days post-exposure, of which 46 were annotated with BlastX search. Included among the annotated, upregulated genes were a lectin involved in the inflammatory response, a protease inhibitor, membrane receptors including a toll-like receptor, and genes associated with cytoskeletal structure [31]. In a separate study [32], challenged a strain of Eastern oyster selected for survival in a high-disease environment [33] with *P. marinus* and assessed differences in gene expression between infected and control groups at 30 d post-exposure with a 12K microarray developed from published EST sequences. Genes associated with the regulation of oxidative stress, apoptosis, and host defense (a galectin, TNF receptor, and a cathepsin among them) were represented among the eighty-five annotated, differentially expressed genes in both lightly and heavily infected challenged oysters. While these studies identify genes whose role in *P. marinus* infection merit further investigation, the scope was limited by the genomic resources and technologies available at the time, observations made at a single timepoint late in the infection process, and limited knowledge of the variation in Eastern oyster responses to the disease.

Despite efforts dedicated to revealing the mechanistic bases for *P. marinus* pathogenicity and the Eastern oyster's response to it, results from many of the foundational studies are equivocal, leaving several questions unanswered. Two recent developments provide new opportunities to enhance our understanding of this consequential host-parasite interaction. First, significant advances in characterizing and consistently measuring Dermo-resistant phenotypes within an Eastern oyster breeding population have allowed us to identify variation in oyster ability to avoid *P. marinus* ingestion, eliminate the parasite once established, and survive the infection [34,35]. Second, high-quality genomic resources now available for the Eastern oyster (e.g. Refs. [24,36] enable high-resolution, global assessment of oyster gene expression patterns in response to *P. marinus* exposure. Together, these developments can be leveraged to generate transcriptome profiles to better understand host responses to the parasite and isolate the genetic and molecular mechanisms underlying observed phenotypic differences among hosts. Similar approaches have been used to investigate the response to Newcastle Disease virus (NDV) in susceptible and resistant inbred chicken lines [37], Infectious Pancreatic Necrosis virus (IPNV) pathogenesis in resistant and susceptible families from an Atlantic salmon breeding program [38], and the effects of the haplosporidian parasite, *Bonamia ostreae*, on two European oyster stocks, one naïve to the disease and the other residing in a long-term affected area [39].

In this study, we used RNAseq and differential expression analysis to 1) generate global gene expression profiles for resistant and susceptible Eastern oyster families and 2) identify differentially expressed transcripts between control and *P. marinus*-challenged oysters within each family at three time points post-injection. Functional enrichment analysis of differentially expressed transcript lists for each within-family comparison was performed to gain further insight into phenotype-specific transcriptomic responses to the parasite over time and highlight putative transcripts and molecular pathways associated with Dermo resistance. Characterization of genome-wide differences in expression among hosts with divergent resistance phenotypes will not only enhance our general understanding of host-parasite interactions in this system, but also contribute to more effective disease management strategies including selective breeding for Dermo resistance.

2. Materials and methods

2.1. Disease challenge and RNAseq design

Disease challenge methods, including oyster source, pedigree and health history, animal care, exposure, and sampling protocols are described in detail in Ref. [35]. In brief, full sibling oyster families from the Aquaculture Genetics & Breeding Technology Center's (ABC) selective breeding program were challenged with either 5×10^6 *P. marinus* cells g^{-1} wet tissue weight (injected treatment) or artificial seawater (control treatment) via injection in adductor muscle tissue. For each treatment, N = 56 oysters per family were maintained in separate, quarantined, recirculating aquarium systems for six weeks and monitored daily for survival. In addition, six oysters per family per treatment were censored at 6 h, 36 h, 7 d, and 28 d post-injection to confirm successful parasite exposure (6 h), track changes in parasite load over time, and characterize global gene expression patterns (36 h, 7 d, and 28 d). Mantle, gill, and rectum tissues from each censored oyster were preserved separately in RNAlater (Invitrogen, Waltham, MA, United States) according to the manufacturer's instructions. Parasite load in censored oysters, expressed as log parasites g^{-1} wet tissue weight, was quantified at the Aquatic Diagnostics Laboratory (ADL; Roger Williams University, Bristol, RI) using DNA extracted from

rectum and/or mantle tissue with the real-time quantitative PCR (qPCR) assay developed by Ref. [40]. Relative resistance phenotypes for each family were determined based on survival and parasite elimination rate measurements.

Our global gene expression analysis focused on mantle tissues collected from two families identified as susceptible (low survival and low parasite elimination rate, family 242) and resistant (high survival and moderate parasite elimination rate, family 266) to *P. marinus* infection in the laboratory disease challenge [35]. Tissues collected from a minimum of five oysters per family, per treatment at the 36 h, 7 d, and 28 d timepoints (N = 62) were included in the analysis. We selected samples with consistent parasite loads for each group (e.g. 266_injected_36 h) to minimize within group variation in parasite-induced expression. Although hemocytes are the primary immune effector cells in oysters and play a prominent role in this host-parasite interaction, we characterized transcriptomic response in mantle tissue because 1) the small size of challenged oysters (shell height mean, standard error = 28.1 ± 0.48 mm) precluded collection of sufficient hemolymph volumes for subsequent RNA extraction and sequencing library preparation and 2) hemocytes are well distributed throughout the mantle and associated mucosal secretions [15].

2.2. RNA isolation, library preparation and sequencing

Archived tissue (5–10 mg per sample) was homogenized in 750 µl TRI Reagent® (MilliporeSigma, Burlington MA, U.S.A) with an Omni Tip homogenizer (Omni International, Kennesaw GA, U.S.A.) and total RNA extracted following the manufacturer's protocol. RNA extracts were subsequently treated with DNA-free™ DNase (Invitrogen, Waltham MA, U.S.A) and quantified with both the NanoDrop™ 8000 and the Qubit™ RNA HS Assay Kit (Thermo Scientific, Waltham MA, U.S.A.). Because molluscan 28s and 18s rRNA fragments are electrophoretically indistinguishable [41], RNA integrity (RIN) could not be calculated by the Agilent 2100 Bioanalyzer (Agilent, Santa Clara CA, U.S.A.). Instead, RNA quality assessment was based on the lack of degradation observed with traditional agarose gel electrophoresis as in Ref. [42].

Individual sequencing libraries were prepared for each sample included in the analysis. Tagged libraries were constructed according to the Illumina TruSeq Stranded mRNA (Illumina, San Diego CA, U.S.A.) protocol with approximately 0.5 µg DNAse-treated RNA. Equimolar amounts of uniquely-tagged libraries were pooled for sequencing such that one individual (biological replicate) per group (e.g. 266_injected_36 h, 266_control_36 h, 242_injected_36 h, 242_control_36 h, etc.) was represented in each pool. Library construction and sequencing of samples occurred in two batches during the fall of 2015 and fall of 2017. In 2015, a maximum of 24 individuals were included in each pool and pools were run on duplicate sequencing lanes to achieve the desired number of reads per individual and serve as technical replicates. Since no differences were observed between technical replicates in 2015, pools consisting of no more than 12 individual libraries were sequenced on a single lane in 2017. All pools were paired-end sequenced on an Illumina HiSeq 2500 instrument with the standard v4 2 × 125 kit, samples de-multiplexed, and raw sequencing reads QC'ed with FastQC.

2.3. RNASeq

To quantify global gene expression patterns in each study sample, sequence data were analyzed in CLC Genomics Workbench version 11 (https://www.qiagenbioinformatics.com/). In addition to FastQC analysis, we used CLC to trim reads with low quality (< Q20) and/or ambiguous bases and remove fragments < 50 bp. Trimmed reads were then mapped to the *C. virginica* representative genome assembly-3.0 (NCBI; GCA_002022765.4) using the RNA-Seq Analysis tool and default parameters except for the length fraction setting, which was reduced from 0.8 to 0.6 to address the high level of polymorphism observed in the Eastern oyster [24]. Reads that mapped as broken pairs and/or mapped to > 10 locations within the genome were not included in subsequent analyses to avoid confounding differential expression signals [43]. We obtained transcript-wise expression counts and applied the 'PCA for RNA-Seq' tool in CLC to assess whether the different library prep/sequencing dates resulted in a batch effect and to identify sample outliers, both of which can obscure differential expression patterns. A strong batch effect was detected (Supplementary file 1) and was included in the experimental model for differential expression analysis.

2.4. Differential expression, annotation and enrichment analyses

The R package DESeq2 v 1.22.1 [44] was used for differential expression analysis of transcript count data based on Log2 fold change (LFC) in expression between experimental groups. Transcripts with < 10 read counts across all samples being compared were considered not expressed. Because LFC estimates for transcripts with low counts are inherently noisy and prevent biologically meaningful ranking of differentially expressed transcripts in a data set, we applied approximate posterior estimation for a generalized linear model (apeglm) shrinkage to our LFC estimates. Apeglm shrinkage applies a heavy-tailed Cauchy prior distribution for effect size, which reduces variance without over shrinking large LFC changes [45]. We identified transcripts with LFC ≤ −1 or ≥1 and Benjamini and Hochberg's adjusted p-value to control false discovery rate ≤0.05 as significantly differentially expressed. To assess baseline expression differences between the resistant and susceptible families, we considered only control treatment samples within the experimental model: batch + family. Genetic differences in transcriptomic response to parasite exposure were characterized by identifying differentially expressed transcripts between control and injected groups for each family at each timepoint separately (e.g. 242_injected_36 h vs 242_control_36 h) using the model: batch + treatment. Results from these comparisons were visualized in Venn diagrams constructed with the R package Goplot v 1.0.2 [46].

Functional interpretation of our significantly differentially expressed transcript lists was achieved by annotation with Gene Ontology (GO) terms and GO term enrichment analysis in Blast2GO Pro v 5.2.4 using a standard workflow [47]. GO terms mapped to expressed transcripts were evaluated for annotation quality using the default parameters aside from the GO weight, which was increased from 5 to 15, prior to assigning annotation. We then merged our results with the results from an InterProScan search (v 5.26–66.0) to augment the number of annotated expressed transcripts. GO terms overrepresented in differentially expressed transcripts relative to all expressed transcripts in each comparison described above were identified by running Fisher's exact tests in Blast2GO. Those with an FDR-corrected p-value ≤ 0.05 were significantly enriched. Because lists of enriched GO terms are often long and difficult to interpret, we used Revigo with default parameters to condense them in a meaningful way. Rather than reducing lists by removing specific terms and keeping only general GO terms, Revigo reduces GO term lists based on semantic similarity, which provides a measure of their functional similarity [48].

3. Results

3.1. Resistant and susceptible phenotypes

Oyster survival was at or near 100% for both families when injected with sterile seawater. When injected with the parasite, family 242 experienced higher mortality (25%) than family 266 (10%) during the experimental period (Fig. 1A). With respect to parasite load, we did not detect a significant difference between families shortly (36 h) after injection (mean log *P. marinus* cells g^{-1} wet weight of 5.49 ± 0.32 and 5.58 ± 0.35 in families 242 and 266 respectively); however, by 28 d post-injection, *P. marinus* load was significantly lower in family 266 (3.99 ± 0.46 cells g^{-1} wet tissue weight) compared to family 242

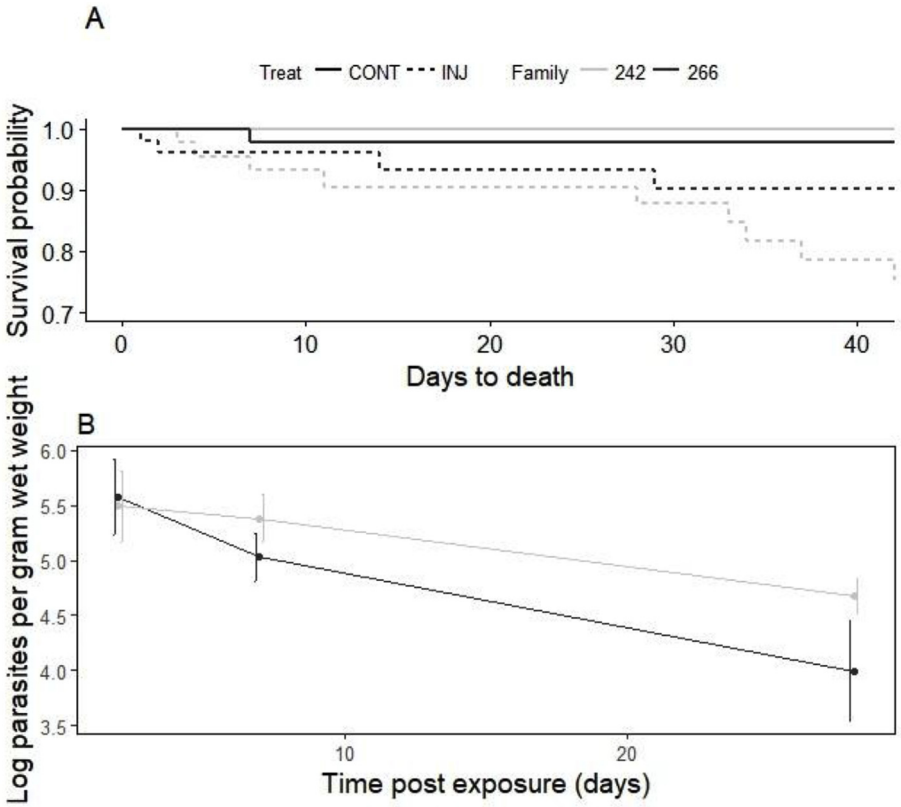

Fig. 1. Family-specific phenotypic responses to *P. marinus* exposure. (A) Survival probabilities in control and injected treatments during a 42-day laboratory challenge experiment. (B) Mean parasite load (± SE) in injected oysters measured at 36 h, 7d, and 28d post-injection with *P. marinus*.

(4.68 ± 0.16 cells g^{-1} wet tissue weight) (Fig. 1B). Our phenotypic measurements of parasite load over time and survival indicate that both families became infected with the parasite and that family 266 was better able to eliminate it and resist its negative effects.

3.2. Sequencing and read mapping

A total of 1,193,638,038 raw paired-end reads were sequenced in this study. The average number of reads from each sample grouping (family_treatment_timepoint) ranged from 15.5 million (266_INJ_7 d) to 22 million (242_INJ_28 d) reads. Variation observed in raw read counts among individuals within a group was largely driven by sequencing date. Sequence data was high quality. Approximately 97% of reads had a Q score > 30 and 98% of reads were retained in the data set following adapter trimming and filtering (Table 1). Depending on the sample grouping, between 74 and 79% of all reads mapped to the *C. virginica* genome as complete pairs (Table 1) which falls well within the expected range for uncontaminated, accurately-sequenced samples [49]. An additional 12.6–15.7% of reads from each group mapped to the genome as broken pairs, but they were not included in the transcript quantitation for differential expression analysis because they are often reads that mapped outside of the estimated pair distance and pair distance information increases the confidence that a read has been assigned to the correct location [50]. All raw sequence reads affiliated with this study have been deposited in the NCBI short read archive under accession # PRJNA590205.

3.3. Differential expression analysis

Approximately 86% of genome features (51,570/60,201 transcripts)

Table 1
Sequencing and mapping statistics averaged across individuals within each sample grouping.

Family	Treatment	Time point	Number of replicates	Average raw read count	% raw reads with Q > 30	Average post trimming/ filtering read counts	% pairs mapped	% broken pairs mapped (not counted)	% pairs not mapped (not counted)
242	Control	36 h	5	20,002,520	96.7	19,693,971	76.0	13.1	10.9
242	Control	7 d	6	19,354,296	97.2	19,034,866	77.2	12.6	10.2
242	Control	28 d	6	18,472,499	96.9	18,169,682	76.7	13.9	9.3
242	Injected	36 h	5	19,975,426	97.0	19,630,257	75.0	14.4	10.6
242	Injected	7 d	5	20,757,244	97.0	20,437,196	77.9	12.9	9.2
242	Injected	28 d	5	21,880,351	93.3	21,524,124	78.9	13.2	7.9
266	Control	36 h	5	18,305,270	97.0	18,011,720	76.3	14.0	9.7
266	Control	7 d	5	17,601,066	96.0	17,136,223	74.3	15.7	10.0
266	Control	28 d	5	19,295,562	95.8	18,528,220	77.6	14.2	8.2
266	Injected	36 h	5	20,287,946	96.6	19,911,514	76.3	14.1	9.7
266	Injected	7 d	5	15,576,292	97.0	15,308,892	77.0	14.3	8.7
266	Injected	28 d	5	19,653,777	96.7	19,313,400	77.8	13.8	8.4

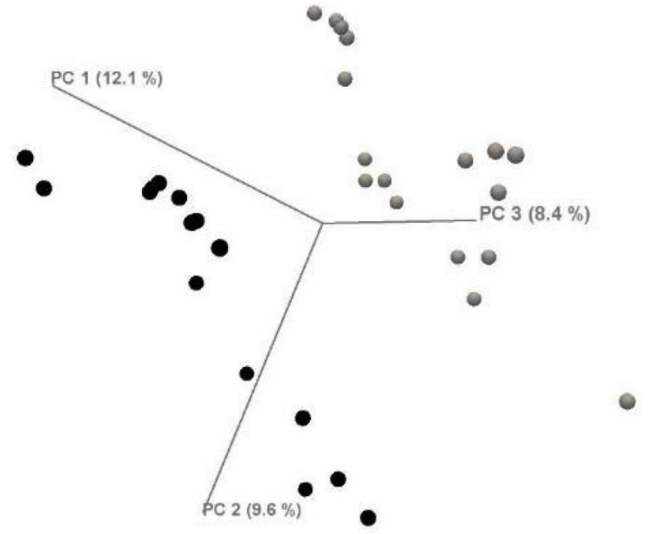

Fig. 2. 3D PCA plot generated using individual expression profiles in CLC Genomics Workbench and showing clear differences in baseline gene expression between families. Grey points correspond to the susceptible family (242) and black points correspond the resistant family (266).

were considered expressed in our data set. Significant differences in baseline expression between the two families were revealed when we compared all control samples among families. A total of 3185 transcripts were differentially expressed between the resistant (266) and susceptible (242) family; 2014 upregulated and 1171 downregulated. A Principle Component Analysis (PCA) of the gene expression data for all control samples clearly showed significant clustering by family along PC 1, 2, and 3 which explain 12.1, 9.6, and 8.4% of the variation in the data respectively (Fig. 2).

While inherent genomic differences between families surely contribute to the varied Dermo-resistance phenotypes, family-specific responses to *P. marinus*, assessed by comparing expression patterns among control and injected oysters at multiple timepoints, more directly address the mechanistic basis of resistance. As expected, the susceptible and resistant families exhibited strikingly different transcriptomic responses to the parasite over a 28-day time period (Fig. 3). At 36 h post-exposure, we detected 278 transcripts in the resistant family that were differentially expressed in the injected treatment relative to control and the majority (183) were upregulated. A similar magnitude response was observed in the same family at 7 d post-exposure

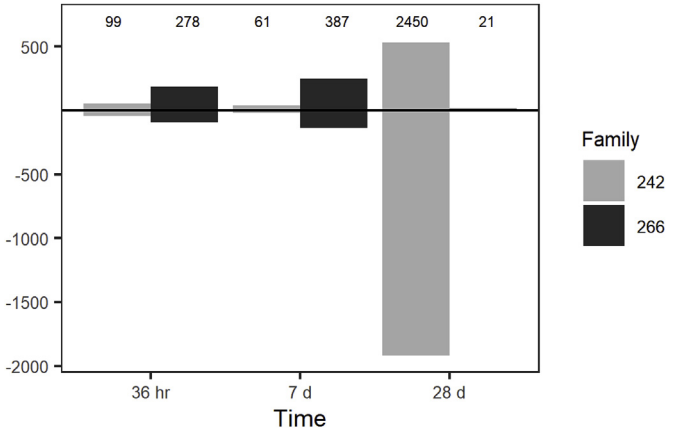

Fig. 3. Up- and down-regulated transcripts relative to control for each family at 36 h, 7 d, and 28 d post-exposure. The total number of differentially expressed transcripts is noted above each bar. Transcripts with an LFC > 1 or < -1 at an FDR-corrected p-value of 0.05 were considered differentially expressed.

(387 differentially expressed transcripts; 249 upregulated, 138 downregulated); however, by 28 d post-exposure, the expression of only 21 transcripts differed between injected and control groups. In contrast, relatively few transcripts differentially expressed between control and injected treatments were detected at the early timepoints in the susceptible family (99 and 61 at 36 h and 7 d respectively), but the expression of 2450 transcripts varied significantly by treatment at 28 d and 1918 of those transcripts were downregulated (Fig. 3).

We saw surprisingly little overlap in differentially expressed transcripts among timepoints within each family, suggesting a dynamic host response to the parasite and underscoring the value in assessing expression patterns at different stages of infection. No transcripts were shared across all three timepoints in either family. In the resistant family, the largest number of transcripts were shared between the 36 h and 7 d timepoints (46 upregulated and 3 downregulated; Supplementary file 2). Only 15 transcripts were shared between 36 h and 28 d in the susceptible family. Interestingly, those transcripts were expressed in opposite directions at the two timepoints (Fig. 4, Supplementary file 3).

3.4. Annotation and enrichment analysis

Of the 51,570 expressed transcripts in our data set, 7873 (15%) remained 'uncharacterized' following homology searches against the SwissProt and NCBI non-redundant protein databases and 31,160 (60%) were assigned GO terms after functional annotation in Blast2GO using relatively strict annotation criteria [47]. The percentage of 'uncharacterized' transcripts within comparison-specific differentially expressed transcript lists (e.g. 242_36 h) ranged from 28 to 39%. We were able to assign at least one GO term to between 53 and 70% of the differentially expressed transcripts identified in each comparison. We evaluated GO term enrichment in upregulated and downregulated transcripts relative to all expressed transcripts separately for each comparison and identified overrepresented GO terms within differentially expressed transcript lists in four out of 12 assessments. At 36 h post-infection, 28 GO terms were enriched among the upregulated transcripts in the resistant family (266) and zero GO terms were enriched among the downregulated transcripts. Again at 7 d, no significant GO enrichment was observed among downregulated transcripts in the resistant family, but 14 GO terms were enriched among upregulated transcripts. Eight enriched GO terms were identified among upregulated transcripts and 72 among downregulated transcripts at 28 d post-exposure in the susceptible family (242).

Significantly enriched GO terms among transcripts upregulated at the 36 h timepoint in the resistant family were associated with the molecular functions "peptidase inhibitor activity" and "molecular function regulator" and the biological process "regulation of proteolysis." Within "regulation of proteolysis", the GO terms "negative regulation of peptidase activity", "regulation of proteolysis" and "negative regulation of molecular function" were the most highly enriched (Fig. 5A). At 7 d post-injection, biological processes, "oxidation reduction process" and "protoporphyrinogen IX biosynthesis", were equally significantly enriched among upregulated transcripts while "peptidase inhibitor activity", "succinyltransferase activity", and "oxidoreductase activity" were equally enriched molecular functions (Fig. 5B).

GO term enrichment was only observed among differentially expressed genes at 28 d post-injection in the susceptible family. Those associated with the biological process "G-protein coupled receptor signaling pathway", the molecular functions "transmembrane signaling activity" and "actin filament binding", and the cellular components "membrane" and "actin cytoskeleton" were overrepresented among upregulated transcripts. Among down-regulated transcripts, the most significantly enriched GO terms were affiliated with molecular functions "microtubule motor activity", "calcium ion binding", and "extracellular ligand-gated channel activity" and cellular components

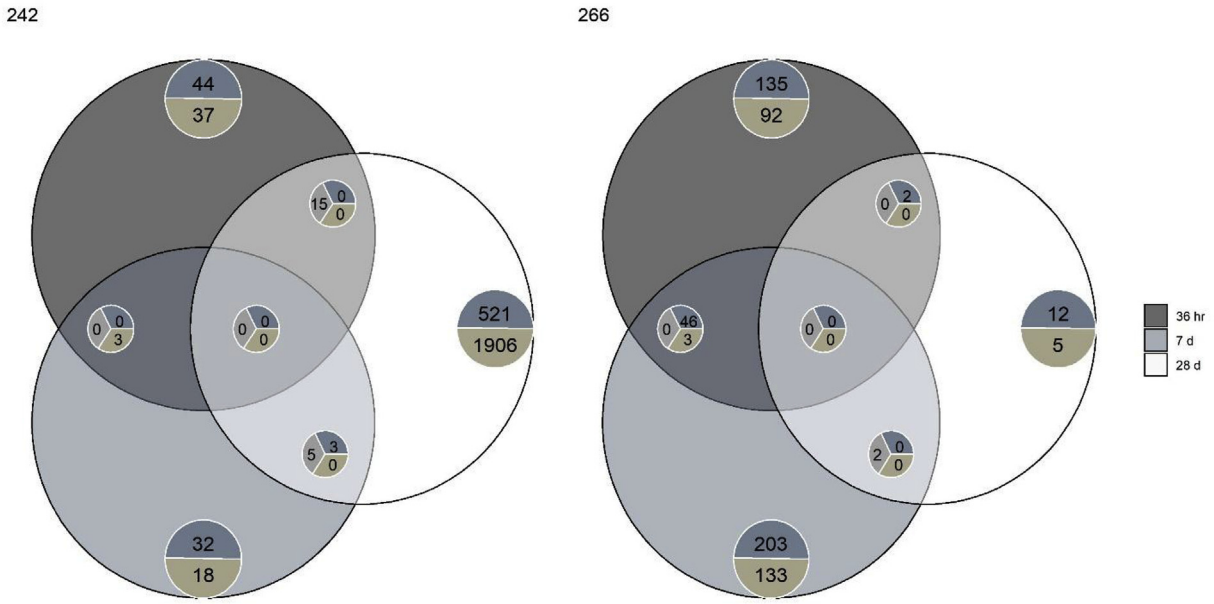

Fig. 4. Overlap in differentially expressed transcripts between time points within each family. Larger insets represent the number of upregulated (top number) and downregulated (bottom) number transcripts unique to each time point. Smaller insets represent the number of shared upregulated, shared downregulated, and shared but expressed in opposite direction transcripts between time points.

"cilium" and "cell projection" (Fig. 6A). Ninety-two percent of the biological process terms overrepresented in the downregulated transcripts for the susceptible family at 28 d fell under the more general GO term "microtubule-based process" (Fig. 6B).

4. Discussion

In order to gain a better understanding of *P. marinus* pathogenicity and the infection process, as well as the Eastern oyster response, we compared global gene expression patterns and differential expression in injected and control oysters from two families that varied in genetic resistance to the parasite. The resistant family (266) exhibited significantly higher survival and parasite elimination rates than the susceptible family (242). Phenotypic differences among families were reflected in their highly divergent transcriptomic responses to the parasite. A greater understanding of the contrasting responses to *P. marinus* in families 266 and 242 provides valuable insight into the functional basis of Dermo resistance and will enable more efficient genetic improvement of resistance traits.

Within 36 h post-injection, we observed a focused response to *P. marinus* in the resistant family. Enriched biological processes and molecular functions among differentially expressed genes were primarily associated with "regulation of proteolysis" and "peptidase inhibitor activity". Included among the most upregulated, characterized, transcripts (LFC between 2.5 and 9.7) were cystatin-A-like, BPTI/Kunitz domain-containing protein 4-like, four-domain proteases inhibitor-like isoform X1, eppin-like, kunitz-type serine protease inhibitor PILP-3-like, kielin/chordin-like, and metalloproteinase inhibitor 3-like proteins, all of which are protease inhibitors. The *P. marinus* genome codes for more than 500 protease genes and proteases often function as virulence factors for pathogenic parasites. In turn, host protease inhibitors can neutralize pathogen virulence by inactivating parasite proteases, making them an important component of the host immune response [51]. [52] were the first to identify protease inhibitors in oysters of the genus *Crassostrea* and [53] found that inhibitor activity was negatively correlated with Dermo disease intensity and mortality. Similarly, specific protease inhibitors purified from oyster plasma, namely CvSI-1, have been shown to limit *P. marinus* proliferation *in vitro* and be more highly expressed in eastern oysters selected for Dermo resistance [54]. Resistance has also been statistically associated with two mutations in the CvSI-1 gene sequence [55,56]. Despite the demonstrated role of CvSI-1 in this host-pathogen interaction and its putative role in Dermo resistance, it was not among the transcripts differentially expressed in response to *P. marinus* in our study. The failure to identify this important transcript in our analysis may be due to localized expression. We profiled gene expression in mantle tissue; however [54], found that CvSI-1 is primarily expressed in the digestive gland with expression levels three to four orders of magnitude higher than in any other tissue. In addition, given that CvSI-1 expression varies by genotype but not Dermo exposure status, it is likely a constitutively expressed gene and would not be detected in a differential expression analysis [54]. These conclusions are supported by our mantle transcriptome profiles for resistant and susceptible control oysters. CvSI-1-like genes were detected in both families, but at very low expression levels. Nevertheless, our results show a variety of upregulated protease inhibitors at 36 h post-injection and underscore their importance in the early response to *P. marinus* in the resistant oyster family.

Significant protease inhibition continued through 7 d post-injection in the resistant oyster family. Many upregulated protease inhibitors overlap with those overexpressed at 36 h (see Supplementary file 2), while others, such as serine protease inhibitor CvSI-2 are unique to 7 d. However, the biological processes "oxidation-reduction process" and the molecular function "oxidoreductase activity" were also significantly enriched. Specific upregulated transcripts (LFC $>$ 2) annotated with these GO terms include peroxidase, dual oxidase, and cytochrome P450 27C1-like proteins. As described in detail by Ref. [57] and summarized by Ref. [6]; production of cytotoxic reactive oxygen intermediates by hemocytes is an integral component of the bivalve immune system. Upon recognition, attachment, and endocytosis of pathogens, Eastern oyster hemocytes take up O_2 and produce superoxide anions that are converted to hydrogen peroxide. Peroxidase enzymes subsequently break down peroxide into cytotoxic hydroxyl radicals that kill pathogens. Recent characterization of dual oxidases in mammals, sea urchins, and nematodes indicate that dual oxidases are the source for hydrogen peroxide used in host defenses along mucosal surfaces and in extracellular matrix modifications [58]. Earlier work investigating ROS activity in hemocytes collected from both healthy and Dermo-infected oysters showed that introducing zymosan, a phagocytosis stimulant, to

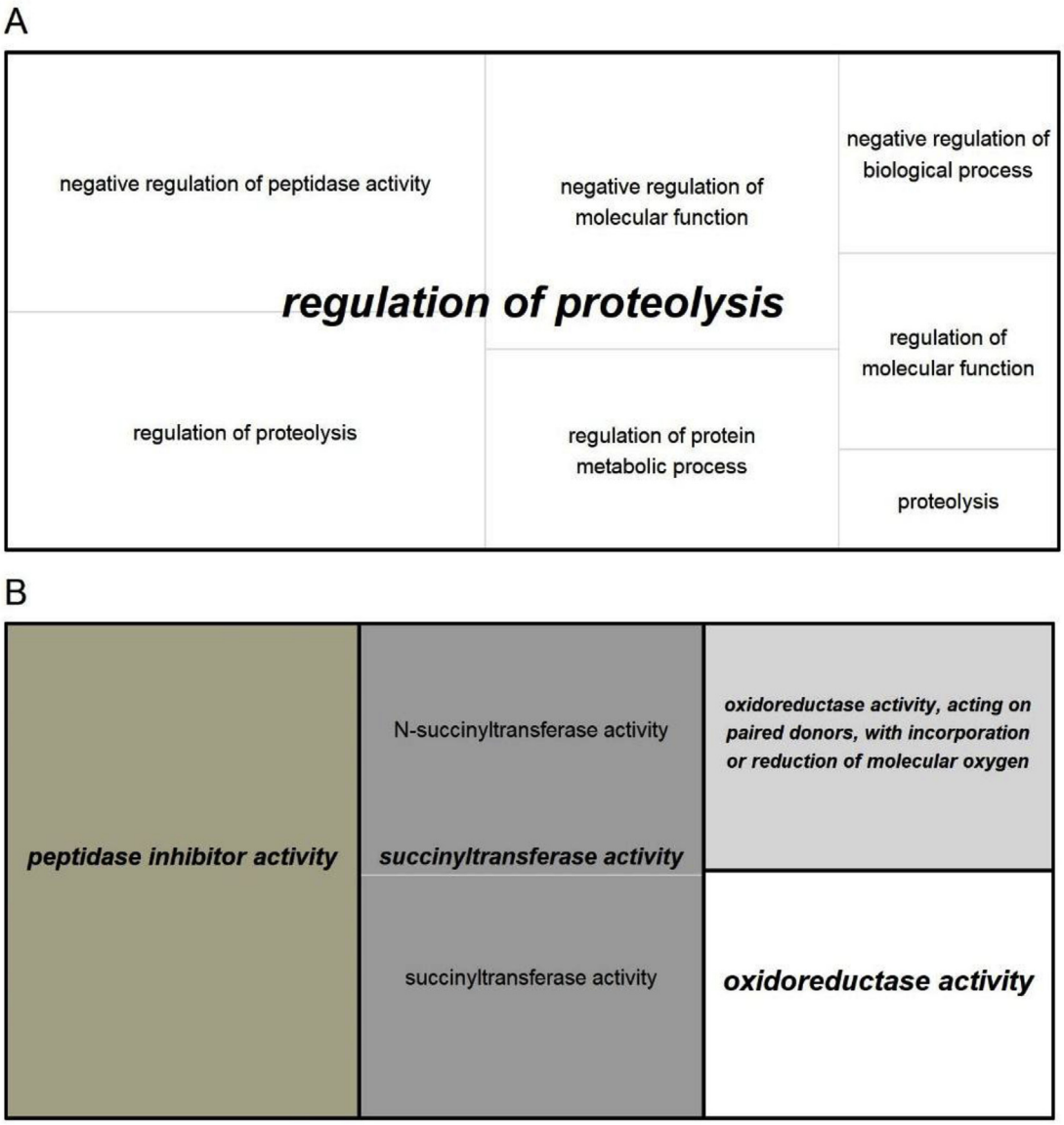

Fig. 5. Treemap summary of GO enrichment analysis results generated in Revigo for upregulated transcripts in the resistant family at early time points. (A) significantly enriched biological processes at 36 h post-injection with *P. marinus*, and (B) significantly enriched molecular functions at 7 d post-injection with *P. marinus*. Box color indicates relatedness among enriched GO terms while box size reflects the significance level of enrichment. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

cultured hemocytes resulted in up to six-fold increases in ROS over baseline levels while the addition of live *P. marinus* cells to matched hemocyte cultures led to no difference, or even a slight decline, from baseline. The author concluded that *P. marinus* somehow inhibits ROS activity and its ability to inhibit contributes to the parasite's pathogenicity [13,59]. While Anderson's conclusions contradict our results of successful inhibition of *P. marinus* virulence factors (ECPs containing serine proteases) and the realization of phagocytosis and parasite elimination in the resistant family, it is important to note that the oysters used by Anderson studies were wild, unselected, oysters likely susceptible to Dermo infection. A microarray-based gene expression analysis of the Eastern oyster response to *P. marinus* challenge using the Rutgers NEH line selected for high survival and disease resistance in Delaware Bay for over 15 generations found genes associated with oxidative stress to be upregulated in infected oysters at 30 d post- *P. marinus* exposure [32].

In contrast, a relatively weak, poorly-coordinated transcriptomic response to *P. marinus* was observed in the susceptible family immediately after injection. Although our functional annotation and enrichment analyses did not identify significantly overrepresented GO terms at 36 h post-injection, several immune-related transcripts were highly upregulated (LFC $\geq$ 3) including fibrinogen C domain-containing protein 1, complement C1q, caspase 9, and tetraspanin 9. Many invertebrate fibrinogen domain-containing proteins have been described as lectin-like pattern recognition receptors that agglutinate invading pathogens [60]. Complement C1q is also involved in pathogen agglutination and lysis [61], while Caspase 9 is an initiator of apoptotic pathways that has been associated with host-parasite interactions in many bivalve molluscs [62,63]; and [64]. In a recent study, exposure to *P. marinus* promoted increased tetraspanin expression in Eastern oyster hemocytes, thereby increasing cell motility [65]. A tetraspanin gene identified in the suminoe oyster, *Crassostrea ariakensis* was suggested to play a role in innate immunity due to its localization in hemocyte granules important for phagocytosis [66]. Taken together, the upregulation of these genes in the susceptible oyster family at the early stages of *P. marinus* infection reflects the existing paradigm for this specific host-parasite interaction. Upon infection, oyster hemocytes are induced to move toward the pathogens, agglutinate and internalize

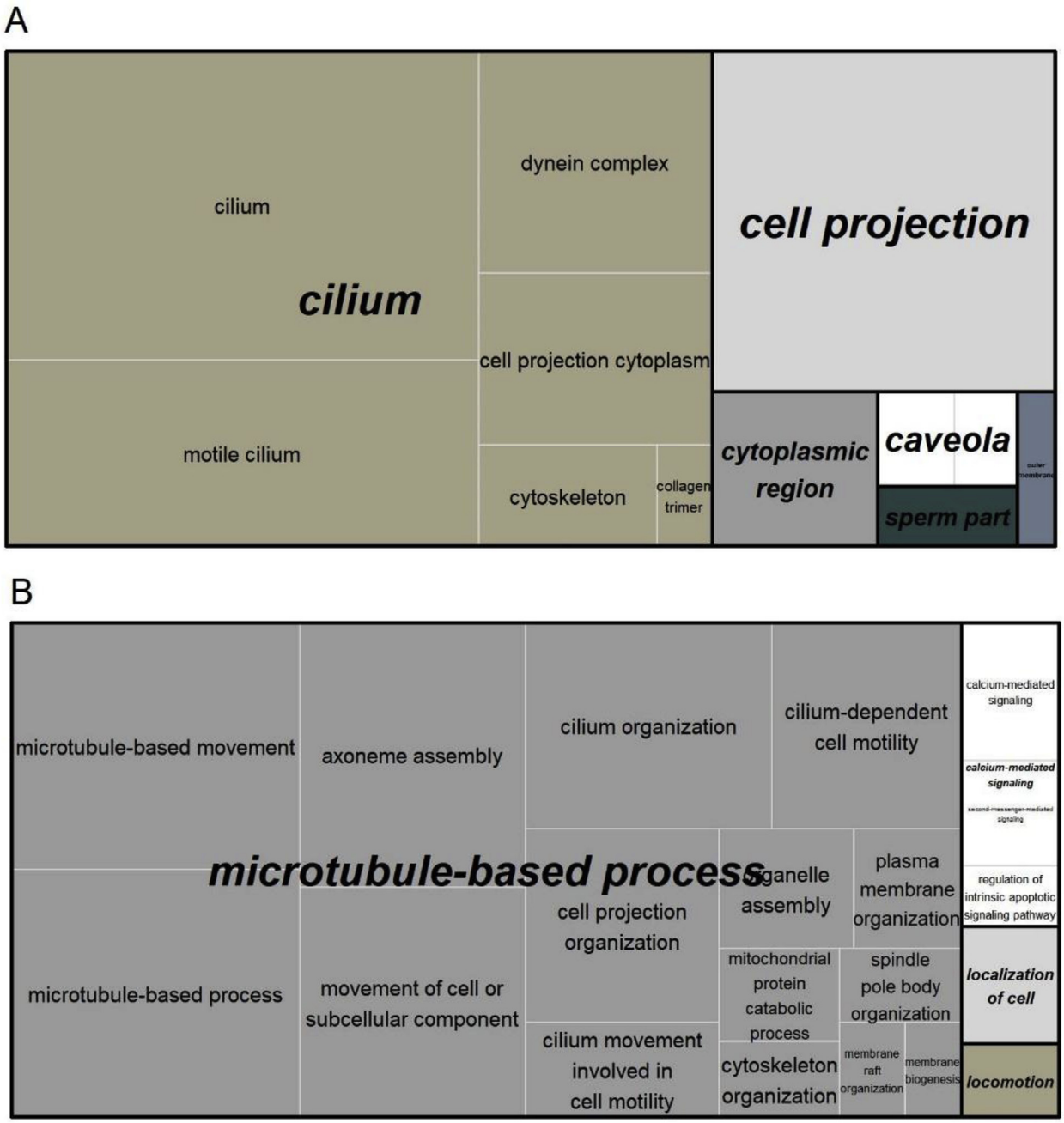

Fig. 6. Treemap summary of GO enrichment analysis results generated in Revigo for downregulated transcripts in the susceptible family at 28 d post-injection with *P. marinus*. (A) significantly enriched cellular components and (B) significantly enriched biological processes. Box color indicates relatedness among enriched GO terms while box size reflects the significance level of enrichment. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

them, and then attempt controlled cell-death, likely via the intrinsic mitochondrial pathway, to eliminate the pathogen and/or reduce its spread [67]. However, *P. marinus* virulence factors (e.g. serine proteases) allow the parasite to evade the host response, persist, and spread [51]. It is important to note that not a single protease inhibitor was among the transcripts differentially expressed between control and exposed susceptible oysters.

By 28 d post-injection a handful of protease inhibitors were modestly upregulated (LFC $<$ 2.4) in injected oysters relative to control in the susceptible family; however, GO terms associated with "negative regulation of proteolysis" and "protease inhibitor activity" were not significantly enriched among differentially expressed transcripts. Rather, the most overrepresented GO term in this data set included the biological process "G-protein coupled receptor signaling activity" and the molecular function "transmembrane signaling activity". Signal transduction is a critical element of the innate immune response in all bivalve molluscs and G-protein coupled receptors are a large family of transmembrane signaling proteins that function in a wide array of physiological processes, including immune system regulation and inflammation [61]. While specific G-coupled receptor protein roles are not well-characterized in bivalves, their importance in the immune response is supported by their upregulation in response to *P. marinus* (this study) as well as their upregulation in Pacific oyster juveniles exposed to oyster species herpes virus (OsHV-1) [28].

Among transcripts significantly downregulated in the susceptible family at 28 d post-injection, cytoskeletal elements and microtubule-

based processes were highly represented. Cytoskeletal functions including the maintenance of cell shape, facilitation of intracell transport, movement, and cell division are critical for survival and many pathogens co-opt host features to their own advantage. Yet the mechanisms by which parasites manipulate host cytoskeletal components for their own benefit remain elusive [68]. Within the context of host-parasite interaction, it is unclear why parasite intervention would result in the dramatic downregulation of cytoskeletal transcripts, specifically Dynein and Kinesin, in our dataset. Multiple isoforms of both genes, which are heavily involved in mitosis, meiosis, and intracellular transport, exhibited LFC as low as −10.95 in the injected treatment relative to the control. The downregulation of transcripts associated with microtubule-based processes (e.g. cell movement, cytoskeletal organization, etc.) may reflect the negative effects of the parasite on organismal growth. In a recent study comparing the transcriptomes of wild, unselected Pacific oysters and those selected for fast growth, the most significantly enriched GO terms among transcripts upregulated in the selected oysters were “microtubule-based movement”, “microtubule motor activity”, and “dynein complex” [69].

5. Conclusions

The availability of a high-quality genome assembly and Eastern oyster families with divergent Dermo-resistance phenotypes allowed us to harness the power of global transcriptomic and differential expression analyses to characterize host responses to *P. marinus* and gain a deeper understanding of how variation in the host-parasite interaction at the molecular level contributes to Dermo resistance. We observed an immediate, focused response to *P. marinus* by the resistant family. The dramatic upregulation of several transcripts coding for protease inhibitors appears to be essential in defending against potent virulence factors (proteases contained within ECPs) released by the parasite. Early deactivation of *P. marinus* proteases seems to limit parasite proliferation and facilitate the production of cytotoxic ROS that can further reduce parasite densities in the host. No such early, coordinated response was observed in the susceptible family. While we did see upregulation of some protease inhibitor transcripts in the susceptible family at the 28 d time point, the response appears to be too little, too late. Similarly-divergent gene expression patterns have been observed between resistant and susceptible families/lines of other oyster species. Upon exposure to *B. ostreae*, European flat oyster populations with naturally acquired resistance mount a rapid, more intense immune response than naïve populations [39]. Furthermore, the early reduction of OsHV-1 replication via the immediate upregulation of host antiviral pathways was shown to be the most important mechanism of resistance against Pacific Oyster Mortality Syndrome (POMS) in the Pacific oyster [70]. Taken together, these studies indicate that the timing of expression in response to infection is just as, if not more, important for resistance than the genes being expressed. Our comprehensive analysis of transcript expression in response to *P. marinus* infection over time has not only expanded our understanding of the mechanisms underlying Dermo resistance, but also generated a novel list of genes whose role in this host-parasite interaction should be investigated further. Functional characterization and validation of these putative resistance genes can then be incorporated into effective disease management strategies such selective breeding for Dermo resistance.

Author statement

DAP conceived the project, designed the experiment, oversaw all experimental work and sample preparation, assisted with data analysis and wrote the manuscript. MES conducted RNASeq, differential expression, and enrichment analyses and assisted with writing the manuscript.

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Appendix A. Supplementary data

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