Identification of clam plasma proteins that bind its pathogen Quahog Parasite Unknown

Rachel Hartman, Emmanuelle Pales Espinosa and Bassem Allam *

School of Marine and Atmospheric Sciences, Stony Brook University, Stony Brook, NY 11794, USA

*Corresponding Author: Bassem.Allam@stonybrook.edu

Abstract

The hard clam (*Mercenaria mercenaria*) is among the most economically-important marine species along the east coast of the United States, representing the first marine resource in several Northeastern states. The species is rather resilient to infections and the only important disease of hard clams results from an infection caused by Quahog Parasite Unknown (QPX), a protistan parasite that can lead to significant mortality events in wild and aquacultured clam stocks. 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 the host. This study examined the interactions between the clam immune system and QPX cells. First, the effect of clam plasma on the binding of hemocytes to parasite cells was evaluated. Second, clam plasma proteins that bind QPX cells were identified through proteomic (LC-MS/MS) analyses. Finally, the effect of prior clam exposure to QPX on the abundance of QPX-reactive proteins in the plasma was evaluated. Results showed that plasma factors enhance the attachment of hemocytes to QPX. Among the proteins that specifically bind to QPX cells, several lectins were identified, as well as complement component proteins and proteolytic enzymes. Furthermore, results showed that some of these lectins and complement-related proteins are inducible as their abundance significantly increased following QPX challenge. These results shed light on plasma proteins involved in the recognition and binding of parasite cells and provide molecular targets for future investigations of factors involved in clam resistance to the disease, and ultimately for the selection of resistant clam stocks.

Keywords: *Mercenaria*, Plasma, QPX, Proteomics, LC-MS/MS

Introduction

Bivalves are economically and ecologically important in the United States, and in many countries around the world, with the bivalve industry recently valued worldwide at 16 billion US dollars (FAO, 2014). 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 Quahog Parasite Unknown (QPX) being the only pathogen known to significantly affect survivorship. QPX is a protist of the class Labyrinthulomycetes and the order Thraustochytriidae (Whyte et al., 1994). The Labyrinthulomycetes are ubiquitous and diverse, yet remain a poorly understood group of protists (Raghukumar, 2002). The presence of QPX disease in clams has been documented since the 1960s 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 in other locations along the east coast of the United States and Canada as far south as Virginia (Ragone Calvo et al., 1998; Maas et al., 1999). Much of the available information on the parasite involves the effect of clam genetic background and environmental parameters on disease development (Ford et al., 2002; Dahl et al., 2008; Dahl et al., 2010; Dahl and Allam 2015), while limited information exists on host-parasite interactions at the cellular and molecular levels.

Like other bivalve species and invertebrates in general, clam immune response to infections relies on the recognition of the invader via constitutive innate immune effectors, and the subsequent initiation of molecular cascades triggering secondary immune responses (Allam and Raftos, 2015). This initiation step can display some degree of specificity, though not to the extent of vertebrate memory-based immune systems (Mills et al., 2015). In many invertebrates such as arthropods, crustaceans, and mollusks, plasma proteins are involved in non-self recognition process and in the activation of the innate immune system (Dempsey et al., 1996; Garver et al., 2008; Allam and Raftos, 2015).

Previous studies have extensively shown the effects of plasma proteins on the host immune response to a pathogen, including higher immune-related transcript abundance (Genard et al., 2013) and increased abundance of immune-related plasma proteins in host strains (Monroy et al.,

1992; Charlet et al., 1996; Zhang et al., 2008). There is also evidence that exposure to a pathogen increases the immune response to a secondary exposure to the same pathogen (Portela et al., 2013; Pinaud et al., 2016). Recently, the plasma proteins from the snail *Biomphalaria glabrata* that bind to surface and secreted proteins from the parasite *Schistosoma mansoni* have been identified using proteomic methods, and comparisons have been made between the plasma proteins from susceptible and resistant snail strains (Wu et al., 2017). Concerning the interactions between the hard clam and QPX, previous studies showed that clam plasma factors decreased the growth of QPX cells (Anderson et al., 2003), and that QPX challenge resulted in increased clam plasma protein concentrations and an increase in anti-QPX plasma activity, particularly in resistant clam strains (Perrigault and Allam, 2009).

Proteomic methods have become widely used in recent studies due to improved techniques and lower costs. Liquid chromatography-mass spectrometry is a common proteomic method that is often considered the "gold standard" for protein identification and quantification. It involves rapid sequencing of genomic or transcriptomic data using 6 reading frames, which is then compared to a database to identity proteins (Charnot et al., 2017).

The current study aimed to better characterize the molecular immune response of the hard clam to QPX, and to probe the role of plasma proteins in this process. The effect of clam plasma on the binding of hemocytes to parasite cells was evaluated. Plasma proteins that bind parasite cells were then identified and their abundance compared between plasma from naïve clams and clams previously challenged with QPX to contrast baseline and inducible levels of QPX-reactive proteins. Results are discussed in light of current knowledge on the role of plasma proteins in facilitating the neutralization of invading microbes.

Materials and Methods

Clams

Naïve adult clams (70-80 mm in length) were obtained from a commercial source (Frank M. Flower and Sons, Oyster Bay, NY) and were maintained in tanks containing ultraviolet-treated filtered seawater 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, Sustainable Aquatics, Jefferson City, TN) and water in the tanks was continuously aerated and filtered. Following an initial 1-week acclimation period, temperature in the holding tanks was gradually increased to reach 18°C and clams were held under these conditions for 3 days before submitted to the various treatments prior to hemolymph collection.

QPX culture and cell preparation

Two series of QPX (isolate NY0313808BC7) cultures were grown, with one series used for clam challenge experiments and the second series for the isolation of QPX-reactive clam plasma proteins. QPX used for challenge experiments was grown at 21ºC in minimal essential medium supplemented with 10% fetal bovine serum according to Kleinschuster et al. (1998). Exponentially growing cultures were centrifuged and parasite cells resuspended in filtered artificial seawater (FASW, 28 ppt, 0.2 µm filter pore size) at a final concentration of 10^6 cells/mL and used to inject and "prime" naïve clams (see below). Parasite cells for plasma proteins isolation 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 mucosal proteins could interfere with downstream analyses). Cultures (350 mL/replicate; 6 replicate cultures) were incubated for 5 days to reach high cell densities (~10^6 cells/mL) and parasite cells were counted with a hemocytometer. The cultures were centrifuged, pooled, and resuspended in FASW. The cells were then fixed overnight with glutaraldehyde (0.2% final concentration). Cell size of cultured QPX under our experimental conditions typically ranges from 2-40 µm with an average around 25-30 µm. 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).

Effect of clam plasma on QPX-hemocyte interactions

Hemolymph for hemocyte recovery (~150 µl per clam, n = 10 clams) was withdrawn from the adductor muscle (Perrigault and Allam, 2009) directly into an ice-cold 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), which prevented the immediate clumping of hemocytes. The

hemocytes were obtained by centrifugation (300 g, 4 °C, 10 min). Hemolymph for plasma recovery (~350 µl per clam) 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 ice-cold 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 attachment events was determined microscopically after 1 and 3 hours of incubation by counting about 100 QPX cells from each preparation. The percentage of QPX cells with attached hemocytes, determined as parasite cells having hemocytes attached to a minimum of 25% of their cell surface, was contrasted between preparations containing plasma and controls added with FASW. Data were arcsin-transformed and submitted to paired *t*-test comparisons at the standard $p < 0.05$ cutoff.

Challenge Experiment

The objective of this experiment was to determine whether or not QPX-reactive plasma proteins are inducible in response to prior QPX challenge. Clams were challenged by injecting 10^5 QPX cells (in 100 µL FASW) into the heart area according to Dahl and Allam (2007). Control clams received 100 µL FASW (28 ppt) to account for the general response to stress as opposed to the pathogen-specific immune response expected from the experimental group. Once injected, clams were kept out of the water for 1 h before being transferred back to separate tanks (3 "control" and 3 "challenged" tanks containing 10 clams each). The clams were incubated in their tanks for 2 days post-injection to allow the clam proteome to respond to QPX challenge. Since prior studies have shown that, while transcriptional responses can occur rapidly, changes in protein expression following a stimulus can take 2-3 days to be observed in invertebrates (Schoville et al., 2012). After incubation, clams were processed for the isolation of QPX-reactive plasma proteins as described below.

Isolation of proteins bound to QPX

Hemolymph was withdrawn from the adductor muscle of control and challenged clams (Perrigault and Allam, 2009), pooled (10 clams/pool; 3 pools/condition), 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., 2003). Fixed QPX cells were then added to the filtered plasma (~10^6 cells/mL), and incubated for 2 hours at 4ºC (with light shaking) to allow plasma proteins to bind to QPX cells. Following incubation, parasite cells were collected by centrifugation (400 g, 30 minutes, 5ºC), and the supernatant discarded (an aliquot was kept for protein measurement); this contained proteins that did not bind to QPX cells. The cell pellet was washed twice with FASW (9 ppt), to remove weakly-bound/adsorbed proteins, before resuspension in an elution buffer (10 mM EDTA and 1 M NaCl), to release bound proteins. The salinity was lowered for the washes to 9 ppt because a higher salinity may cause the removal of bound proteins before the washing steps were complete. Eluted proteins were then retrieved by centrifugation (400 g, 30 minutes, 5ºC), and the pelleted QPX cells discarded. 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 record 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 cat no. PM030UM). These beads are neutral with no charge, and hydrophobic. The protein concentrations in the eluates were measured using the Bradford Protein Assay (BioRad 5000002) following manufacturer's recommendations and samples were kept at -80 °C until submitted to LC-MS/MS analysis. It should be noted that the isolation of proteins that bind to beads was made for the QPX-challenged group vs. QPX. Plasma samples from control clams incubated with beads were not analyzed because of the low levels of bound plasma protein combined with the high cost of running LC-MS/MS samples.

Proteomics analyses

Samples were analyzed using LC-MS/MS (Liquid Chromatography-Tandem Mass Spectrometry) following the general methods described by Pales Espinosa et al. (2016). Proteins were precipitated by deoxycholate-TCA (Peterson, 1977) and the resultant pellet was dissolved in 20 µl 8 M urea, 25 mM NH_4HCO_3. The protein solution was then subjected to trypsin digestion as follows: reduced in 4 mM DTT (30 min, room temperature), alkylated in 8.4 mM iodoacetamide (30 minutes, room temperature in dark), the urea concentration was reduced to 1.7

196 M and the solution incubated 16 h at 37 ºC in the presence of trypsin (Promega, Gold, Mass
197 Spectrometry Grade, cat# V5280) at >1 µg/40 µg protein. After incubation, the digest was
198 brought to 2 % formic acid (FA) and desalted with Supel-Tips C18 Micropipette Tips (Sigma-
199 Aldrich) using FA containing solutions with varied acetonitrile (ACN) essentially as described in
200 vendor's bulletin. The solvent was removed from the eluted peptides using a vacuum centrifuge
201 and the resultant dried peptides stored at -80 °C. The eluted peptides were dissolved in 2% ACN,
202 0.1% FA (buffer A) for analysis by automated microcapillary LC-MS/MS. Fused-silica
203 capillaries (100 µm inner diameter - i.d.) were pulled using a P-2000 CO_2 laser puller (Sutter
204 Instruments, Novato, CA) to a 5 µm i.d. tip and packed with 10 cm of 5 µm ProntoSil 120-5-
205 C18H (Bischoff Chromatography, Leonberg, Germany) using a pressure bomb. The samples
206 were loaded via a Dionex WPS-3000 autosampler, part of a Dionex Ultimate 3000 system
207 (Germering, Germany). The column was installed in-line with a Dionex LPG-3000
208 Chromatography HPLC pump running at 300 nL min^{-1}. The peptides were eluted from the
209 column by applying a 5-min linear gradient from 0% buffer B (98% ACN, 0.1% FA) to 10%
210 buffer B, followed by a 120 min linear gradient from 10% buffer B to 45% buffer B. The
211 gradient was switched from 45% to 80% buffer B over 10 min. Finally, the gradient was changed
212 from 80 % buffer B to 0 % buffer B over 10 min, and then held constant at 0 % buffer B for 20
213 more minutes. The application of a 2.2 kV distal voltage electrosprayed the eluting peptides
214 directly into an LTQ Orbitrap XL ion trap mass spectrometer (Thermo Fisher, San Jose, CA)
215 equipped with a nano-liquid chromatography electrospray ionization source. Full mass spectra
216 (MS) were recorded on the peptides over a 400 to 2000 *m/z* range at 60,000 resolution, followed
217 by top-five MS/MS scans in the ion-trap. Charge state dependent screening was turned on, and
218 peptides with a charge state of +2 or higher were analyzed. Mass spectrometer scan functions and
219 HPLC solvent gradients were controlled by the Xcalibur data system (Thermo Fisher, San Jose,
220 CA). MS/MS spectra were extracted from the RAW file with ReAdW.exe
221 (http://sourceforge.net/projects/sashimi). The resulting mzXML data files were searched with
222 GPM X!Tandem against a combined *M. mercenaria* (Wang et al., 2016b) and QPX (Rubin et al.,
223 2015) proteome database. Proteins identified as deriving from fixed QPX cells were removed
224 from downstream statistical analysis. Protein expression levels were quantified using normalized
225 spectral counts, with a cutoff of one peptide and two spectral counts. The data were normalized
226 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) and Significance Analysis of Microarray (SAM) analyses were used to identify proteins differentially abundant in samples from the different treatments following the approach described by Roxas and Li (2008).

Results

Effect of clam plasma on QPX-hemocyte interactions

A significant increase in the attachment of hemocytes to QPX cells was measured in samples containing plasma after 3 hours of incubation ($p = 0.03$, paired *t*-test, $n = 10$; Figure 1).

Measurement of proteins that attach to QPX cells

Total protein concentrations for each treatment and at each washing step are shown in Figure 2. For control clams (injected with seawater), protein concentration in spent plasma from the binding assay following incubation with fixed QPX cells was 576 µg/ml. Protein concentration in washing solutions decreased to 85 µg and 34 µg/ml following the first and the second wash, respectively. Protein concentration in the eluates increased to 150 µg/ml indicating the efficiency of the elution step in releasing bound proteins. Protein concentration using plasma from challenged clams incubated with fixed QPX cells followed the same trend and decreased from 548 µg/ml in spent plasma to undetectable levels following the second wash (below the detection limit estimated by the manufacturer at 1.25µg/ml) followed by release of bound proteins during the elution step (115 µg/ml). Similarly, protein concentration using plasma from challenged clams incubated with beads also showed a decrease during the washing steps, but in this case no increase was noted 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 were bound to the beads.

Identification of proteins that attach to QPX cells

The LC-MS/MS proteomic analysis yielded 361 clam proteins and 333 QPX proteins (see Supplemental Table for full annotated list) from the 9 samples (3 plasma samples from control and 3 plasma samples from challenged clams incubated with QPX, and 3 plasma samples from

challenged clams incubated with beads). In general, more proteins were identified in samples derived from challenged and control groups incubated with fixed QPX cells than from the group incubated with beads. Proteins identified in all or nearly all of the samples included ribosomal proteins, proteases, heat shock proteins, and cytoskeletal proteins. QPX proteins identified included actin, tubulin, and ribosomal proteins; these were not considered in the SAM analyses.

Injection of live QPX cells into clam circulatory system 2 days prior to plasma collection caused a significant increase in nine plasma proteins that bind fixed QPX cells (Figure 3 and Table 1). These included key immune proteins such as pattern recognition receptors (the complement related proteins c1q and factor H-like, the lectin echinoidin, von Willebrand factor-related proteins and an immunoglobulin-domain containing HSPC-like protein), as well as a protease and a protease inhibitor. In contrast, none was significantly more abundant in the control group as compared to challenged clams.

Among challenged clams, seventeen proteins were identified to bind more significantly to fixed QPX cells than to beads (Figure 4 and Table 1). These included several proteins shown above to be overexpressed following QPX challenge such as c1q and complement factor H-like, the lectin echinoidin, the von Willebrand factor-related protein, as well as the protease 10 and an undescribed protein. Additional proteins that bound QPX cells significantly more than beads but that were not induced following challenge include the complement factors H and C3, a lactose-binding protein and several enzymes.

Discussion

QPX disease has been a problem for the hard clam industry since its discovery in the 1960s. 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 most prominent clam response to the disease is an infiltration of infected tissues by hemocytes and encapsulation of parasite cells (Dahl et al., 2008, 2010). The current study shows that plasma factors enhance hemocyte interactions with the parasite, in agreement with earlier studies showing the central role of plasma proteins in facilitating encapsulation of non-self entities in other invertebrate

species (Yu and Kanost, 2004; Wang et al., 2013b; Jin et al., 2013). Results also allowed the identification of several pattern recognition receptors (PRR) that specifically bind QPX cells and that are overrepresented following clam challenge with the parasite. In fact, most of the proteins that specifically bind QPX or that are overrepresented following challenge have well-established immunological functions including recognition of non-self entities, induction of innate immune responses, and membrane association.

A complement 1q (c1q) domain-containing protein, a complement factor H-like protein, and a 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 complement component system (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 complement component system of the hard clam could recognize and respond to the presence of PAMPs present on QPX cells, as recently suggested for the Pacific oyster in response to LPS exposure (Wang et al., 2017). Specifically, the complement component C3, which plays an essential role in the activation of the complement system and functions as an opsonin, has been detected in oyster plasma and was shown to be overexpressed following injection with LPS (Wang et al., 2017). Studies in bivalves have shown upregulation of other complement related factors, including c1q domain-containing transcripts, following challenge with pathogens suggesting these proteins play an important role in pathogen recognition (Liu et al., 2014; Jiang et al. 2015; Wang et al., 2017), though the molecular interactions between different members of the complement cascade are not entirely understood.

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 (Sanggaard et al.,

2016) and have been shown to be overexpressed in response to heavy pathogen infections in shrimps (Goncalves et al., 2014). von Willebrand factors have been described as serine protease domains involved in the activation of the complement component system in the oyster *C. gigas* (Wang et al., 2017), indicating a possible similar function in *M. mercenaria*. In mammals, von Willebrand proteins act as co-factors for the cleavage of complement proteins and its enrichment in QPX may be the result of its co-location with some of these proteins (e.g. C3). 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 was likely enriched in this case due to recognition of 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). 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., 2013a; 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). This protein contains a CUB domain, which is found in the complement C1 system, indicating a possible complement cascade-activation function. An astacin-like metalloproteinase was found to be highly expressed in the hemocytes of the pearl oyster (*Pinctada fucata*) and was suggested to play a role in wound

healing and cell proliferation (Xiong et al., 2006). The blastula protease 10-like isoform detected here was also found to contain a PAN domain, 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.

A PAN domain-containing uncharacterized protein was also identified as being more abundantly bound to QPX than beads, and more abundantly in the challenged group than the control group bound to QPX. As discussed above, PAN domains mediate protein interactions with other proteins or carbohydrates and therefore may have functioned in pathogen recognition in this case. Huang et al. (2015) found PAN domains in fibrinogen related proteins (FREPs) in oysters, and FREPs are prominent PRRs in mollusks (Portet et al., 2017).

A basement membrane-specific heparan sulfate proteoglycan core protein was identified as more abundant in the challenged group. Heparan sulfates have a wide range of functions in invertebrates such as cellular adhesion and anti-clotting (Gomes et al., 2009). 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 heparan sulfate proteoglycan protein. Ig domain-containing proteins are very diverse in invertebrates and often function as PRRs, sometimes initiating the complement system (Buchmann, 2014; Wang et al., 2016a). It is therefore likely that the overrepresentation of this protein in the challenged group represents an indication of the heightened immune response following exposure to QPX.

An inter-alpha-trypsin inhibitor heavy chain H3-like protein was also found in higher abundance bound to QPX in the challenged group than controls. 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). 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 in this study, while not used in statistical analyses, were shown to include several proteases, suggesting that the clam protease inhibitor identified here could possibly be involved in the inhibition some of QPX membrane- or mucus- bound proteases.

Finally, a few clam enzymes were also shown to be overrepresented among plasma proteins bound to QPX as compared to beads. These included a catalase, an aldehyde dehydrogenase, a malate dehydrogenase and a nidogen-like protein with a phospholipase A2 domain. These proteins are not know to function as PRRs although their ability to bind QPX may reflect the presence of their specific substrates in parasite cells.

Conclusions

Our results demonstrated that exposure of QPX to plasma enhance the attachment of hemocytes to parasite cells. Several plasma proteins, including many PRRs, were shown to bind to QPX cells more efficiently than to beads. These proteins included complement proteins, lectins and enzymes, many of which are known to be associated with the activation and functioning of the complement component system. Further research using different microbes (pathogens and commensals) as affinity matrices is needed to determine if some of the proteins identified here represent a specific response to QPX or not. In parallel, our results showed an increase in the abundance of QPX-reactive proteins following challenge with the parasite. These findings suggest that pre-exposure to QPX could (1) increase the general immune response, (2) enhance the specific expression of QPX- reactive proteins, or (3) increase the affinity of pre-existing QPX-reactive proteins. Unraveling these non-mutually exclusive scenarios requires targeted additional investigations. Given the devastating impact of QPX disease on clam stocks, understanding the effects of prior QPX exposure on the clam immune system may provide innovative means to mitigate QPX disease.

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Table 1. Differentially abundant proteins identified in the different treatments. Symbols in the "Significance" column indicate statistically different levels of proteins recovered from fixed QPX cells for plasma originating from challenged or control clams (*), and of proteins recovered from fixed QPX cells or beads for plasma originating from challenged clams (#).

Accession	Protein Description	Domain Description	Significance
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	*

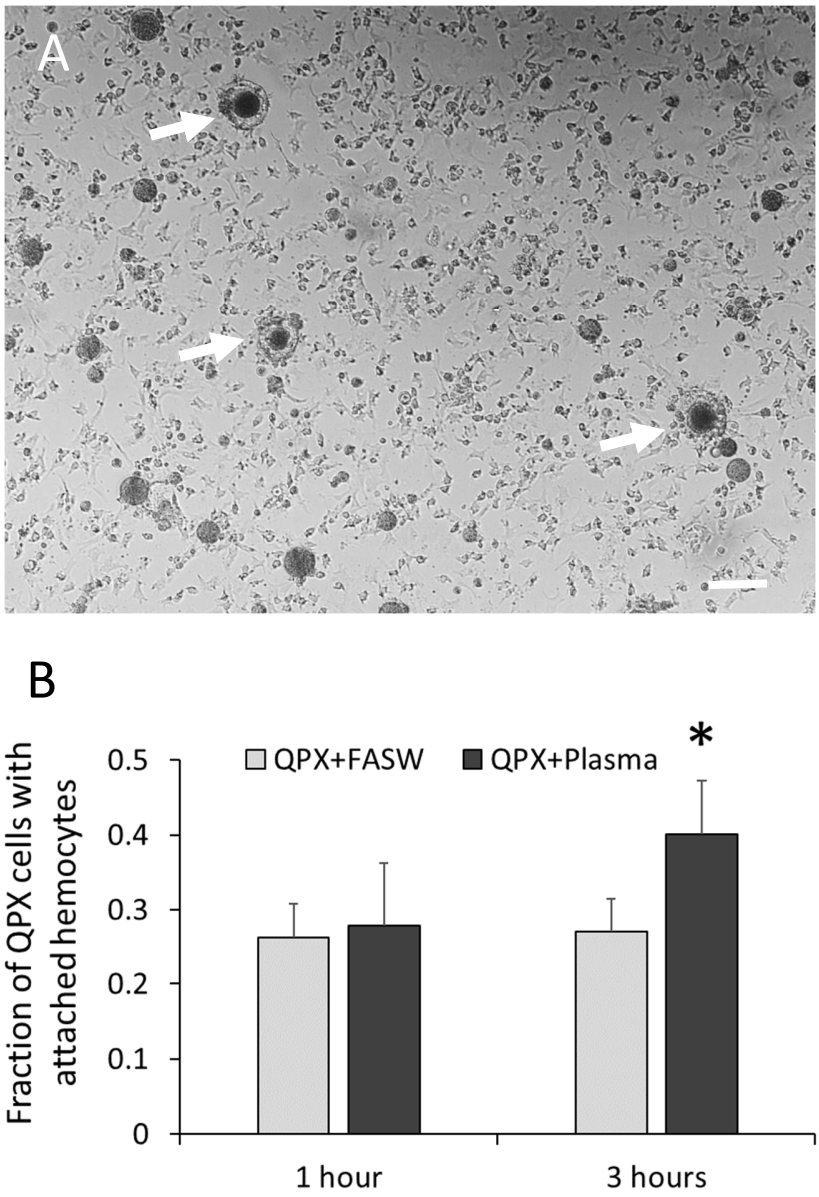

Figure 1. Attachment of clam hemocytes to fixed QPX cells. A. Micrographs showing attachment events (denoted by arrows, scale bar = 50 µm). B: Effect of plasma on the attachment frequency (mean ± standard deviation). *: significantly higher than FASW controls ($p = 0.03$, paired *t*-test, n = 10 clams/data point).

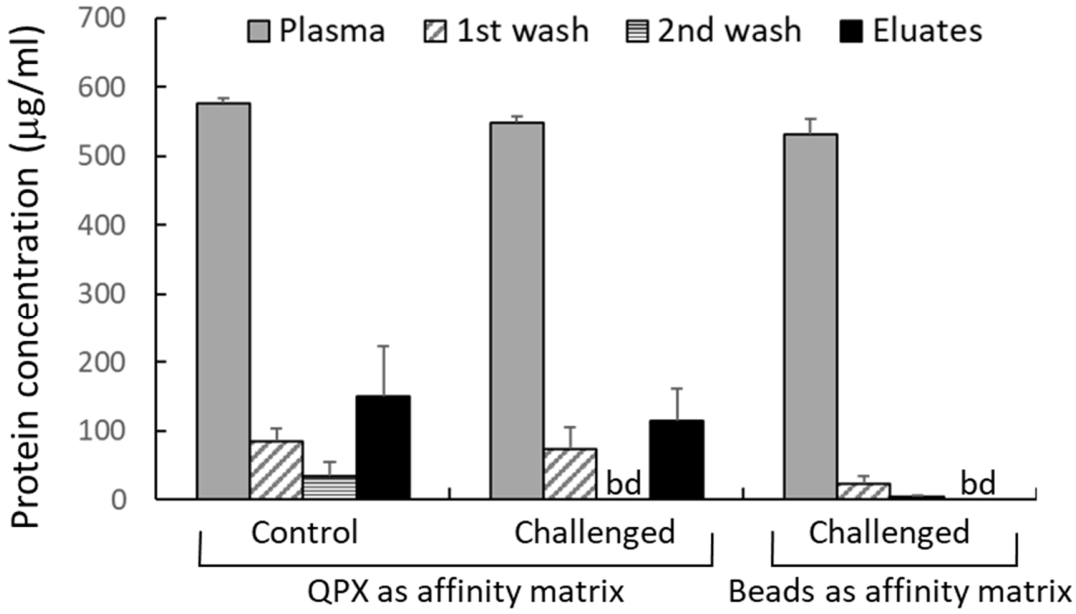

Figure 2. Protein concentrations measured during each stage of the plasma protein binding assay. Plasma from challenged (injected with QPX) or control (injected with seawater) clams was incubated with fixed QPX cells or beads before assessment of protein concentrations in the washing solutions or in the final eluates. Mean ± standard deviation, n = 3 pools/data point. bd: below detection limit.

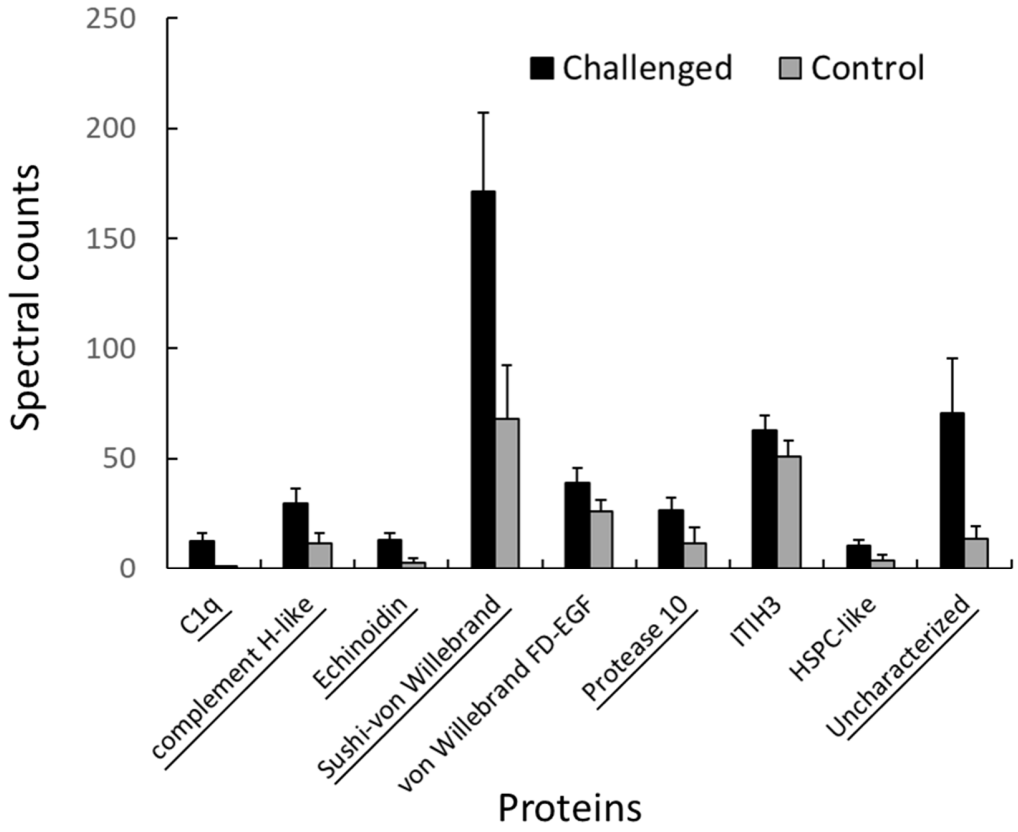

Figure 3. Proteins (mean spectral counts ± standard deviation, n = 3 pools/data point) that were differentially represented in eluates recovered from fixed QPX cells for plasma originating from challenged and control clams. Underlined proteins are those also shown to significantly bind more to fixed QPX cells as compared to beads (see Figure 4). Full protein and domain descriptions are given in Table 1.

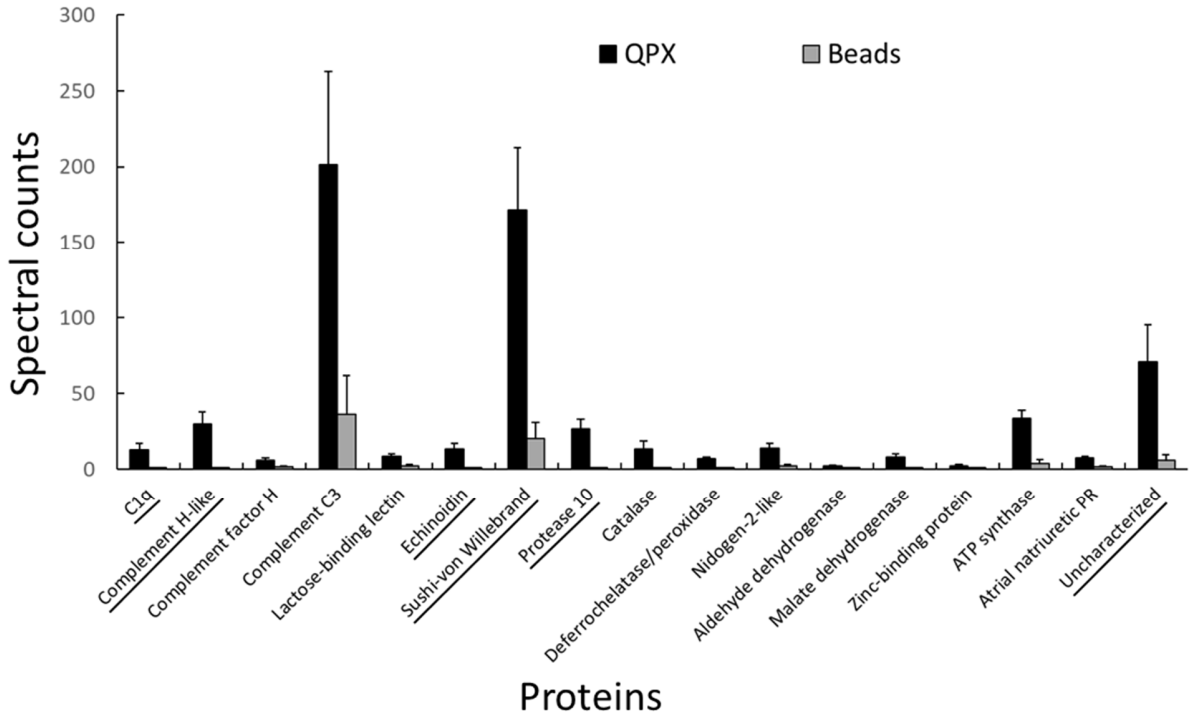

Figure 4. Proteins (mean spectral counts ± standard deviation, n = 3 pools/data point) shown to differentially bind fixed QPX cells and beads. Full protein and domain descriptions are given in Table 1. Underlined proteins are those also shown to be significantly induced following QPX challenge (see Figure 3). Full protein and domain descriptions are given in Table 1.

Plasma proteins from the hard clam (*Mercenaria mercenaria*) adhere to the cell membrane of the pathogen Quahog Parasite Unknown