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ORIGINAL PAPER

Immune response and mechanical stress susceptibility in diseased oysters, *Crassostrea virginica*

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Abstract Eastern oysters, *Crassostrea virginica*, naturally infected with the parasite *Perkinsus marinus* were subjected to a mechanical stress by centrifugation, and immune parameters, pathological conditions, and gene expression of selected transcripts were compared to uninfected controls. Immune parameters were assessed by flow cytometry, pathology and parasites by histotechnology and fluid thioglycollate assays, and gene expression by quantitative RT-PCR. Irrespective of mechanical stress, an increased number of hemocytes were observed in *P. marinus*-infected oysters that corresponded to increased expression of genes that have been shown to be involved in inflammation and apoptosis, two processes associated with regulating immune cell populations. Mechanically stressed, diseased oysters showed histological gill abnormalities and aggregations of hemocytes in tissues not seen in stressed, uninfected oysters. Expression of a high-mobility group protein and hemocyte phagocytosis were significantly upregulated upon mechanical stress only in uninfected oysters. The results of this study demonstrate the role of inflammation in the oyster immune response including possible underlying molecular mechanisms. Furthermore, this study highlights the importance of considering mechanical stressors when characterizing oyster immune function.

Keywords Oyster · Stress · Gene expression · Immune · Hemocyte

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Introduction

The eastern oyster, *Crassostrea virginica* (Gmelin), is a mollusk found along the east coast of the USA that has significant ecological and economic standing. For over 50 years, disease caused by *Perkinsus marinus* (Phylum Apicomplexa) has had detrimental effects on populations of this species. *P. marinus* was first described by Mackin et al. (1950) as *Dermocystidium marinum*, an agent causing extensive oyster mortalities in the Gulf of Mexico. Soon after, *P. marinus* was discovered in Chesapeake Bay (Andrews and Hewatt 1957) and in Delaware Bay a few years later (Ford and Haskin 1982). In the 1990s, *P. marinus* started northward range extension into the Long Island Sound, Rhode Island, Massachusetts, and Maine (Brousseau 1996; Ford 1996, Karolus et al. 2000; Kleinschuster and Parent 1995). There are no reports of *P. marinus* in eastern oysters on the west coast of the USA where early importations of *C. virginica* from the east coast have established a limited aquaculture industry. Initial infections are observed in the stomach and intestine epithelia of the oyster (Mackin 1962). As disease progresses, parasites are ingested by hemocytes and disseminated throughout the tissues (Perkins 1976). Histological presentation of

P. marinus induced disease consists of infiltration of hemocytes into connective tissue, atrophy of digestive tubules, and tissue lysis. *P. marinus* infections also result in reduced shell growth, reproductive output, and eventually death (Ford and Tripp 1996).

The immune system of an oyster is limited to an innate response comprising cellular and humoral components. Hemocytes are the primary defense cells, found in the hemolymph and interstitial spaces. Hemocytes are involved in inflammation, wound repair, phagocytosis, and oxidative burst activity. *P. marinus* is readily recognized and phagocytosed by oyster hemocytes, although phagocytosis is not able to completely destroy all pathogens (Anderson 1996). Phagocytosis is facilitated by galactose-containing surface proteins on the parasite that bind to complementary oyster-cell lectins (Vasta et al. 1995). Engulfed *P. marinus* have a mechanism to evade intracellular destruction by host reactive oxygen intermediates (ROIs). *P. marinus* produces iron-based superoxide dismutase (SOD), which scavenges host ROIs, allowing survival within the host (Ahmed et al. 2003). Other specific immune responses that have been examined in relation to *P. marinus* infection include protease inhibitor production (Oliver et al. 2000), inflammation (Ford and Tripp 1996), and apoptosis (Hughes et al. 2010). Some mechanisms have been identified as playing an important role in *P. marinus* response; however, a comprehensive understanding is lacking. Furthermore, there is a limited understanding of how a subsequent stress affects the immune response of parasitized oysters.

The goal of this study was to determine the physiological response of the eastern oysters to *P. marinus* infection using complementary approaches focused on tissue, cellular, and molecular effects. We also set out to determine how a subsequent mechanical stress would affect the physiology of infected oysters. Mechanical stress was used as it has been previously demonstrated to elicit a neuroendocrine change similar to other environmental stressors (Lacoste et al. 2001) and has been shown to alter immune function (Lacoste et al. 2002). Furthermore, mechanical stress is often encountered in aquaculture. The immune response is described through histopathological analysis, hemocyte population characterization, and gene expression analysis. Genes selected for analysis were based on their diverse putative roles in immune function coupled with the limited functional understanding of these genes in bivalves. This study provides important insight into the immune response of oysters, particularly with respect the effects of multiple stressors. This knowledge improves our basic understanding of innate immune function as well as having practical implications for aquaculture production of oysters.

Methods

Sampling

Eastern oysters, *C. virginica* (108 mm, SD 9.4 mm), potentially infected with *P. marinus*, were collected on the east coast of the USA from Milford Harbor, Connecticut (CT). Uninfected oysters, *C. virginica* (98 mm, SD 6.7 mm), were obtained from the west coast of the USA from a commercial shellfish farm in Shelton, Washington (WA). Twenty oysters were collected from each site. Oysters from CT were held at 17°C for 4 weeks (5th March till 5th April) in raceways supplied with continuous flow, unfiltered seawater from Milford Harbor. Oysters were shipped overnight from WA and analyzed the day they arrived. The approximate water temperature that the WA oysters were harvested from was 13°C. CT oysters were kept in a Styrofoam cooler overnight at the Milford Laboratory as the WA oysters were being shipped to provide similar conditions prior to the experiment. Following this period, oysters originally from CT and WA were divided into two groups and half ($n = 10$) were exposed to acute mechanical stress. Specifically, oysters were centrifuged for 5 min at 1,000 rpm ($350\times g$), a procedure shown to induce biochemical stress responses (Lacoste et al. 2001). Oysters were placed with their dorsal–ventral axis perpendicular to the axis of rotation. Hemolymph was immediately harvested with needle and syringe from the adductor muscle, through a notch clipped in the shell edge, and placed on ice. Each hemolymph sample was divided for flow-cytometric analysis of hemocytes and quantitative reverse transcription polymerase chain reaction (RT-PCR). All analyses were carried out on an individual basis. Directly following hemolymph sampling, tissue was taken for histological analysis.

Disease diagnosis and histopathology

Gross and histological examinations were performed to determine disease status in all individuals. *P. marinus* was diagnosed from anal–rectal tissues using Ray's fluid thioglycollate medium (RFTM) containing 2% NaCl and supplemented with a final concentration of 50 units of penicillin G and 50 μg of streptomycin sulfate per ml of medium (Bushek et al. 1994). The tissues were incubated in dark for 8 days at room temperature. The intensity of the infection in the oyster was rated using the Mackin Scale (0–5) (Mackin 1962). A value of zero was given if no hypnospores were present and a value of five when the infection was considered heavy. The values for all animals in a sample were averaged to determine the weighted prevalence value. Four-millimeter cross sections of the same oysters, including digestive diverticula, gills and

mantle, were excised and fixed in Davidson's fixative for 48 h at 4°C. Samples were dehydrated and embedded in paraffin. Sections of 5 μm were stained using hematoxylin–eosin (Howard et al. 2004). Histological slides were examined to detect any infectious organisms (viruses, bacteria, or parasites) or any histopathological change, such as inflammatory response, degeneration, cell and tissue death, growth derangement, and hemodynamic and fluid derangement.

Hemocyte characteristics

Hematological characteristics, including counts of live and dead granular and agranular hemocytes, relative sizes of hemocytes, and internal complexities of these hemocyte classes, were recorded using the flow-cytometric methods described in Hégaret et al. (2003a). One immune function variable, phagocytosis of fluorescent microbeads, was quantified (Hégaret et al. 2003b). A FACScan flow-cytometer (B-D BioSciences, San Jose, CA) was used for hemocyte analyses, and cytometric data were processed using WinMDI 2.8. Two-way ANOVAs were carried out to determine significant differences ($p < 0.05$).

Gene expression analysis

To characterize the effects of *P. marinus* and physical stress on immune physiology, quantitative RT-PCR was carried out on hemocytes from all oysters. RNA was extracted from hemocytes using Tri-Reagent (MRC), and samples were treated with DNase (Ambion, Turbo DNA-free) to remove any possible genomic-DNA carryover. RNA was then transcribed to cDNA using AMV reverse transcriptase (Promega). For gene expression analysis, a set of genes involved in immune response was selected. Quantitative PCR reactions (Brilliant SYBR Green QPCR Master Mix Kit, 1-Step, Stratagene) were carried out in an Opticon2 Continuous Fluorescence Detection System (Bio-Rad). Each 25-μl reaction contained 2× Master Mix, 0.04 μM of each primer, 2 μl of sample, and sterile water. Primer sequences and gene names are provided in Table 1. Reactions were carried out as follows: 10 min of initial denaturation at 95°C, 40 cycles of 30 s denaturation at 95°C, 1 min of annealing at 55°C, and 30 s of extension at 72°C, with fluorescence measured at the end of annealing and extension steps. Following 1 min of denaturation at 95°C, melting curve analysis was performed by increasing the temperature from 55 to 95°C at a rate of 0.2°C s^{-1}, measuring fluorescence every 0.5°C. A single product was observed in all melt curve analyses, and quantitative PCR products were sequenced to verify product identity. All samples were run in duplicate.

Analysis of PCR data was carried out based upon the kinetics of individual PCR reactions using Real-time PCR Miner v2.1 (Zhao and Fernald 2005). All data were normalized to corresponding 18S RNA values and expressed as fold increases over the minimum. All data were then log-transformed [natural log (value +1)] for statistical analysis. Two-way ANOVAs were carried out to determine significant differences in expression ($p < 0.05$). The factors examined included infection status and exposure to mechanical stress (PASW Statistics 18).

Results

Parasites and pathology

Prevalence of *P. marinus* in CT samples was 85% and the weighted prevalence was 1.3. Oyster samples from WA contained one thioglycollate-positive specimen with a very light infection. The thioglycollate assay is considered specific for the *Perkinsus* family that contains several species in addition to *P. marinus*, which infects oysters

Table 1 Primer sequences used for quantitative RT-PCR analysis

Gene [accession no.]	Primer sequences	Blastx hit organism [*E*-value]
Cytokine-induced apoptosis inhibitor (CIAPIN) [DQ403192]	TCTGTCGCATTCTGAAACCAAATGG, TGCCACGTATTCCTGGGATTTATCC	*Bos taurus* [2e-24]
Lysosomal phospholipase (LP) [DQ889751]	TTTGCCCCAATCGTGAACGC, AAACAACACCCAGACTGTCCCCTG	*Homo sapiens* [7e-64]
Cathepsin L (CatL) [EU437741]	ACAGGGCTGGAAGGAGTTCAAGAT, ATGTCCTGTTCAGGCCGTTGTA	*Pinctada fucata* [2e-43]
High-mobility group protein (HMGP) [EU437744]	CAAAGAAGGCCGGCAAACCCATAA, ATTCTTGCCCTTCATCTTGGCCCT	*Saccostrea kegaki* [8e-39]
18S Ribosomal RNA [L78851]	AATACATGCAACGAAGCTCCGACC, TTTCTCATGCTCCCTCTCCGGAAT	n/a

E-values are provided for significant Blastx hits as well as corresponding organism

only on the east coast of the USA. Uninfected specimens from CT (3) and an infected specimen (1) from WA were omitted from the following data. Mechanically stressed *P. marinus*-infected specimens had unusual gill lesions that were not present in any other oysters. A normal gill of the eastern oyster is composed of two demibranchs, and each demibranch is composed of two lamellae. The lamellae consist of a series of folds, plicae that contain several filaments. There is an intraplical sinus within the plicae that opens into the hemolymph sinuses inside each filament (Fig. 1a, b). Stressed, infected specimens had dilated sinuses within the plicae and filaments that gave the gills an edematous appearance (Fig. 1c, d). There was an apparent increase in hemocytes in the sinuses of the affected specimens.

Hemocyte characteristics

Most hemocyte parameters varied with regard to *P. marinus* infection status. Hemolymph from *P. marinus*-infected oysters had a significantly larger number of both granular and agranular hemocytes (Table 2). In contrast, uninfected oyster hemocytes were significantly larger in overall size (Table 2). There was no significant effect of mechanical stress on hemocyte number or size (data not shown), and percentages of dead hemocytes in the different treatments were in the range of 3–5% with no significant contrasts. Hemocytes from oysters without *P. marinus* infections had more phagocytic hemocytes than infected oysters, but only after mechanical stress (one-way ANOVA contrasting infected and uninfected oysters after mechanical stress, $p < 0.05$) (Fig. 2).

Gene expression analysis

There was not a significant main effect of mechanical stress on gene expression across all samples (Table 3). There was a significant result for the interaction term for HMGP expression indicating that the infection status influenced expression levels when oysters were subjected to mechanical stress (Table 3; Fig. 3d). *P. marinus* infection was associated with significant upregulation of three

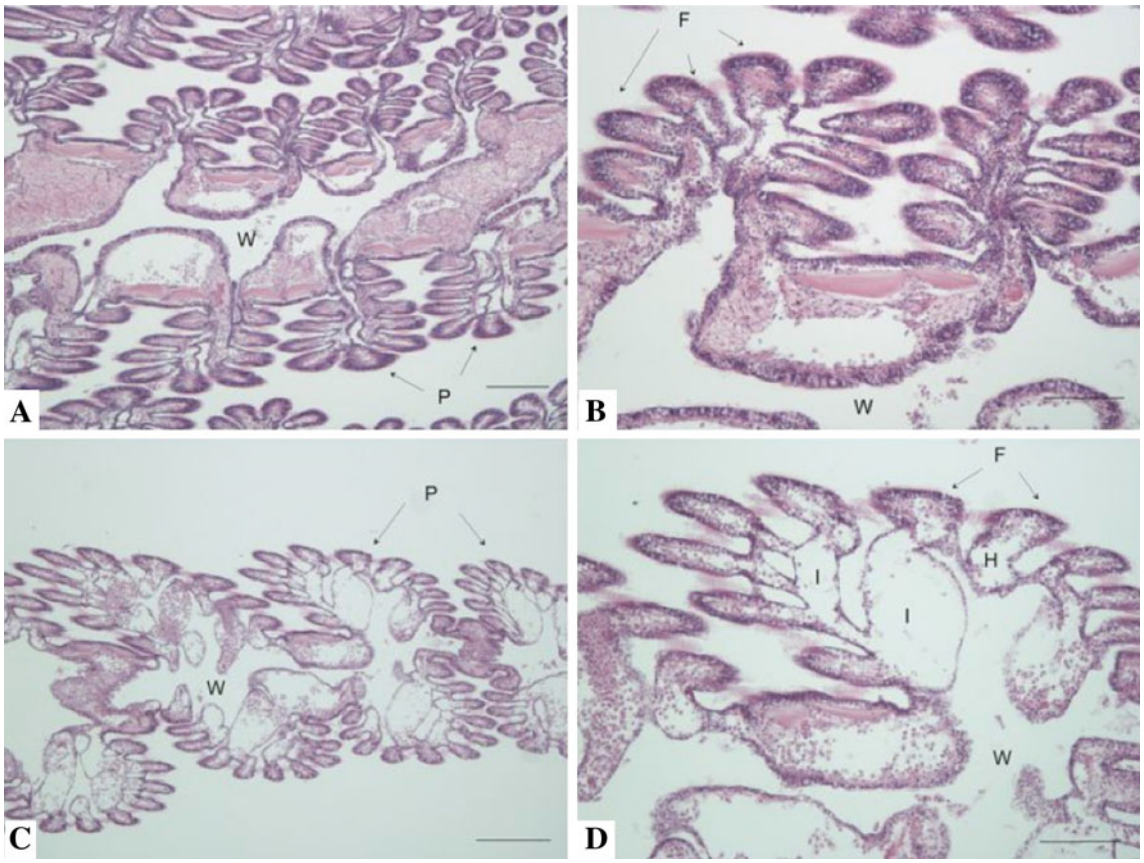

Fig. 1 Cross sections of gills from the eastern oyster, *C. virginica*, harvested in CT. Paraffin sections, hematoxylin–eosin. **a** Unstressed *P. marinus*-infected oyster. *Scale bar*, 250 μm. **b** Unstressed *P. marinus*-infected oyster. Higher magnification of (**a**). *Scale bar*, 100 μm. **c** Stressed *P. marinus*-infected oyster. *Scale bar*, 250 μm. **d** Stressed *P. marinus*-infected oyster. Higher magnification of (**c**). *Scale bar*, 100 μm. *I* intraplical sinuses, *F* filaments, *H* hemolymph sinus of filaments, *P* plicae, *W* water tube

Table 2 Hematology of oysters, *C. virginica*, from two cohorts, infected with *P. marinus* ($n = 17$) and uninfected ($n = 19$), determined by flow cytometry (mean ± SE)

	P. marinus infected	Uninfected
Granular cells × 10^6 ml^{-1}	0.214 ± 0.0556	0.0614 ± 0.014
Agranular cells × 10^6 ml^{-1}	2.27 ± 0.458	0.586 ± 0.065
Granular cell diameter in μm	8.52 ± 0.30	11.4 ± 0.20
Agranular cell diameter in μm	5.31 ± 0.10	6.29 ± 0.31

No effect of mechanical stress was observed; therefore, data from stressed and unstressed were combined

All comparisons of *P. marinus*-infected and uninfected variables presented in this table are significantly different

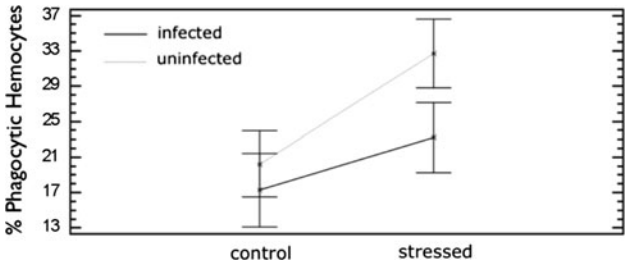

Fig. 2 Percentage of phagocytic hemocytes in *P. marinus*-infected ($n = 17$) and uninfected oysters ($n = 19$). A significant difference was observed after mechanical stress only in uninfected oysters (ANOVA $p < 0.05$)

transcripts with homology to a cytokine-induced apoptosis inhibitor (CIAPIN) ($p < 0.05$), lysosomal phospholipase (LP) ($p < 0.001$), and cathepsin L (CATL) ($p < 0.001$) (Fig. 3). A high-mobility group protein (HMGP) was expressed at a significantly lower level in *P. marinus*-infected oysters as compared to uninfected oysters ($p < 0.001$) (Fig. 3d).

Discussion

The current study was undertaken to examine the host response in oysters (*C. virginica*) infected with *P. marinus*, alone and combined with the effects of mechanical stress.

Irrespective of mechanical stress, an increased number of hemocytes were observed in *P. marinus*-infected oysters, which corresponded to increased expression of genes that have been shown to be involved in inflammation and apoptosis, two processes associated with regulating immune cell populations. Another key finding of this work was that exposure to mechanical stress resulted in histological gill abnormalities and edema in *P. marinus*-infected oysters that was not seen in other oysters. One of the most intriguing findings was that hemocytes from infected oysters did not have elevated rates of phagocytosis following mechanical stress, as did uninfected oysters.

The prevalence of *P. marinus* in CT samples was high, 85%, allowing comparison with uninfected oysters. A significant difference between these oysters was an elevated number of hemocytes in the *P. marinus*-infected samples. This was quantified by flow cytometry and observed by histological examinations of the gill hemolymph sinuses. These results are similar to what has been described previously, as increased number of hemocytes is associated with an inflammatory response, one of the main characteristics of *P. marinus* infection (Ford and Tripp 1996). In mammals, upstream regulators of inflammation are phospholipases A2 (PLA2s), which hydrolyze arachidonic acid. Prostaglandins, primary mediators of inflammation, are synthesized from arachidonic acid. In the current study, a phospholipase with significant homology to vertebrate PLA2s was expressed at a higher level in hemocytes from infected oysters, compared to uninfected oysters (Fig. 3b), consistent with observed inflammation and suggesting a conserved role for prostaglandins in invertebrates.

The protease cathepsin L is also involved in the inflammatory response. Cathepsin L has been shown to be able to generate kinins from kininogens, indicating that this protease could act as a kininogenase at inflammatory sites (Desmazes et al. 2003). Several cathepsins have been described previously in molluscan shellfish, usually related to development (Liu and Warner 2006; Donald et al. 2003) and environmental-contaminant exposure (Venier et al. 2006). In the present study, CATL expression was strongly associated with the number of circulating granulocytes and was upregulated in response to *P. marinus* intensity (data

Table 3 Two-way ANOVA analysis output values for gene expression analysis

	CIAPIN			LP			CATL			HMGP		
Factor	*df*	*F*	*p*	*df*	*F*	*p*	*df*	*F*	*p*	*df*	*F*	*p*
P. marinus infection	1	4.795	0.037	1	21.606	0.000	1	12.239	0.001	1	18.714	0.000
Mechanical stress	1	0.211	0.650	1	2.731	0.108	1	0.674	0.418	1	3.626	0.067
Interaction	1	0.000	0.982	1	1.070	0.090	1	0.007	0.933	1	5.939	0.021

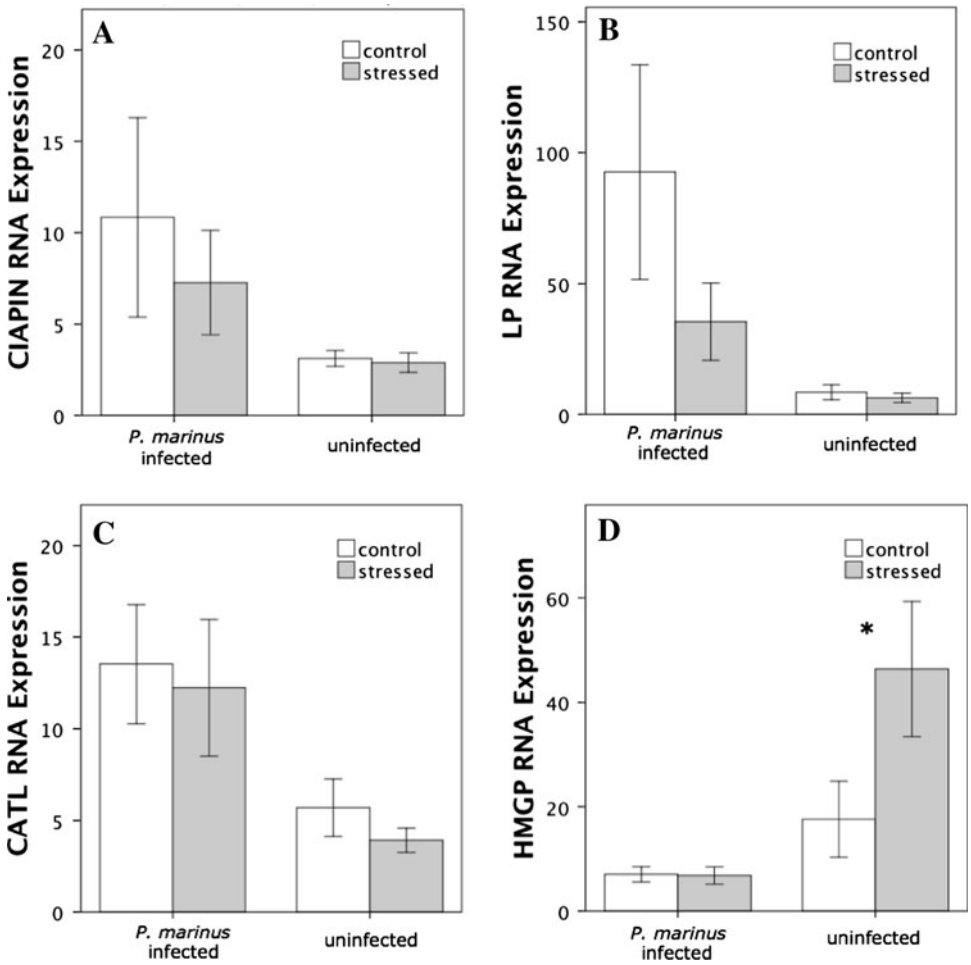

Fig. 3 Average expression levels of *C. virginica* cytokine-induced apoptosis inhibitor (CIAPIN) (**a**), lysosomal phospholipase (LP) (**b**), cathepsin L (CATL) (**c**), and high-mobility group protein (HMGP) (**d**) in hemocytes from *P. marinus*-infected and uninfected oysters exposed to mechanical stress (*gray bars*) and not exposed to mechanical stress (*white bars*). Values are expressed as fold increase over minimum. *Error bars* indicate standard error. Expression level is significantly different between *P. marinus*-infected and uninfected oysters for all genes examined. There was no main effect of mechanical stress for any gene examined; however, there was a significant interaction effect for HMGP, designated with an *asterisk*

not shown and Fig. 3c respectively). To our knowledge, this is the first report of CATL being involved in bivalve immune function.

The increased number of hemocytes in oysters with *P. marinus* infection could also be related to the ability of the pathogen to regulate hemocyte numbers. According to Sunila and LaBanca (2003), the increased number of hemocytes is attributable to prevention of hemocyte apoptosis by *P. marinus*. Recently, Hughes et al. (2010) demonstrated that *P. marinus* was able to decrease apoptosis in oyster cells. In this study, expression of a cytokine-induced inhibitor of apoptosis (CIAPIN) homolog was increased in *P. marinus*-infected oysters consistent with this explanation (Fig. 3a). Although there was a greater number of hemocytes, the size of hemocytes in infected oysters was significantly smaller. In this study, we are not able to assess the rates of hemocyte differentiation, growth, or apoptosis directly to determine which mechanism(s) contribute to this observed difference.

To better understand the relationships between physical stress, *P. marinus* infection, and the immune response, half of the oysters were exposed to acute mechanical stress by centrifugation. Severe edema was observed only in the infected oysters subjected to mechanical stress. Edema in the gill lesions was extracellular, in the sinuses of the plicae and filaments. The cascade of events during an inflammatory response in oysters is poorly understood, but we hypothesize that the inflammatory response induced by *P. marinus* infection was associated not only with increased numbers of hemocytes, but also with increased permeability of gill epithelial cells that allowed intake of water in the gills during mechanical stress. Changes in osmolarity related to salt balance across epithelial cell membranes probably contributed to the mechanism. Edema was possibly mediated by occlusion of hemocyte vessels, an event reported to occur during *P. marinus* infection (Ford and Tripp 1996) that would prevent outflow of water from the gill sinuses.

In terms of hemocyte immune function, mechanical stress increased hemocyte phagocytosis only in uninfected oysters. This increase in hemocyte phagocytosis would be expected in response to tissue damage caused by normal

cell disruption. Lacoste et al. (2002) also observed an increase in phagocytic hemocytes following mechanical stress in oysters, though they did observe a decrease in phagocytosis during the stress event. While it is likely that severe mechanical stress could have a negative impact on oyster physiology, our results suggest that occasional mechanical stress may increase immune response by stimulating phagocytosis in healthy oysters. More studies, however, would be needed to determine if the increased number of phagocytic hemocytes would be available to respond to a parasite or pathogen as opposed to removing any damaged cells.

Mechanical stress was also associated with increased expression of HMGP, only in uninfected oysters (Fig. 3d). Similar to phagocytosis, HMGP proteins are important in immune response and for removing damaged cells (Dumitriu et al. 2005). In the current study, HMGP expression decreased in oysters infected with *P. marinus*. The differential expression pattern in uninfected oysters could indicate a negative impact of *P. marinus* infection on oysters' ability to effectively respond to cell damage. This process would have implications in an aquaculture setting, as well as the wild capture fisheries, as a mechanism by which diseased shellfish are less likely to recover when subjected to mechanical stress.

In summary, the results of the current study correspond with previous findings on the role of inflammation in the oyster immune response while providing insight into the molecular mechanisms associated with this response. It is also likely that the increased hemocyte presence in infected oysters could be related in part to apoptosis inhibition by *P. marinus*. The combination of stressors in the current study suggests modulating effects of *P. marinus* infection, including increased susceptibility to edema and decreased ability to repair tissue damage.

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