Transcriptomic, proteomic, and functional assays underline the dual role of extrapallial hemocytes in immunity and biomineralization in the hard clam *Mercenaria mercenaria*

Caroline Schwaner[1], Sarah Farhat[1], John Haley[2], Emmanuelle Pales Espinosa[1], Bassem Allam[1]*

[1]School of Marine and Atmospheric Sciences, Stony Brook University, Stony Brook, NY, United States
[2]Stony Brook University Biological Mass Spectrometry Center, Stony Brook Medicine, Stony Brook, NY, United States

*** Correspondence:**
Bassem Allam
bassem.allam@stonybrook.edu

Keywords: clams, biomineralization, immunity, extrapallial fluid, hemolymph, hemocytes

Abstract

Circulating hemocytes in the hemolymph represent the backbone of innate immunity in bivalves. Hemocytes are also found in the extrapallial fluid (EPF), the space delimited between the shell and the mantle, which is the site of shell biomineralization. This study investigated the transcriptome, proteome, and function of hemocytes found in the EPF and hemolymph in the hard clam *Mercenaria mercenaria*. Total and differential hemocyte counts were similar between EPF and hemolymph. Overexpressed genes in the EPF were found to have domains previously identified as being part of the "biomineralization toolkit" and involved in bivalve shell formation. Biomineralization related genes included chitin-metabolism genes, carbonic anhydrase, perlucin, and insoluble shell matrix protein genes. Overexpressed genes in the EPF encoded proteins present at higher abundances in the EPF proteome, specifically those related to shell formation such as carbonic anhydrase and insoluble shell matrix proteins. Genes coding for bicarbonate and ion transporters were also overexpressed, suggesting that EPF hemocytes are involved in regulating the availability of ions critical for biomineralization. Functional assays also showed that Ca^{2+} content of hemocytes in the EPF were significantly higher than those in hemolymph, supporting the idea that hemocytes serve as a source of Ca^{2+} during biomineralization. Overexpressed genes and proteins also contained domains such as C1q that have dual functions in biomineralization and immune response. The percent of phagocytic granulocytes was not significantly different between EPF and hemolymph. Together, these findings suggest that hemocytes in EPF have dual functions of biomineralization and immunity.

Introduction

Bivalves have an open circulatory system populated by hemocytes, the quintessential component of bivalve immunity. Hemocytes function in several additional physiological processes including wound healing, biomineralization, nutrition, and transport (Cheng, 1981; Cheng, 1984; Canesi et al., 2002; Mount et al., 2004). Hemocytes in the hemolymph, the blood of bivalves, are well studied and known to serve as the main constituent of innate immunity (Song et al., 2010; Canesi et al., 2002; Allam & Raftos, 2015; Burgos-Aceves & Faggio, 2017). Hemocytes are also found in the extrapallial fluid (EPF), an aqueous microenvironment between the mantle and the shell which is the site of shell formation. While hemocytes are believed to play roles in biomineralization (Mount et al., 2004; Huang et al., 2018), there is much less known about functional differences between cells present in EPF and those in the circulatory system.

Differences between populations of hemocytes have primarily focused on morphological diversity and classification into the subpopulations granulocytes and agranulocytes (Foley and Cheng, 1977; Allam et. al 2002; Chang et al., 2005). These classifications are based on size and presence or absence of granules. While it is known that granulocytes are more phagocytic than agranulocytes (Wang et al., 2017a), functional differentiation of hemocyte populations, and specifically differentiation between hemocytes from different fluids, lags behind. Studies that have examined functional differences between hemocytes from hemolymph and EPF did not employ omic approaches (Allam & Paillard, 1998; Lau et al., 2017). With the advent of omic technologies, it is now possible for molecular determination of functions of hemocytes between different fluids, addressing this long-standing question. The genome of *Mercenaria mercenaria*, the hard clam, has recently been assembled at a chromosome level and genes were annotated (Farhat et al., 2021) facilitating the ability to classify genes and proteins of hemocytes into functional groups. Molecular differentiation of hemocytes from hemolymph and EPF can elucidate their specialized roles.

The extrapallial space, between the mantle epithelium and the shell, is the site of shell biomineralization (Wheeler, 1992). The classical theory of biomineralization is that it is controlled by mantle tissue, which secretes organic materials such as shell matrix proteins and inorganic ions creating the EPF. There is now evidence to suggest that hemocytes function in this process, specifically in sequestering calcium (Ca^{2+}) and carbonate (CO_3), providing a replenishing supply of Ca^{2+} which is a major constraint during calcification (Mount et al., 2004; Huang et al., 2018). Mount et al. (2004) proposed that a special type of hemocyte contains Ca^{2+} positive granular contents and calcium carbonate ($CaCO_3$) crystals and can function in biomineralization; however, the role of hemocytes in biomineralization is still not well understood. Regulation of pH in the EPF is critical to maintain calcification, and evidence suggests marine invertebrates can elevate their pH in this calcifying fluid relative to the seawater and increase $CaCO_3$ saturation through ion transport (Al-Horani et al., 2003; Trotter et al., 2011; Ries, 2011; Holcomb et al., 2014). Ivanina et al., (2017) demonstrated over expression of ion transporters in hemocytes, suggesting hemocytes are involved in ionic regulation to promote calcification in the EPF.

In addition to participating in biomineralization, hemocytes in the EPF perform phagocytosis and produce a range of humoral factors (Allam & Paillard, 1998; Lau et al., 2017), suggesting EPF might serve other biological processes such as defense. The extrapallial space is an active site for microbial colonization and multiple infections affecting bivalves are initiated in this area. This is the case for juvenile oyster disease (JOD) and brown ring disease (BRD), which are bacterial diseases that respectively affect *Crassostrea virginica* (eastern oyster) and *Ruditapes philippinarum* (Manila clam), and are often associated with localized hemocyte infiltration to mantle surfaces and the EPF, suggesting these areas have localized-immune response at the site of infection (Allam and Pales Espinosa, 2016). Despite these preliminary investigations, additional research is needed to allow the identification of molecular pathways that enable hemocytes in the EPF to contribute to biomineralization while still fulfilling their immune functions.

This study used functional assays, transcriptomics, and proteomics to characterize hemocytes from hemolymph and EPF in *M. mercenaria,* and to determine potential specialization with regard to processes such as biomineralization, ion regulation, and immunity.

Materials and Methods

Animals

Adult (50-70 mm) *M. mercenaria* were obtained from a commercial source (Frank M. Flowers & Sons Inc., Oyster Bay, NY). Clams were scrubbed to remove debris and epibionts upon arrival. They were maintained in 4 aerated aquaria (salinity 30 ppt, Temp 25°C, pH 7.8) and fed daily with commercial diet (LPB Frozen Shellfish Diet, Reed Mariculture, City, CA, USA). Body fluids were collected 5-7 days later.

EPF and hemolymph collection

EPF and hemolymph samples were individually collected (Figure 1) from 20 clams (5 per aquarium). EPF was collected by drilling a hole into the external shell face using a round dental bur as described by Allam and Paillard (1998). When the EPF volume from one shell was not sufficient for all downstream analyses (< 1 ml), EPF was collected from both valves of each clam and pooled. A second hole was drilled above the anterior adductor muscle and hemolymph was withdrawn using a syringe. Approximately 1-1.5 ml of each fluid type was collected, and all samples were held on ice. Quality of the EPF was checked by examination of mantle integrity after opening the valves, if the mantle was compromised, a new clam was processed.

Each fluid type was aliquoted, and 100 µl was saved for functional assays and diluted 1:4 with ice cold filtered artificial seawater (FASW 30 ppt). The remaining fluid was centrifuged (800g, 4°C, 10 min). The supernatant was transferred to a new collection tube and protease inhibitor cocktail (SIGMA*FAST*™ Protease Inhibitor Tablets; 50 µl/m1) was added. The pelleted cells and tubes containing supernatant were flash frozen and stored at -80°C.

Functional assays

Flow cytometry (BD FACSCalibur) was used to assess a series of hemocyte activities as described below. Most of these assays rely on the assessment of fluorescent signals emitted by hemocytes following the addition of dyes that target specific pathways or molecules. For all these assays, a minimum of 1,000 hemocytes were assessed (often > 3,000), and data was compared between EPF and hemolymph using nested ANOVAs. Data was generated from a total of 20 clams, with five individual clams sampled from four replicate tanks (n=4).

<u>Total and viable cell counts</u>: Propidium Iodide (PI; ThermoFisher) was added at the final concentration of 20 µg/ml, incubated for 10 min in the dark at room temperature (RT) before flow cytometry readings. PI cannot enter viable cells, but binds to DNA in dead cells, consequently making dead cells more fluorescent in the orange (FL2) channel. Agranulocytes and granulocytes were separated based on light forward (FSC) and side (SSC) scatter parameters and treated separately for the calculation of the percent of dead cells in each hemocyte subpopulation.

<u>Intracellular pH</u>: Fluid samples were transferred to sealed 0.5 ml microcentrifuge tubes to minimize gas exchange and then immediately centrifuged (800g, 4°C, 10 min). The supernatant was removed and the pellet was resuspended in FASW containing 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM; Sigma) at a final concentration of 1µM. Samples were incubated at RT in the dark for 10 min before reading on the flow cytometer.

Ca^{+2} *measurements*: Relative Ca^{+2} contents in hemocytes were assessed using Fluo-3 (ThermoFisher), a dye that show increase in green fluorescence (FL1 channel) intensity with increasing levels of Ca^{+2}. Fluo-3 was added at a final concentration of 5 µM and incubated at RT in the dark for 20 min before sample reading.

Phagocytosis: Yellow-green latex beads (2 µm; Sigma) were added to samples (1:10 hemocyte:bead ratio) and incubated at RT for 1 hr before sample reading. Hemocytes associated with beads were considered phagocytic.

RNAseq library preparation, sequencing, and analysis

RNA was extracted using TRIzol Reagent (Invitrogen, ThermoFisher). DNA was removed using DNA-free^{TM} Kit (Ambion), following manufacturer's instructions. After checking the quality and quantity of RNA (Nanodrop), samples derived from EPF and hemolymph from the same 8 clams were selected for sequencing. Extracted RNA was sent for sequencing to Novogene Corporation (UC Davis, Sacramento, California). 1 µg RNA per sample was used as input material. Sequencing libraries were generated using NEBNext® Ultra^{TM} RNA Library Prep Kit for Illumina® (NEB, USA), following manufacturer's instructions and with indices added for demultiplexing of samples. Libraries were sequenced on Illumina platform (Novaseq 6000) and 150 PE reads were generated. Novogene performed quality control tests, and cleaned reads were used in downstream analyses. RNASequencing of the EPF generated 351,049,084 PE reads (41,512,382 - 47,766,790/library) and 355,059,084 PE reads for hemolymph (41,115,505-48,985,418/library). Cleaned sequence reads were trimmed based on quality scores (limit 0.05), ambiguous nucleotides (max 2 nucleotides per sequence), and adapters (CLC workbench (version 11.0.1 (https://digitalinsights.qiagen.com)). Reads were mapped on the predicted genes annotated on the *M. mercenaria* genome (Farhat et al., 2021) using CLC workbench. Bam files were sorted and indexed, and read counts were generated using Samtools idxstats (Li, 2011) on Stony Brook University's high-performance computing cluster SeaWulf. Raw read counts were analyzed with R (3.6.3) and the package *DESeq2* from Bioconductor (Love et al., 2014) to perform differential gene expression analysis. Significant differences in gene expression between EPF and hemolymph were identified with a cut-off threshold of adjusted p-value <0.05 after Benjamini-Hochberg correction for multiple comparisons and log fold change > |2|. Principal component analysis was performed to visualize gene expression differences, a volcano map was created to visualize fold change (Figure S1), and heat map was generated for the top 100 DEGs between EPF and hemolymph (Figure S2). Genes translated into proteins (Farthat et al., 2020) were aligned to the KEGG database using BLAST with a minimum e-value of 10^{-5}, keeping the best match per protein with a score greater than 90% identity. Functional annotation (domain identification and three best matches on NR were from (Farhat et al., 2021). GO terms and KEGG pathways were assigned to DEGs (Figure S3-S8).

Proteomics

Cell-free hemolymph and EPF were solubilized in 5% SDS, 100mM TEAB, 10mM DTT at 55°C for 30 min. Reduced cysteines were alkylated with 20mM iodoacetamide for 30 min at RT in the dark and proteins were acidified with phosphoric acid. Then proteins were micro precipitated with 90% methanol, 50mM TEAB, and bound to S-Trap solid phase cartridges as described elsewhere (Zougman et al. 2020). Protein precipitates are washed with 90% methanol, 50mM TEAB and digested with trypsin at 47ºC for two hours before eluted with sequential 50mM TEAB, 0.2% formic acid and 50% acetonitrile (ACN), 0.2% formic acid elution steps by centrifugation (4000g 1 min) each.

Peptides were analyzed by C18 reverse phase LC-MS/MS. HPLC C18 columns were prepared using a P-2000 CO2 laser puller (Sutter Instruments) and silica tubing (100µm ID x 20 cm) and were self-packed with 3u Reprosil resin. Peptides were separated using a flow rate of 300 nl/minute, and a gradient elution step changing from 0.1% formic acid to 40% ACN over 90 minutes, followed 90% ACN wash and re-equilibration steps. Parent peptide mass and collision-induced fragment mass information were collected using an orbital trap (Q-Exactive HF; Thermo) instrument followed by protein database searching using Proteome Discoverer 2.4 (Thermo). Electrospray ionization was achieved using spray voltage of ~2.2 kV. Information-dependent MS and MS-MS acquisitions were made using a 100 ms survey scan (m/z 375 – 1400) at 60,000 resolution, followed typically by 'top 20' consecutive second product ion scans at 15,000 resolution. Peptide and spectra false discovery rates were set to 0.05. Protein and RNA datasets were integrated and analyzed using JMP10 (SAS) (Table S1-S2). The resulted proteins were mapped to the predicted proteins annotated from the whole genome (Farhat et al., 2021).

RESULTS

Functional assays

Total hemocyte count (nested ANOVA; n=4; p-value=0.46) and the percentage of agranulocytes and granulocytes (nested ANOVA; n=4; p-value=0.73) did not differ significantly between EPF and hemolymph (Figure 2). Cell viability was not different between granulocytes collected from both body fluids (nested ANOVA; n=4; p-value 0.14) but agranulocytes were more viable in the hemolymph as compared to EPF (ANOVA; n=4; p-value=0.022) (Figure 2).

There were no differences in fluorescence intensity after staining with BCECF-AM, suggesting intracellular pH was similar between agranulocytes (p=0.49) or granulocytes (p=0.51) from both fluids (Figure 3). Relative fluorescence intensity of Fluo-3, which is indicative of intracellular Ca^{2+} contents, was significantly higher in granulocytes from the EPF as compared to hemolymph (nested ANOVA; n=4; p-value=0.026) while there were no differences for agranulocytes (nested ANOVA; n=4; p-value=0.14) (Figure 3). The percentage of phagocytic agranulocytes was higher in EPF than hemolymph (p<0.001; nested ANOVA; n=4) but there were no significant differences for granulocytes (p-value=0.4) (Figure 3).

Differential gene expression

Gene transcription profiles were analyzed for hemocytes collected from the EPF and hemolymph. Principal component analysis showed separation in gene expression between both fluids (Figure 4). 190 genes were differentially expressed (Figure 4), with 113 of these displaying higher expression in EPF as compared to hemolymph (Table S3).

Genes with higher expression in EPF were surveyed for their associated functions. There were 37 genes overexpressed in EPF associated with biomineralization (Figures 5-6). These included 6 carbonic anhydrase genes (CAs), 6 chitin metabolism genes, tyrosinase, and 2 genes with Von Willebrand factor-A (VWA) domains which all make up the "basic tool kit" for molluscan biomineralization (Arivalagan et al., 2017). Other important genes for biomineralization that were overexpressed in EPF included 7 insoluble shell matrix proteins (ISMPs), perlucin, a gene with Whey Acidic Protein (WAP) domain, a gene with epidermal growth factor (EGF) domain, 4 genes with Ca^{2+} binding domains, 3 collagen, type VI, alpha genes, 2 alkaline phosphatase genes, 2 laminin alpha genes, and a xylosyltransferase. Genes related to transport were also overexpressed in EPF including 3 ion transporters, 4 solute carriers, and 2 bicarbonate exchange genes. While some domains have been traditionally associated with immune functioning, they also are known to function in biomineralization. There were 9 genes that could be important for biomineralization and/or immunity. For example, hemocytes collected from EPF showed an overexpression of genes with C1q domain, proteases, and protease inhibitors, including genes with kazal domains and metalloprotease domains, which might function in immunity or biomineralization. Seven genes were related to immune processes including lysozyme, a gene with Ig-like domain profile, a gene with immunoglobulin domain, and a cell death abnormality protein. While not as common, remaining genes had functions related to signaling, RNA processing, metal ion binding, cytoskeleton, digestion, and oxidoreductase activity. 38 genes did not have a known function.

On the other hand, 77 genes had higher expression in hemolymph as compared to EPF (Figure 5). Of these, 11 genes were associated with immune response including 2 universal stress proteins, a gene with tumor necrosis factor (TNF) domain, a vitelline membrane outer layer protein 1, 3 peroxidase genes, macroglobulin, autocrine proliferation repressor protein A-like, and claudin (Figure 5). Genes related to cytoskeleton were the next most prevalent group, with 9 genes including 2 tubulin beta genes, 2 myosin regulatory light chain genes, a microtubule-associated protein futsch-like isoform, 2 myosin heavy chain genes, and 2 calmodulin genes. Overexpressed transcripts also included 7 genes with Ca^{2+} binding domains and Ca^{2+} ion binding functions. Other notable functional groups included transport, signaling, and protein binding. Finally, there were 2 genes that might be important to biomineralization or immunity including genes with C1q domains, and 2 genes linked to biomineralization. 19 genes overexpressed in hemolymph did not have known function.

Proteomics

10,383 peptides were identified corresponding to 1,602 proteins (Table S1). Protein expression was compared between EPF and hemolymph and filtered for log2foldchange > |0.8| resulting in 284 proteins with significantly greater abundance in EPF and 329 higher in hemolymph (Table S2).

Convergence between proteomic and transcriptomic results was noted 171 genes had higher expression in EPF as compared to hemolymph at both the protein and mRNA levels (Table 1, Table S2, Figure 7), or had positive fold change by RNASeq and were significant at the protein level (Table 1, Table S2). Proteins were also filtered to include those with more than one peptide matching to a protein. On the other hand, genes related to chitinase and CA as well as genes with Ca^{2+} binding domains and c1q domains were overexpressed in the hemolymph proteome as compared to EPF (Table 2). Proteins related to cytoskeleton were particularly overexpressed in the hemolymph proteome, specifically actin-myosin cytoskeleton (Table S2). There were also several immune proteins including heat shock proteins, toll-like receptors, tumor necrosis factor, defensin, lysozyme, galectin, cathepsin, and large subunit GTPase 1 (Table S2).

Discussion

Prior investigations of hemocytes in bivalves focused on circulating hemocytes in the hemolymph and their role in immunity, overlooking hemocytes in other body fluids such as EPF. Recently, studies have begun to address this gap in knowledge; however, the approaches have mainly focused on morphological and physiological differences between hemocyte groups (Wang et al., 2012; Lin et al., 2013; Lau et al., 2017; Nguyen et al 2020) and have not applied holistic omic tools. This study used functional assays, transcriptomics, and proteomics to further reveal major functions of hemocytes from different fluids and found evidence to support the role of EPF hemocytes in both immunity and biomineralization. In addition, EPF hemocytes appeared to function in ion transport, which could be important for buffering the impact of altered environmental conditions, such as ocean acidification (Hendriks et al., 2015; Ramesh et al., 2017). While hemocytes present in hemolymph primarily expressed genes and proteins important for immune response and cytoskeleton, there was evidence to suggest they also contribute to biomineralization with specific genes putatively involved in shell formation, specifically Ca^{2+} binding and transport.

Although evidence suggests hemocytes from hemolymph play a role in shell formation, this study highlights the importance of the EPF in biomineralization, with convergence of evidence from all analyses. The majority of genes and proteins with higher expression in the EPF compared to hemolymph were related to shell formation. Arivalagan et al., (2017) proposed a basic tool kit that is required for shell formation. This “tool kit” consists of proteins with the functional domains tyrosinase, CA, chitin-binding-2, and Von Willebrand factor-A (Arivalagan et al., 2017). In addition to the proteins explicitly included in the basic toolkit, Arivalagan et al., (2017) identified additional proteins that are important for the nucleation and arrangement of $CaCO_3$ polymorphs found in the shell. These included EGF and WAP domain-containing proteins (Arivalagan et al., 2017). In this study, genes with higher expression in EPF included CA, chitin metabolism and binding genes, tyrosinase, and genes with VWA, EGF and WAP domains. Interestingly, genes with WAP and VWA domains, CA, and chitinase were significantly enriched at both the mRNA and protein level. While not expressly listed in the basic tool kit, other important biomineralization genes were also found overexpressed at both the mRNA and protein level in EPF. For instance, ISMPs were significantly higher in both the transcriptome and proteome of EPF. Insoluble shell matrix proteins (ISMPs), including chitin, create a framework guiding shell formation (Marie et al, 2012). Xylosyltransferase was also significantly higher at both mRNA and protein level. This gene functions in proteoglycan synthesis, and proteoglycans are involved in $CaCO_3$ based biomineralization (Arias & Fernandez, 2008). Similarly, perlucin functions in nucleation of $CaCO_3$ crystals (Weiss et al., 2000; Schwaner et al., unpublished), and our results showed this gene to be significantly overexpressed (by RNASeq) and displayed higher relative spectral counts (proteomics, although not significantly overexpressed) in EPF as compared to hemolymph.

It should be noted that hemocytes from hemolymph also contained genes related to biomineralization that were overexpressed at the mRNA and protein levels, including chitinase and at the protein level CA. Previous studies have shown that hemocytes migrate into the EPF from other areas of the body (Li et al., 2016; Huang et al., 2018) during infection (Allam et al. 2014) and shell damage (Allam et al., 2006; Sillanpää et al., 2016). Hemocytes can transport calcite crystals to the site of calcification and are involved in biomineralization (Mount et al., 2004). Chitin has been suggested to be directly involved in crystal packing and orientation for creation of the foliated microstructure of the inner shell layer, suggesting chitin plays an important role in shell formation (Chan et al., 2018). Studies have shown that *C. gigas* (Pacific oyster) hemocytes transport Ca^{2+} ions after induced shell damage (Sillanpää et al., 2016). As mentioned before, a CA was also higher in hemolymph as compared to EPF and it is believed that CAs can play a role in regulating intracellular Ca^{2+} content of hemocytes (Wang et al., 2017b). This supports the idea that hemocytes transport Ca^{2+} to the area of calcification and might be further involved in biomineralization.

During biomineralization, mantle tissue regulates Ca^{2+} turnover, necessitating active ion transportation between cells. Ion transporters, bicarbonate transporters, and genes related to ion channel activity are all important for biomineralization. Bicarbonate transporters can supply dissolved inorganic carbon at the site of calcification, regulate pH, or transport Ca^{2+} (Bhattacharya et al., 2016). Yarra et al. (2021) investigated the transcriptomes of *Pecten maximus* (great scallop), *C. gigas*, and *Mytulis edulis* (blue mussel) during shell repair and proposed the upregulation of bicarbonate transporters was to regulate the availability of bicarbonate ions to the site of calcification. Genes overexpressed in EPF that could potentially be involved in calcification by transporting ions included ion transporters, solute carriers, and bicarbonate exchange genes. Similarly, ion transporters, solute carriers, and Ca^{2+} binding domains were overexpressed in the EPF proteome. Ca^{2+} content was higher in granulocytes from the EPF than hemolymph, which supports the idea that hemocytes serve as a source of Ca^{2+} during biomineralization. In addition to transporting Ca^{2+} to the site of calcification, some of the aforementioned genes/proteins might be important for acid-base regulation. CA was included in the "tool kit" for biomineralization, because of its role in concentrating inorganic carbon in shell fluid, but it is also very important in acid-base regulation (Le Roy et al., 2014). However, there were no differences in intracellular pH between hemocytes from EPF and hemolymph. Similar pH was reported between EPF and hemolymph in *R. philippinarum* (Rahmani et al., 2020) and *M. edulis* (Thomsen et al., 2010; Heinemann et al. 2012). Instead of raising pH at the site of mineralization to achieve a microenvironment super saturated with respect to carbonate, the overexpression of ion transporters in EPF might be primarily functioning in transportation of Ca^{2+} or CO_3. Granulocytes had higher intracellular pH and calcium content than agranulocytes, further supporting the role of granulocytes as the main hemocyte subpopulation involved in biomineralization.

Like CA, some genes/proteins can have multiple functions. For example, Arivalagan et al., (2017) found immune associated domains in the shell formation proteins. Previous studies in clams showed that complement 1q domain- containing (c1qDC) protein and other complement components are overexpressed in the plasma proteome of clams infected with the fungus-like pathogen *Mucochytrium quahogii* (formerly QPX, Hartman et al., 2018). In fact, complement proteins can function as pattern recognition receptors to identify pathogens and can initiate innate immune response (Tang et al., 2005; Liu et a., 2014; Hartman et al., 2018). In particular, the calcium-dependent lectin-like c1qDC proteins display a marked expansion in bivalve mollusks (primarily to enable a tailored immune response to various microbes; Gerdol et al., 2015; Farhat et al., in press) and have been strongly suggested to be involved in biomineralization as their expression increase after shell damage (Yarra et al., 2021). A similar role for c1qDC in biomineralization has also been suggested in zebrafish (Holubowicz et al., 2017). Proteases and protease inhibitors, both associated with immunity, have also been identified as shell matrix proteins (Arivalagan et al., 2017). Gerdol et al., (2011) demonstrated overexpression of proteases in hemocytes during microbial invasions (Gerdol et al., 2011), and metalloprotease domain is a conserved disintegrin domain involved in the inflammatory response (Arivalagan et al., 2017). Disintegrin and metalloprotease with thrombospondin motifs 16 was overexpressed in the EPF compared to hemolymph at the mRNA level and trypsin- serine protease was over expressed at the protein level. Protease inhibitors are involved in the inflammatory response (Arivalagan et al., 2017); however, protease inhibitors were also identified as potential shell proteins (Arivalagan et al., 2017) and have been associated with the fibrous organic matrix between aragonite crystals in *Pinctada fucata* (pearl oyster; Kubota et al., 2017). A serine protease inhibitor with kazal domain and a tissue inhibitor of metalloproteases were overexpressed at the mRNA level and kazal type serine protease inhibitor at the mRNA and protein level in the EPF.

While some immune related genes may serve dual functions in biomineralization and immune response, there were several additional genes most commonly associated with innate immunity. In the EPF, lysozyme and genes with Ig-like domain profile and immunoglobulin domain were over expressed at both the mRNA and protein levels. Proteins with tumor necrosis factor domains, stress 70 protein, autophagy-related protein, ficolins, and CD9 antigen isoform were significantly higher in the EPF proteome as compared to hemolymph. Previous studies have demonstrated that EPF hemocytes are involved in phagocytosis (Lau et al, 2017; Huang et al., 2018). There were no significant differences in phagocytosis for granulocytes, which are the most common population of hemocytes performing phagocytosis, between EPF and hemolymph in this study. Total hemocyte count and the percentage of granulocytes and agranulocytes did not differ between EPF and hemolymph. Microbes and pathogens have been documented to infiltrate EPF from the seawater (Huang et al., 2018) and many pathogens of bivalves can disrupt shell formation (Elston et al., 1999) or lead to shell diseases (Zannella et al., 2017). JOD and BRD are 2 bacterial diseases that begin in the periostracum at the edge of the mantle, leading to an inflammatory response characterized by an increase in hemocyte counts in the EPF and alterations in hemocyte activity at the infection loci (Allam et al 2014). Allam et al. (2014) showed differences in transcription profiles between hemocytes in the circulatory system compared to hemocytes from EPF in clams infected with BRD.

It is already well established that hemocytes in hemolymph are main components of immune response in bivalves (Chu, 1988; Canesi et al., 2002; Pruzzo et al 2005; Matozzo et al., 2007; Allam & Raftos, 2015; Lau et al., 2018a; Lau et al., 2018b). Both the transcriptome and proteome generated in the current study reflected this, with many immune related genes having higher expression at both the mRNA and protein levels in hemolymph as compared to EPF. Many genes and proteins were associated with signaling. Signaling pathways, including pathogen recognition receptors or toll-like receptors, are an important part of the immune response. Heat shock and stress proteins (higher expression in hemolymph) in bivalves play a role in immune response by contributing to signaling (Fabbri et al., 2008) and these pathways were overexpressed in hemolymph. Engulfment of foreign particles during phagocytosis is mediated by the cytoskeleton, specifically the actin-myosin contractile system (Allen & Aderem, 1995). Hemocytes of the Manila clam had increased expression of cytoskeleton related genes during pathogen exposure (Brulle et al., 2012). A transcriptomic analysis of hemocytes in *C. gigas* found granulocytes with higher expression of genes related to regulation of actin cytoskeleton supporting evidence that granulocytes function in phagocytosis and engulfment of pathogens (Mao et al., 2020).

Together, these findings demonstrate functional specialization of hemocytes between EPF and hemolymph. Variation in the expression of biomineralization-related genes and proteins between these fluids, along with differences in calcium content, underline the role of EPF in shell formation. Hemocytes in the hemolymph played an auxiliary role, by transporting Ca^{2+} into the extrapallial cavity. This compartmentalization may allow the use of hemolymph as an important conveyer of elements (e.g. Ca^{2+}) towards calcification sites, while the specific hemocyte activities in EPF may create the microenvironment conducive to biomineralization. Future work is required to further clarify the role of hemocytes in biomineralization, such as their relationship with mantle-mediated calcification. Similarly, studies are needed to determine whether hemocytes in EPF represent a specialized fraction of hemocytes commonly present in the circulatory system or if their unique transcriptomic signature results from a “maturation” process that they undergo when they migrate to the extrapallial space. This study underscores the well-established role of circulatory hemocytes in bivalve defense, while also demonstrating that peripheral compartments including the extrapallial cavity contain hemocytes that function in immune protection of their compartment. Functional analysis of genes that might be important for both biomineralization and immunity, such as genes with c1q and kazal domains, should be further investigated to better elucidate whether a dual function in immunity and biomineralization is truly the case for the same genes or whether different members of these diversified gene families play different roles.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Funding

This research was supported by New York Sea Grant program (projects R/XG-24 and R/XG-32).

Acknowledgements

We are grateful for the members of the Marine Animal Disease Laboratory at Stony Brook University, including Michelle Barbosa, Chris Brianik, Ray Czaja, and Sabrina Geraci-Yee for their help in processing clams and performing functional assays. The authors would also like to thank Frank M. Flower and Sons for providing experimental clams.

Author contributions

EPE and BA led the conceptualization and secured the funding. CS contributed to investigation, methodology, formal analysis, and writing the original draft. SF and JH processed the sequencing data and performed analyses. All authors participated in manuscript revision.

Figures and tables

Figure 1. Hemolymph was collected through a puncture drilled over the anterior adductor muscle (A) and (B). The extrapallial fluid (EPF) was collected through a puncture made in the central part of the shell (C). (D) is a schematic representation of a clam cross section that indicates the position of the EPF between the shell and the mantle.

Figure 2. Count and viability (mean ± standard error) of agranulocytes and granulocytes collected from hemolymph and EPF *: significant difference between agranulocytes from both fluids (p=0.022; nested ANOVA; n=4).

Figure 3. Relative fluorescence intensity of (A) BCECF-AM (indicator of intracellular pH) and (B) Fluo-3 (indicator of Ca^{2+}), and phagocytic activity (C) for agranulocytes and granulocytes from EPF and hemolymph. Mean ± standard error. *: significantly higher in EPF as compared to hemolymph (p=0.026 in B and p<0.001 in C; nested ANOVA; n=4).

Figure 4. Principal component analysis of the normalized RNAseq data generated from EPF and hemolymph.

Figure 5. Genes overexpressed in EPF (blue) and hemolymph (red) grouped by their putative functions.

Figure 6. Biomineralization-related genes overexpressed in EPF. "+" above the bar indicates that the corresponding genes were also over expressed in the proteome of the EPF (see below).

Figure 7. Proteins overexpressed in EPF (blue) and hemolymph (red) grouped by their putative functions.

Table 1. Representative differentially expressed genes (DEG) displaying higher expression at the mRNA and protein level in EPF as compared to hemolymph with their corresponding log2 fold change at mRNA and protein levels.

ID	**Description**	**DEG**	**Protein**	**Functional Group**

mRNA.chromosome_10.203.1	WAP domain	6.5	0.87	Biomineralization
mRNA.chromosome_15.1545.1	VWA domain	5.4	1.16	Biomineralization
mRNA.chromosome_6.686.2	CA	3.05	1.24	Biomineralization
mRNA.chromosome_ 1.3030.1	CA	5.69	3.32	Biomineralization
mRNA.chromosome_18.1170.1	ISMP	9.94	1.61	Biomineralization
mRNA.chromosome_18.1174.1	ISMP	7.64	2.72	Biomineralization
mRNA.chromosome_8.1704.1	Xylosyltransferase	2.81	2.87	Biomineralization
mRNA.chromosome_2.2742.1	EF-hand calcium-binding domain	4.48	2.4	Biomineralization
mRNA.chromosome_12.552.1	Chitinase	0.4	1.2	Biomineralization
mRNA.chromosome_9.114.1	Kazal domain	8.96	0.94	Biomineralization/Immunity
mRNA.chromosome_4.844.1	Kazal domain	6.71	3.32	Biomineralization/Immunity
mRNA.contig_2790.1.1	C1q domain	0.76	1.76	Biomineralization/Immunity
mRNA.chromosome_4.347.1	C1q domain	0.4	1.00	Biomineralization/Immunity
mRNA.chromosome_15.1559.1	Trypsin-7	0.4	0.98	Biomineralization/Immunity
mRNA.chromosome_5.362.1	CD109 antigen	6.54	1.01	Biomineralization/ Immunity
mRNA.chromosome_13.323.1	Lysozyme C	7.34	2.22	Immunity
mRNA.chromosome_3.1492.1	Complement C1q tumor necrosis factor-related protein 4-like	5.42	1.23	Immunity
mRNA.chromosome_15.1205.2	70 kDa neurofilament protein-like isoform X1	2.42	1.88	Immunity
mRNA.chromosome_9.485.1	Stress-70 protein	1.08	3.32	Immunity
mRNA.chromosome_16.1096.1	Ficolin-1-like	0.04	3.32	Immunity
mRNA.chromosome_19.2906.1	Ficolin-2-like	0.73	0.83	Immunity
mRNA.chromosome_18.298.4	Ig-like domain	0.26	3.32	Immunity

Table 2. Biomineralization-related genes displaying higher expression in the hemolymph proteome with their corresponding log2 fold change at mRNA and protein levels

ID	**Description**	**DEG**	**Protein**	**Functional Group**
mRNA.chromosome_9.1851.1	Chitinase	4.46	1.02	Biomineralization
mRNA.chromosome_6.1443.1	Carbonic anhydrase	0.44	0.91	Biomineralization

mRNA.chromosome_11.1876.1	Sarcoplasmic calcium-binding protein	4.03	1.10	Ca binding
mRNA.chromosome_2.1676.1	Calcineurin-binding protein	4.59	1.8	Ca binding
mRNA.chromosome_13.726.1	Sodium/calcium exchanger regulatory protein 1-like, RS-rich protein-1	5.47	1.47	Ca binding
mRNA.chromosome_16.1880.1	C1q domain	1.01	0.91	Biomineralization/Immunity
mRNA.chromosome_12.764.1	C1q domain	0.87	2.06	Biomineralization/Immunity
mRNA.chromosome_16.407.1	C1q domain	0.13	0.93	Biomineralization/Immunity
mRNA.chromosome_18.740.1	C1q domain	0.52	0.86	Biomineralization/Immunity
mRNA.chromosome_10.106.1	C1q domain	0.41	0.84	Biomineralization/Immunity

References

Al-Horani, F.A., Al-Moghrabi, S.M., de Beer, D. (2003). Microsensor study of photosynthesis and calcification in the scleractinian coral, *Galaxea fascicularis*: active internal carbon cycle. J. Exp. Mar. Biol. Ecol. 288:1–15. doi:10.1016/S0022-0981(02)00578-6

Allam, B., and Paillard, C. (1998). Defense factors in clam extrapallial fluids. Dis. Aquat. Org. 33:123-128.

Allam, B., Ashton-Alcox, K.A., Ford, S.E. (2002). Flow cytometric comparison of hemocytes from three species of bivalve mollusks. Fish Shellfish Immunol. 13:141-158. doi:10.1006/fsim.2001.0389

Allam, B., Paillard, C., Auffret, M., Ford, S.E. (2006). Effects of the pathogenic *Vibrio tapetis* on defense factors of susceptible and non-susceptible bivalve species: II. Cellular and biochemical changes following in vivo challenge. Fish Shellfish Immunol. 384-397. doi:10.1016/j.fsi.2005.05.013

Allam, B., Pales Espinosa, E., Tanguy, A., Jeffroy, F., Le Bris, C., Paillard, C. (2014). Transcriptional changes in manila clam *Ruditapes philippinarum* in response to brown ring disease. Fish Shellfish Immunol. 41(1):2-11. doi:10.1016/j.fsi.2014.05.022

Allam, B., and Raftos, D. (2015). Immune responses to infectious disease in bivalves. J. Invertebr. Pathol. 131:121-136. doi:10.1016/j.jip.2015.05.005

Allam, B., and Pales Espinosa, E. (2016). Bivalve immunity and response to infections: Are we looking at the right place? 53:4-12. doi:10.1016/j.fsi.2016.03.037

Allen, L.H., and Aderem, A. (1995). A role for MARCKS, the alpha isozyme of protein kinase C and myosin I in zymosan phagocytosis by macrophages. J. Exp. Med. 182: 182–829. doi:10.1084/jem.182.3.829

Arias, J.L., and Fernandez, M.S. (2008). Polysaccharides and proteoglycans in calcium carbonate-based biomineralization. Chem. Rev. 108(11): 4475-82. doi:10.1021/cr078269p

Arivalagan, J., Yarra, T., Marie, B. Sleight, V.A., Duvernois-Berthet, E., Clark, M.S., et al. (2017). Insights from the Shell Proteome: Biomineralization to Adaptation. Mol. Biol. Evol. 34(1): 66–77. doi: 0.1093/molbev/msw219

Bhattacharya, D., Agrawal, S., Aranda, M., Baumgarten, S., Belcaid, M., Drake, J.L., et al. (2016). Comparative genomics explains the evolutionary success of reef-forming corals. Elife 5:13288.

Brulle, F., Jeffroy, F., Madec, S., Nicolas, J.L., Paillard, C. (2012). Transcriptomic analysis of *Ruditapes philippinarum* hemocytes reveals cytoskeleton disruption after in vitro *Vibrio tapetis* challenge. Dev. & Comp. Immunol. 38(2): 368-376. doi:10.1016/j.dci.2012.03.003

Burgos-Aceves, M.A., and Faggio C. (2017). An approach to the study of the immunity functions of bivalve hemocytes: Physiology and molecular aspects. Fish Shellfish Immunol. 67:513-517. doi:10.1016/j.fsi.2017.06.042

Canesi, L., Gallo, G., Gavioli, M., Pruzzo, C. (2002). Bacteria–hemocyte interactions and phagocytosis in marine bivalves. Microsc. Res. Tech. 57(6):469–476. doi:10.1002/jemt.10100

Chang, S.J., Tseng, S.M., Chou, H.Y. (2005). Morphological Characterization via Light and Electron Microscopy of the Hemocytes of two Cultured Bivalves: A Comparison Study between the Hard Clam (*Meretrix lusoria*) and Pacific Oyster (*Crassostrea gigas*). Zool. Stud. 44(1):144-153.

Chan, V.B.S, Johnstone, M.B., Wheeler, A.P., Mount, S. (2018). Chitin Facilitated Mineralization in the Eastern Oyster. Front. Mar. Sci. doi:10.3389/fmars.2018.00347

Cheng, T.C. (1981). Bivalves. 233-300 in N.A. Ratcliffe and A.F. Rowley, (eds). In Invertebrate Blood Cells, Academic Press, New York.

Cheng T.C. (1984) A Classification of Molluscan Hemocytes Based on Functional Evidences. Invertebrate Blood. Comparative Pathobiology. 6. doi:10.1007/978-1-4684-4766-8_5

Chu, F.L.E. (1988). Humoral Defense Factors in Marine Bivalves. VIMS Books and Book Chapters. 121.

Elston, R., Frelier, P.F., Cheney, D. (1999). Extrapallial abscesses associated with chronic bacterial infections in the intensively cultured juvenile pacific oyster *C. gigas*. Dis. Aquat. Org. 37:115–12

Fabbri, E., Valbonesi, P., Franzellitti, S. (2008). HSP expression in bivalves. ISJ. 5:135-161.

Farhat, S., Tanguy, A., Pales Espinosa, E., Guo, X., Boutet, I., Smolowitz, R., et al. (2020). Identification of variants associated with hard clam, *Mercenaria mercenaria*, resistance to Quahog Parasite Unknown disease. Genomics 112(6): 4887-4896.

Farhat, S., Bonnivard, E., Pales Espionsa, E., Tanguy, A., Boutet, I., Guiglielmoni, N., et al. (2021). Comparative analysis of the *Mercenaria mecenaria* genome provides insights into the diversity of transposable elements and immune molecules in bivalve mollusks. BMC Genomics. In press.

Foley, D.A., and Cheng, T.C. (1977). Degranulation and other changes of molluscan granulocytes associated with phagocytosis. J. Invertebr. Pathol. 29, 321-325. doi:10.1016/S0022-2011

Gerdol, M., Manfrin, C., De Moro, G., Figueras, A., Novoa, B., Venier, P., et al. (2011). The C1q domain containing proteins of the Mediterranean mussel *Mytilus galloprovincialis*: a widespread and diverse family of immune-related molecules. Dev Comp Immunol. 35:635–643. doi:10.1016/j.dci.2011.01.018

Gerdol, M., Venier, P., Pallavicini, A. (2015). The genome of the Pacific oyster *Crassostrea gigas* brings new insights on the massive expansion of the C1q gene family in Bivalvia. Dev. Comp. Immunol. 49(1):59–71. doi:10.1016/j.dci.2014.11.007

Hartman, R., Pales Espinosa, E., Bassem, A. (2018). Identification of clam plasma proteins that bind its pathogen Quahog Parasite Unknown. Fish Shellfish Immuol. 77:214-221. doi:10.1016/j.fsi.2018.03.056

Heinemann, A., Fietzke, J., Melzner, F., Böhm, F., Thomsen, J., Garbe-Schönberg, D., et al. (2012). Conditions of *Mytilus edulis* extracellular body fluids and shell composition in a pH-treatment experiment: Acid-base status, trace elements and δ11B. Geochem. Geophy. 13(1). doi:10.1029/2011GC003790

Hendriks, I.E., Duartea, C.M, Olsen, Y.S., Steckbauer, A., Ramajoa, L., Moore, T.S., et al. (2015). Biological mechanisms supporting adaptation to ocean acidification in coastal ecosystems Estuar. Coast. 152: LA1-A8. doi: 10.1016/j.ecss.2014.07.019

Holcomb, M., Venn, A. A., Tambutte, E., Tambutté, S., Allemand, D., Trotter, J., et al. (2014). Coral calcifying fluid pH dictates response to ocean acidification. Sci. Rep. 4:5207. doi:10.1038/srep05207

Hołubowicz, R., Wojtas, M., Taube, M., Kozak M. ,Ożyhar, A., Dobryszycki, P. (2017). Effect of calcium ions on structure and stability of the C1q-like domain of otolin-1 from human and zebrafish. 284(24):4278-4297. doi:10.1111/febs.14308

Huang, J., Li, S., Liu, Y., Liu, C., Xie, L., Zhang, R. (2018). Hemocytes in the extrapallial space of *Pinctada fucata* are involved in immunity and biomineralization. Sci. Rep. 8(4657). doi: 10.1038/s41598-018-22961-y

Ivanina, A.V., Falfushynska, H.I., Beniash, E., Piontkivska, H., Sokolova, I.M. (2017). Biomineralization-related specialization of hemocytes and mantle tissues of the Pacific oyster *Crassostrea gigas*. J. Exp. Biol. 220(18): 3209-3221. doi:10.1242/jeb.160861

Kubota, K., Tsuchihashi, Y., Kogure, T., Maeyama, K., Hattori, F., Kinoshit, S., et al. (2017). Structural and functional analyses of a TIMP and MMP in the ligament of *Pinctada fucata*. J. Struct. Biol. 199(3):216–224. doi:10.1016/j.jsb.2017.07.010

Lau, Y.T., Sussman, L., Pales Espinosa, E., Katalay, S., Allam, B. (2017). Characterization of hemocytes from different body fluids of the eastern oyster *Crassostrea virginica*. Fish Shellfish Immunol. 71:372-379. doi:10.1016/j.fsi.2017.10.025

Lau, Y.T., Snatos, B., Barbosa, M., Pales Espinosa, E., Allam, B. (2018a). Regulation of apoptosis-related genes during interactions between oyster hemocytes and the alveolate parasite *Perkinsus marinus*. Fish Shellfish Immunol. 83:180-189.

Lau, Y.T., Gambino, L., Santos, B., Pales Espinosa, E., Allam, B. (2018b). Transepithelial migration of mucosal hemocytes in *Crassostrea virginica* and potential role in *Perkinsus marinus* pathogenesis. J. Invertebr. Pathol. 153:122-129. doi: 10.1016/j.jip.2018.03.004

Le Roy, N., Jackson, D.J., Marie, B., Ramos-Silva, P., Marin, F. (2014). The evolution of metazoan alpha-carbonic anhydrases and their roles in calcium carbonate biomineralization. Front Zool. 11(1):75.

Li, S., Liu, Y., Liu, C., Huang, J., Zheng, G., Xie, L., Zhang, R. (2016). Hemocytes Participate in Calcium Carbonate Crystal Formation, Transportation and Shell Regeneration in the Pearl Oyster *Pinctada fucata*. Fish & Shellfish Immunol. 51:263-270. doi:10.1016/j.fsi.2016.02.027

Lin, T., Yao, Z., Hao, Z., Lai, Q., Zhou, K. (2013). Classification of granulocytes in three bivalve species using neutral red staining. J. Shellfish Research. 32(3):861-865. doi:10.2983/035.032.0330

Liu, H.H., Xiang, L.X., Shao, J.Z. (2014). A novel Cl q-domain-containing (C1 qDC) protein from *Mytilus coruscus* with the transcriptional analysis against marine pathogens and heavy metals, Dev. Comp. Immunol. 44 (1):70–75. doi:10.1016/j.dci.2013.11.009

Love, M.I., Huber, W., Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15(550). doi: 10.1186/s13059-014-0550-8

Mao, F., Wong, N.K., Lin, Y., Zhang, X., Liu, K., Huang, M., et al. (2020). Transcriptomic Evidence Reveals the Molecular Basis for Functional Differentiation of Hemocytes in a Marine Invertebrate, *Crassostrea gigas*. Front. Immunol. doi:10.3389/fimmu.2020.00911

Marie, B., Joubert, C., Tayale, A., Zanella-Cleon, I., Belliard, C., Piquemal, D., et al. (2012). Different secretory repertoires control the biomineralization processes of prism and nacre deposition of the pearl oyster shell. Proc. Natl. Acad. Sci. U. S. A. 109(51): 20986–20991. doi:10.1073/pnas.1210552109

Matozzo, V., Rova, G., Marin, M.G. (2007). Hemocytes of the cockle *Cerastoderma glaucum*: Morphological characterization and involvement in immune responses. Fish & Shellfish Immunol. 23(4):732-746. doi: 10.1016/j.fsi.2007.01.020

Mount, A. S., Wheeler, A.P., Paradkar, R.P., Snider, D. (2004). Hemocyte-Mediate Shell Mineralization in the Eastern Oyster. Science. 304(5668):297-300. doi:10.1126/science.1090506

Nguyen, D.H., Donghong, N., Chen, Z., Peng, M., Tran, N.G., Li, J. (2020). Classification and morphology of circulating hemocytes in the razor clam *Sinonovacula constricta*. Aquaculture and fisheries. doi:10.1016/j.aaf.2020.06.007

Pruzzo, C., Gallo, G., Caesi, L. (2005). Persistence of *vibrios* in marine bivalves: the role of interactions with hemolymph components. Environ. Microbiol. 7(6): 761-772. doi:10.1111/j.1462-2920.2005.00792.x

Rahmani, A., Mathien, C., Bidault, A., Le Goïc, N., Pichereau, V. (2020). External pH modulation during the growth of *Vibrio tapetis*, the etiological agent of brown ring disease. J. Appl. Microbiol.,129(1): 3-16. doi:10.1111/jam.14699

Ramesh, K., Hu, M.Y., Thomsen, J., Bleich, M, Melzner, F. (2017). Mussel larvae modify calcifying fluid carbonate chemistry to promote calcification. Nat. Commun. 8(1709). doi:10.1038/s41467-017-01806-8

Ries, J. B. (2011). A physicochemical framework for interpreting the biological calcification response to CO_2-induced ocean acidification. Geochim. Cosmochim. Acta. 75:4053–4064.doi: 10.1016/j.gca.2011.04.025

Sillanpää, J.K., Ramesh, K., Melzner, F., Sundh, H., Sundell, K. (2016). Calcium mobilization following shell damage in the Pacific oyster, *Crassostrea gigas*. Mar. Genomics. 27:75-83. doi: 10.1016/j.margen.2016.03.001

Song, L., Wang, L., Qiu, L., Zhang, H. (2010). Bivalve Immunity. In: Soderhall K. (eds) Invertebrate Immunity. Advances in Experimental Medicine and Biology. Springer, pp. 44–65. doi: 10.1007/978-1-4419-8059-5_3

Tang, Y.T., Hu, T.H, Arterburn, M., Boyle, B., Bright, J.M., Palencia, S., et al. (2005). The complete complement of C1q-domain-containing proteins in Homo sapiens. Genomics. 86(1):100–111. doi:10.1016/j.ygeno.2005.03.001

Thomsen, J., Gutowska, M. A., Saphorster, J., Heinemann, A., Trubenbach, K., Fietzke, J., et al. (2010). Calcifying invertebrates succeed in a naturally CO_2-rich coastal habitat but are threatened by high levels of future acidification. Biogeosciences. 7:3879–3891. doi:10.5194/bg-7-3879-2010

Trotter, J., Montagna, P., McCulloch, M., Silenzi, S., Reynaud, S., Mortimer, G., et al. (2011). Quantifying the pH 'vital effect' in the temperate zooxanthellate coral *Cladocora caespitosa*: validation of the boron seawater pH proxy. Earth Planet. Sci. Lett. 303:163–173.

Wang, Y., Hu, M., Chiang, M.W.L, Shin, P.K.S., Cheung, S.G. (2012). Characterization of subpopulations and immune-related parameters of hemocytes in the green-lipped mussel *Perna viridis*. Fish Shellfish Immunol. 32(3):381-390. doi:10.1016/j.fsi.2011.08.024

Wang W., Li, M., Wang, L., Chen, H., Liu, Z., Jia, Z., et al. (2017a). The granulocytes are the main immunocompetent hemocytes in *Crassostrea gigas*. Dev. Comp. Immunol. 67:221-228. doi: 10.1016/j.dci.2016.09.017

Wang, X., Wang, M., Jia, Z., Song, X., Wang, L., Song, L. (2017b). A shell-formation related carbonic anhydrase in *Crassostrea gigas* modulates intracellular calcium against CO_2 exposure: implication for impacts of ocean acidification on mollusk calcification. Aquat. Toxicol. 189:216-228. doi:10.1016/j.aquatox.2017.06.009

Weiss, I.M., Kaufmann, S., Mann, K., Fritz, M. (2000). Purification and Characterization of Perlucin and Perlustrin, Two New Proteins from the Shell of the *Mollusc Haliotis laevigata*. Biochem. Biophys. Res. Commun. 267(1):17-21. doi:10.1006/bbrc.1999.1907

Wheeler, A.P. (1992). Mechanisms of molluscan shell formation. Calcification in Biological Systems. CRC Press, Boca Raton, FL.

Yarra, T., Blaxter, M., Clark, M.S. (2021). A Bivalve Biomineralization Toolbox. Mol. Biol. Evol. 38(9):4043-4055. doi: 10.1093/molbev/msab153

Zannella, C., Mosca, F., Mariani, F., Franci, G., Folliero, V., Galdiero, M., et al. (2017). Microbial Diseases of Bivalve Mollusks: Infections, Immunology and Antimicrobial Defense. Mar Drugs. 15(6): 182. doi:10.3390/md15060182

Zougman, A., Wilson, J.P., Banks, E.R. (2020). A simple serum depletion method for proteomics analysis. Biotechniques. 69(2):148-151. doi: 10.2144/btn-2020-0017