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Investigating cellular and molecular interactions between the hard clam (*Mercenaria mercenaria*) and its pathogen Quahog Parasite Unknown (QPX)

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Abstract of the Thesis

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Quahogs, or hard clams (*Mercenaria mercenaria*) are an economically important species of clam. They are often used for human consumption, but can also be indicators of water quality because of the bioaccumulation of contaminants within filter feeders. They are found abundantly on the east coast of the United States and Canada, and actively aquacultured from Massachusetts to Florida. The hard clam is a robust species with few naturally-occurring diseases. The most well-known disease of the hard clam is Quahog Parasite Unknown (QPX), a unicellular parasite found on the east coast of North America from Virginia up into Canada, causing well-documented mortality events. Though the presence of QPX disease has been documented since the 1960s, little information is available on cellular and molecular interactions between the parasite and clams.

This research project targets the interactions between the clam immune system and QPX cells. The clam immune response to QPX consists of an inflammatory response that often involves the encapsulation of the parasite by clam immune cells, or hemocytes. This research identified the clam plasma proteins involved in the initial immune response to QPX and evaluated the role of plasma proteins in initiating the encapsulation response. The effect of exposure of clams to QPX on encapsulation rate and on the expression of plasma proteins that bind parasite cells was also determined. Results allowed the identification of proteins that

recognize and bind parasite cells, providing molecular targets for future investigations of factors involved in clam resistance to the disease, and ultimately for the selection of resistant clam stocks.

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Chapter I Background

Bivalves are economically and ecologically important in the United States, and in many countries around the world. They are consumed as seafood in large quantities, and the bivalve industry was recently valued worldwide at 16 billion US dollars (FAO, 2014). Bivalves are also important in that they bioaccumulate environmental contaminants and can be used as biomarkers for pollution (Calabrese et al., 1983; Chiarelli and Roccheri, 2014). Most bivalves are also filter-feeders, which by definition can filter contaminants and microbes from water (Hood et al., 1983; Doré and Lees, 1995; Sze and Lee, 1995). Bivalves are also important for the flux of dissolved nutrients and gases across the sediment-water interface (Dame et al., 1984; Doering et al., 1987). Since they serve human interest, bivalves have been studied extensively. Bivalve pathogens and the immune responses to those pathogens have taken the forefront of recent research, since methods for researching this topic are more readily available, and could directly impact the profitability of the shellfish industry. Quahogs, or hard clams (*Mercenaria mercenaria*) are an economically important species of clam found abundantly on the east coast of the United States and Canada, with aquaculture efforts existing from Massachusetts to Florida. They are a relatively robust species with one pathogen known to significantly affect survivorship, Quahog Parasite Unknown (QPX).

QPX is a protist of the class Labyrinthulomycetes and the order Thraustochytriidae (Whyte et al., 1994). The Labyrinthulomycetes are an extremely common and diverse, yet poorly understood group of protists, frequently associated with the decomposition of organic matter (Raghukumar, 2002). The presence of QPX disease in clams has been documented since the 1960s. It was first identified in New Brunswick, Canada (Drinnan and Henderson, 1963), and later in Massachusetts in the late 1990s (Smolowitz et al., 1998). It has since been found on the east coast of the United States and Canada as far south as Virginia (Ragone Calvo et al., 1988; Maas et al., 1999). In aquaculture, high densities of clam stocks can increase the prevalence of QPX disease, and seed clams brought in from locations free of QPX are more susceptible to the disease (Ford et al., 2002; Dahl et al., 2008; Dahl et al., 2010; Dahl and Allam 2015).

QPX is a species-specific pathogen, known at this time to only infect the hard clam *M. mercenaria*. Disease has traditionally been detected in clams using histology (Drinnan and Henderson, 1963, Ragone Calvo et al., 1988, Smolowitz et al., 1998; Dove et al., 2004), but due to the limitations of these techniques a qPCR protocol has been developed (Liu et al., 2009). Symptoms of QPX disease can vary, but include nodules with high densities of QPX cells, yellowing of the clam tissue, and receding of the mantle tissue, though histological and qPCR confirmation are required to diagnose QPX disease (Ragone Calvo et al., 1998; Smolowitz et al., 1998). Little is understood about the mechanisms of infection of clams by QPX or the clam immune response to the pathogen, while more is understood about the environmental factors affecting the relationship between the host and the pathogen.

QPX optimal growth *in vitro* is in the temperature range of 20-23^{o}C, but low incidence of QPX disease is observed in this temperature range (Dahl et al., 2011). QPX is thought to be a facultative parasite that infects the host when optimal conditions for disease development are prevailing. In fact, the definition of "optimal conditions" is not well understood, but previous studies showed that clam immunity is compromised at colder temperatures (e.g. 13^{o}C) leading to an increase in QPX disease severity (Dahl et al., 2011). At this colder temperature, clams have a lower total hemocyte count and lower rate of immune activities by hemocytes (Perrigault et al., 2011). At warmer temperatures of around 20^{o}C, the clam is able to mount an immune response against QPX, which can be effective enough to lead to partial or complete healing from the disease (Dahl et al., 2011). Clams are also more susceptible to QPX disease at higher salinities of 30 psu compared to lower salinities of 17 psu (Perrigault et al., 2012). It is also important to note that these factors may vary between strains of clam, as some clam stocks are more susceptible to QPX than others (Dahl et al., 2008; Perrigault and Allam, 2012).

The innate immune system is the only line of defense for mollusks, as they lack an adaptive, or acquired, immune system (i.e. immune memory) (Charlet et al., 1996). The innate immune system functions include recognition of self from non-self, and the elicitation of the different immune responses (Iwanaga and Lee, 2005). In clams and other bivalves, the immune system relies on the humoral responses and the cellular responses (Allam and Raftos, 2015). The humoral responses involve factors present in the plasma (e.g. lectins, antimicrobial proteins) that aid in immunity. The cellular responses involve the antimicrobial activities of immune cells

called hemocytes (e.g. granulocytes), which are capable of several responses such as encapsulation, phagocytosis, production of reactive oxygen species, and cytotoxicity to invaders (Lemaitre et al., 1996; Roch, 1999; Allam and Raftos, 2015). Proteins present in the plasma are believed to greatly influence the types of immune responses exhibited. These molecules may also be responsible for some degree of primitive immune memory in invertebrates (Dempsey et al., 1996; Allam and Raftos, 2015).

Although poorly understood, there is evidence of some level of immune memory in invertebrates, though lacking the mechanisms of the vertebrate adaptive immune system (Little and Kraaijeveld, 2004; Kurtz, 2005; Allam and Raftos, 2015). The fruit fly, *Drosophila sp.* was found to have increased gene expression of immune-related genes after microbial infection, and this increase was sustained in the long-term post-infection, though appeared to be nonspecific (Lemaitre et al., 1997). Kurtz and Franz (2003) found that, in copepods, reinfection by a parasite occurred less frequently when the parasite antigens were similar to parasites from previous infections. Pham et al. (2007) discovered that in *Drosophila*, a sublethal bacterial infection protected the host from a later lethal infection of the same bacterium, but not against other pathogens suggesting a relatively high degree of specificity. Netea et al. (2011) suggest that invertebrates possess a "trained" immune system, which differs from adaptive immunity. A trained immune system would demonstrate a heightened immune response in the presence of previously-encountered pathogen-associated molecular patterns (PAMPs), though it does not display the high degree of specificity or long-lasting effects found in a true adaptive immune system.

Encapsulation is a common immune response exhibited by invertebrates in response to pathogens, in particular those that are too large to be phagocytosed (Nappi, 1975; Allam and Raftos, 2015). Encapsulation can involve several steps depending on the host organism, but generally, hemocytes surround the invader with an envelope or capsule. The hemocytes then can die and degrade, leading to the release of destructive enzymes, or can actively produce and release enzymes and other antimicrobial molecules, causing the death or incapacitation of the invader (Johansson et al., 2000). Previous studies on encapsulation mechanisms in invertebrates have mainly focused on insects and crustaceans, though some studies have been conducted on mollusks, especially gastropods (Mohamed et al., 2011). In one of the few studies involving bivalves, an encapsulation experiment was conducted using the mussel *Perna veridis*. The study

showed that the hemocytes preferentially encapsulated beads with a positive surface charge (Jayaraj et al., 2009). Another study found that the presence of the plasma of the clam, *Meretrix casta*, caused an increased rate of encapsulation. The authors also suggested that hemocytes might secrete these encapsulation-facilitating factors in response to pathogens (Meena et al., 2010).

In the highly studied immune systems of insects, pattern recognition receptors (PRRs) in the insect plasma (and also PRRs present on hemocytes) recognize and bind to PAMPs on the pathogen cell membrane. Lectins and other similar molecules are often involved in recognition of PAMPs in insects (Ling and Yu, 2006). It is important to understand these interactions in each individual case because the pathway of encapsulation can be variable. For example, melanization, or the encapsulation of a pathogen within a coat consisting of melanin, is a step present in this process for some organisms such as insects, but is not always observed in other organisms (Christiansen et al., 2005). In addition, while encapsulation can be initiated within 24 hours of infection in some organisms, in others such as the snail *Lymnaea glabra* the process can take up to 60 days after infection to initiate (Rondelaud et al., 1987).

In the case of QPX disease, parasite encapsulation by clam hemocytes seems to represent the primary defense response since phagocytosis of QPX is rarely observed, as QPX cells are much larger than the hemocytes (Smolowitz et al., 1998). QPX is also thought to be capable of resisting host clam immune factors. QPX produces dense mucus that appears to contribute to QPX pathogenic properties (Anderson et al., 2003a). For example, antimicrobial factors present in the plasma are effective against QPX only when the mucus layer surrounding the parasite is washed away, suggesting that the mucus can shield the pathogen from the host immune factors. The secretome of QPX has been recently shown to contain a high number of peptidases and hydrolases, which can contribute to pathogenicity (Rubin et al., 2015).

Especially in cases involving economically important species, understanding hosts' immune responses to infections is crucial. Identifying the expressed proteins involved in clam immunity towards QPX could enable the selection of resistant clams for aquaculture purposes. If highly resistant strains of clams produce a certain plasma protein more than less-resistant clams, this phenotype could be selected for. For both parts of this study, the differences in immune response between clams challenged with QPX and control clams were comparatively assessed in order to contrast constitutive and induced hemocyte responses and immune proteins.

The first part of this study focused on identifying the clam plasma proteins that bind to QPX during initial host-pathogen interactions. The plan for this was to first develop a method for growing QPX with limited mucus production, as proteins in the mucus could skew the results. Next, clam plasma would be incubated with fixed QPX cells, the bound proteins eluted, and the eluted proteins identified through proteomic methods. The second part of the study evaluated the effects of clam plasma on parasite encapsulation by hemocytes. Hemocytes from naïve or from clams previously challenged with QPX were incubated with fixed QPX cells in presence or absence of plasma, and encapsulation was measured over time. Combined, these two experiments were intended to help elucidate the role of plasma and the effects of pathogen challenge on the humoral and cellular responses of the hard clam.

Chapter II Plasma Protein Identification

Background

Invertebrate immune systems lack acquired immunity and rely solely on innate immunity. This involves recognition of a pathogen and the subsequent initiation of protein cascades triggering secondary immune responses (Lemaitre et al., 1996). This initiation step can display some degree of specificity, though not to the degree of mammalian immune systems (Lemaitre et al., 1997). In many invertebrates such as arthropods, crustaceans, and mollusks, plasma proteins can be involved in the recognition and initiation processes of the innate immune system (Dempsey et al., 1996; Garver et al., 2008). These processes are well-documented in arthropods and are becoming better understood in mollusks.

Previous studies show the effect of host plasma proteins on the immune response to a pathogen. Genard et al. (2013) discovered that the larvae of the pacific oyster *Crassostrea gigas* exhibited higher transcript abundance of immune-related plasma proteins when challenged with bacteria. Charlet et al. (1996) isolated and identified plasma proteins present in the blue muscle *Mytilus edulis* following bacterial challenge, and found antimicrobial peptides that appeared to homologous to those found in arthropods. In the snail *Biomphalaria glabrata,* researchers found that susceptible strains produced more carbohydrate binding proteins than resistant strains (Monroy et al., 1992). Another study focused on fibrinogen-related proteins (FREPs), discovered that susceptible snails increased FREP expression levels in the plasma in response to exposure to the trematode *S. mansoni*, while resistant snails exhibited a FREP expression level increase in the plasma to a lesser degree (Zhang et al., 2008). A recent study in *B. glabrata*, showed that challenge with a parasite completely prevented later infection by the same parasite and that the effects of the challenge lasted the lifespan of the organism (Portela et al., 2013), while a related study (Pinaud et al., 2016) determined that immune-related plasma proteins such as C-type lectins, agglutination factors, and peroxidases were upregulated in response to the secondary parasite challenge.

Anderson et al. (2003b) exposed QPX cells to filtered plasma and observed that plasma factors resulted in a significant decrease in growth of QPX cells, indicating the presence of anti-

QPX plasma factors. Perrigault and Allam (2009) found an increase in plasma protein concentration and reduction in anti-QPX plasma activity following challenge with QPX. The current research sought to increase the limited understanding of the molecular immune response of the hard clam to the pathogen QPX. The goal was to identify clam plasma proteins that recognize and bind QPX cells. Further, the study compared plasma from naïve clams and clams previously challenged with QPX to contrast baseline and inducible levels of QPX-reactive proteins. Certain immune-related proteins were identified bound to QPX in higher abundance than to a bead control, and in higher abundance from challenged than naïve clam plasma.

Methods

QPX Cultures and Cell Preparation

Parasite cells were grown in 0.3% yeastolate medium (BD Biosciences, USA, Cat. 255772) (Rubin et al., 2015) and incubated at room temperature on a shaker. Preliminary experiments showed that these conditions reduce the production of mucus by QPX cells compared to the routinely used minimal essential media (abundant mucus proteins could interfere with downstream analyses). Cultures (350 mL) were incubated for 5 days to reach high cell densities (~10^6 cells/mL). Parasite cells were counted with a hemocytometer. The cultures were centrifuged, pooled, and resuspended in filtered artificial seawater (FASW; 28 ppt). The cells were then fixed with 0.2% final concentration glutaraldehyde, with fixation occurring overnight. Glutaraldehyde was removed by washing the cells several times with FASW by centrifugation, and cells were refrigerated until use (no more than 24 hours after fixation).

Isolation of Proteins Bound to QPX

Hemolymph was withdrawn from the adductor muscle of 10 clams (Perrigault and Allam, 2009), pooled, and hemocytes were pelleted by centrifugation (200 g, 15 minutes, 5ºC). The supernatant was recovered and filtered through a 0.8 μm filter to eliminate cell debris (similar to methods described in Anderson et al., 2003b). Fixed QPX cells were then added to the filtered plasma, allowing plasma proteins to bind to QPX cells. Following incubation (2 hours, 4ºC, light shaking), parasite cells were collected by centrifugation (400 g, 30 minutes, 5ºC), and the supernatant discarded, as this contained proteins that did not bind to QPX cells. The cell pellet was washed twice with FASW (9 ppt), before resuspension in an elution buffer (10 mM

EDTA and 1 M NaCl) to release bound proteins. The two washes with FASW (9 ppt) were found to remove unbound proteins while still eluting proteins at the final elution step, in preliminary experiments. The salinity was lowered for these washes to 9 ppt because a higher salinity may remove bound proteins before the washing steps were complete. Eluted proteins were then retrieved by centrifugation (400 g, 30 minutes, 5^{o}C), eliminating the pelleted QPX cells. At each stage (prewash- pure plasma, 1st wash- 9 ppt FASW, 2nd wash- 9 ppt FASW), 1 mL of supernatant was retrieved and the protein concentration was measured to observe the change in protein concentration over time. In parallel, control preparations were made to evaluate nonspecific binding of plasma proteins by replacing QPX cells with synthetic beads in the same size range of QPX cells (31.4 μm, Polymethyl Methacrylate Latex, MAGSPHERE lot no. PM30095-0515). The beads were neutral with no charge, and hydrophobic. The protein concentrations in the eluates were measured using the BSA Protein Assay (BioRad) following manufacturer's recommendations. After assay optimization, the challenge experiment was conducted to evaluate change in plasma proteins following clam exposure to QPX.

Challenge Experiment

The goal of this experiment was to determine whether or not the clam plasma proteins binding to QPX were different between naïve clams and those challenged with QPX. Clams were obtained from a commercial shellfish company in Oyster Bay, NY, and were acclimated in a tank with ultraviolet filtered seawater for one week at 13°C and 28 ppt (conditions similar to those measured in the clam collection area). Clams were fed daily with algae (DT's Live Marine Phytoplankton, Sycamore, IL) and water in the tanks was continuously filtered (recirculating and aerated), maintained at 18°C and 28 ppt. Clams were allowed to acclimate to the new temperature for 3 days. Three of these tanks represented the experimental, challenged group while the other three represented the control, non-challenged group.

QPX culture for injections was grown according to Kleinschuster et al. (1998) and diluted with FASW (28 ppt) to a final concentration of 10^6 cells/ml. 100 μl of QPX cell suspension was injected into the adductor muscle of each of the 30 experimental clams. Injected clams were maintained outside of their tanks for 1 hour, and then returned. The 30 control clams were each injected with 100 μl FASW (28 ppt), to account for the general immune response to stress as opposed to the QPX-specific immune response expected from the experimental group.

The clams were left in their tanks for 2 days post-injection to allow the clam proteome to respond to QPX challenge. It has been shown that while transcriptional changes occur within hours, changes in protein expression following a stimulus can take 2-3 days to be observed in invertebrates (Schoville et al., 2012). Clams were processed for the isolation of plasma proteins that bind QPX as described above. For each replicate (1 pool of plasma from 10 clams, 6 total replicates), 350 ml cultures were grown at room temperature on a shaker, for a total of 2.1 L culture. All clams were processed on the same day. Final protein concentrations were measured using the BSA protein assay (BioRad) and samples were kept at -80 °C until transportation to the proteomics facility at Stony Brook University for proteomic analysis. Samples were analyzed using 1-dimension LC/MS-MS (Liquid Chromatography/Mass Spectrometry-Tandem Mass Spectrometry) and peptides identified using a combined *M. mercenaria* (Wang et al., 2016b) and QPX (Rubin et al., 2015) transcriptome databases following the general methods described by Pales Espinosa et al. (2016). Protein domains identified from the databases were annotated using the Hidden Markov Model algorithm (InterPro), and the ProDom, PIRSF, TIGRFAM, Coils, SMART, ProSitePatterns, Pfam, SUPERFAMILY, and Gene3D libraries. Overall protein descriptions were obtained using Blast2Go. Proteins identified as deriving from fixed QPX cells were removed from downstream statistical analysis. Protein expression levels were quantified using normalized spectral counts, with a cutoff of one peptide and two spectral counts. The data were normalized by dividing the number of spectral counts for each protein by the total number of spectral counts within the sample. The normalized protein abundance data were analyzed in MultiExperiment Viewer (MeV). Significance Analysis of Microarray (SAM) analyses were used to identify proteins significantly more or less abundant for the different treatments (Roxas and Li, 2008).

Results

The total protein concentrations for each treatment and at each washing step are shown in Figure 1. The concentrations for the treatments of plasma incubated with fixed QPX cells showed a decrease from the prewash step (spent plasma from the binding incubation) to the 1st washing step (9 ppt FASW), a smaller decrease to the 2nd wash (9 ppt FASW) and then an increase during the elution step (elution buffer). The treatments of plasma incubated with beads show a decrease from the prewash step to the 2nd wash step, and no increase during the elution

step. These results show that measurable proteins were bound to the fixed QPX cells and were recovered during the elution step, but few proteins, if any, were bound to the beads (below the detection limit of the BCA method).

The LC/MS-MS proteomic analysis yielded 361 identified clam proteins and 333 identified QPX proteins (see Supplemental Table for full annotated list) from the 3 experimental replicates (plasma from challenged clams incubated with QPX cells), 3 control replicates (plasma from naïve clams incubated with QPX cells), and 3 bead replicates (plasma from challenged clams incubated with beads). Plasma incubated with beads from the control clams was not analyzed because with of the low levels of bound plasma protein combined with the high cost of running LC/MS-MS samples. In general, more proteins were identified from the experimental and control groups incubated with the fixed QPX cells than from the group incubated with beads. Proteins identified in all or nearly all of the samples included ribosomal proteins, many types of proteases, heat shock proteins, and cytoskeletal proteins. QPX proteins identified included actin, tubulin, and ribosomal proteins; these were not considered in the SAM analyses.

SAM results are shown in Table 1 with fold change in protein abundance plotted in Figure 2. Nine proteins were found to be significantly more abundantly bound to QPX in the challenged group than the control group, while none were significantly more abundant in the control group. These include a lectin (echinoidin-like), complement 1q (c1q) and H proteins, and an immunoglobulin-domain containing protein. Seventeen proteins were identified to be significantly more abundant in the challenged group bound to QPX than to beads, while none were identified as significantly more abundant bound to beads than to QPX. Significant proteins include several among those described in the previous section such as in c1q, C-type lectins, and enzymes.

Discussion

QPX disease has been a problem for the hard clam industry since its discovery in the 1960s and remains a problem today. The understanding of the host-parasite interactions on a molecular level is still poor, and increased research efforts in this area have taken the forefront of QPX research. The current project used a proteomic approach to identify constitutive and inducible plasma proteins that bind to QPX cells. The SAM comparison of the plasma from

challenged clams incubated with QPX to plasma incubated with beads allowed the identification of 17 proteins with higher abundance in the group exposed to QPX (Figure 2), many with immune-related functions. Among these were C-type lectins, c1q and H-like complement factor proteins, complement component C3 proteins, and sushi von Willebrand proteins. Also present were several enzymes such as malate dehydrogenase, catalase, and aldehyde dehydrogenase. The SAM comparison of challenged clams and control clams resulted in 9 proteins with higher abundance in the challenged clam group (Figure 2). Virtually all of these have immunological functions including recognition of non-self entities, induction of innate immune responses, and membrane association.

A complement 1q (c1q) protein, a complement factor H-like protein, and complement component C3 protein were identified as more abundantly bound to QPX than beads, with the same c1q and factor H-like proteins identified as more abundantly bound to QPX in the challenged than control group. Complement proteins often function as pattern recognition receptors (PRRs) to identify pathogen-associated molecular patterns (PAMPs) present on the cell surfaces of pathogens and foreign invaders, and can also function to initiate the primitive complement component system in invertebrates (Tang et al., 2005). Complement component proteins can initiate various innate immune responses including inflammation, phagocytosis, and encapsulation (Liu et al., 2014). This suggests that the primitive complement component system of the hard clam could generally recognize non-self cells or possibly recognize and respond to the presence of PAMPs present on QPX cells. Studies in bivalves have shown upregulation of c1q domain-containing transcripts following challenge with pathogens, suggesting these proteins play an important role in pathogen recognition and responses, though these processes are not entirely understood (Liu et al., 2014; Jiang et al. 2015).

The complement component C3 protein identified as more abundantly bound to QPX than beads, contained an alpha-2 macroglobulin domain. Alpha-2 macroglobulin is a protease inhibitor that functions to regulate the prophenoloxidase system in invertebrates, an important immunological pathway, and also degrades foreign proteases (Shanthi and Vaseeharan, 2014), so enhanced levels of alpha-2 macroglobulin may suggest binding of these proteins to extracellular proteases produced by the parasite, if the binding substrate was present on the parasite extracellular membrane.

A sushi von Willebrand factor type A protein was also identified with higher abundance bound to QPX than beads, and in the challenged compared to control group bound to QPX. A von Willebrand factor D and EGF-containing protein isoform with a Sushi/CCP/SCR domain profile were also identified with higher abundance in the challenged group bound to QPX. In invertebrates, von Willbrand factors are often involved in hemolymph clotting (Dheilly et al., 2013; Sanggaard et al., 2016) and have been shown to be overexpressed in response to heavy pathogen infections in shrimp (Goncalves et al., 2014). The function of this protein could be pathogen recognition. In the challenged group, these binding proteins may have been more abundant due to a general heightened immune response resulting from the QPX challenge.

An echinoidin-like protein and a lactose-binding lectin I-2 like protein were also identified as more abundantly bound to QPX than beads, and also bound to QPX in the challenged group than the control group. Echinoidin is a C-type lectin (CTL) identified from the sea urchin, which is believed to be involved in cell adhesion and has several homologous sequences in other organisms (Takahashi et al., 1985; Giga et al., 1987; Vasta et al., 1999). CTLs are a family of PRRs functioning in carbohydrate recognition and recognition of PAMPs to distinguish self from non-self. They are capable of recognizing a wide range of pathogens (Cambi et al., 2005; Li et al. 2015). This protein likely was likely enriched in this case due to recognition of the PAMPs on the QPX cells. In previous studies in bivalves, CTL transcripts were found to be upregulated in response to bacterial challenge, and could recognize a broad range of bacteria (Li et al. 2015), indicating that these proteins may recognize many different microbes and may not be pathogen-specific. Previous studies also suggested the involvement of CTLs in "immune priming", where a prior encounter with a pathogen can induce upregulation of CTLs in a later exposure to the same pathogen (Wang et al., 2013b; Allam and Raftos, 2015), though further research will be needed to determine if this is the case in the hard clam response to QPX.

Another protein present in higher abundance bound to QPX than beads, and in the challenged group than control bound to QPX, was a blastula protease 10-like isoform. This protein is an astacin metalloprotease, which display various functions including tissue and cellular degradation (Lhomond et al., 1996; Gallego et al., 2005; Modica et al., 2015) Interestingly, an astacin-like metalloproteinase was found to be highly expressed in the hemocytes of the pearl oyster (*Pinctada fucata*), and contained a C-terminal with possible

functions in wound healing and cell proliferation (Xiong et al., 2006). The blastula protease 10-like isoform was also found to contain a PAN domain in this case, which can have immune functions. In general, PAN/apple domains mediate protein-protein and protein-carbohydrate interactions. They are also notably found in plasminogens, hepatocyte growth factors, coagulation factors, and plasma prekallikreins (Tordai et al, 1999). Such a protein could be involved in the recognition of pathogens and the degradation of the pathogen cell membranes.

There was an uncharacterized protein identified more abundantly bound to QPX than beads, and more abundantly in the challenged group than the control group bound to QPX. A PAN domain was also identified in this protein, and since the overall protein description is uncharacterized the function will be predicted from the domain. PAN domains, as previously described, mediate protein interactions with other proteins or carbohydrates. PAN domain-containing protein families can also be associated with the formation and degradation of blood clots, an inflammatory immune response. On the other hand, Gong et al. (2012) identified proteins containing PAN/apple domains in the parasite *Toxoplasma gondii* that were thought to enable proliferation of the parasite. Since PAN domains enable protein-protein and protein-carbohydrate interactions, they are able to bind to many different types of molecules and may have functioned in pathogen recognition in this case. Huang et al. (2015) found PAN domains in fibrinogen related proteins (FREPs) in oysters, which were thought to function in cell signaling. The presence of this protein in significantly higher abundance bound to QPX compared to beads and more abundantly bound to QPX in the challenged group, suggests involvement in pathogen recognition.

A basement membrane-specific heparin sulfate proteoglycan core protein was identified as more abundant in the challenged group. Heparin sulfates have a wide range of functions in invertebrates such as cellular adhesion and anti-clotting (Gomes et al., 2009), and are commonly used by humans in cardiovascular disease treatments (Iozzo and San Antonio, 2001). The function of these proteins can vary significantly since the side chains determine the nature and function of the overall protein (Volpi et al., 1998). An immunoglobulin (Ig) domain was identified in this particular heparin sulfate proteoglycan protein. Ig domain-containing proteins are a group of highly diverse proteins found in invertebrates and vertebrates. They can function as antibodies, specializing in identifying and neutralizing pathogens, though not all Ig-domains function this way. In humans and higher vertebrates, Ig domains can be part of the adaptive

immune system and can exhibit some form of memory in regards to specific antigens. Exposure to one antigen increases the ability to fight off the same antigen in the future (Borghesi and Milcarek 2006). Ig domains can also function to initiate the complement component system (Wang et al., 2016a). Invertebrates are capable of producing many diverse classes of immunoglobulin-like proteins to increase the possibility of an effective immune response to many types of invaders and pathogens (Buchmann, 2014). Knocking out immunoglobulin-containing genes has also been shown to decrease the ability to fight off infection in invertebrates (Garver et al., 2008). In crustaceans, Dscam, an Ig domain-containing PRR, was found to be induced following pathogen exposure (Jin et al., 2013b). In gastropods (snails), it was found that fibrinogen-related protein (FREP) domains, involved in recognition of non-self, contained Ig domains as well (Knight and Adema, 2009). The FREP domains in these animals were diverse, with expression changing depending on the pathogen encountered, suggesting a high degree of immune specificity (Adema, 2015; Adema and Loker, 2015). Diverse FREPs were also identified in oysters (Huang et al.; 2015). It should be noted that some proteins contain Ig domains associated with C-type lectins, which were similarly structured to the Ig-containing FREP domains (Dheilly et al., 2014). For hard clams, fibrinogen-related proteins were found to be overexpressed when challenged with QPX (Wang et al., 2016c), which could support the higher abundance of Ig domains found in the challenged group in this study, though no FREP domains were identified with significantly different abundance between the challenged and control groups. Since the heparin sulfate proteoglycan core protein was basement-membrane specific and contained an Ig domain, it is possible this protein is involved in cell signaling and adhesion.

In response to pathogens, invertebrate immune systems can release reactive oxygen species (ROS) to damage the pathogens. Catalase, aldehyde dehydrogenase, and zinc transport system proteins (with a copper/zinc superoxide dismutase domain), which are all important in destroying excess ROSs to avoid host cellular damage (Wang et al., 2013c; Wang et al., 2013d; Stephens-Camacho et al.; 2015), were identified as significantly more abundantly bound to QPX than beads. In insects, certain enzymes act as innate immune factors in response to pathogen stimulation (Kanost and Jiang, 2015), and others were also identified to be overexpressed in the hemolymph following pathogen exposure in the abalone, suggesting an immune-related function (Bathige et al., 2015). As binding plasma proteins and immune factors in this case, these proteins

likely functioned in pathogen degradation. Aldehyde dehydrogenase can also function to damage cell membranes (Pedrini et al., 2013). A nidogen-like protein was identified with a phospholipase A2 domain. Phospholipase A2 domains and Dyp-type peroxidase domains can aid in protection and immune response against stress, and can also be involved in cell signaling (Balsinde et al., 2002; Ahmad et al., 2014; Tomanek, 2015). Malate dehydrogenases may function in metabolic stress responses in invertebrates. (Zhang et al., 2015).

An atrial natriuretic peptide receptor with a PLAT/LH2 domain was identified bound more abundantly to QPX than beads, which is a protein involved in the binding of atrial natriuretic factor, a hormone involved in blood pressure (Bold, 1985). Polycystin/lipoxygenase/α-toxin (PLAT) domains are believed in humans to function in membrane-protein and protein-protein interactions (Bateman and Sanford, 1999). In nematodes, proteins containing these domains are believed to function in intracellular signaling pathways (Hu and Barr, 2005). In sea urchins, PLAT/LH2 domains were found intracellularly in sperm plasma membrane receptors functioning as receptors for egg jelly (Gunaratne et al., 2007). In this case, the function of these proteins could be PAMP recognition or cell signaling.

An inter-alpha-trypsin inhibitor heavy chain H3-like protein was found in higher abundance bound to QPX in the challenged group than control. This protein is known to function as a protease inhibitor, as well as an extracellular matrix stabilization factor. In humans, it is known to be downregulated in cancerous tissues (Himmelfarb et al., 2004). Though there are few studies on the function of this protein in invertebrates, it has been identified in the snail *Biomphalaria glabrata* as a protease inhibitor (Mitta et al., 2004). In the brain of *Octopus vulgaris*, a vault domain was identified in a protein complex involved in post-translational protein modification (Maio et al., 2013). In this study, it is possible that the vault protein was acting as a protease inhibitor against the proteases present in the QPX mucus or produced by the QPX cells. QPX is known to secrete several different types of enzymes involved in pathogenesis, and the secretome has been characterized (Rubin et al., 2015). The QPX proteins identified from the elution, while not used in statistical analyses, were compared to the proteins characterized from the QPX secretome. Hydrolases, heat shock proteins, metallopeptidases, and antioxidants were matched to the QPX secretome, suggesting that the vault inter-alpha-trypsin protease inhibitor identified from the clam could possibly have functioned in inhibiting some of QPX membrane- or mucus- bound proteases.

Conclusions

The significantly higher abundance of immune-related proteins bound to QPX than to beads suggests recognition of the QPX cells. These proteins included complement proteins, enzymes, and lectins. Further research is needed to determine the differences in plasma protein binding to QPX as opposed to other pathogenic or non-pathogenic microbes. This would clarify whether or not the increase in abundance is QPX-specific, a general response to the presence of a pathogen, or even a response to the presence of a living foreign microbes, not necessarily pathogenic.

Plasma proteins found to be bound to QPX cells in higher abundance with prior challenge to QPX than the control group included immune-related domains involved in signaling the complement C3 system, signaling via protein-carbohydrate/protein interactions, and pathogen recognition. This could indicate that challenge with QPX could increase the general immune response if the immune system encounters the pathogen at least two days in the future. Further research is needed to determine the duration of the elevated immune response, i.e. if similar results occur 1-week post-challenge, 1-month post-challenge, etc. Future research is also needed to determine if the elevated immune response is observed only with QPX challenge or if similar results are obtained from challenge with other organisms (e.g. oyster protistan pathogen *Perkinsus marinus* or bacterial challenge).

Given the devastating impact of QPX disease on clam stocks, understanding the molecular response of the clam to QPX infection is increasingly important in particular due to the continuous economic growth of the clam aquaculture industry. Understanding the effects of prior QPX exposure on the clam immune system may provide innovative means to mitigate QPX disease, particularly if resurgences threaten the profitability of this growing industry.

Chapter III Encapsulation Experiments

Background

Encapsulation is a major mechanism used by the innate immune system response in which blood cells (e.g. hemocytes) surround and eliminate a pathogen. This process can vary between organisms, but in general the hemocytes surround the pathogen to form a capsule, which releases various antimicrobial factors to attack the invader (Cheng and Garrabrant, 1977). In many cases, melanization occurs, in which the capsule produces melanin to aid in the destruction of the pathogen (Johansson et al., 2000; Christensen et al., 2005; Lavine and Strand, 2002). Plasma proteins are known to play a role in encapsulation initiation (Ao et al., 2007), though this role has not been studied in hard clams.

In insects, the encapsulation process is well understood, and studies exist showing the importance of plasma proteins. Ao et al. (2007) showed that C-type lectins, a common group of plasma proteins, acted as a PRR for encapsulation in *Drosophila*. In the wax moth, *Galleria mellonella*, hemocyte-microbe adhesion was shown to be initiated by secondary signaling plasma proteins (Lapointe et al., 2012). Ling and Yu (2006) discovered that in the tobacco horn worm immulectins, common plasma proteins, were crucial in pathogen recognition and encapsulation initiation. In the Manila clam, *Tapes philippinarum*, encapsulation was found to be a response to three different parasites (Lee et al., 2001). In the estuarine clam, *Meretrix casta*, the effects of different suspension media on the encapsulation of sepharose beads by clam hemocytes were tested (Meena, et al., 2010). It was discovered that hemocyte suspension in clam plasma and suspension in hemocyte-conditioned media both significantly increased the encapsulation frequency, but hemocyte lysate media decreased encapsulation frequency compared to an iso-osmotic buffer control (Meena et al., 2010).

As discussed in the background chapter, a main immune response to QPX infection is the encapsulation of parasite cells by clam hemocytes. The objective of this experiment was to determine whether or not QPX encapsulation frequency is affected by the presence of clam plasma, and whether or not prior exposure to QPX has an effect on encapsulation frequency. These experiments required the use of live hemocytes harvested from individual clams. Since primary cultures of hemocytes are difficult to maintain (Cao et al., 2003), all hemolymph was

harvested and used in experiments the same day, with retrieval methods optimized to minimize hemocyte damage. Previous studies have tested encapsulation response in vitro with similar methods. Jayaraj et al. (2009) incubated washed hemocytes from the mussel *Perna viridis* with sepharose beads with varying characteristics. Meena et al. (2010) compared encapsulation frequency of sepharose beads when suspended in different media types, including plasma. The current experiment expanded upon this by determining the effect of plasma from naïve and QPX-challenged clams on encapsulation frequency of the pathogen QPX and contrasted this to the encapsulation of synthetic beads.

Methods

QPX cultures and cell preparation

QPX cultures were grown, cells recovered, and fixed similar to methods described in Chapter II. Fewer fixed QPX cells were required for this experiment, therefore only 40 mL culture was grown to a cell density of ~10^6 cells/mL, and fixed with 0.2% glutaraldehyde in FASW.

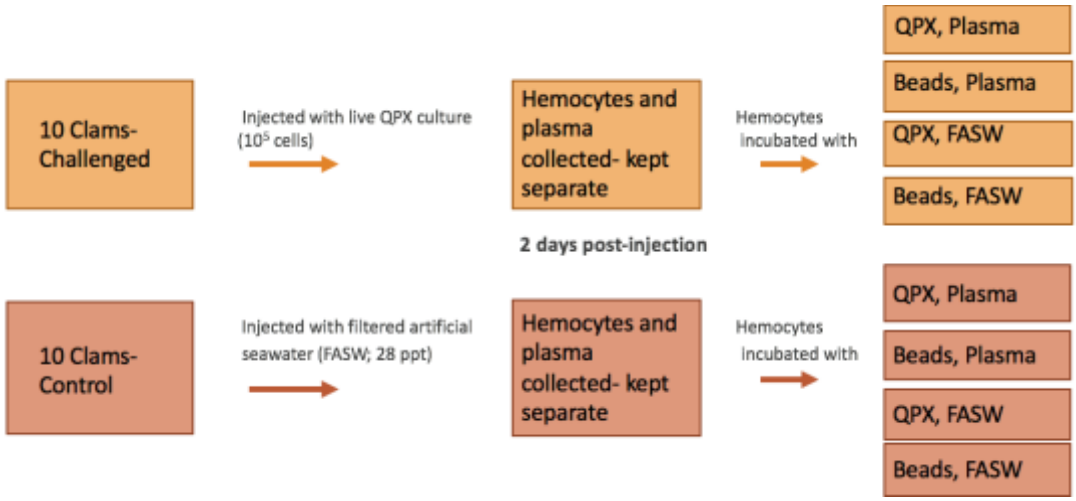
Challenge experiment

This experiment was conducted similarly to the plasma protein assay (Chapter II) in that there was an experimental, challenged group injected with QPX and a control group injected with FASW. These two groups consisted of 10 clams each. Hemocytes collected from each individual clam were divided into 4 different aliquots that were incubated with fixed QPX cells or beads, in the presence or absence of plasma. Encapsulation assays were initiated two days post-injection (as in Chapter II), which was determined from previous literature to be an adequate time for the proteome to respond to challenge.

Encapsulation assay

Hemolymph for hemocyte recovery (~150 µl per clam) was aspirated directly into an anticoagulant solution (14.4 g $NaH_2PO_4 \cdot 2H_2O$, 2.6 g $Na_2HPO_4 \cdot H_2O$, 25 g NaCl, 10 g EDTA; 1 L distilled water; pH 7.4; Allam and Paillard, 1998) . The hemocytes were obtained by centrifugation (300 g, 4 ^oC, 10 min). Hemolymph for plasma recovery was aspirated separately from the same clam, with no anticoagulant, filtered through a 0.8 µm syringe filter to eliminate

cell debris, and held on ice. Hemocytes were washed twice with FASW (28 ppt) and resuspended in either ice cold FASW or the previously recovered filtered plasma. Fixed QPX cells (diluted to ~10^4 cells/mL) were incubated with hemocytes (~10^5 cells/mL, either suspended in FASW or filtered plasma) yielding a 10/1 hemocyte/QPX ratio. Incubations were performed in 96-well plates in the dark, and the frequency of encapsulation events was determined microscopically at different time intervals (1 hr, 3 hrs, 6 hrs, and 24 hrs). Control treatments were incubated with synthetic beads (31.4 μm, diluted to ~10^4 cells/mL) in place of fixed QPX cells to account for non-specific encapsulation activity. Since preliminary experiments showed that encapsulation in this case does not involve melanization, events with greater than 75% coverage by hemocytes were considered true encapsulation events. Events with less than 75% coverage by hemocytes were considered to be associations rather than true encapsulation events. The following flowchart represents the design of the challenge experiment.



Encapsulation did not meet the normality and homogeneity of variance assumptions for parametric testing. Therefore, the data were submitted to nonparametric testing considering the time points separately. Friedman's repeated measures ANOVA and post-hoc Wilcoxon signed rank tests were conducted to determine the significant time points and the significant comparisons at the standard $p < 0.05$ cutoff. Separate Wilcoxon signed rank tests were conducted to consider every possible comparison at every time point, when Friedman's tests determined the overall time point insignificant. Mann-Whitney U tests were conducted to compare encapsulation frequency between the challenged and control groups.

Results

Figure 3 shows examples of encapsulation of fixed QPX cells and beads as they were observed in the experiments after 1-hour incubation.

The significant results from the Friedman's repeated measures ANOVA, Wilcoxon signed rank tests, and Mann-Whitney U tests are summarized in Table 2. The time point T24 in the control group was found to be significant, with post-hoc tests showing that incubations with QPX and seawater exhibited higher encapsulation frequency than beads and seawater. The time point T3 was found to be significant in the challenged group, with post-hoc tests showing incubation with beads and plasma had higher encapsulation frequency than QPX and plasma. Separate Wilcoxon tests for all possible comparisons at all individual time points also show that, in the challenged group at time points T3 and T6, beads incubated with plasma had a greater encapsulation frequency than QPX incubated with plasma. At T1 in the challenged group, QPX incubated with seawater had a greater frequency than QPX incubated with plasma. In the control group at time point T24, QPX incubated with seawater had a greater frequency than beads incubated with seawater. Mann-Whitney U tests for comparisons between the control and challenged group at all time points showed that at time point T1, the challenged group had a greater encapsulation frequency than the control group when comparing QPX incubated with seawater.

Discussion

The objectives of this experiment were to determine the role of plasma proteins in the encapsulation rate of fixed QPX cells and beads by *M. mercenaria* hemocytes, and to determine if prior QPX challenge affects the encapsulation frequency. Analyses were conducted considering the time points separately, using nonparametric methods.

Effects of Plasma on Encapsulation Frequencies

At time point T3 and T6 in the challenged group, beads incubated with plasma showed a higher encapsulation frequency than QPX with plasma, which was unexpected from this experiment. This could be due to the hyperactivation of hemocytes in the presence of the PAMPs on the QPX cells. It has been shown that overstimulation of the innate immune system in crustaceans can trigger self-harming responses such as release of cytotoxic molecules from

hemocytes, hemocyte clotting, and immune exhaustion (Apines-Amar and Amar, 2015; Solidum et al., 2016). In earth worms, overstimulation of hemocytes can cause antimicrobial activities to fail (Rorat et al., 2013). In this experiment, the decreased encapsulation frequency observed from the combined plasma and QPX exposure was possibly not a response to the plasma factors, but an inhibitory effect of QPX PAMPs on the hemocytes themselves.

Another possibility is that the QPX exposure triggered the programmed cell death (apoptosis) of the clam hemocytes. Apoptosis can be triggered by many stimuli such as pathogen exposure, environmental factors (e.g. pesticides), or cell damage (Ray et al., 2013; Gervais et al., 2015). Challenge experiments in other invertebrates have shown that microbial exposure can induce apoptosis in hemocytes or overexpression of apoptosis-related transcripts (Xu et al., 2014; Ye et al., 2016). This response could help limit pathogen proliferation. In the future, hemocyte apoptosis could be evaluated in response to QPX and plasma incubation.

Pathogens have also been shown to be capable of mimicking the characteristics of host cells, effectively preventing non-self recognition (Kawasaki et al., 2013). For example, some bacterial pathogens are capable of creating a capsule of polysaccharides that resembles the surfaces of different types of host cells, preventing pathogen recognition (Cress et al., 2014). In oysters, the parasite MSX (*Haplosporidium nelsoni*) was found to have cell surface receptors that inhibit recognition by host hemocytes, limiting the phagocytosis of MSX cells (Kanaley and Ford, 1990). Future experiments could determine the similarities between the QPX cell receptors and host cell receptors to determine if any pathogen mimicry is taking place, causing the failure of hemocytes to encapsulate QPX cells.

Many previous studies have shown that host plasma can facilitate encapsulation of beads and pathogens in insects and crustaceans (Yu and Kanost, 2004; Meena et al., 2010; Wang et al., 2013a; Jin et al., 2013a; Hu et al., 2014), which was the case in this experiment only for beads and not when combined with pathogen exposure. The situations described above could provide reasoning behind these results.

Effects of Prior QPX Challenge on Encapsulation Frequency

Results of statistical analyses showed higher encapsulation frequency in the challenged group at time point T1 for QPX incubated with seawater. Challenge with a pathogen has been shown to increase the hemocyte encapsulation response and general hemocyte activities in

gastropods (Loker, 2013) and crustaceans (Silva-Aciares et al., 2013). These effects were not as statistically significant as the effects of plasma and particle type described above, but it is possible that the QPX-challenged clams exhibited a higher encapsulation response.

Conclusions

Encapsulation rates were examined considering incubation time, particle type, the effects of plasma, and prior QPX challenge. Hemocyte encapsulation was significantly lower when QPX was incubated with plasma as opposed to beads incubated with plasma, indicating possible inhibitory effects of the overstimulation of hemocytes in response to the PAMPs on the QPX cells. It is also possible that apoptosis of hemocytes was stimulated in response to QPX, or that the QPX cells were not being recognized by the clam hemocytes. These differences were not observed from the incubation with seawater, so it is likely to be an effect of the combination of plasma incubation and QPX. Prior QPX challenge was found to increase encapsulation rates in all samples except QPX incubated with plasma. This is in accordance with previous studies showing the challenge of a host with a pathogen increases hemocyte immune responses.

Understanding encapsulation of QPX by the hard clam hemocytes is important due to past extensive mortality events caused by QPX. Future studies could further separate the effects of hemocyte activation from the effects of plasma factors, and determine the effects of fixed QPX cells on the hemocytes specifically. The effects of the fixative used in the experiment (glutaraldehyde) should also be evaluated. To avoid the interactions of so many different variables, future studies could seek to examine one variable at a time. For example, the particle type could be kept constant for all treatments because either using only QPX or only beads would eliminate particle size variability. Jayarj et al. (2009) used uniformly-sized sepharose beads in all treatments, but changed the characteristics of the beads. Another factor that could be examined in the future is clam strain. Previous studies have shown that susceptible and resistant clam strains exhibit different levels of immune transcripts including several recognition and binding factors (Wang et al., 2016b). Additionally, previous literature was used as a guideline to define an encapsulation event, as it was unclear whether only events with 100% coverage by hemocytes should be considered true encapsulation, or if incomplete coverage could also be accepted (Bayne et al., 1980; Soderhall, et al., 1984; Aladaileh et al., 2007). So as not to further complicate the results, >75% hemocyte coverage was considered a true encapsulation event,

while any events <75% were considered associations. Future research could take associations into consideration as well as true events.

Chapter IV Overall Thesis Conclusions

Innate immune mechanisms have been brought to the forefront of current research efforts, particularly considering economically important species of invertebrates such as the hard clam. Past clam mortality events from the parasite QPX have increased the need for more specialized research into host-pathogen interactions. This thesis examined the interactions between the hard clam and QPX during the initial immune response, first by identifying and characterizing clam plasma proteins binding to QPX, and second by examining the effects of plasma proteins on parasite encapsulation. The effects of prior QPX challenge on these processes were also evaluated.

Plasma proteins binding to QPX in the initial immune response included PRRs such as C-type lectins, complement component proteins, and proteases and enzymes. These proteins could function in a variety of ways, including non-self recognition, clotting, pathogen degradation, and cell signaling. Prior QPX challenge also affected the abundance of these plasma proteins, suggesting that the clam immune system could be primed by prior pathogen exposure. The overall abundance of immune factors present in the host plasma could be increased, resulting in a higher quantity of immune factors capable of binding to the parasite cells in the event of a secondary exposure.

The effects of plasma proteins and parasite challenge on the encapsulation response were also evaluated. The reduction of encapsulation under the conditions of combined plasma protein and QPX incubation showed that the hemocytes could potentially be hyperactived and inhibited by these factors. Previous studies show that some pathogens can negatively affect hemocytes by stimulating processes such as apoptosis, overproduction of reactive oxygen species, and general immune exhaustion. Some pathogens can even mimic the membrane patterns of the host cells, effectively protecting them from the host immune system. QPX challenge was shown to have a positive impact on the encapsulation of QPX cells. This is in accordance with previous studies, and is further evidence of a possible capability to boost or prime the hard clam immune system with prior pathogen exposure.

These two experiments show the importance of plasma proteins in the immune response to the pathogen QPX. The response could be pathogen or microbe-specific, as differences were observed comparing the responses of fixed QPX cells to the bead control. Prior QPX challenge

also had an impact in both experiments. The increased abundance of plasma proteins bound to fixed QPX cells and the increased encapsulation frequencies observed suggest possible immune system priming capabilities. The limitations of both experiments must also be taken into account. For example, both studies used QPX cells fixed with glutaraldehyde, and while the cells were washed several times, there was likely some amount of glutaraldehyde leeching and possible altering of the plasma proteins and/or hemocytes. There are also the limitations of comparing QPX cells with a variable size range to the beads, which were consistently 31.4 μm.

Future studies could be conducted to validate these results and to tease apart the different observed responses. These experiments could be conducted using a different parasite not pathogenic towards the hard clam, to determine if the responses are QPX or microbe specific. The hypothesis of hemocyte hyperactivation could be tested by assays measuring hemocyte activity. Clam strains naïve and resistant to QPX, and more pathogenic QPX strains could also be tested. These future studies could also provide evidence for a possible trained or primed immune system in the hard clam, and could provide valuable information in the event of future QPX disease outbreaks.

Figures

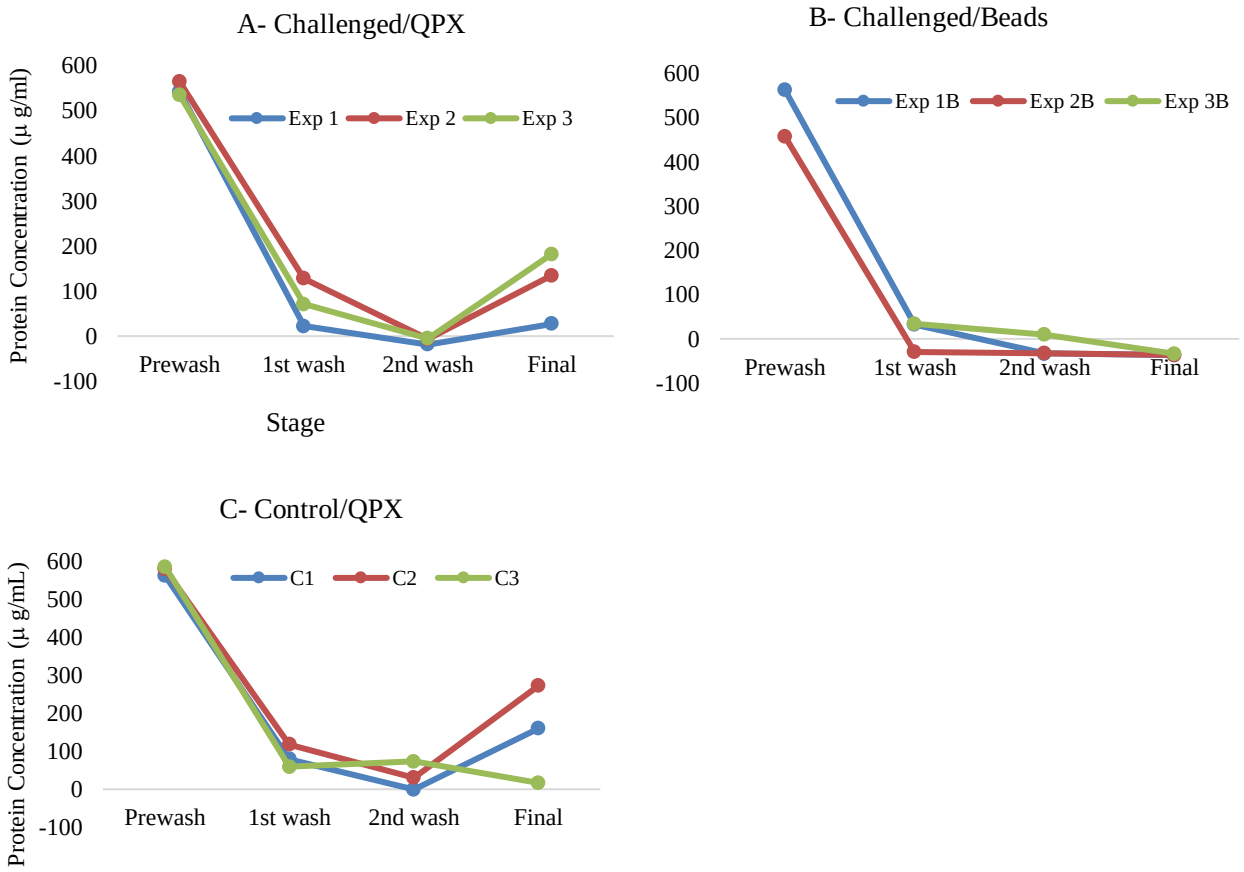


Figure 1. Protein concentrations measured during each stage of the plasma protein binding assay. Plasma from challenged (injected with QPX; A and B) or control (injected with seawater; C) clams was incubated with fixed QPX cells or beads before assessment of protein concentrations in the washing solutions or in the eluates (final).

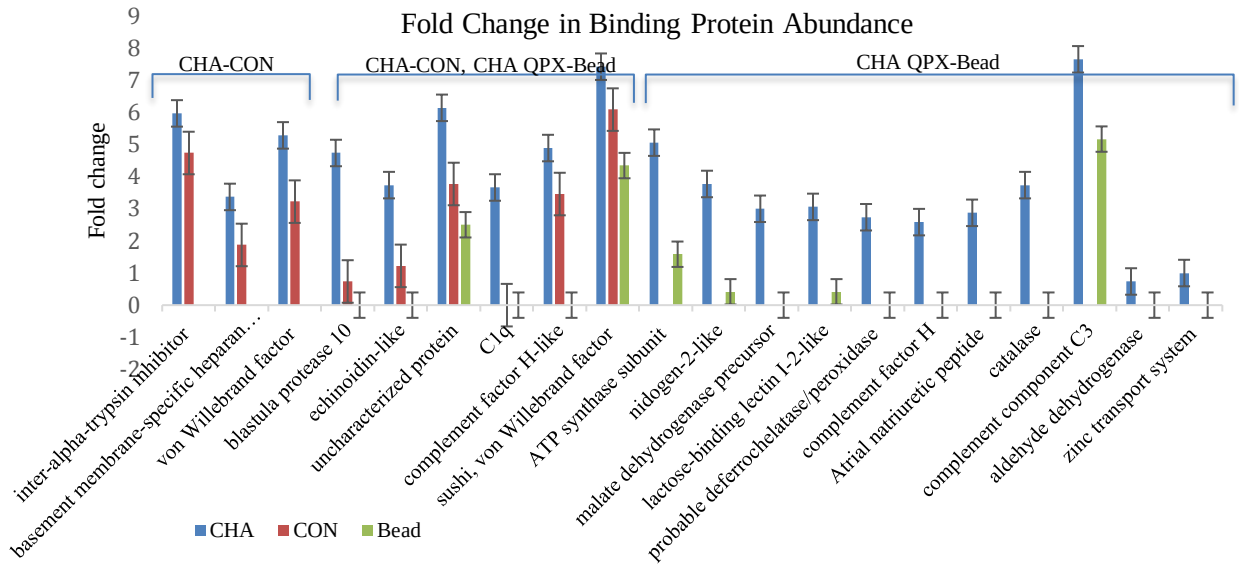
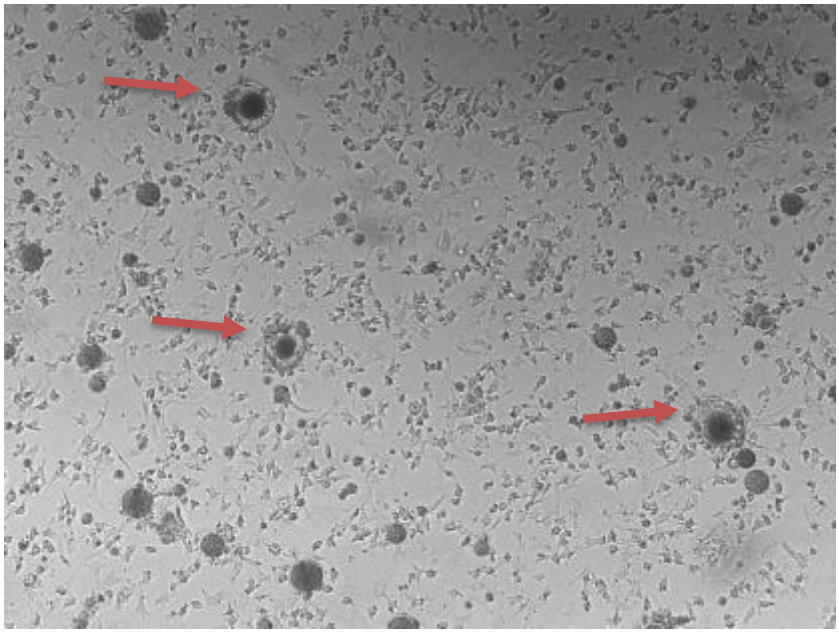


Figure 2. Fold change differences in binding protein abundance (SAM analyses, $\log_2$(mean spectral counts)) between challenged (CHA) and control (CON), and challenged QPX and beads (CHA QPX-Bead) with standard error bars. Full protein and domain descriptions can be found in Table 1.

A



B

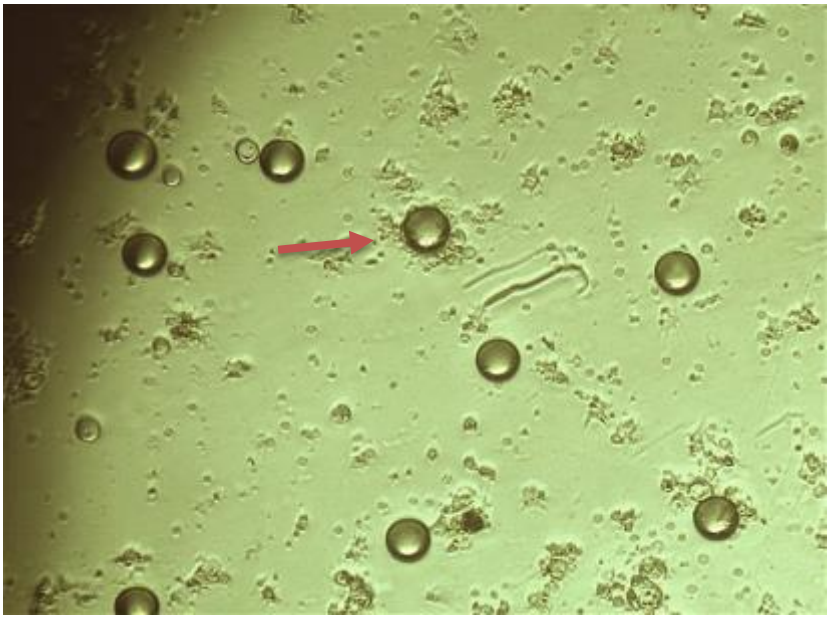


Figure 3. Encapsulation of fixed QPX cells (A) or beads (B) after 1 hour. Red arrows indicate encapsulation events.

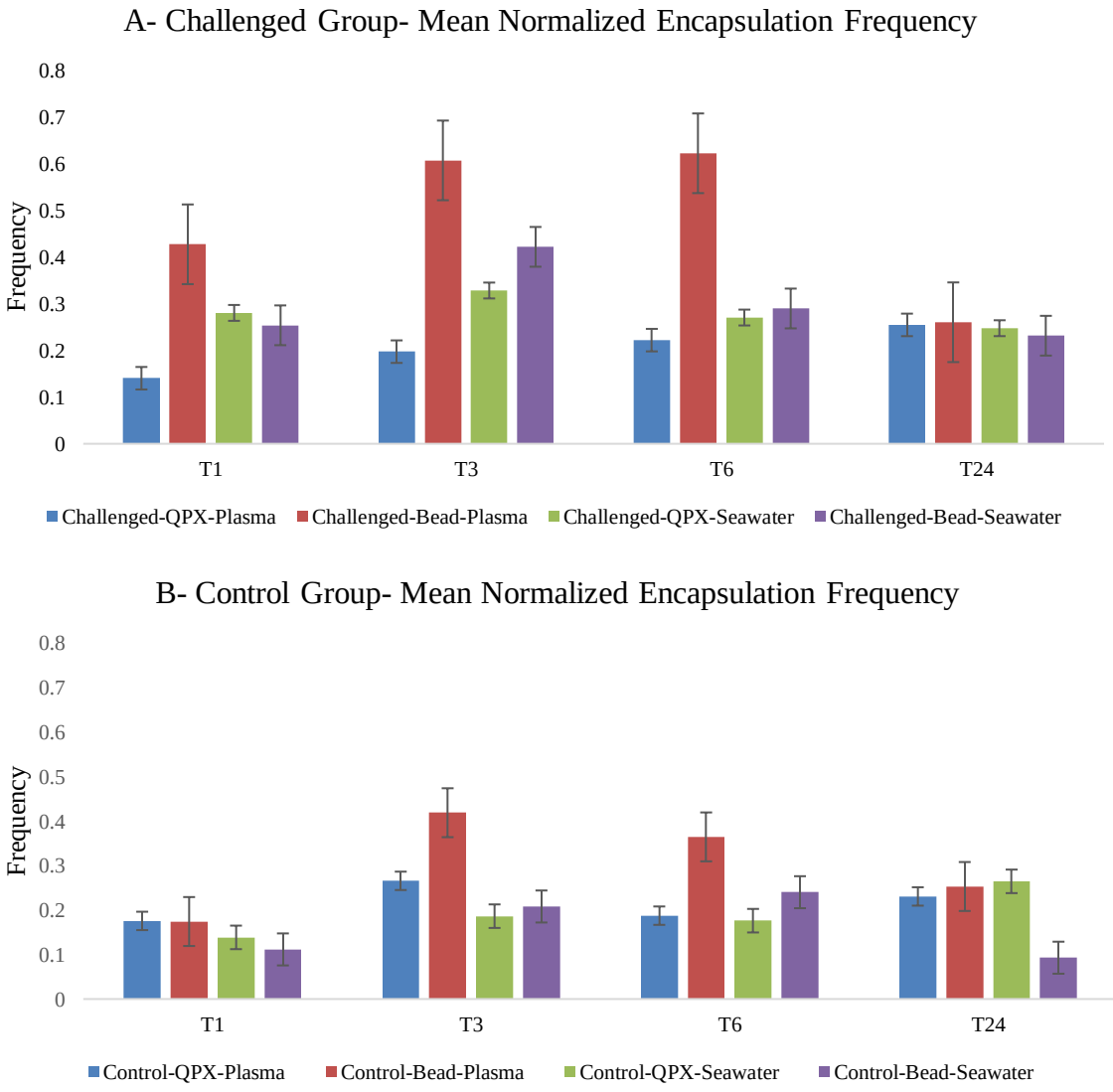


Figure 4- Untransformed, mean normalized encapsulation frequency (+/- standard error) for each time point (T1, T3, T6, T24) for the challenged group (A) and control group (B).

Tables

Table 1. Significant proteins from SAM analysis for Challenged QPX vs. Challenged Beads (*) and Challenged QPX vs. Control QPX (#).

Accession	Protein Description	Domain Description	
comp167747_c0_seq1_1	blastula protease 10-like isoform X3	PAN domain	*#
comp176879_c0_seq3_1	echinoidin-like	C-type lectin (CTL) or carbohydrate-recognition domain (CRD)	*#
comp179726_c0_seq1_6	uncharacterized protein LOC105342476	PAN domain	*#
comp180034_c0_seq1_6	C1q	C1q domain	*#
comp186855_c0_seq9_5	complement factor H-like	PLAT/LH2 domain	*#
comp188664_c0_seq2_6	sushi, von Willebrand factor type A, EGF and petraxin domain-containing protein 1 isoform X3	Domain abundant in complement control proteins; SUSHI repeat; short complement-like repeat (SCR)	*#
comp156933_c0_seq1_5	ATP synthase subunit beta, mitochondrial	atpD: ATP synthase F1, beta subunit	#
comp164993_c0_seq1_4	nidogen-2-like	Phospholipase A2 domain	#
comp166738_c1_seq1_3	malate dehydrogenase precursor	MDH_euk_gproteo: malate dehydrogenase, NAD-dependent	#
comp171627_c0_seq1_4	lactose-binding lectin I-2-like	C-type lectin (CTL) or carbohydrate-recognition domain (CRD)	#
comp174455_c1_seq1_1	probable deferrochelatase/peroxidase YfeX	Dyp_perox_fam: Dyp-type peroxidase family	#
comp186855_c0_seq14_5	complement factor H	Sushi repeat (SCR repeat)	#
comp186855_c0_seq4_5	Atrial natriuretic peptide receptor	PLAT/LH2 domain	#
comp188259_c0_seq2_1	catalase	Catalase	#
comp188894_c0_seq1_1	complement component C3	Alpha-2-macroglobulin family	#
comp190658_c0_seq6_5	aldehyde dehydrogenase family 16 member A1-like	Aldehyde dehydrogenase family	#
comp177355_c4_seq1_3	zinc transport system substrate-binding protein	Copper/zinc superoxide dismutase (SODC)	#
comp167670_c0_seq1_5	inter-alpha-trypsin inhibitor heavy chain H3-like	Vault protein Inter-alpha-Trypsin domain	*
comp169403_c1_seq3_4	von Willebrand factor D and EGF domain-containing protein-like isoform X3	Sushi/CCP/SCR domain profile.	*
comp174465_c0_seq1_5	basement membrane-specific heparan sulfate proteoglycan core protein-like isoform X16	Immunoglobulin	*

Table 2. Significant comparisons of normalized encapsulation frequency from nonparametric Friedman's repeated measures ANOVA on ranks with post-hoc Wilcoxon signed rank test (A), separate Wilcoxon signed rank tests for all possible comparisons (B), and Mann-Whitney U Tests for between-group comparisons (C). Red text signifies variable with significantly higher encapsulation frequency.

A) Friedman's repeated measures ANOVA significant comparisons			**Post-hoc Wilcoxon signed rank test**	**p-value**
Time Point	Group	p-value	Comparison	0.005
T24	Control	0.04	QPX-SW-B-SW	0.018
T3	Challenged	0.05	QPX-P-B-P	

B) Wilcoxon signed rank tests- within control and challenged groups

Time Point	Group	Comparison	p-value
T24	Control	QPX-SW-B-SW	0.005
T1	Challenged	QPX-P-QPX-SW	0.028
T3	Challenged	QPX-P-B-P	0.018
T6	Challenged	QPX-P-B-P	0.021

C) Mann-Whitney U tests- between challenged and control groups

Time Point	Comparison	p-value
T1	CHA-QPX-SW- CON-QPX-SW	0.028

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