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Molecular characterization of clam (*Mercenaria mercenaria*) immune responses against Quahog Parasite Unknown (QPX): Effect of host and environmental factors

A Dissertation Presented

by

Kailai Wang

to

The Graduate School

in Partial Fulfillment of the

Requirements

for the Degree of

Doctor of Philosophy

in

Marine Sciences

(Concentration – Marine biology)

Stony Brook University

May 2016

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Abstract of the Dissertation

Molecular characterization of clam (*Mercenaria mercenaria*) immune responses against Quahog Parasite Unknown (QPX): Effect of host and environmental factors

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2016

Quahog Parasite Unknown (QPX) is a fatal protistan parasite that infects wild and cultured hard clams (*Mercenaria mercenaria*) along the northeastern coast of the US. The severity of clam mortality events and resulting economical losses caused by QPX emphasize the urgent need for the development of effective disease control measures, which require a good understanding of disease pathobiology and host resistance mechanisms. This dissertation explored clam immunity and host and environmental factors that affect *M. mercenaria* resistance against QPX infection. The investigation of molecular immune mechanisms using multiple high-throughput transcriptomic methods revealed an extraordinary complexity of *M. mercenaria*'s defense system, featured by highly diversified immune recognition receptors and pathways involved in immune cell activation, signal transduction, wound repair and apoptosis. Results also showed that QPX infection induces a tailored immune response in clams with focal up regulation of variable pattern recognition receptors (e.g. lectin, fibrinogen-related proteins, complement-1q-domain containing proteins) and signaling networks involved in the activation of focal adhesion, inflammation, apoptosis and extracellular killing, in contrast to a systemic response characterized by an overall immune suppression and up regulation of stress proteins. Comparative analysis between resistant and susceptible clam strains highlighted a transcriptome-wide deficiency in QPX-responsive immune factors in susceptible clams, suggesting a primary role of host genetic make-up in QPX resistance. Furthermore, results showed that temperature influences disease development mainly through the alteration of the expression of immune genes. Low temperatures resulted in the suppression of the clam immunome thus facilitating the

establishment of QPX infection, while higher temperatures supported much higher levels of immune gene expression, which were correlated with host resistance. Finally, given the importance of temperature in regulating disease development, the study evaluated the role of heat shock treatments on the progress of pre-established infections. Results showed a reduction of QPX disease in clams submitted to temperature shocks. Best mitigation results were found in conditions with short exposures to moderately elevated (27ºC for 2 hours) temperatures. The heat shock treatment has great potential for field-application in hard clam fisheries. Overall, the findings of this dissertation represent a valuable addition to the currently limited molecular data on *M. mercenaria*, and contribute to a better understanding of the mechanisms of clam resistance to QPX. The results provide solid guidelines and preliminary data that facilitate future research targeting clam selective breeding and the development of disease mitigation strategies.

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Acknowledgments

This dissertation would not have been possible without the help and support from generous people around me and because of whom my graduate experience has been one I will cherish forever. Although this dissertation bears my name, many people have contributed to it.

First and foremost, I would like to express my deepest gratitude to my advisor, Professor Bassem Allam, for his excellent guidance, caring, patience, and invaluable insights throughout all the stages of my research and writing of this dissertation. He provided unreserved support in every aspect of my Ph.D. research, patiently corrected my writing and generously paved the way for my development as a researcher.

I would also like to thank my committee members, Dr. Anne McElroy, Dr. Jackie Collier, Dr. Susan Ford and Dr. Arnaud Tanguy, who have given me generous advice and help in various ways. I would like to extend my appreciation to Dr. Emmanuelle Pales Espinosa, who has provided me good advice and taught me patiently new experimental techniques.

I am fortunate enough to have been able to work in this loving environment of SoMAS and the Marine Animal Disease Lab. My research projects would not have been accomplished without the help and support from all my lab colleagues: Ewelina Rubin, Joyce Lau, Chris Del Castillo, Melissa Ouvrard, Mickael Perrigault, Soren Dahl and Sue Pawagi.

I am grateful to all my wonderful friends Jin Gao, Yuan Liu, Zhenrui Cao, Xia Hua, Tiantian Tang, for their care and encouragements, which helped me adjust to my new life in this new place quickly and happily. I value their friendship deeply and greatly.

My special thanks to New York Sea Grant and CSC foundation, who have provided generous scholarships and a thesis completion award that allowed me to overcome financial difficulties and finish my Ph.D. work.

Last, but not least, my deepest gratefulness goes to my beloved family, my dearest parents and my loving husband, who have gave me unconditional love and support all the time, despite the long distance.

Chapter 1 Introduction

1.1. The hard clam

The hard clam *Mercernaria mercenaria* is an ecologically- and economically- important marine bivalve species that is widely exploited along the northeastern coast of the United States. *M. mercenaria* is also known as quahog, but other commercial names also exist based on shell size, such as littlenecks (40-50 mm), topnecks (50-70 mm), cherrystones (70-80 mm), or chowder clams (>90 mm) (Ritchie, 1977). Hard clams live in soft sandy bottoms of shallow coastal bays from the Gulf of St. Lawrence, Canada, to the Atlantic coast of Florida, and support a robust wild capture fishery from Cape Cod to New Jersey.

As suspension-feeders, hard clams play a vital role in coastal ecosystems. They participate actively in the benthic-pelagic coupling, supplying the benthic food webs and contributing to nutrient cycling by directing particulate organic matter toward the benthos (Dame and Gardner, 1993). By their filtering activity, hard clams can control phytoplankton populations by removing suspended particles, thus reducing turbidity and improving water quality (Newell, 2004). This in turn enhances the growth of sea grass beds and benthic vegetation, which serve as habitat for diverse groups of fish and invertebrates (Newell and Koch, 2004; Phelps, 1994).

Aside from its ecological value, the hard clam is one of the most important commercial marine species supporting a robust fishing industry across the Atlantic coastal states, particularly in New York State where it occupies the highest rank for dockside value of marine products. In the past century, New York State was once the largest producer of hard clams in the U.S., and more than half of the nation's hard clams were from Great South Bay. In 2014, US hard clam fisheries produced 7.9 million pounds landings valued at US$48.9 million, of which the harvest from New York State alone accounted for 25% of the nation's total production. However, in the past decades, several large-scale clam mortality episodes were reported in association with a protistan parasite called QPX (Quahog Parasite Unknown). These disease outbreaks have severely impacted the NY hard clam fishery, causing tremendous losses and inflicting important restrictions on the Shellfish Transplant Program since 2002. The Shellfish Transplant Program allowed harvesting hard clams from uncertified waters in Raritan Bay (off Staten Island) before transfer and depuration in certified waters of the Peconic Bay for cleansing bacteria to meet the food safety standards before entering the market, which used to contribute about 50% of New York State's annual hard clam production. The program was suspended in 2002 following the detection of QPX in Raritan Bay in order to prevent the introduction of the parasite into the Peconic Bay, before it was re-opened in 2005 but with restrictions on fishing sites and methods of clam deployment.

1.2. QPX disease in hard clams

Since its first reported outbreak in the late 1950s in New Brunswick, Canada (Drinnan and Henderson, 1963), QPX was linked to mass mortality in aquacultured and wild clam populations at various locations along the eastern coast of North America (Calvo et al., 1998; Dove et al., 2004a; Ford et al., 2002; MacCallum and McGladdery, 2000; Smolowitz et al., 1998).

Morphological and molecular evidence classified QPX as a member of the Thraustochytrid family under the phylum Labyrinthulomycota (Collado-Mercado et al., 2010; Maas et al., 1999; Qian et al., 2007b; Ragan et al., 2000; Stewart and Creese, 2002; Stokes et al., 2002; Whyte et al., 1994). Thraustochytrids are a group of single-celled, fungal-like, marine protists associated with decaying vegetation, shells, and detritus (Raghukumar, 2002). Some nonpathogenic thraustochytrids can survive in marine aggregates, and QPX has also been detected in marine snow in areas subjected to disease outbreaks (Lyons et al., 2005). This provides evidence of the ability of QPX to survive and spread between epizootics and strongly suggests that QPX is an opportunistic pathogen.

QPX has three life stages: thallus (2-10 µm in diameter), sporangium (10-15 µm in diameter) and mature sporangium (18-25 µm in diameter). All three basic stages can be seen in tissue lesions in infected clams as well as QPX cultures established *in vitro* using minimal essential media (MEM). The thallus stage is represented by single-nucleate cells that can grow and develop into the sporangium stage. The sporangium stage, which lacks a well-defined membrane-bound nucleus, undergoes an endosporulation process resulting in the development of mature sporangia containing 20 to 40 endospores (immature thalli) enclosed by basophilic cell walls (Dove et al., 2004a; Smolowitz et al., 1998). The parasite produces abundant mucoid secretions both *in vitro* and *in vivo* as evidenced by histopathological examinations of infected hard clams (Smolowitz et al., 1998). Histologically, the mucoid secretions are indicated by clear, cell-free areas surrounding the parasite, which is likely the result of mucoid materials being removed during histological processing (Smolowitz et al., 1998). Alcian Blue/Periodic Acid Schiff stain has confirmed that the mucoid secretions of QPX are comprised of mucopolysaccharides that form a mucofilamentous net structure (Calvo et al., 1998). It has been suggested that the mucoid secretions of QPX are for protection purposes and can be related to the virulence of QPX (Anderson et al., 2003b).

QPX lesions seem to initiate in clam mantle, gill, and siphon tissues, indicating a direct acquisition of the parasite from seawater during suspension-feeding (Smolowitz et al., 1998). Typical symptoms of QPX disease include massive inflammation in infected tissues. This may result in the swelling of impacted organs, such as the gills and mantles, altering clams ability to close their shells and to extract food and oxygen through gill pumping (Dove et al., 2004a; Smolowitz et al., 1998). Inflammatory lesions are usually multifocal and often granulomatous. Clam hemocytes are usually unable to phagocytize QPX cells because parasite cells are comparatively larger in size and surrounded by a thick layer of protective mucus. During infections, the mucoid substance is also thought to protect QPX by isolating the cells from recognition by clam antimicrobial proteins and preventing phagocytosis by clam hemocytes (Anderson et al., 2003b). Nevertheless, clam hemocytes infiltrate the infection site and usually surround parasite cells, possibly releasing reactive oxygen species and other toxic agents leading to local tissue destruction and formation of granulomas (Smolowitz et al., 1998; Anderson *et al.,* 2003). This massive host response may have two fates, either successfully leading to the degradation of parasite cells and clam recovery, or failing to sufficiently control parasite

proliferation. In the later scenario, proliferating parasites can cause irreversible alterations leading to clam death (Anderson et al., 2003).

1.3. Variability of hard clam susceptibility to QPX

Significant differences in susceptibility to QPX infection have been observed in hard clam broodstocks with different genetic and geographic origins. In general, clam broodstocks from southern states (e.g. South Carolina and Florida) were shown to be more susceptible to the infection as compared to northern broodstocks (New Jersey, New York and Massachusetts), likely as a result of lacking pathogen exposure in their environment since QPX has never been detected in warm coastal waters further south than Virginia, whereas severe QPX disease outbreaks were frequently reported throughout the northeast (particularly between NJ and MA) and higher selective pressure may have been exerted by QPX on clam populations in northern locations (Calvo et al., 2007; Calvo et al., 1998; Ford et al., 2002).

The field study by Ford et al. (2002) was the first to show the relationship between clam genotype and QPX susceptibility. They reported significant mortality due to QPX infection in southern clam seeds (SC) but not in local NJ clams planted in New Jersey. The authors concluded that clam susceptibility to the infection might result from an "unfavorable genotype-environment interaction" (Ford et al., 2002). This observation was also supported by subsequent field studies performed throughout the northeast (Calvo et al., 2007; Dahl et al., 2010; Kraeuter et al., 2011). For example, the study by Calvo et al. (2007) compared the growth, mortality and QPX prevalence of 5 hard clam strains (originating from FL, SC, VA, NJ, and MA) deployed at 2 sites in Virginia and New Jersey for 2.5 years. The southern strains (FL and SC) exhibited significantly higher QPX prevalence and mortality than northern strains (NJ and MA) at both sites. The mortality, interestingly, correlated well with QPX prevalence. In the field study of Dahl et al. (2010), FL and NY aquaculture clam strains and a NY wild type strain were deployed in NY water during summertime and disease development was evaluated during the subsequent 16 months. Throughout the experiment, wild type NY clams did not show detectable signs of QPX infection, whereas the FL aquaculture strain acquired QPX infection almost immediately after deployment (within 7 weeks) and the NY aquaculture strain started showing QPX infection only the following summer. Both NY strains showed higher survivorship than FL strains and the results provided evidence for the potential utilization of local clam broodstocks to mitigate the disease. Similarly, Kraeuter et al. (2011) investigated the performance of clams originating from MA, NJ and SC that were deployed in Massachusetts and New Jersey. Their results resembled the observation by Calvo et al. (2007) with the highest QPX disease development and lowest survival in SC seed at both sites. These combined data suggest that host genetic variation due to geographic origins represents a determinant of clam susceptibility to QPX disease, with strains of southern origin being significantly more susceptible than those of northern origin. However, the natures of these postulated genetic variables were unknown.

Questions have been raised however whether higher susceptibility observed in southern seeds could be the result of poor adaptation to colder water temperatures in northern deployment sites (Calvo et al., 2007; Ford et al., 2002). In this scenario, deployment of southern clams in

northern waters would submit clams to stressful cold conditions thus compromising their defense mechanism against the parasite. However this scenario seems to be, at least partially, discredited by field observations of Dahl et al. (2010) showing that the southern strain they used (FL) readily acquired QPX during summer after the clams had only been exposed to the warmest temperatures for that field site. Additionally, laboratory transmission studies by Dahl et al. (2008) also suggest that the greater susceptibility of southern clam strains toward QPX disease is not derived from their poor acclimation, but could be rather linked to genetically-mediated differences in clam resistance to QPX.

1.4. Effects of environment factors on QPX disease

Among all the contributing factors to QPX disease, environment plays a predominant role that cannot be neglected. As a matter of fact, the outbreak of an infectious disease is actually the result of interplay of three sets of factors: host-related factors, pathogen factors, and environmental factors that could favor either the host or the pathogen. Disease generally occurs when susceptible hosts are exposed to virulent pathogens under environmental conditions that are either stressful to the host, or beneficial to the pathogen, or a combination of both.

Being an opportunistic pathogen, QPX is widely distributed in enzootic areas. It has been detected in a wide variety of substrates including seawater, sediments, macroalgae, marine aggregates, sea grasses and many invertebrate species. Its presence in the environment also shows seasonal patterns with high frequency of positive seawater and algae samples during spring and high frequency of positive invertebrate samples during fall (Gast et al., 2008; Lyons et al., 2005). However, QPX was rarely found in areas with low salinity and its distribution appears to be restricted to euhaline and mesohaline waters. *In vitro* investigations showed that QPX tolerates salinities as low as 15 psu where it barely survives without growth (Perrigault et al., 2010). Brothers et al. (2000) also reported extremely low growth rate of *in vitro* QPX cultures maintained at 20 psu. Similarly, temperature has been shown to significantly regulate QPX growth and survival with optimum *in vitro* growth between 20 to 23 °C and parasite mortality above 32 °C (Brothers et al., 2000; Perrigault et al., 2010). In fact, these conditions for QPX *in vitro* growth correspond well with temperature and salinity ranges observed in enzootic areas. Additionally, different QPX isolates showed best performance at temperature and salinity combinations similar to their geographical origins, suggesting specific adaptations developed by the organism to different locations (Perrigault et al., 2010). Therefore, environmental factors such as temperature and salinity appear to directly affect the distribution of the parasite, subsequently determining disease risk in different areas.

On the other hand, the effectiveness of the host defense system against QPX infection is also highly regulated by environmental factors. Previous laboratory challenge experiments demonstrated that cold temperature is more suitable for disease development (Dahl et al., 2011), with disease prevalence, intensity and disease-related mortality significantly higher in clams maintained at 13 °C as compared to those held at 21 or 27 °C. Similar trends of disease development at low temperature were also found in naturally infected clams maintained under laboratory conditions. Interestingly, this exacerbation of QPX disease at low temperature is

consistent with the reduced overall immune competency and low anti-QPX activity found in these "cold stressed" clams, as reported by Perrigault et al. (2011). This suggests that the reduction of host immunity due to stressful environment may provide a window for the parasite to establish itself. In the same study, highest levels of anti-QPX activity were observed in plasma from clams held at 21 °C which corresponds to the optimal temperature for hard clams. Anti-QPX activity in plasma was lower in clams maintained at 27 °C as compared to those held at 21 °C, however disease failed to develop and signs of healing were observed in previously infected clams held at 27 °C suggesting that this temperature was detrimental to the parasite (Perrigault et al., 2011). In contrast, low anti-QPX activity in plasma and high disease development were observed in clams held at 13 °C suggesting that this temperature is more stressful to the host than to the parasite. These results provide a functional explanation to field observations showing that QPX disease is associated with clams growing in northern states, especially among those southern clam strains that are readily infected when grown in the colder waters of northern locations.

Salinity has been shown to be another important environmental factor that affects QPX disease development in *M. mercenaria*. Perrigault et al. (2012) investigated the effect of salinity (by comparing 17 and 30 psu) on hard clam immune response and QPX disease development and reported higher disease development and mortality in clams held at high salinity (30 psu). In addition, these authors provided evidence for the modulatory effect that salinity has on clam immunity. However, modulation caused by salinity was less substantial and came secondary when compared to that mediated by temperature. Considering the typical field distribution of QPX in euhaline areas (Calvo et al., 1998) and its reduced *in vitro* growth under low salinities (Perrigault et al., 2010), it is possible to attribute disease development at 30 psu to the higher vitality (growth and possibly virulence) of the parasite at this salinity as compared to 17 psu. Indeed, reduced QPX performance at moderate or low salinity may allow the host to mount a defense response to limit disease development and initiate healing.

It should be noted that field observations show a seasonal pattern of QPX disease prevalence in Raritan Bay that often peaks in early summer, in conjunction with a dramatic reduction in dissolved oxygen in enzootic waters. This pattern suggests a possible role of dissolved oxygen as another environmental factor modulating disease outbreaks. Also, field observations have shown that QPX-related mortalities vary according to clam position in the intertidal zone suggesting a relationship between disease development and periods of air exposure (Calvo et al., 2007; Ford et al., 2002). This, again, implies a link between dissolved oxygen and QPX disease since air exposed clams are unable to acquire oxygen from seawater and become subjected to hypoxic/anoxic conditions. It should be noted that anoxia is supposed to be a stressful condition to both the pathogen and the host. As a matter of fact, *in vitro* investigations have showed suppression of QPX growth under anoxic conditions (Perrigault et al., 2010). Alternatively, extended periods of low oxygen usually cause clams to remain closed, possibly giving longer time for QPX retained in the mantle cavity to colonize clam tissues and establish infection. However, there is no available information on how hard clam immune system responds to hypoxia and how dissolved oxygen levels influence the development of QPX disease. Further

investigations are needed to establish the relationship, if any, between dissolved oxygen and QPX disease.

1.5. Host-pathogen interactions during QPX infection

Like other invertebrates, bivalves lack an adaptive immune system and rely solely on innate immune factors to fight invaders. The shell and mucus that cover the soft body of mollusks serves as external protective barriers for the underlying tissues from physical damages, fluid loss and infections. The internal defense of mollusks requires the cooperation of both cellular and humoral defense mechanisms to jointly fight invaders. Hemocytes are the primary effectors of cell-meditated immune responses in bivalves. They are activated through a complex molecular signaling cascade upon detection of non-self materials, leading to phagocytosis or encapsulation of the foreign entities, as well as production of reactive oxygen species (ROS) (Anderson, 1994; Pipe, 1992). Humoral factors such as lectins, lysozymes, defensins and peptidases work synergistically with cellular components to recognize, bind and degrade invading microorganisms (Allam et al., 2001). In fact, the antimicrobial constituents associated with plasma and hemocytes are actually present in all tissues since bivalves have an open circulatory system.

Cellular and molecular interactions between QPX and the hard clam are not very well understood. Histopathology examination of infected clams demonstrated the capability of the host to mount a defense reaction characterized by an intense inflammatory response, encapsulation and sometimes phagocytosis of parasite cells. The presence of dead and necrotic QPX cells in infected clams suggests that the host defense mechanisms are sometimes effective in neutralizing the parasite and initiating the healing process (Calvo et al., 1998; Dahl et al., 2008; Dove et al., 2004a). Additionally, extracts from clam tissues have been shown to contain factors that inhibit the *in vitro* growth of QPX (Perrigault and Allam, 2009). Interestingly, inhibitory activities were measured in extracts from tissues where infection initiates (e.g. mantle, gill) implying that defense mechanisms can be mounted quickly upon contact with the parasite. Furthermore, differences in QPX inhibitory activities were detected between QPX-resistant (NY) and -susceptible (FL) clam strains, with higher anti-QPX activities in NY clams as compared to FL strains. This observation implies that the southern clam stocks may be deficient of certain QPX-inhibitory mechanisms, putting them at higher risk of being infected by this parasite (Perrigault and Allam, 2009). However, the exact nature of the anti-QPX factors remains unknown. Transcriptional changes in clams in response to QPX infection have been investigated by suppression subtractive hybridization (SSH) and quantitative PCR (Perrigault et al., 2009c), and genes related to signal transduction, stress response and immunity were identified in the SSH library providing a preliminary inspection of factors potentially involved in anti-QPX mechanisms.

On the other hand, parasite cells produce factors that are toxic to clam hemocytes. Histological observations have suggested that mucus secreted by QPX contains virulence factors that protect the parasite from host defense mechanisms (Smolowitz et al., 1998). *In vitro* investigations demonstrated that mucus also protects QPX from clam humoral defense factors

present in plasma and therefore is crucial for parasite establishment (Anderson et al., 2003a). The cytotoxicity of QPX toward *M. mercenaria* hemocytes has been associated with virulence factors secreted by QPX in extracellular products (ECP), which can be further regulated by clam factors such as tissue extracts and plasma (Perrigault and Allam, 2009). Bioinformatics and proteomic analyses have explored the molecular composition of QPX virulence factors (Rubin et al., 2015; Rubin et al., 2014), and demonstrated an important role of serine type peptidases, particularly the subtilisin-like peptidases in parasite infectiveness, as they were shown to digest clam plasma proteins likely facilitating QPX proliferation inside the host. The comparative transcriptomic study also showed a strong influence of QPX genetic and geographic origins on the expression of virulence proteins, likely driven by the adaptation of different QPX strains to local environmental conditions (Rubin et al., 2014).

1.6. Objectives of this study

Severe clam mortality events and resulting economical losses caused by QPX highlight an urgent need for the development of disease mitigation and control measures. A thorough understanding of QPX disease biology and host resistance mechanisms would provide a solid ground for the establishment and evaluation of disease control and prevention strategies. Research presented in this dissertation aimed at enhancing the knowledge of two essential aspects of QPX disease in hard clams including 1) a thorough characterization of the clam immunome and an in-depth assessment of clam immune responses to QPX, and 2) an evaluation of the effect of environmental factors on clam molecular immunity and host-parasite interactions. The following studies have been conducted to serve this overall aim:

1. Sequencing and characterization of *M. mercenaria* transcriptome using next generation sequencing (NGS) methods to identify immune-related genes and pathways involved in clam defense in response to pathogen challenge;
2. Molecular characterization of *M. mercenaria* focal and systemic immune responses to QPX infection using RNASeq technology;
3. Profiling and contrasting transcriptomic alterations in resistant and susceptible *M. mercenaria* in response to temperature and QPX infection using oligoarray methods (DNA chips);
4. Development and evaluation of QPX disease mitigation methods based on heat-shock treatments.

Results of these investigations have provided valuable information on the molecular, physiological and ecological aspects of clam defense mechanisms and response to QPX infection. These investigations also substantially enhanced the limited molecular data available on *M. mercenaria*. Information generated from these studies will help unveil the genetic basis of clam resistance to QPX disease and permits future research on clam selective breeding and the development of disease mitigation strategies.

Chapter 2 High-throughput sequencing unravels the diversity of immune transcripts in the hard clam *Mercenaria mercenaria*

Abstract

The hard clam (*Mercenaria mercenaria*) is an ecologically- and economically-important bivalve species that supports a large aquaculture industry along the east coast of North America. However, in conjunction with the rapid growth of its culture in recent years, infectious diseases of larvae, juveniles and adult clams have brought about dramatic economic losses. Unfortunately, our understanding of *M. mercenaria* immune mechanisms remains scarce and fragmented, highlighting the critical need for a thorough probing of the molecular mechanisms of its immune responses in order to develop disease control strategies and prevent future losses. In this study, we characterized a normalized transcriptome of immune-stimulated *M. mercenaria* using high-throughput sequencing. A diverse set of transcripts with putative immune functions was identified, including various pathogen recognition receptors and putative members of several immune processes and signaling pathways. This study represents the first characterization of the *M. mercenari*a immunome. Results provide an enriched source of transcriptomic data for the study of clam immune responses during host-pathogen interactions. This valuable information highlights the diversity and evolution of innate immune mechanisms and opens the way for future studies on the biological bases for disease resistance in clams.

Keywords

Mercenaria mercenaria; Innate immunity; Normalized transcriptome; Fibrinogen domain; Sequence diversity

2.1. Introduction

The hard clam *Mercernaria mercenaria* is a bivalve species with important commercial value in the United States. It is widely exploited along the Atlantic coasts of North America from the Maritime Provinces of Canada to Florida. *M. mercenaria* is often considered as a robust bivalve species. However, in conjunction with the rapid growth of its culture in recent years, infectious diseases of larvae, juveniles and adult clams have brought about dramatic economic losses. Among the major disease agents affecting the species, *Vibrio* and QPX (quahog parasite unknown) are the most notorious ones (Ford et al., 2002; Lyons et al., 2007; Maas et al., 1999; Ragan et al., 2000; Smolowitz et al., 1998; Stokes et al., 2002; Utsalo et al., 1988). Although specific immune responses are not present in mollusks, their innate immune system, constituted by circulating hemocytes and a large variety of molecular effectors, represents an efficient barrier that responds to microbial invaders after recognition of microbe-specific molecular signatures (Soderhall, 2010; Song et al., 2010). However, the nature and functions of many immune effectors and pathways have not been fully elucidated in these animals. A better understanding of the molecular mechanisms of bivalve immune responses would be of critical importance to the shellfish industry for improving disease control and development of pathogen resistant breeds.

Recent advances in next-generation sequencing (NGS) techniques have dramatically increased the sequencing throughput and enabled more cost-efficient genomic analysis allowing the characterization of transcriptomes from non-model species. As a consequence of NGS development, genomic information on bivalves has witnessed exponential-growth over the past few years, advancing our knowledge of the molecular basis for biological processes with commercial or ecological importance. For example, genome sequencing and assembly have been completed in the Pacific oyster *Crassostrea gigas* (Zhang et al., 2012a), the blue mussel *Mytilus galloprovincialis* (Nguyen et al., 2014), the pearl oyster *Pinctada fucata* (Takeuchi et al., 2012) and the zebra mussel *Dreissena polymorpha* (Penarrubia et al., 2015), leading to the identification of gene markers and molecular processes highly relevant to growth, immunity, shell formation, stress tolerance and population differentiation. However, most of the available genomic/transcriptomic information in the class Bivalvia is from the Mytilid and Ostreid families, while data available for other important bivalve species such as *M. mercenaria* are still limited. So far, genomic information available in public database for *M. mercenaria* is limited and poorly annotated, underlining the urgent need for enhancing genomic resources for the species.

In this study, we constructed a normalized cDNA library using the Roche-454 GS FLX pyrosequencing platform with clams submitted to different pathological challenges to identify transcripts potentially involved in clam immunity. By combining immune stimulation and duplex specific nuclease (DSN) digestion normalization, it enabled the identification of rare or constitutively lowly expressed transcripts that could have escaped

detection with conventional methods. The cDNA normalization reduces the risk of sequencing only highly abundant transcripts, thus enhancing the chance of identifying low expressed transcripts that may have escaped detection in traditional cDNA libraries. In fact, the mRNA copies per gene differ from a few to several tens of thousands in eukaryotic cells (Alberts, 1994). Such dramatic difference in mRNA abundance adds to the complexity of trancriptome analysis since sequenced libraries are highly skewed toward those abundant mRNAs. Normalization of cDNA produced from mRNA before sequencing is a method that decreases the prevalence of highly abundant transcripts and equalizes the numbers of gene transcript in a cDNA sample. This in turn dramatically increases the efficiency of sequencing by enhancing the discovery of rare transcripts. By sequencing this normalized *M. mercenaria* transcriptome, an enriched set of transcripts associated with host-pathogen interactions was identified. This information was then discussed with a specific focus on the diversity and evolution of innate immune mechanisms, setting a robust background for future studies on molecular features associated with disease resistance in clams.

2.2. Materials and methods

2.2.1. *Mercenaria mercenaria*

M. mercenaria were obtained from commercial shellfish farms in New York (NY, 54±7 mm in length) and Florida (FL, 45±4 mm). These aquaculture clam broodstocks display differential susceptibility to QPX infections in both laboratory settings and in the field (Dahl et al., 2008; Dahl et al., 2010; Perrigault and Allam, 2012; Perrigault et al., 2009a). Clams were maintained in re-circulated filtered seawater tanks at 18 °C with continuous aeration for 2 weeks and fed daily with commercial algae (DT’s Live Phytoplankton, Sycamore, IL). A sub-sample (30 clams/stock) was checked for pre-established QPX infections using standard qPCR techniques (Liu et al., 2009) by the end of acclimation, and all tested clams were negative.

2.2.2. QPX and bacterial cultures

QPX strain NY0313808BC7 was isolated from nodules of infected New York clams (Qian et al., 2007a) and subcultured in Minimal Essential Medium (MEM) supplemented with 10% fetal bovine serum (Kleinschuster et al., 1998). QPX cultures were initiated in 25-cm^2 flasks and incubated at 23 °C for 2 weeks. QPX cells were harvested by centrifugation (15 min at 900 g) and were resuspended in filtered artificial seawater (FASW). The cell concentration of the QPX suspension was measured using Neubauer chamber and subsequently adjusted to 1×10^6 cells ml^{-1}. *Vibrio parahaemolyticus* was initially isolated from wild oysters growing in Long Island Sound (New York, USA). The bacterial suspension was made in FASW from a 48 h culture of *V. parahaemolyticus* grown on marine agar (Difco BD, USA). The suspension was adjusted to 1×10^8 bacteria ml^{-1} by dilution with FASW before using for immune stimulation experiment.

2.2.3. Immune stimulation and *M. mercenaria* RNA extraction

M. mercenaria were challenged with QPX and the opportunistic pathogen *V. parahaemolyticus* to increase the representation of immune-related transcripts. FL and NY clams were challenged with either bacteria (1×10^7 cfu in 100 µl) or QPX (1×10^5 cells in 100 µl) injected into the heart area. Following injection, clams were maintained out of the water for 1 h before being returned into their respective tanks. At 24 and 72 h post injection, both QPX- and bacteria-challenged clams were collected (3 NY and 4 FL per pathogen per time point) to allow for the identification of transcripts with different temporal expression patterns. Hemolymph was withdrawn from each individual clam and centrifuged at 4 °C, 800×g for 10 min to retrieve hemocyte pellets. Gill tissues were also collected from each clam by dissection after hemolymph sampling. Following tissue collection, hemocyte pellets and gills were individually homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) immediately and total RNA was extracted according to the protocol supplied by the manufacturer. All RNA samples were subsequently processed with further clean-up and on-column DNase digestion to improve purity and eliminate possible genomic DNA residues using the QIAGEN RNeasy Kits according to the supplier's instructions. After quality assessment and concentration measurement of the RNA by spectrophotometry using NanoDrop® ND1000 (Thermo Scientific, Wilmington, USA), all RNA samples were pooled with the same amount of RNA (1µg/sample) from each sample to ensure sequence diversity.

2.2.4. Construction of normalized cDNA library and sequencing

The pooled total RNA was reverse-trancribed to cDNA using the MINT cDNA synthesis kit (Evrogen, Moscow, Russia) according to the manufacturer's manual. Resulting cDNA was subsequently amplified and normalized using the DSN (Duplex-Specific Nuclease) method (Shagin et al., 2002; Zhulidov et al., 2005; Zhulidov et al., 2004). The method involves denaturation-reassociation of cDNA and a subsequent digestion with a DSN enzyme. The enzymatic degradation occurs primarily on the faction of highly abundant cDNA, resulting in a significant increase in the relative abundance of lowly expressed transcripts. The preparation of the normalized cDNA library was carried out by the commercial transcriptome service provider Evrogen (Moscow, Russia). The normalized cDNA library was sequenced using the Roche 454 GS-FLX pyrosequencing platform, which represents a good method for species with limited genomic resources as it generates long reads that can be easily assembled. The sequencing run was performed at Genome Quebec Innovation Center at McGill University (Montreal, Canada) with the Titanium (454 Life Sciences, Branford, Connecticut, USA) reagents.

2.2.5. Assembly and functional annotation

Pyrosequencing raw data were retrieved from the Roche quality control pipeline using the default settings. Sequences were trimmed and screened prior to assembly by Seqclean (http://compbio.dfci.harvard.edu/tgi/software/) software to remove primers,

adaptors, and poly A/T tails sequences. Reads smaller than 40 bp after the quality control and cleaning were excluded from further sequence assembly and annotation. Cleaned sequencing reads were assembled using the MIRA assembler (Chevreux et al., 2004). A total of 59,036 *M. mercenaria* transcripts were presented in this normalized transcriptome.

The sequence annotation was performed using Blast2GO (http://www.blast2go.org/) with a semi-automated functional annotation based on sequence homology searches. Putative gene identities were obtained by BLASTx search against the National Center for Biotechnology Information's (NCBI) non-redundant sequences (nr) database with a cut off value of 10e-3. Protein domain search and enzyme annotation were also performed with Blast2GO using the InterProScan and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis modules. Putative gene functions were predicted by sequence similarity searches against the Gene Ontology database (GO, http://www. geneontology.org/). GO annotation terms were deduced from the BLAST and InterProScan results and assigned to each mapped transcript with sorted subcategories for “molecular function”, “cellular component” and “biological process”. Default values in Blast2GO were used to perform the analysis and GO level 2 was selected to construct the GO category summary charts. The KEGG Orthology (KO) terms were also assigned to the sequences using the online KEGG Automatic Annotation Server (KAAS, http://www.genome.jp/kegg/kaas/) with bi-directional best-hit method.

2.2.6. Identification of immune-related transcripts

The identification of transcripts putatively involved in immune responses was based on 1) presence of immune-related keywords in the sequence BLAST hit descriptions; 2) assigned GO annotation terms at level 2, 3 and 4 that belong to immune-related categories; 3) presence of immune-related functional protein domains by InterPro Scan prediction. The list of immune keywords was summarized in reference to the molecules with reported immune functions or associations with host defense in published literatures. Selected immune-related sequences were mapped to KEGG immune pathways using KAAS and compared with the reference pathway maps.

2.2.7. Sequence diversity analysis of fibrinogen related proteins (FREPs)

Diversity of fibrinogen (FBG) domain sequences was analyzed and compared between *M. mercenaria* and other molluscan species. For example, human FBG domains have 40 highly conserved residues (100% conservation) that function in pathogen recognition during immune defense (Kairies et al., 2001). Sequences of *M. mercenaria* FBG domains were therefore compared in reference to the human FBG for the assessment of diversity. The diversity of *M. mercenaria* FBG domains was visualized using the program WebLogo (http://weblogo.berkeley.edu/), which graphically represents the alignment of individual amino acid residues. Conserved residues in *M. mercenaria* FBG domain were further compared with FBG sequences available in the NCBI databases for *C. gigas*, *Crassostrea virginica* and *Strongylocentrotus purpurat*us.

A phylogenetic analysis was carried out to compare FBGs in *M. mercenaria* with those from other molluscan species. A total of 397 *C. gigas*, 150 *L. gigantea*, 139 *B. glabrata* and 35 *M. edulis* FBG sequences were obtained from NCBI and used in our phylogenetic analysis, for a total of 807 sequences including the 86 *M. mercenaria* sequences. The FBG domains of all selected molluscan sequences were aligned with MUSCLE (http://www.ebi.ac.uk/Tools/msa/muscle/) and neighbor joining phylogenetic trees were constructed using TreeBeST (http://treesoft.sourceforge.net/treebest.shtml).

2.3. Results and discussion

2.3.1. Transcriptome analysis

A comprehensive transcriptome dataset for *M. mercenaria* was constructed. RNA used for the generation of the normalized library was pooled from different tissues of immune-stimulated *M. mercenaria* originating from stocks susceptible or resistant to QPX disease, in order to enrich the representation of immune-related genes that are usually expressed at low levels in mollusks (Dheilly et al., 2015; Philipp et al., 2012). Sequencing and assembly statistics are summarized in Table 2.1. The assembly generated a total of 59,036 transcript sequences, with 10,613 contigs and 48,423 singletons (Table S2.1). The contigs refer to those sequences assembled from at least 2 reads. The average length of assembled contigs was 1,068 bp, with an n50 value of 961 bp (Table 2.1). The remained unassembled reads represented singletons and ranged from 42 to 4,936 bp, with n50 value of 481 (Table 2.1). The overall size distribution of the *M. mercenaria* transcriptome is summarized in Figure 2.1, with the transcripts length ranging from 42 to 6633 bp, with an average length of 622 bp and n50 value of 511 bp.

The BLASTx search against NCBI nr database returned 16,166 transcripts (27.4%) with blast hits (cutoff E-value = 10e-3, Table S2.1). Distribution of BLAST top-hit species listed in Figure 2.2A. The species with the most sequence matches was *Branchiostoma floridae*, with 2,315 hits. The top-matched molluscan species was the bivalve *Ruditapes philippinarum*, with 215 hits and ranked 14th. Other molluscs, including *Crassostrea gigas, Mytilus galloprovincialis*, *Aplysia californica* and *Halitotis discus* appeared at rank 21, 23, 30 and 32 with respectively 156, 131, 96 and 92 hit occurrences. Overall, 9.86 % (1,594) of all the annotated transcripts matched sequences from the phylum Mollusca, in which 7.29 % (1,178) belonged to the class *Bivalvia* (Figures 2.2B and C). Interestingly, a noticeable fraction of the transcripts was homolog to sequences of chordates, arthropods, hemichordates and gastropods (Figures 2.2B and C), and only 622 sequences from the *Bivalvia* class matched to order *Veneroida* (Figure 2.2D). Observations similar to ours have been reported in other genomic and transcriptomic studies in bivalves (Huan et al., 2012; Moreira et al., 2012a; Philipp et al., 2012), underlining the abundance and complexity of genomics data from model species as opposed to very low representation and annotation quality of bivalve transcripts archived in public databases. Aside from animals (90.76 %), some transcripts had top-hits matching sequences from plants (1.35 %), fungi (1.04 %), protists (1.30 %), bacteria

(2.23 %), archaea (0.23 %), viruses (0.05 %) and undefined sequences (1.08 %).

2.3.2. Functional annotation

A total of 6,658 (11.3%) sequences have been assigned with at least one Gene Ontology (GO) term, which were further categorized into the 3 generic functional groups of "biological process", "molecular function" and "cellular component" at the GO level 2 (Figure 2.3). Within the "biological process" category, "cellular process" and "metabolic process" were the most represented terms with 2,173 and 1,892 transcripts, respectively. They were followed by the terms "biological regulation" and "response to stimulus", which included 956 and 563 transcripts. The term "immune system process" had a representation of 66 transcripts. This comparatively high representation of transcripts associated with stress and immune response might be the result of the immune stimulation procedure implemented prior to the tissue sampling and transcriptome normalization. Mollusks are known to express some defense genes at very low level and to notably induce the expression only following stimulation (Dheilly et al., 2015; Philipp et al., 2012), so the microbial challenge may have induced the expression of those transcripts that are constitutively low or even not expressed. On the other hand, the normalization may have significantly increased the representation of transcripts with low abundance in the transcriptome. With regard to the "molecular functions" category, the GO terms containing the most transcripts were "binding" with 2,354 transcripts, "catalytic activity" with 1,866 transcripts and "structural molecular activity" that included 198 transcripts. As for the "cellular components" category, the highest number of transcripts was assigned GO terms of "cell" and "organelle" with 1,892 and 1,367 transcripts in each group respectively. The "macromolecular complex" immediately followed and contained 897 transcripts.

The functional protein domain prediction was performed using InterProScan searches, which resulted in 28,273 transcripts assigned putative functional domains. This protein domain prediction has greatly facilitated our search for candidate immune related sequences in complement with the information given by GO terms and BLAST hit descriptions. For example, the candidate immune-related sequences assigned to the GO term "immune system process" mainly consisted of toll-like receptor (TLR) -like transcripts (Philipp et al., 2012). It should be noted that searches based on immune-related keywords heavily rely on annotation information retrieved by the BLAST hit, which has great limitations for the identification of immune-related candidate sequences in non-model species like *M. mercenaria* given the poor nature of clam genomic databases. However, with the integration of protein domain searches into our approach, those disadvantages were circumvented and the searching capability has been improved for the identification of immune-related transcripts.

The protein domains used for transcripts identification and the number of occurrence for each domain are summarized in Figure 2.4. The selected protein domains mainly featured pattern recognition receptors (PRRs) involved in pathogen recognition and

immune process regulation. The immunoglobulin-like (Ig) fold and C-type lectin (CLECT) were the most represented immune related domains in *M. mercenaria* transcriptome, which were followed by the complement component C1q (C1q) and fibrinogen (FBG) domains. Additionally, tumor necrosis factor (TNF) and Toll-interleukin-1 receptor (TIR) domains were also among the most abundant categories among immune-related domains.

2.3.3. Immune-related transcripts in *M. mercenaria*

Candidate immune-related sequences were identified and selected by referring to the combined source of BLAST annotation, GO terms and functional protein domains. Transcripts were then examined for their possible role in immune pathways by integrating information with KEGG pathway maps to gather a more comprehensive overview of *M. mercenaria* transcriptional response to microbial challenge. A vast majority of sequences was found to cluster into some well-understood, fairly conserved immune pathways, such as the toll-like receptors (TLR) pathway, complement pathway and apoptosis pathway, suggesting the immune mechanisms present in *M. mercenaria* likely have a very ancient origin and evolutionarily conserved. In addition, sequences matching components of immune-related signaling pathways were also abundantly identified in our database. These pathway elements are well known as mediators that transduce the extra-cellular immune recognition signals into the intra-cellular cascades to regulate downstream defense processes and the production of immune effectors. Sequences involved in the immune system are discussed with regard to their putative functions and associated pathways. Special focus was given to PRRs and immune signaling mediators that play key roles in the innate defense mechanisms.

2.3.3.1. Immune-related pathways and signaling cascades

The host defense system is activated when innate immune cells detect pathogen presence or tissue injury. Surveillance mechanisms involve the binding of pattern recognition receptors (PRRs) to pathogen ligands or host-derived damage patterns. Upon the detection of danger, signals are transduced by a cascade of biochemical reactions through multiple immune pathways, which trigger the launch of cellular defense responses, promoting the gene transcription of inducible immune-related proteins, and initiating hemocyte recruitment.

The signaling network of the immune system consists of two components, namely, the inter-cellular communication and intra-cellular signaling pathways. Inter-cellular communication is mediated by various cytokines, chemokines, growth factors, and their associated receptors, while the intra-cellular signaling pathways are downstream of immune receptors and consist of adapters, GTPases, caspases, kinases, and transcription factors. Several signaling components have been identified in *M. mercenaria* transcriptome following pathogenic stimulation, including components from the

complement cascade, toll-like receptor (TLR) pathway, nuclear factor κB (NF-κB) pathway, mitogen-activated protein kinase (MAPK) pathway and apoptosis cascade.

The complement system is an ancient pro-inflammatory system that, in vertebrates, consists of over 30 small plasma proteins that function synergistically to distinguish and eliminate pathogens. Three pathways, namely the classical pathway, the alternative pathway and the lectin pathway can initiate the complement system in these organisms leading to the augmentation of immune responses and the activation of cell-killing membrane attack complex (MAC). The complement system was once considered exclusive to the vertebrates, however recent discoveries of complement factors in mollusk genomic and transcriptomic studies have supported the presence of this defense mechanism in lower animals, such as bivalves (Moreira et al., 2012a; Philipp et al., 2012; Zhang et al., 2013a), highlighting the ancient origins of this immune mechanism (Pinto et al., 2007). Our sequencing results allowed the identification of a large set of complement pathway components in *M. mercenaria* transcriptome (Figure 2.5, Supplementary file 2.2), with the most abundant sequences being complement system initiator molecules, particularly cell surface receptors such as the C1q domain containing proteins (C1qDC), complement factor C3 and mannose-binding proteins (MBL).

C1q is the key component of the classical complement pathway that also serves as a link between innate and acquired immunity due to its great binding diversity enabled by the highly adaptive globular C1q domain. The C1q domain was also found in many non-complement proteins, involved in a broad range of processes including apoptosis, inflammation, cell adhesion and cell differentiation (Ghai et al., 2007). Proteins containing C1q domains have been abundantly identified in different bivalves including oysters (Zhang et al., 2013a), clams (Moreira et al., 2012a), scallops (Zhang et al., 2008) and mussels (Gerdol et al., 2011; Gestal et al., 2010; Philipp et al., 2012), with some of these proteins exhibiting microbe binding functions similar to lectins. In *M. mercenaria*, we identified a total of 153 C1q-like or C1q domain-containing transcripts, indicating the great expansion of C1qDCs in this species. Since adaptive immunity and mechanisms producing somatically diversified antibodies are absent in invertebrates, maintaining a diverse pool of germline-coded recognition molecules is of critical importance for invertebrates to keep effective immune surveillance functions. Therefore, an expansion of pathogen recognition proteins like C1qDCs would significantly enhance the pool of recognizable targets, thus enabling the host to respond against a broad range of antigens.

The C3 complement factor is a group of thioester-containing proteins (TEP) that react with amino or hydroxyl groups of pathogen cell surface molecules. The binding of C3 to pathogens rapidly initiates and amplifies immune response via the alternative complement pathway. The C3 factor also functions as the central element of the complement system that bridges the alternative pathway with the lectin/classical pathway via the complement component C5. The complement C3 orthologs and their downstream components have been previously identified in bivalves (Moreira et al., 2012a; Philipp et al., 2012; Prado-Alvarez et al., 2009), supporting the existence of immune activation

pathways that are analogous to the complement system in lower invertebrates. In this study, 15 sequences homologous to the C3 complement factor and thioester-containing proteins were identified (Supplementary file 2.2). In addition, other complement pathway components such as factor B, C2, C4 and C5 that directly interact with C3 have also been identified (Figure 2.5, Supplementary file 2.2).

Among the components that are known to form membrane attack complex (MAC), only the complement factor C6 was identified in our library (Figure 2.5, Supplementary file 2.2). The remaining components C7, C8 and C9 were not identified neither by annotation nor via protein domain analysis. It should be noted that the presence of these MAC factors has only been reported in the deuterostome, such as sea urchins and tunicates (Azumi et al., 2003; Multerer and Smith, 2004), while their absence has been commonly noted in lower marine invertebrate lineages (Castillo et al., 2009; Miller et al., 2007; Moreira et al., 2012a; Philipp et al., 2012). Overall, current data suggest that bivalve's primitive complement system is fairly different from that possessed by vertebrates. While the complement system has been extensively characterized in the vertebrates and higher invertebrates, the composition and immune functions of bivalve complement system remain unclear and require further characterizations.

The TLR signaling pathway is a central signaling junction that links signal transduction through both intra- and inter-cellular pathways. It is an evolutionarily conserved pathway that plays pivotal role in the innate immunity. The TLR pathway is activated when foreign compounds bind to TLR receptors located on the outer membrane of immune cells. The danger signals are then transferred into the cell interior, and are amplified via an array of biochemical reactions with adapters, kinases and GTPases. The TLR pathway also communicates with its downstream intra-cellular signaling cascades, namely NF-κB, MAPK, apoptosis and complement pathways, and initiates a comprehensive host defense response against the invader. Many key elements of the TLR pathway have been recently identified in bivalves following immune stimulation. These include the myeloid differentiation primary response protein 88 (MyD88), tumor necrosis factor receptor–associated factors (TRAFs), IL-1 receptor-associated kinase (IRAK) and the adapter-like protein (TIRAP) (He et al., 2013; Tanguy et al., 2004; Venier et al., 2011a; Wang et al., 2011b). In our study, a large set of interrelated pathway components belonging to the TLR-signaling pathway has been identified (Figure 2.6). For example, we identified 59 sequences containing the toll/interleukin-1 receptor domain (TIR, IPR000157), which covered the protein homologues to TLR1-3, TLR6-9 and TLR13. In addition, a majority of elements downstream of the PRRs have also been found, however, displaying a fairly low level of sequence diversity (as opposed to the TLRs) with either 1 sequence or only a few isoforms identified (Supplementary File 2.2). This observation is in conformity with the “funnel” model described for innate immune signaling, where the molecular complexity is significantly reduced from the cell-surface (receptor) level to the nucleus (transcription factor) level, so as to sort the numerous initial signals into a limited number of “simplified” pathways (Sansonetti, 2006).

The NF-κB pathway (Supplementary file 2.2) is a key regulator of bivalve immune system. Components of this pathway were first described in *C. gigas* (Montagnani et al., 2004) and later revealed in all studied bivalves (Moreira et al., 2012a; Philipp et al., 2012; Tanguy et al., 2013a; Zhang et al., 2013a; Zhou et al., 2013). The NF-κB pathway activates the transcription of genes participating in the stress and immune responses. As a downstream element of the TLR signaling pathway, the key component NF-κB is only released from the inactive heterodimer NF-κB /IκB following the activation of TLR pathway cascade reactions. The active NF-κB is then translocated to the nucleus to perform its function as a transcription factor. Many processes downstream of the PRRs are mediated by NF-kB activation, such as cell proliferation, cell survival and immunity. NF-κB may also function as an inhibitor of serine proteases and metalloproteinases, which are two key virulence factors commonly associated with pathogens (Johnson, 2013). Similar to other molluscan NF-κB molecules, the *M. mercenaria* fragments also contained the conserved Rel homology domain (RHD, IPR000451) with the characteristic amino acid sequence of the DNA recognition loop and nuclear localization sequences (De Zoysa et al., 2010; Goodson et al., 2005; Jiang and Wu, 2007; Montagnani et al., 2004; Philipp et al., 2012).

A set of transcripts associated with MAPK pathways has been identified in our study following immune stimulation (Supplementary file 2.2). The MAPK signaling cascades are involved in a variety of critical cell processes, such as cell proliferation, cell death and cellular immunity. MAPKs have three well-characterized subfamilies, namely the extracellular signal-regulated kinases (ERKs), c-Jun amino-terminal kinases (JNKs) and p38-MAPKs (Johnson and Lapadat, 2002). These evolutionarily-conserved enzymes connect cell-surface receptors to critical regulatory targets within cells. Homologues of MAPK pathway components and their associated regulators have been recently identified in bivalves, including *C. virginica*, *R. philippinarum*, *M. galloprovincialis* and *Meretrix meretrix* (Huan et al., 2012; Kang et al., 2006; Moreira et al., 2012a; Tanguy et al., 2004; Zhang et al., 2013a). The triggers that were shown to activate bivalve MAPK pathways include environmental stress, infection and tissue damage (Betti et al., 2006; Ciacci et al., 2010; Yao and Somero, 2012) and our results support the conserved functions of these components as main mediators of stress-response.

Additionally, components of the JAK/STAT pathway were also identified in our study (Supplementary file 2.2). The JAK/STAT pathway responds to chemical signals and is involved in the regulation of chemotactic effectors of the immune system. JAK proteins are activated by cytokine receptors and phosphorylate STAT proteins, which then translocate into the nucleus and modulate the expression of various genes involved in immune reactions. Signals recognized by the receptors include cytokines, such as interferon, interleukin, growth factors, and other chemical messengers. However, due to the wide structural discrepancies between vertebrate and invertebrate cytokines, only a small number of cytokine sequences have been identified in invertebrates by traditional sequence analysis methods using mammalian cytokine databases as references (Hibino et

al., 2006; Huang et al., 2008; Philipp et al., 2012). In agreement with other bivalves, we were unable to identify any sequences matching interferons (IFN) or containing an IFN-domain. However, interferon regulatory factors (IRF) and other interferon-inducible proteins were abundantly detected in *M. mercenaria*. Similarly, we were able to identify two *M. mercenaria* sequences putatively encoding for interleukins 16 (IL16) and 17 (IL17), as well as three IL-related receptors (Supplementary file 2.2). The putative IL16 showed a high similarity to that identified in *S. purpuratus* and contained a PDZ domain (IPR) typical of IL16. In contrast, IL-17 seems to be fairly well conserved since homologues were identified in many other organisms including *C. gigas*, *M. edulis*, *S. purpuratus*, as well as in *Xenopus tropicalis*.

Interestingly, *M. mercenaria* transcriptome also contained components from Notch and Wnt pathways (Supplementary file 2.2 and 2.3), which have been rarely studied in invertebrates. Despite their well described roles in regulating the activation, proliferation and fate of immune cells in mammals (Radtke et al., 2010; Staal et al., 2008b; Zhou et al., 2009), the Notch and Wnt signaling pathways have only been reported to regulate embryo development in mollusks (Nakamoto et al., 2010).

Apoptosis represents an important aspect of the immune system. It plays significant roles in the control of the immune response, regulating self- and non-self recognition, and initiating cytotoxic killing. Given the terminal nature of this process, apoptosis is tightly-controlled and the regulatory pathways are evolutionarily highly conserved (Degterev and Yuan, 2008; Quistad et al., 2014). Apoptosis pathways have been extensively investigated in a variety of invertebrates including bivalves (Sokolova, 2009; Terahara and Takahashi, 2008). Interspecies comparisons have found that the apoptotic pathways are simplified in lower animals and display increasing complexity toward higher animal lineages (Devitt and Marshall, 2011). In *M. mercenaria*, multiple transcripts of the major components of the apoptosis pathway have been identified, even though results suggest a simplification of this pathway as compared to the standard KEGG pathway (human as reference; Figure 2.7). For instance, various members of the TNF receptor, BCL2 and apoptosis inhibitor (IAP) families were identified (Supplementary file 2.2). In addition, caspase-like transcripts containing caspase specific domains (Peptidase C14, IPR011600) have also been identified. Some of the caspase sequences also contain additional Death (IPR011029) or CARD domains (IPR001315), implying multiple roles for these molecules, possibly as pathway initiators or effectors. The diversity of caspase transcripts suggests complex apoptosis regulatory mechanisms in *M. mercenaria*.

2.3.3.2 Immune recognition receptors

The key process to activate the immune system is the pathogen recognition, which relies on distinguishing between self and non-self. Like all invertebrates, clam innate immune system depends on receptors and secreted proteins that recognize a large collection of conserved microbial molecular signatures shared by many pathogens. These immune receptors are encoded by genes and are inherited through the germline (Janeway

and Medzhitov, 2002). Several groups of different pattern recognition receptors (PRRs) have been detected in the *M. mercenaria* transcriptome (Figure 2.4, Supplementary file 2.4), including lectins, toll-like receptors (TLRs), C1q domain containing proteins (C1qDCs), fibrinogen related proteins (FREPs), scavenger receptors (SRs), peptidoglycan recognition proteins (PGRPs), leucine-rich repeats (LRRs) and lipopolysaccharide binding proteins (LBPs). These PRRs are widely recognized for their roles in immune protection in mollusks and other invertebrate species.

Lectins

Lectins are a large family of carbohydrate-recognition proteins that play crucial roles in innate immunity. Lectins recognize and bind directly to specific carbohydrate structures on the surfaces of microbial cells and initiate a subsequent series of rapid defense reactions, such as pathogen precipitation, immobilization, cell lysis, or participate in the opsonization of microbes to promote their phagocytosis by hemocytes (Vazquez-Mendoza et al., 2013; Wang et al., 2013). Several types of lectins (C-type lectin, sialic acid binding lectin, fucolectin and galectin) have been identified in *R. philippinarum* (Bulgakov et al., 2004; Kim et al., 2008; Moreira et al., 2012a), *Mytilus edulis* (Tanguy et al., 2013a; Tanguy et al., 2013b), *M. galloprovincialis* (Venier et al., 2009; Venier et al., 2011a) and *C. virginica* (Zhang et al., 2013a) following pathogen stimulation. The critical involvement of lectins in bivalve immune responses has also been characterized in many studies. For example, lectins mediate pathogen recognition and opsonization in the scallop *Chlamys farreri* (Yang et al., 2010a), and enhance cell adhesion and pathogen encapsulation by hemocytes in the scallops *Argopecten irradians* (Wang et al., 2012b) and *C. farreri* (Yang et al., 2010a). In addition, harmful immune complexes (e.g. inflammatory agents and lysosomal products) produced during host response can also be recognized by lectins to facilitate their removal from the inflammation site and prevent host damage (Gazi and Martinez-Pomares, 2009).

In our study, a large number of putative lectin sequences was found in *M. mercenaria*, including 201 C-type lectins (CLECT, calcium-dependent carbohydrate-binding lectins), 19 galectins (GLECT) and members of other subgroups such as selectin, fucolectin and lactose-, mannose- or sialic acid-binding lectins. CLECT contain one or more C-type lectin domains (CTLDs, IPR001304) that are capable of binding a variety of ligands, including carbohydrates associated with pathogen surface, glycolipids and glycoproteins. The CLECTs are highly diverse both structurally and functionally, as demonstrated in many species ranging from *C. elegans* to Humans (Dodd and Drickamer, 2001). Our data show that *M. mercenaria* possesses a large pool of CLECTs that contained either a single or multiple CTLDs in conjunction with various other functional domains (supplementary file 2.4). This versatility of CLECT structures potentially enables a diversification of the recognition targets and may allow the fine-tuning of the binding functions, as reported in human and other animal species where CLECT were shown to differentiate endogenous ligands and pathogenic carbohydrates allowing the

concomitant regulation of immune homeostasis and pathogen killing (Drickamer and Taylor, 2015; McGreal et al., 2004).

On the other hand, galectins also displayed some sequence diversity in *M. mercenaria* although at a lower scale as compared to CLECT. Galectin is a family of lectins characterized by a conserved carbohydrate recognition motif and specific β-galactoside-binding domain (IPR001079, Figure 2.4). Galectins have multiple immune regulatory functions and play roles in diverse immune processes, such as pathogen recognition (as PRRs), cell migration and apoptosis (as cytokine-like modulators), or as immuno-modulators of inflammatory responses during infections (Liu, 2005). Galectins identified in our study were mostly tandem repeat galectins and generally lacking the classical signal peptides (Supplementary file 2.4), which suggest they mainly function as soluble mediators synthesized and localized intracellularly. However, recent research indicated that galectins stored in the cytoplasm could be either passively released by dying cells or actively secreted by inflammatory cells upon pathogen-induced tissue damage (Sato et al., 2009). The exported galectins participate in the regulation of host immune homeostasis and mediate intracellular signaling pathways (Vasta et al., 1999). Interestingly, since galectins are dominantly found in infection foci, they are considered as damage-associated molecular pattern (DAMP) candidates that orchestrate the innate immune responses. More intriguing, recent studies have reported the presence of conserved 'galectin-like' domains in certain pathogens including helminths and protistan parasites, suggesting that they could also serve as potential virulence factors used by pathogens to manipulate the host immune system and influence the outcome and course of infection (Davicino et al., 2011). Overall, the variety of lectins observed in our study may indicate diversified sugar-binding specificity and functional interplay among different lectin types to neutralize microbial invaders.

Toll-like receptors (TLRs)

TLRs constitute an ancient family of pattern recognition receptors that are activated by the stimulation with pathogen-associated molecular patterns (PAMPs) (Qiu et al., 2007). They play key roles in innate immunity by mediating the detection of non-self substances and activation of downstream host defense responses via TLR signaling pathways. Structurally, TLRs consist of several extracellular leucine-rich repeat (LRR, IPR001611, Figure 2.4) solenoids, a trans-membrane domain, and a highly conserved intra-cytoplasmic domain known as the toll/interleukin 1 receptor (TIR, IPR000157, Figure 2.4) (Mikami et al., 2012; Offord et al., 2010; Zhang et al., 2011a). In *M. mercenaria*, 59 and 57 sequences were predicted to containing TIR and LRR domains (Figure 2.4). These typical protein domains enable the membrane localization and downstream signal transduction of TLRs (Mikami et al., 2012; Zhang et al., 2011a). The end processes induced by TLR signaling include phagocytosis of pathogens, cell adhesion as well as recruitment and proliferation of immune cells (Takeda, 2004). Bivalve TLRs were shown to be regulated by pathogenic stimuli as demonstrated in *C. farreri* (Qiu et al., 2007), *C. gigas* (Tanguy et al., 2004), *Mya arenaria* (Mateo et al.,

2010), *M. galloprovincialis* (Venier et al., 2011b) and *M. mercenaria* (Perrigault et al., 2009c). The sequence diversity of TLR family members have been reported in several bivalve species (Moreira et al., 2012a; Philipp et al., 2012; Tanguy et al., 2013a; Zhang et al., 2013a). In our study, multiple putative Toll-like receptor family members were identified, including TLR1 to 9, TLR13 and TLR18 (Figure 2.6, Supplementary file 2.4). Among these, TLR1 and 2, and TLR2 and 6 form membrane-bound heterodimers that recognize and are activated by various microbial components such as peptidoglycans and lipoproteins (Takeda, 2004). TLR4 and TLR5 respectively recognize bacterial lipopolysaccharide and flagellin, both of which represent bacterial virulence factors (Takeuchi and Akira, 2010). TLR3, 7, 8 and 9 stimulate innate immune responses upon recognizing pathogen-derived nucleic acids, such as viral ssRNA, unmethylated CpG DNA and dsRNA. TLR13 recognizes conserved bacterial 23S ribosomal RNA (rRNA) sequences (Oldenburg et al., 2012) and TLR18 also plays a role in innate immunity by acting as a pattern recognition receptor that directs the expression of peptides active against Gram-negative bacteria (van der Sar et al., 2006).

Scavenger receptors (SRs)

Scavenger receptors (SR) are surface PRRs, usually located on phagocytes, that are able to recognize endogenous or microbial-derived lipoproteins (Mukhopadhyay and Gordon, 2004). SRs represent a large family containing gene members that are structurally unrelated. For example, 218 SR cysteine rich (SRCR, IPR001190) -domain containing sequences were identified in the sea urchin genome (Multerer and Smith, 2004) and 270 SRCR domain-containing sequences were identified in amphioxus genome (Huang et al., 2008). In our study, 24 sequences containing SRCR domains were detected. While this number suggests a lower expansion of the family as compared to sea urchin and amphioxus, it is close to that reported in humans where a total of 16 SRCR domain-containing genes were identified.

Peptidoglycan recognition proteins (PGRPs)

PGRPs specifically bind peptidoglycans, which are a major component of the bacterial cell wall. PGRPs exert bactericidal activities by inducing oxidative stress and cell wall hydrolysis. In addition, PGRP also influence host-pathogen interactions by participating in inflammatory processes (Royet et al., 2011). In bivalves, the anti-microbial activity of PGRP has been confirmed in many species including *A. irradians* (Ni et al., 2007), *C. farreri* (Yang et al., 2010b), *Solen grandis* (Wei et al., 2012) and *C. gigas* (Iizuka et al., 2014). Following immune challenge, 10 sequences containing PGRP domain (IPR006619) were identified in *M. mercenaria* (Supplementary file 2.4), further supporting the important involvement of PGRPs in immune regulation and response during host defense against microorganisms.

Bactericidal/permeability-increasing proteins (BPIs) and lipopolysaccharide-binding proteins (LBPs)

BPIs and LBPs are important PRRs that are involved in the innate defense against

bacterial pathogens. These proteins share basic architectures and residues that allow them to recognize lipopolysaccharides (LPS), a major virulence factor and cell membrane component of Gram negative bacteria (Krasity et al., 2011). The interaction initiates the activation of various host defense mechanisms, and the induction of these proteins in response to pathogen stimulation were commonly observed in bivalves (Gonzalez et al., 2007; Zhang et al., 2011b). Two sequences with homology to BPI and 3 sequences homologous to LBP were found in our study (Supplementary file 2.4).

Leucine-rich repeats (LRRs)

Besides the abovementioned PRRs, we have also identified 57 LRR domain-containing transcripts (Supplementary file 2.4), all of which encoded proteins containing no other domains but LRRs. This structural feature is similar to the LRR-containing proteins observed in vertebrates, such as the human CD14 and variable lymphocyte receptors (VLRs), which are typical LRR-only proteins with crucial immune functions (Palaniyar et al., 2002). This is in contrast to previous studies in invertebrates where the LRR-containing proteins often contain DEATH and/or NACHT domains at the N-terminal regions, as shown in sea urchin *S. purpuratus* (Hibino et al., 2006) and amphioxus *B. floridae* (Huang et al., 2008).

Fibrinogen related proteins (FREPs)

The FREPs are a group of proteins that contain fibrinogen or fibrinogen-like domains. Members of this family play important roles as pattern recognition receptors and activate major host defense mechanisms such as the complement system and immune signaling pathways. The molluscan FREPs family consists of a large collection of highly variable receptors that are capable of recognizing different antigens (Mone et al., 2010). A very diverse set of FREP sequences have been identified in our study following immune challenge.

2.3.3.3. Sequence diversity of FBG domains in M. mercenaria FREP superfamily

We have identified a total of 86 transcripts of the FREP superfamily containing at least one FBG domain (Supplementary file 2.4). Some of these transcripts also contained sequences of transmembrane regions or signaling domains. The sequence diversity analysis of FBG domains revealed considerable high polymorphisms in translated amino acid sequences (Figure 2.8A). However, residues that form FBG functional domains appeared comparatively more conserved, such as the polymerization pocket, the Ca^{2+} (ion) binding site and the gamma-gamma dimer interface (polypeptide binding site). These conserved sites are critical in mediating protein-protein or protein-carbohydrate interactions (Doolittle et al., 1998; Hermans and McDonagh, 1982; Marucco et al., 2013). In fact, sequence diversity of FREPs has been widely observed in mollusks where previous genome and transcriptome studies have identified 150 sequences with FBG domains in *Lottia gigantea*, 139 in *B. glabrata*, 397 in *C. gigas* and 35 in *M. edulis*. Compared to these species, the number of FREPs identified in *M. mercenaria* is moderate,

however the polymorphic levels of conserved residues (in reference to the human FBG domains) were relatively high in *M. mercenaria* as compared to other invertebrate species (Figure 2.8B). The phylogenetic analysis clustered a majority of *M. mercenaria* FREPs together first, which then converged into adjacent clades mostly with *C. gigas*, suggesting the close evolutionary relationship between the two bivalve species (Figure 2.9). Some sequences also clustered with *L. gigantea* and *M. edulis*. Only a few *M. mercenaria* FREPs were adjoining *B. glabrata*, of which the sequences almost formed their own clades entirely.

FREPs have been recognized as important antigen recognition receptors of the innate immune response in vertebrates and invertebrates (Hanington and Zhang, 2011; Lu and Le, 1998; Zhang et al., 2009a). In fact, the high variability of FREP sequences has implied a great capacity and flexibility of these proteins to respond to a diverse and ever-evolving pathogen repertoire (Mone et al., 2010). Previous studies have reported that FREP families share highly conserved C-terminal FBG domains but vary significantly at N-terminal regions (Zhang and Loker, 2004). However, for many bivalve FREPs, a relatively high level of polymorphism also exists in the FBG domains (Huang et al., 2015; Romero et al., 2011; Zhang et al., 2013a). Our study also demonstrated the diversity of the FBG domains in *M. mercenaria*. Although generally conserved at the domain feature sites, the remaining amino acid residues of FBG are considerably more variable as compared to humans and other invertebrates, even oysters. The highly diversified sequences of immune receptors and effectors are believed to serve as the fundamental mechanism for invertebrates to gain higher levels of immune specificity. Multiple variants of FREPs sequences translate into protein end products with diversified spatial conformations at the antigen-binding sites, potentially expanding the pool of antigens recognized by the immune system and enhancing the binding specificity of certain types of pathogens. It is thus likely that FREPs sequence diversity noted in *M. mercenaria* is developed as a feedback to the selective pressure they face in an environment containing rich, complex and changing populations of microorganisms and potential pathogens. Such challenges trigger the selection for an expansion and diversification of immune recognition factors, allowing the host to respond to fluctuating and unpredictable environmental variations.

Moreover, the clustering of *M. mercenaria* FREPs on the phylogenetic tree seems to suggest that the sequence variants are derived from the expansion and diversification of *M. mercenaria*-specific genes, perhaps under the pressure of their microbe-rich habitat. On the other hand, the highly conserved amino acid residues noted at the FBG domains may be critical for maintaining the structural integrity and biological function of FREPs. The mechanism for FREP diversification has been scrutinized in *B. glabrata* where it was reported that the variants were somatically generated via point mutation, alternative splicing and recombinatorial processes, via mechanisms similar to those allowing the generation of antibody diversity in vertebrates (Zhang and Loker, 2003; Zhang, 2004). Although the underlying biology remains unclear in *M. mercenaria*, our findings of

polymorphic and diversified immune receptor sequences provide additional support to the recent speculations of the existence of an ancient yet sophisticated specific innate immunity in some invertebrates (Huang et al., 2008; Mone et al., 2010; Romero et al., 2011; Soderhall, 2010). The germline-encoded diversity of innate immunity may have been more complex and developed earlier than previously suggested and before vertebrates evolved somatic diversity-based adaptive immunity (Schulenburg et al., 2007). However, further research is necessary to fully understand the mechanisms generating FREP diversity in *M. mercenaria*, and the roles of these molecules in immunity and adaptation to a dynamic repertoire of pathogens.

2.3.3.4 Other immune related transcripts

Probing the transcriptome of immune-stimulated *M. mercenaria* allowed the identification of a range of additional immune effectors such as protease inhibitors, lysozymes, antimicrobial peptides and heat-shock proteins. Protease inhibitors represent an important arm of the innate immune system as they protect hosts against pathogen-derived proteases. Being a well-recognized form of virulence factors, pathogen proteases facilitate the infection by inactivating host proteinaceous immune factors. Many bivalves are known to employ protease inhibitors as an effective means to neutralize pathogen proteases (La Peyre et al., 2010; Wang et al., 2008; Xue et al., 2006; Yu et al., 2011). A total of 41 transcripts with homology to cystein-, serine-, and metallo-protease inhibitors and Kunitz- or Kazal- type protease inhibitors were detected (Supplementary file 2.5).

Antimicrobial peptides (AMPs) are small, cationic peptides that typically alter the permeability of microbial cell membranes resulting in microbial lysis. Bivalve AMPs have been purified from hemocytes and were shown to display microbicidal and immunoregulatory functions against various pathogens (Charlet et al., 1996; Defer et al., 2013; Gestal et al., 2007; Gueguen et al., 2006). We were able to detect 20 transcripts in *M. mercenaria* (Supplementary file 2.5) that were homologous to defensins previously identified in bivalves (e.g. hemocyte defensin, mantle defensin and big defensin).

Lysozyme is an antibacterial molecule with bacteriolytic activity that has been extensively studied in mollusks (Van Herreweghe and Michiels, 2012). The invertebrate specific type-i lysozymes have been characterized in bivalves and were shown to display significantly higher activities than lysozyme families from other organisms (Xue et al., 2010; Yue et al., 2011; Zhao et al., 2010). In our study, 11 transcripts with homology to various types of lysozymes were detected in *M. mercenaria* including several members of type-i lysozymes (Supplementary file 2.5)

Heat shock proteins (HSP) are a group of stress response molecules that have been well studied in bivalves (Fabbri et al., 2008; Roberts et al., 2010; Yik Sung, 2013). Functioning as molecular chaperones, HSPs also participate in innate immune responses as mediators of immune pathways, such as the apoptosis pathway and NF-κB pathway (Roberts et al., 2010). HSPs were one of the most represented categories of immune and stress genes with a total of 140 sequences identified. Most of the HSPs presented in our

dataset were HSP70, which are the most fundamental regulators of normal protein synthesis within cells. However, other less common HSPs, such as small HSPs (HSP10, HSP12, HSP22, HSP40) and some members from the HSP90 family were also represented (Supplementary file 2.5).

2.4. Conclusions

This study represents the first assessment of the *M. mercenaria* immune transcriptome (immunome). By sequencing this normalized transcriptome, we were able to generate sequence information on clam immune factors in a highly efficient manner, resulting in the identification of a greatly diverse set of putative immune transcripts. This new resource will serve as a rich dataset to facilitate discovery of new genes and for the identification of genetic markers for selective breeding. This new dataset also provides a reference to develop comparative transcriptomic studies (microarray, gene expression) in *M. mercenaria*. The results allowed the characterization of immune pathways activated following microbial stimulation, such as the complement cascade, the TLR and apoptosis pathways, and pathways mediating immune signal transduction. This information allows a better understanding of the defense mechanisms employed by *M. mercenaria* to maintain homeostasis in their microbial-rich habitat. In addition, we were also able to identify several immune molecules or pathway components that are rarely described in previous bivalve studies, such as some complement factors, Toll-like receptors, and the Notch and Wnt pathway components. Moreover, results highlighted the expansion and diversification of sequences encoding immune recognition proteins (PRRs), including lectins, C1qs and FREPs. The relatively high diversity of FREPs indicates the comprehensive defense mechanisms displayed by *M. mercenaria*, which may be a key to pathogen resistance. Together, our results illustrate the extraordinary variability and complexity of innate recognition molecules in *M. mercenaria* that likely react to a large repertoire of PAMPs. This may represent a primary strategy for clam adaptation to an environment with high microbial diversity and density. Overall, our findings provide insights into the function and evolution of bivalve immune system.

Table 2.1. Summary of the assembly and annotation results.

	Contigs	Singletons
Length range	56-6,633	42-4,936
Average length	1,068	524
N50 length	961	481
Total transcritps	59,036	
	Counts	**Percentage**
With BLAST match	16,166	27.4%
With assigned GO	6,658	11.3%
With assigned KO	8,844	15.0%
With InterProScan matches	28,273	47.9%
With KEGG enzyme code matches	1,824	3.1%

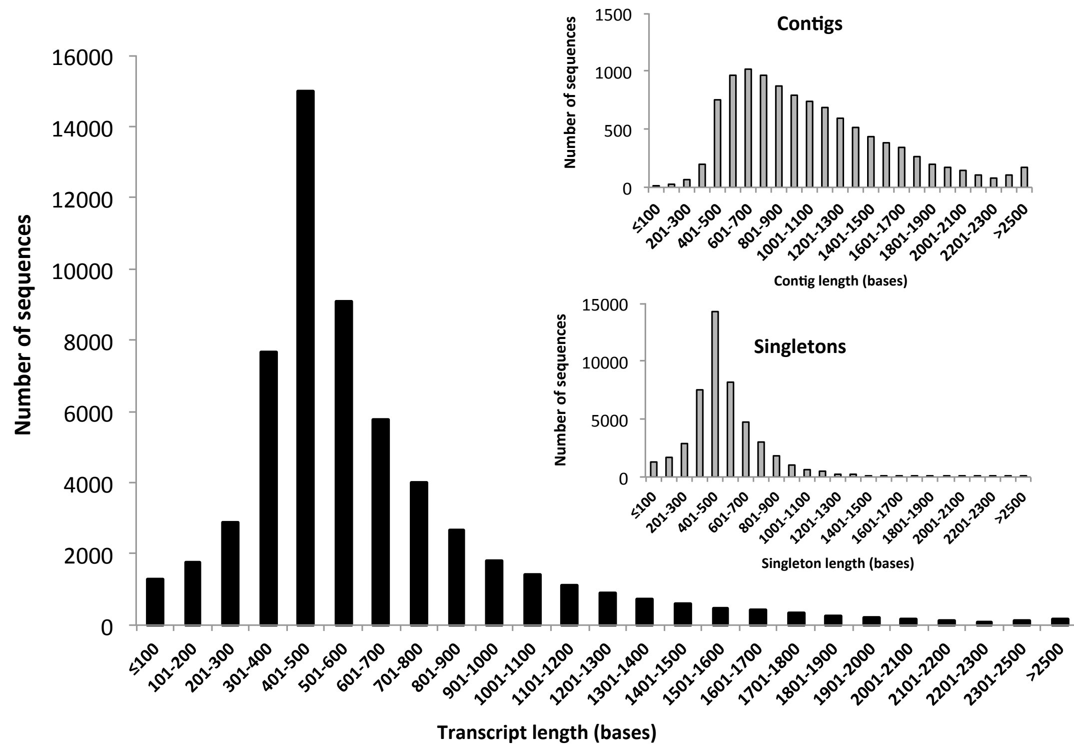


Figure 2.1. Size frequency distribution of the *Mercenaria mercenaria* normalized transcriptome assembly. Inserts: size frequency distribution of assembled contigs (top) and singletons (bottom). For dataset details see Supplementary file 2.1.

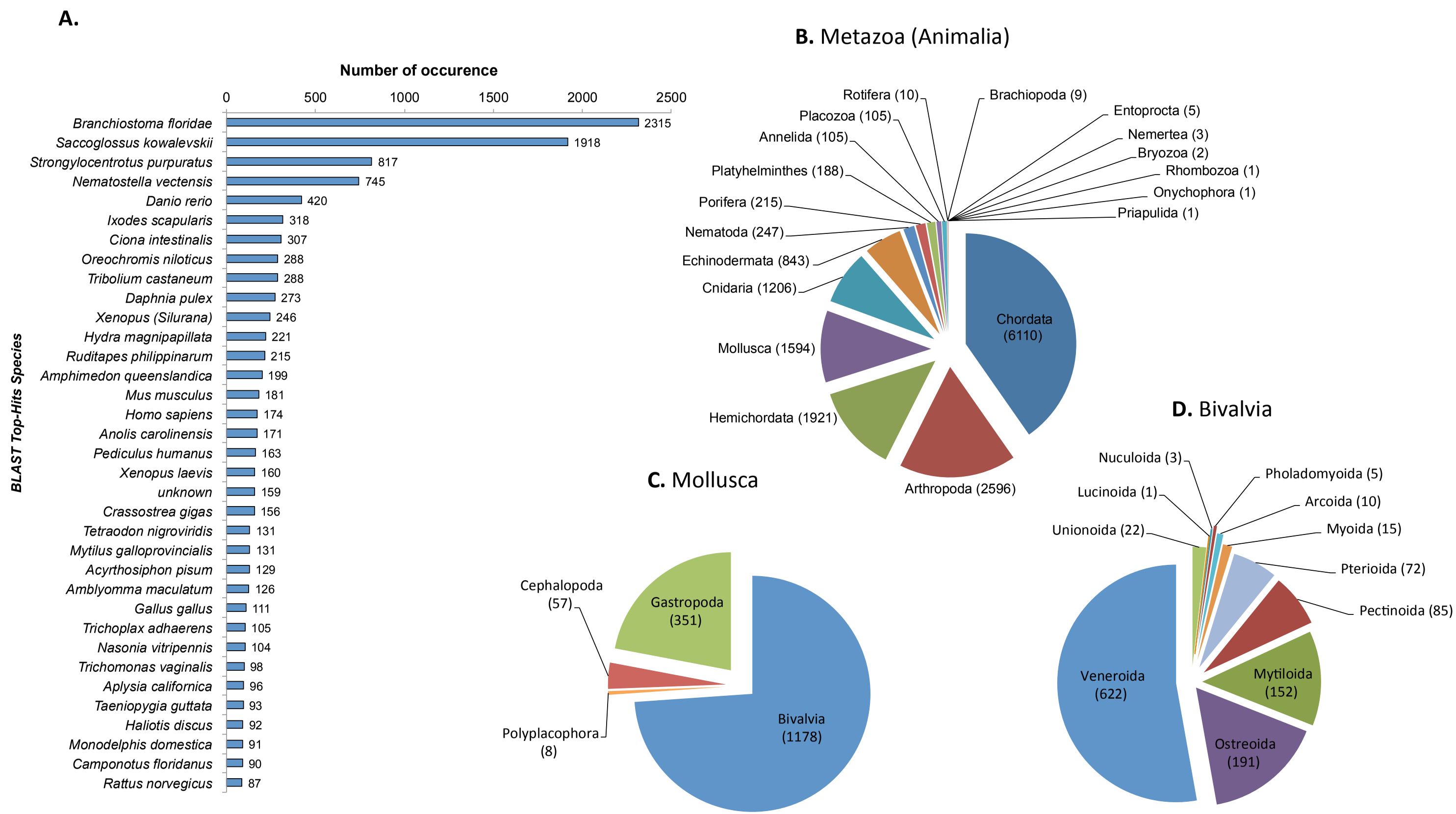


Figure 2.2. Taxonomy classification and top species distribution of annotated sequences generated by BLASTx. Numbers on the bar graph and pie charts indicate the number of occurrences for each species or taxonomic group. A: The top 35 species hit by BLASTx. B: BLASTx hit distribution by Phylum of the Kingdom Metazoa (Animalia). C: BLASTx hit distribution by Class in the Phylum Mollusca. D: BLASTx hit distribution by Order in the Class Bivalvia.

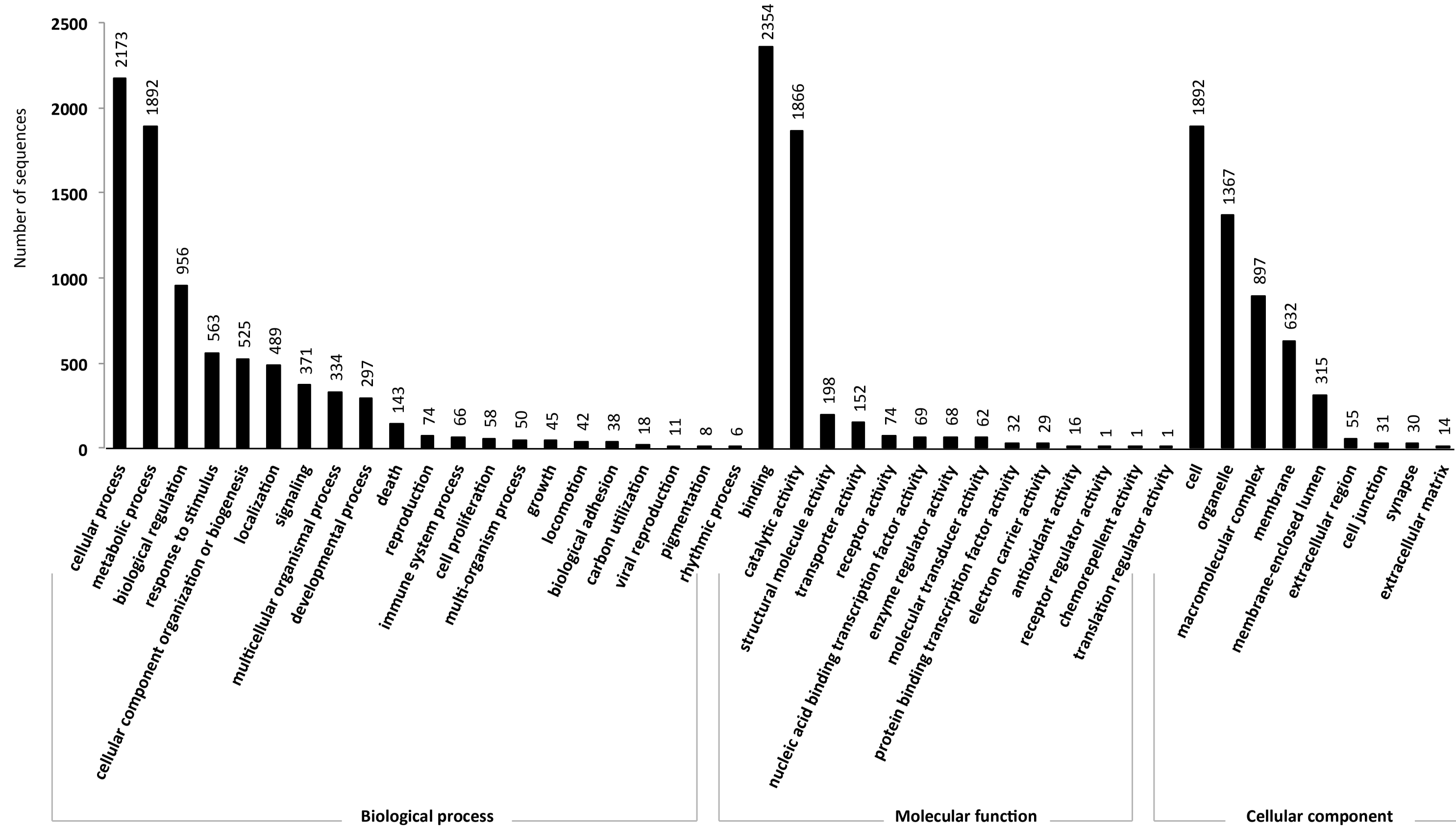


Figure 2.3. Classification of annotated sequences by GeneOntology Terms at level 2. GO terms of biological process, molecular function and cellular component were assigned to annotated sequences. Numbers above each column refer to the occurrence of the GO annotation term at level 2.

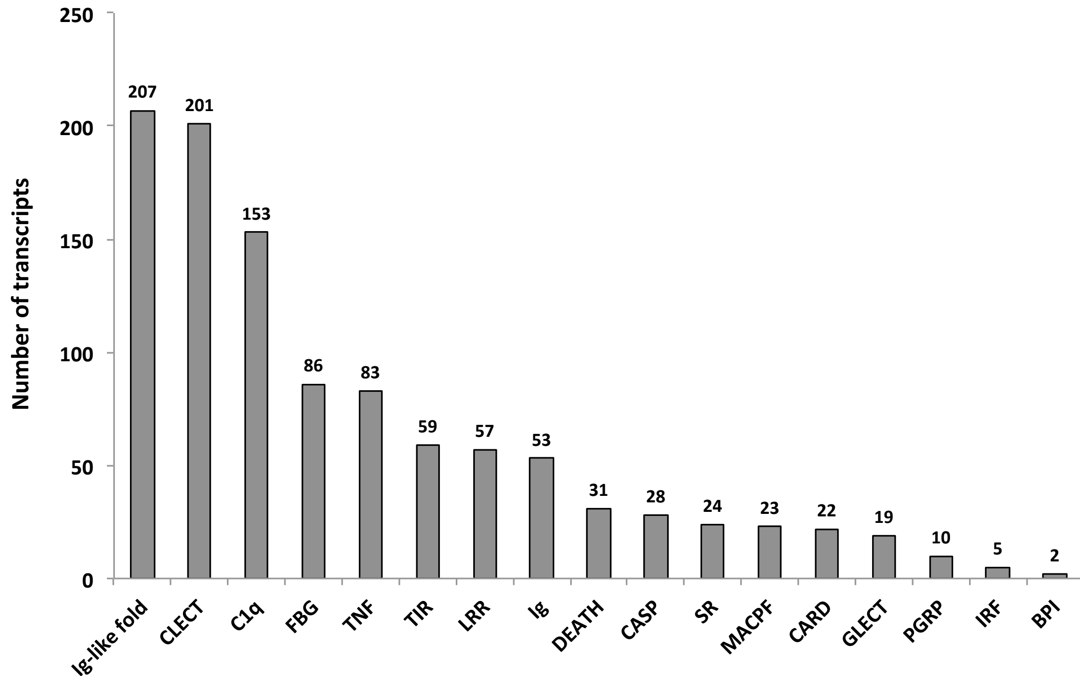


Figure 2.4. A high diversity of protein domains with functions related to immunity was detected in *M. mercenaria* transcriptome. The histogram represents the number of transcripts with immune-related protein domains annotated by InterProScan. Ig-fold: Immunoglobulin-like fold (IPR013783); CLECT: C-type lectin (IPR001304); C1q: complement component C1q domain (IPR001073); FBG: fibrinogen-related domains (IPR002181); TNF: tumor necrosis factor family (IPR006052); TIR: toll-interleukin 1-receptor (IPR000157); LRR: leucine-rich repeat (IPR001611); Ig: immunoglobulin, (IPR003599); DEATH: DEATH domain, found in proteins involved in cell death (IPR000488); CASP: caspase (IPR029030); MACPF: membrane-attack complex/perforin (IPR020864); SR: scavenger receptor cystein-rich domain (IPR017448); CARD: caspase recruitment domain (IPR001315); GLECT: galectin (IPR001079); IRF: interferon regulatory factor (IPR001346); PGRP: animal peptidoglycan recognition protein homolog (IPR006619); BPI: BPI/LBP/CETP: lipopolysaccharide-binding protein (IPR001124 and IPR017942).

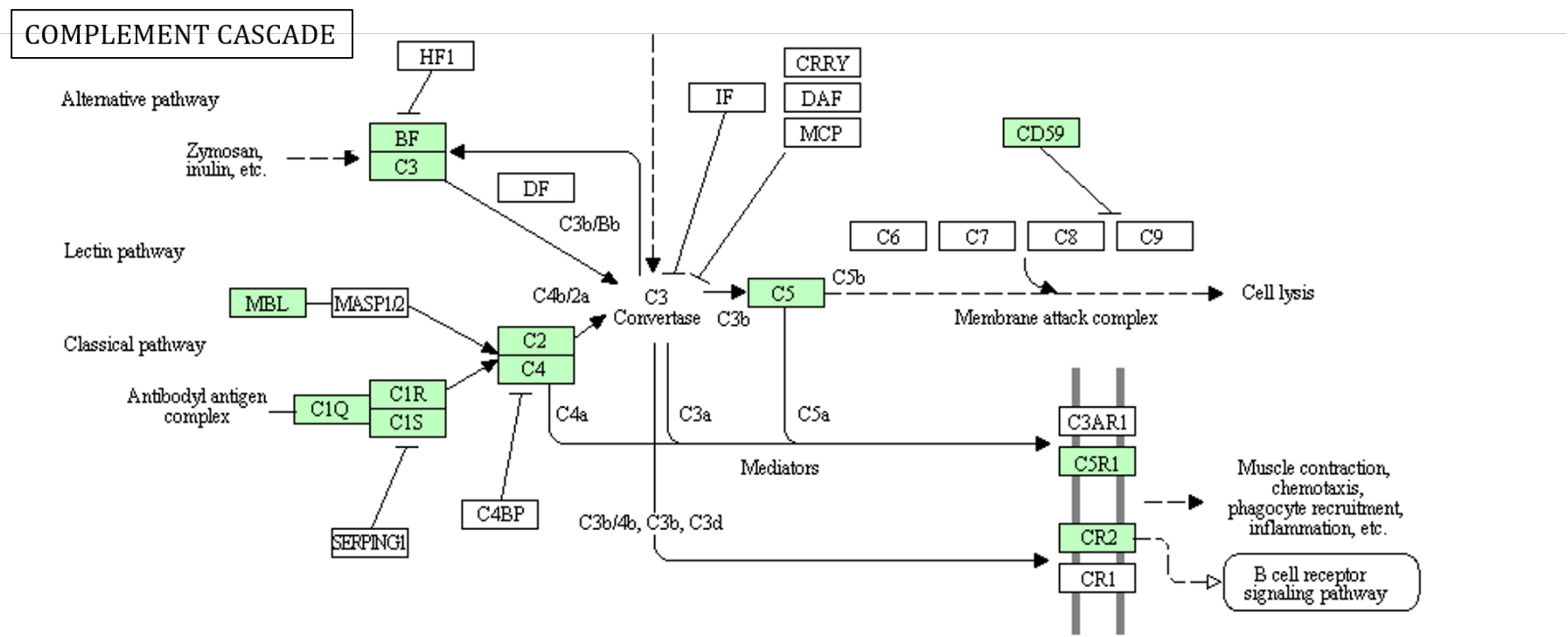


Figure 2.5. Schematic comparison of the complement cascade identified in *M. mercenaria* transcriptome to the KEGG reference pathway. Green boxes indicate pathway components identified in *M. mercenaria* and white boxes the absent ones.

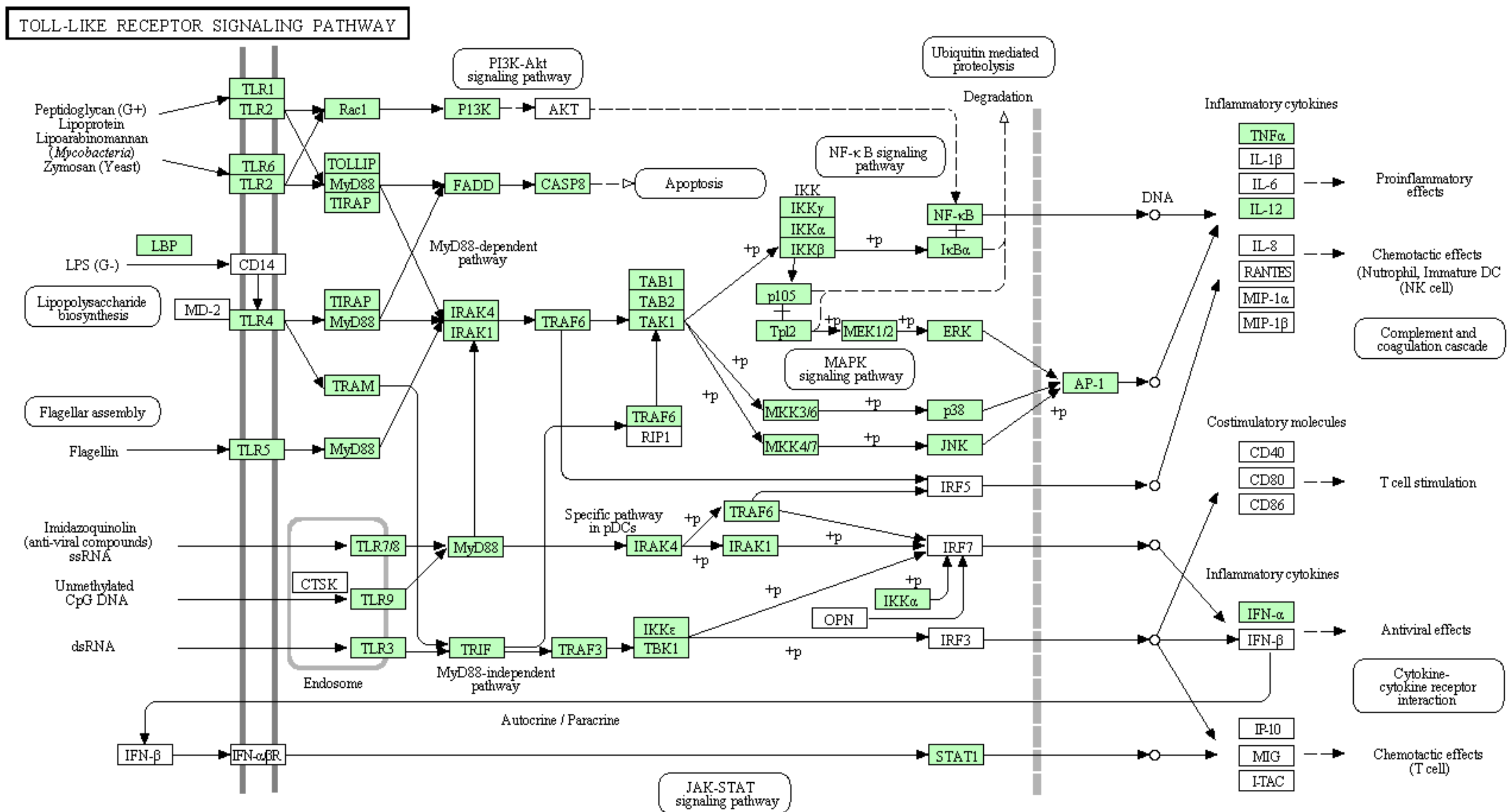


Figure 2.6. Schematic comparison between TLR pathway members identified in *M. mercenaria* transcriptome and the KEGG reference TLR pathway. Green boxes indicate pathway components identified in *M. mercenaria* and white boxes are the absent ones.

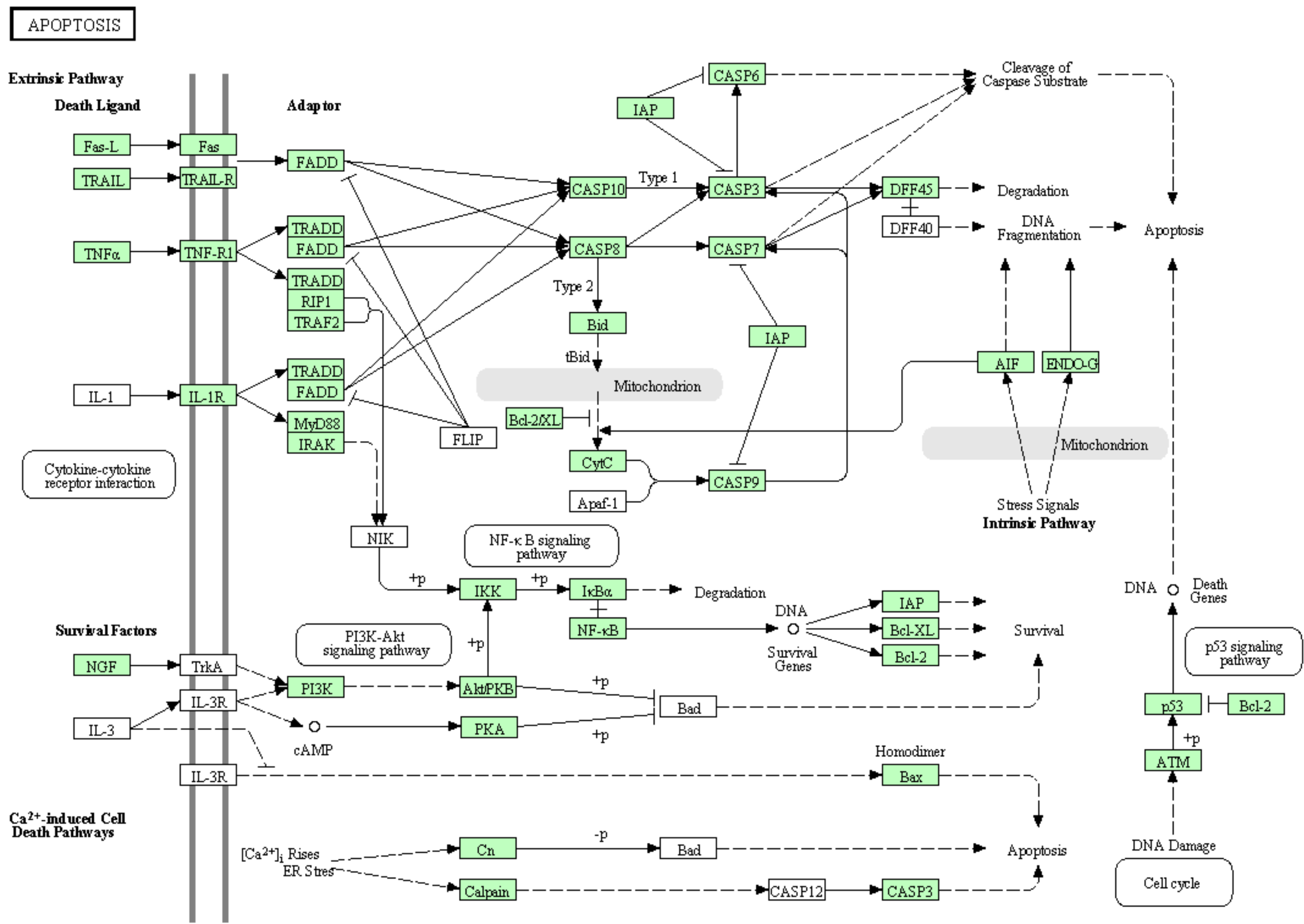
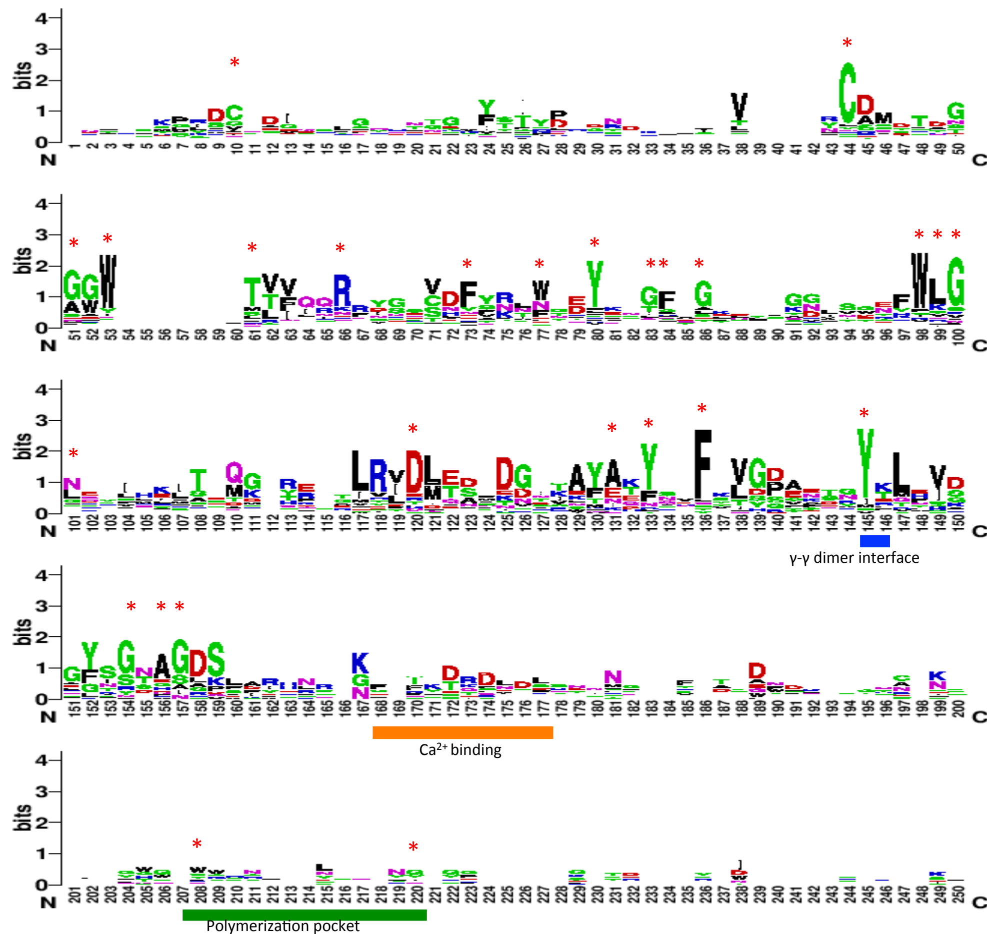


Figure 2.7. Schematic comparison of apoptosis pathway deduced in *M. mercenaria* and the KEGG reference pathway. Green boxes indicate pathway components identified in *M. mercenaria* transcriptome and white boxes are the absent ones.

A.



B.

	10-C	44-C	51-G	53-W	61-T	66-R	73-F	77-W	80-Y	83-G	84-F	86-G	98-W
M. mercenaria (86)	54.0%	78.1%	54.0%	74.7%	83.9%	74.7%	63.2%	51.7%	73.5%	56.3%	57.4%	78.2%	80.5%
C. gigas (205)	28.4%	74.4%	81.2%	82.8%	80.2%	95.3%	93.4%	90.0%	91.6%	94.8%	96.1%	96.1%	96.6%
C. virginica (56)	96.6%	100%	91.4%	91.7%	93.2%	94.4%	86.8%	86.8%	89.7%	94.9%	94.9%	92.5%	92.3%
S. purpuratus (59)	73.8%	90.9%	61.9%	88.4%	84.5%	100%	95.9%	76.0%	96.0%	96.0%	96.0%	96.0%	94.0%

	99-L	100-G	101-N	120-D	131-A	133-Y	136-F	145-Y	154-G	156-A	157-G	208-W	220-G
M. mercenaria (86)	64.7%	78.2%	52.8%	87.3%	82.8%	88.5%	92.4%	94.3%	87.3%	87.4%	87.4%	86.8%	87.4%
C. gigas (205)	53.0%	95.8%	94.2%	90.2%	65.3%	96.7%	90.7%	91.2%	76.5%	70.8%	74.7%	96.0%	56.7%
C. virginica (56)	93.5%	97.5%	88.2%	93.6%	93.5%	95.0%	92.7%	96.8%	88.9%	93.4%	89.2%	97.3%	91.4%
S. purpuratus (59)	89.6%	100%	95.1%	97.1%	86.7%	88.2%	82.4%	89.3%	82.3%	83.9%	82.9%	81.4%	84.4%

Figure 2.8. Diversity of FBG domains in *M. mercenaria*. A: Sequence logos representing the conservation levels of individual amino acid residues of FBG domains. The highly conserved residues in reference to human FBGs are marked with red asterisks. Frequency of each amino acid at that position is indicated by its height. B. The conservation of the invariable positions in human FBG domains among *M. mercenaria*, *C. gigas*, *C. virginica* and *S. purpuratus* (low percentages indicate high variability). The number of FBG domains examined in each species is given between parentheses.

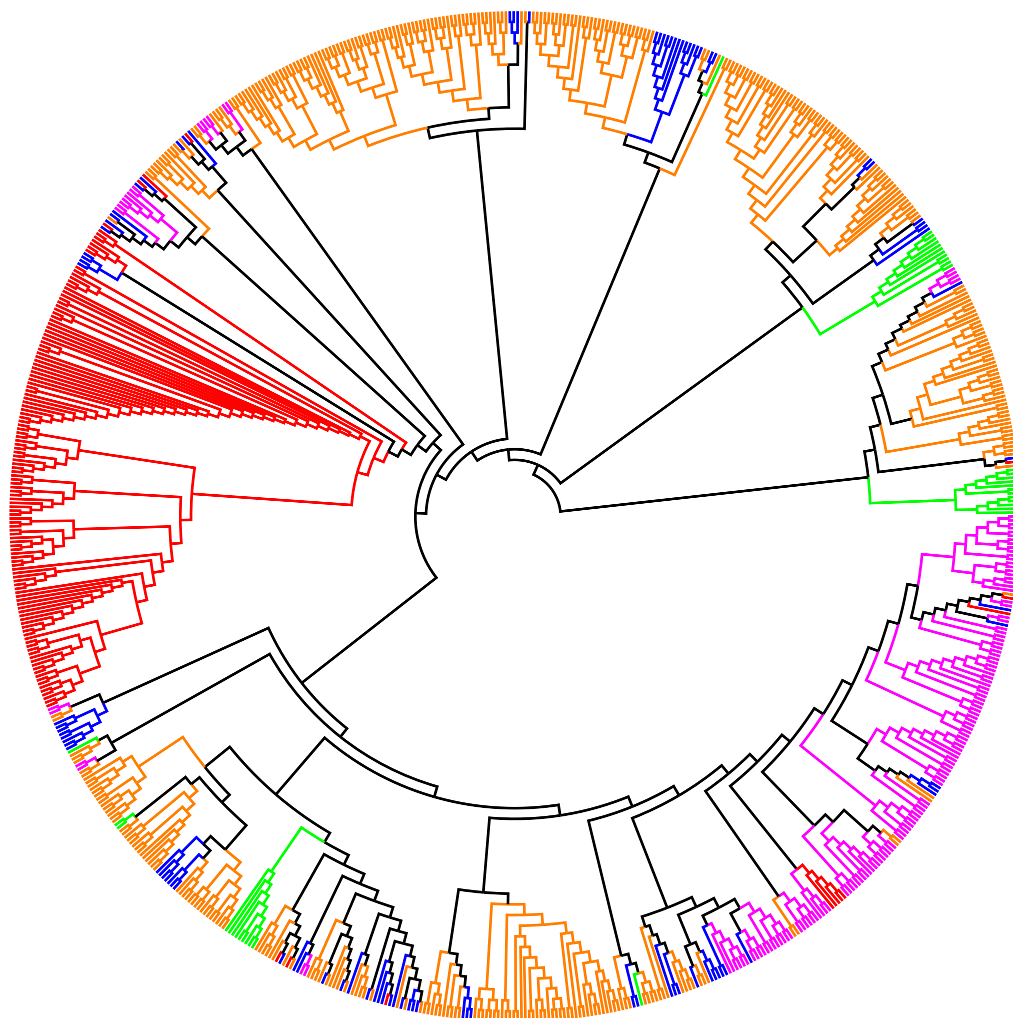


Figure 2.9. Neighbor-joining phylogenetic tree of molluscan FREPs. The statistical frequency and taxon names are not shown. Different colors indicate the FBG domain sequences from different species. *M. mercenaria*: blue, *C. gigas*: orange, *L. gigantea*: pink, *B. glabrata*: red, *M. edulis*: green.

Supplementary files:

Supplementary file 2.1. Sequence summary of the *M. mercenaria* transcriptome. This is a Microsoft Excel Worksheet that contains descriptions and annotation information of individual transcript sequences.

Supplementary file 2.2. Annotation information for putative members of immune-signaling pathways identified in *M. mercenaria*. This is a Microsoft Excel Worksheet that contains descriptions of selected sequences.

Supplementary file 2.3. Schematic comparison of Notch and Wnt pathways in *M. mercenaria* and the KEGG reference pathway. Green boxes indicate pathway components identified in *M. mercenaria* transcriptome and white boxes are the absent ones.

Supplementary file 2.4. Annotation information for putative pattern recognition receptors (PRRs) identified in *M. mercenaria*. This is a Microsoft Excel Worksheet that contains descriptions of selected sequences.

Chapter 3 Clam focal and systemic immune responses to QPX infection revealed by RNA-seq technology

Manuscript published as:

Wang, K., del Castillo, C., Corre, E., Pales Espinosa, E., Allam, B. (2016). Clam focal and systemic immune responses to QPX infection revealed by RNA-seq technology. BMC Genomics 17:146

Abstract

The hard clam *Mercenaria mercenaria* is an important seafood species widely exploited along the eastern coasts of the United States and play a crucial role in coastal ecology and economy. Severe hard clam mortalities have been associated with the protistan parasite QPX (Quahog Parasite Unknown). QPX infection establishes in pallial organs with the lesions typically characterized as nodules, which represent inflammatory masses formed by hemocyte infiltration and encapsulation of parasites. QPX infection is known to induce host changes on both the whole-organism level and at specific lesion areas, which imply systemic and focal defense responses, respectively. However, little is known about the molecular mechanisms underlying these alterations.

RNA-seq was performed using Illumina Hiseq 2000 (641 Million 100 bp reads) to characterize *M. mercenaria* focal and systemic immune responses to QPX. Transcripts were assembled and the expression levels were compared between nodule and healthy tissues from infected clams, and between these and tissues from healthy clams. *De novo* assembly reconstructed a consensus transcriptome of 62,980 sequences that was functionally-annotated. A total of 3,131 transcripts were identified as differentially expressed in different tissues. Results allowed the identification of host immune factors implicated in the systemic and focal responses against QPX and unraveled the pathways involved in parasite neutralization. Among transcripts significantly modulated upon host-pathogen interactions, those involved in non-self recognition, signal transduction and defense response were over-represented. Alterations in pathways regulating hemocyte focal adhesion, migration and apoptosis were also demonstrated.

Our study is the first attempt to thoroughly characterize *M. mercenaria* transcriptome and identify molecular features associated with QPX infection. It is also one of the first studies contrasting focal and systemic responses to infections in invertebrates using high-throughput sequencing. Results identified the molecular signatures of clam systemic and focal defense responses, to collectively mediate immune processes such as hemocyte recruitment and local inflammation. These investigations improve our understanding of bivalve immunity and provide molecular targets for probing the biological bases of clam resistance towards QPX.

Keywords: Hard clam, *Mercenaria mercenaria*, QPX, RNAseq, Immune response, Focal, Systemic

3.1. Background

The hard clam, *Mercernaria mercenaria*, is an ecologically- and economically-important marine bivalve species that thrives along the northeastern coasts of the United States and Maritime Canada. In the past few decades, the hard clam industry has been severely impacted by a protistan parasite called QPX (Quahog Parasite Unknown), which is responsible for mortality episodes in both wild and cultured clam populations (Anderson et al., 2003b; Dove et al., 2004b; Ford et al., 2002; MacCallum and McGladdery, 2000; Ragone Calvo et al., 1998; Smolowitz et al., 1998; Whyte et al., 1994). QPX is believed to be an opportunistic pathogen and has been detected in a wide variety of environmental substrates including sediments, marine aggregates and other organic matrices (Gast et al., 2008; Liu et al., 2009; Lyons et al., 2005). Interestingly, previous reports highlight the ability of QPX to sustain very low abundance in clams without causing disease outbreaks until it encounters hosts with reduced immunity or following shifts of environmental conditions that add to the virulence of the parasite, under which conditions QPX can take advantage to establish infection sometimes leading to severe clam mortality events (Ford et al., 2002; Perrigault et al., 2011).

Lesions caused by QPX, usually associated with the presence of nodules, are commonly found in pallial tissues, such as alongside the inner rim of the mantle or at the base of the siphon (Smolowitz et al., 1998; Whyte et al., 1994). These places are widely considered as the portal of entry for QPX cells acquired from the surrounding environment during suspension-feeding (Dove et al., 2004b; Ragone Calvo et al., 1998). The QPX nodules represent inflammatory masses containing both parasite cells and abundant clam hemocytes, resulting from a series of comprehensive host immune responses induced by the infection that leads to massive focal hemocyte infiltration, parasite encapsulation and partial necrosis of the affected area (Whyte et al., 1994). Like other invertebrates, the hard clam lacks the specific immune responses and their defense mechanism mainly relies on the effectors of innate immunity, which is mediated by circulating hemocytes and highly diversified humoral antimicrobial factors. These cellular and humoral immune components work in a synergistic way to initiate the recognition, segregation and ultimately elimination of pathogens and other non-self entities (Bayne, 1990; Canesi et al., 2002). The launching of innate immune responses involves myriad cellular and humoral events modulated not only at the infection sites (focally) but also at a larger, whole-organism scale (systemically). In general, the focal response represents the alterations driven by direct host-pathogen interactions at the infection sites where direct cell-cell (e.g. molecular patterns) interactions mediate the response, while the systemic response reflects overall modifications within the host as a result of the ongoing infection and is mainly associated with dynamic changes of circulating hemocytes and their secreted immune mediators.

Most of the previous investigations have solely focused on the systemic response of *M. mercenaria* against QPX during the infection events, where changes in cellular and humoral immune parameters (e.g. anti-QPX activity and lysozyme activity in clam plasma, hemocyte

phagocytic activity, reactive oxygen species (ROS) production, etc.) as well as expression of a limited number of immune-related genes in tissues and circulating hemocytes were assessed (Perrigault and Allam, 2012; Perrigault et al., 2012; Perrigault et al., 2011; Perrigault et al., 2009c). In contrast, no previous studies have focused on the characterization of clam focal response at the infection sites. Given the fact that QPX disease is usually focal with formation of well-delimited lesions, the study of clam immune responses at the infection site in the lesions *per se* is of specific value as it provides insights to better characterize cellular interactions between the hard clam and QPX upon their contact. In this framework, QPX disease in clams offer a unique opportunity to contrast focal and systemic responses against microbial diseases in invertebrates allowing for a more comprehensive understanding of defense strategies used by these animals to fend microbial attacks.

Our study aimed to characterize the gene regulation features of *M. mercenaria* during QPX infection by profiling the transcripts at the infection lesion and compare focal clam responses with systemic responses detected in healthy tissues from infected clams (in addition to a parallel comparison with tissues from healthy clams). This study allowed the identification of factors involved in the interactions with the parasite as well as molecular pathways activated by the host to neutralize QPX.

3.2. Methods

3.2.1. Clam tissue and RNA samples preparation

Adult hard clams (54 ± 5 mm in length, mean ± standard deviation) were collected from a QPX enzootic area in Massachusetts. Clams were shucked and grossly examined for the presence of nodules along the mantle rim. Nodule tissues were dissected and a small piece of each nodule was microscopically examined (fresh mount) for the identification of QPX cells. The positive nodules were then submitted to RNA extraction. Meanwhile, a piece of seemingly healthy tissue that is anatomically symmetrical to the nodule was collected from the same clam (e.g. "healthy" tissue from a diseased clam) and divided into 2 aliquots, with the first aliquot used for QPX detection by qPCR (Liu et al., 2009) and the second used for RNA extraction. Mantle tissues were also collected from "seemingly" healthy clams (no visible nodules) and used for QPX detection and RNA extraction. Following confirmation of disease status by qPCR, the samples were divided into the following 3 categories: (1) infected tissue from a diseased clam, (2) "healthy"(non-nodule) tissue from a diseased clam, and (3) "healthy" tissue from a healthy clam. Total RNA was individually extracted using TRIzol ® Reagent (Invitrogen, Carlsbad, CA, USA). Further RNA clean-up and on-column DNase digestion were performed with RNeasy Mini Kit (Qiagen Ltd., Crawley, UK) according to the manufacturer's guideline. RNA quantity and quality were analyzed on Nanodrop ND-1000 (Thermo Scientific, Wilmington, USA). Only RNA samples with absorption ratios of A260/A280 close to 2.0 were used for RNA-seq analysis.

A total amount of 3 μg RNA per sample was pooled into a total of 3 pools (3 clams per pool) representing each tissue category (9 pools in all). The pooling strategy is shown in Table 3.1.

3.2.2. RNA sequencing, *de novo* assembly and annotation

The sequencing of each pooled RNA sample as a paired end (PE) reads library (100 bp) was performed on Illumina HiSeq 2000 platform at the McGill University and Genome Quebec Innovation Center (Montreal, Canada), producing from 27.1 up to 46.8 millions of reads per sample (Table 3.1). Raw reads were filtered and trimmed according to length and quality score (min length 60 nt, end trimming quality 25, min quality filtering: 20 on 75% of the read length) using the FASTX-Toolkit software v 0.0.13 (http://hannonlab.cshl.edu/fastx_toolkit/). rRNA cleaning was performed using the riboPicker software v 4.0.3 (http://ribopicker.sourceforge.net) (Schmieder et al., 2012) against SILVA database v111. And finally pair retrieval was performed using a homemade python script accessible through the ABiMS platform Galaxy instance (http://galaxy.sb-roscoff.fr). High quality filtered sequence reads from all libraries were combined and subsequently used for de novo assembly. Assembly was based on the de Bruijn graph assembler Trinity (http://trinityrnaseq.sourceforge.net/) (Grabherr et al., 2011) using the default parameters. Assembly quality was controlled by re-mapping raw reads back to the transcripts using bowtie 2 and RSEM within Trinity package scripts. The assembled transcripts with sequence length longer than 200 bp, re-mapping FPKM (fragments per kilobase of transcript per million mapped reads) greater than 1 and isoform discovery level greater than 1% were then considered for the following annotation and transcript abundance quantitation. Annotation of this *de novo* assembled transcriptome was performed using Blast2GO (http://www.blast2go.org/) with a semi-automated functional annotation based on sequence similarity search. Putative gene identities were obtained by Blastx search against National Center for Biotechnology Information (NCBI) non-redundant sequences (nr) database with the E-value threshold setting at 1E-03. Putative gene functions were predicted by sequence similarity search against Gene Ontology (GO, http://www. geneontology.org/) database and assigning GO annotation terms to each mapped transcript. Protein domain search and enzyme annotation were also performed using InterPro scan and the Kyoto Encyclopedia of Genes and Genomes (KEGG). KEGG Orthology (KO) terms and KEGG pathways were also assigned to the assembled sequences using the online KEGG Automatic Annotation Server (KAAS, http://www.genome.jp/kegg/kaas/) using bi-directional best-hit methods (Moriya et al., 2007). This server provides KO annotation and pathway mapping.

3.2.3. Differential gene expression analysis

The consensus transcriptome generated in the previous steps was used as a reference for transcript abundance analysis. Reads from each single library were separately mapped to this reference transcriptome using the “RNA-Seq by Expectation-Maximization (RSEM)” method that is bundled within the Trinity package. The expression level of each transcript was

determined as the total mapped reads count. The differences in gene expression between clam tissue samples (nodule, non-nodule and healthy tissues) were estimated using the DESeq Bioconductor package (https://github.com/Bioconductor-mirror/SESeq/tree/release-3.2) (Anders and Huber, 2010) in R statistical software (R Development Core Team, 2010; http://www.R-project.org). The threshold for defining significant differentially expressed (DE) transcripts between two different conditions (3 replications in each condition) was set as adjusted *p*-value smaller than 0.001 and absolute $\log_2$ (fold change) values greater than 2. Expression patterns of DE transcripts were also analyzed by a K-means clustering method using Euclidean distance based on expression levels over all input samples. For further analysis, only those DE transcripts with annotation were considered as candidates of interest and were subsequently divided into over- and under-expressed groups.

3.3. Results and discussion

3.3.1 Illumina sequencing and *de novo* transcriptome assembly

The main objective of this study was to identify molecular features of *M. mercenaria* in response to QPX infection and to compare the immune-related pathways involved in the lesion-specific focal response with the whole-organism scale systemic response. The high-throughput Illumina RNA sequencing and *de novo* assembly employed in this investigation allowed the construction of the transcriptome in the absence of *M. mercenaria* genome information. A total of 640,596,320 raw reads were generated from the Illumina paired-end sequencing with about 27 to 48 millions paired-end reads generated from each of the 9 sequenced libraries (Table 3.1, Figure 3.1A). The short read sequences generated from this RNAseq project have been deposited at the NCBI short Read Archive database under the SRA accession number SRP068241. Trimming and filtering procedures yielded 606,021,407 clean reads that were used for the *de novo* assembly of consensus transcriptome based on all sequenced RNA libraries in order to maximize the diversity of transcripts. This allowed 90.61 to 92.20% of the reads from the 9 libraries be used for the transcriptome assembly. A total of 62,980 transcripts ranging from 201 to 23,103 bp with average size of 1297.59 bp and median size of 835 bp were produced from the assembly after low FPKM and rare isoforms filtering. The size distribution of all the *de novo* assembled transcripts is shown in Figure 1B. Once the transcriptome was constructed, the 9 libraries were individually remapped to the 62,980 transcripts and resulted in 85.27% to 89.05% of reads remapping. Theses counting data were then used for DE analysis.

3.3.2. Transcriptome functional annotation

The transcriptome annotation performed using Blast2GO returned a total of 19,107 transcripts (30.3%) with significant BlastX matches to other sequences in NCBI nr database (E-value< 10E-03) (Figure 1). Not surprisingly, the top 3 species that had the most similarity to *M. mercenaria* sequences were mollusks with available genomes and included the Pacific oyster, *Crassostrea*

gigas (7,467), followed by the owl limpet *Lottia gigantea* (3,539) and the California sea slug *Aplysia californica* (1,931) (Figure 1C). KEGG Orthology (KO) terms were assigned to 6,425 sequences and reference pathways were mapped to the KEGG database based on the assigned KO terms (Figure 3.1A, Additional file 3.1). A total of 29,815 sequences were identified to match to at least one conserved protein domain in the InterPro database (Figure 3.1A, Additional file 3.1).

Gene ontology (GO) assignments were used to classify functions of the predicted clam proteins. Based on sequence similarity (E-value of 0.001), 13,584 sequences were assigned to at least one GO annotation (Figure 3.1A, Additional file 3.1). As summarized in Figure 3.2, a total of 8,168, 4,600 and 4,231 sequences were respectively categorized into the three main categories: biological process, cellular component, and molecular function at the second functional annotation level. The most dominant terms presented in the three categories are “cellular process”, “metabolic process”, “binding”, “catalytic activity”, “cell”, and “organelle”. Very few transcripts were clustered into “rhythmic process”, “cell killing”, “protein tag”, “channel regulator activity”, “nucleoid” or “virion”. It is noticeable that a good fraction of transcripts were clustered into the immune-related categories of response to stimulus (503), immune system process (43) and biological adhesion (38). Those transcripts were of special interest given that they might be involved in the *M. merceneria* defense and resistance toward QPX infection.

A significant portion (69.7 %) of *M. mercenaria* transcripts did not have BlastX match in NCBI nr database, in agreement with previous transcriptomic studies in mollusks (Adema et al., 2010; Leite et al., 2013; Teaniniuraitemoana et al., 2014; Zhao et al., 2012). Most of the unannotated transcripts may represent transcripts spanning untranslated mRNA regions, or transcripts containing only non-conserved protein domains (Mittapalli et al., 2010; Wang et al., 2004).

3.3.3. Identification of differentially expressed transcripts

The generated transcriptome was used as a reference for downstream investigations of global gene expression in the three different tissues of interest (nodule, non-nodule and healthy) to identify genes associated with *M. mercenaria*’s focal and systemic immune response against QPX. A gene-isoform relationship was estimated using RSEM over Trinity output isoforms. Results showed that about 43% (27,021) of all the transcripts had 1 isoform, 19% (12,307) had 2 isoforms and 38% (23,652) had 3 isoforms, suggesting extensive isoform diversity in *M. mercenaria* transcriptome. Transcript isoform variation could affect mRNA stability, localization and translation, as well as the production of protein variants that differ in localization or function (Pelechano et al., 2013).

By comparing the number of transcripts expressed in each sample, the contribution of specific samples to the analysis can be estimated. The highest number of expressed transcripts was found

in the nodules of infected clams, which were closely followed by that found in the healthy clam samples (Figure 3.3A). The lowest number of expressed transcripts came from non-nodule samples of QPX infected clams, with about 1,500 less transcripts expressed than the other two samples. Read coverage, which is critical in accurate determination of fold change, averaged 477, 422 and 509 reads per transcript for nodule, non-nodule and healthy tissue samples, respectively (Figure 3.3B).

Statistical analysis by DEseq identified 3,131 differentially expressed (DE) transcripts from the pair-wise comparisons ($|\log_2$ (fold change)$| > 2$, adjusted p-value < 0.001) between clam tissue samples (Figure 3.4). In nodules, a total of 829 transcripts, including 408 over-expressed and 421 under-expressed transcripts were identified as compared to non-nodule samples of QPX infected clams. Compared to tissues from healthy clams, 1,591 DE transcripts were identified in nodules with 864 over-expressed and 727 under-expressed transcripts. Similarly, 1,681 DE transcripts were obtained from the comparison between healthy and non-nodule clam tissues, of which 513 and 1,168 were over- and under-expressed, respectively (Figure 3.4, Additional file 3.2 and 3.3). A total of 1,694 of these DE transcripts were annotated by BlastX search against the NCBI nr database (e-value <10E-03), which were further examined for their putative functions during *M. mercenaria* immune response toward QPX. Annotated transcripts were subsequently grouped into curated categories according to their biological functions based on the gene ontology (GO) terms and literature searches highlighting immune functions. DE transcript sets were further examined in reference to the assigned KO terms for the analysis of pathway regulation. Significantly enriched KEGG pathways were identified via the Fisher's exact test ($P<0.01$) (Luo et al., 2009).

Here we specifically focus on DE transcripts drawn from comparisons between "nodule vs. non-nodule" and "non-nodule vs. healthy" tissue samples, which were considered to reflect the transcriptomic changes caused by "focal" and "systemic" immune response of *M. mercenaria* toward QPX infection, respectively. The overview of DE transcripts drawn from these two responses is presented in Figure 3.4 and Additional file 3.2 and 3.3.

3.3.4. Focal response

Clam focal response reflected the alterations caused by direct clam-QPX interaction at the infection site (Additional file 3.2). QPX nodules are inflammatory masses resulting from massive hemocyte infiltration and encapsulation of parasite cells (Smolowitz et al., 1998; Whyte et al., 1994). The processes largely rely on the motility and adhesion properties of hemocytes, which allow these cells to migrate throughout the circulatory system and recruit to the infection site. Hemocytes can sense stimuli in host tissues through an array of cell surface receptors, and use these cues to adjust their behavior (Humphries and Yoshino, 2003). The activation of hemocytes requires the binding of specific ligands to the cell surface receptors, which subsequently initiate the transduction of extracellular signals into the cytoplasm via a variety of signaling pathways, thus inducing a series of hemocyte-mediated immune response such as phagocytosis,

encapsulation (prominent response against QPX in clams), ROS production, as well as secretion of immune effectors and cytokines (Canesi et al., 2006; Hatanaka et al., 2009; Soudant et al., 2013). A collection of DE transcripts involved in these defense processes was identified during focal response, suggesting that strong and complex host-pathogen interactions were taking places inside the QPX lesions (Table 3.2, Additional file 3.2). A large fraction of the DE transcripts of focal response was annotated as receptors or molecules with receptor activities, which putatively contribute to the host defense against QPX as (1) cell surface receptors expressed on hemocytes that mediate the recognition and phagocytosis or encapsulation of foreign entities through microbe associated molecular patterns (MAMP); (2) signaling receptors activating intracellular signaling cascades or (3) the soluble bridging molecules mediating the linkage between MAMPs and hemocytes (Jeannin et al., 2008). Among those receptors, most are identified as pathogen pattern recognition receptors (PRRs), which include the C-type lectins (CTLs), the scavenger receptors (SRs) and the toll-like receptors (TLRs).

The C-type mannose receptor-2 (MRC2) identified during the focal response (Table 3.2) is a member of the C-type lectins (CTLs) superfamily, a large group of Ca^{2+}-dependent carbohydrate-binding proteins that play crucial roles in innate immunity. CTLs recognize pathogens and facilitate their phagocytosis (Vazquez-Mendoza et al., 2013; Wang et al., 2013) or encapsulation (Ao et al., 2007; Wang et al., 2012b; Yang et al., 2010a). MRCs are also key regulators of inflammatory responses and contribute to the removal of harmful inflammatory agents (Gazi and Martinez-Pomares, 2009; Lasky, 1995). The 16-fold over-expression of MRC2 during the focal response suggested that active hemocyte encapsulation and local inflammation was induced by QPX at the infection lesions, which is consistent with the results of histopathological observations (Whyte et al., 1994). Another over-expressed CTL member, the perlucin-like protein, has been previously shown to trigger immune response in Manila clams during microbial infection (Moreira et al., 2014).

Scavenger receptors (SRs) were also among the strongly over-expressed transcripts in infection foci (Table 3.2). These included somatomedin-b and thrombospondin type-1 domain-containing (RPE spondin), insulin-related peptide receptor, hemicentin-1, lysyl oxidase-like protein 2 and mam domain-containing glycosylphosphatidylinositol anchor protein 1. SRs are structurally diverse PRRs that share the common function of recognizing oxidized or acetylated low-density lipoprotein (LDL) (Gordon, 2002). They contribute to innate immunity by recognizing MAMPs and mediating non-opsonic phagocytosis (Jeannin et al., 2008; Liu et al., 2011). They are extensively found on immune cells and are able to interact with both modified-host components and exogenous ligands, which makes SRs a key component in host defense, apoptosis, inflammation and lipoprotein homeostasis (Blumbach et al., 1998; Mukhopadhyay and Gordon, 2004; van der Laan et al., 1999). For example, scallop SRs bind not only to acetylated LDL but also to MAMP including lipopolysaccharides (LPS), peptidoglycans (PGN), mannan and zymosan particles (Liu et al., 2011). The sea urchin genome encodes approximately 150 genes consisting of one or more scavenger receptor cysteine-rich (SRCR) domains (Bowdish

and Gordon, 2009), and the members of this gene family exhibit dynamic shifts in transcription after immune challenge (Mukhopadhyay and Gordon, 2004; Rast et al., 2006).

Our results also show an over-expression of TLR-1 and Toll-8/tollo in nodules (Table 3.2), which is in agreement with previous investigations showing up-regulation of TLRs in *M. mercenaria* mantle following QPX challenge (Perrigault et al., 2009c). TLRs are among the most ancient and conserved PRRs. They are expressed by immune cells and interact with a large variety of MAMPs. Bivalve TLRs have been characterized in the oyster *C. gigas* and the scallop *C. farreri* where they exhibited significant response to LPS stimulation (Qiu et al., 2007; Zhang et al., 2013b). Transcriptional modulation of TLRs has also been reported in *Ruditapes philippinarum* and *Mya arenaria* following MAMPs stimulation and bacterial challenge (Mateo et al., 2010; Moreira et al., 2012a). Interestingly, Toll-8 (Tollo) has been shown to participate in *Drosophila* epithelial immunity where it mediates host cells communication that subsequently activates systemic immune responses (Akhouayri et al., 2011). This suggests that the Toll pathway could be one of the crucial links that allow coordination between focal and systemic immune components during infection.

The QPX nodules are formed as the result of granulomatous inflammation, which is a chronic inflammatory reaction characterized by focal accumulation of activated immune cells to isolate the invading agent (de Brito and Franco, 1994; Williams and Williams, 1983). The formation of granuloma requires local recruitment of hemocytes at the site of infection to execute extracellular defense processes around the invaders (Williams and Williams, 1983). An array of transcripts associated with cell migration, adhesion and proliferation was regulated in nodules, including G-protein coupled receptors (GPCRs) and integrins families (Table 3.2). GPCRs regulate inflammatory response via binding to chemokines and chemoattractants, thus activating pathways mediating hemocyte migration and adhesion (Sun and Ye, 2012). They also activate transcription factors in immune cells, thus modulating the synthesis and secretion of certain pro- or anti-inflammatory substances (Cho and Kehrl, 2009). Integrins represent a major group of cell adhesion mediators (Zhang and Wang, 2012). They not only modulate cell-cell and cell-extracellular matrix adhesion, but also affect multiple signal transduction cascades regulating cell survival and proliferation (Zhang and Wang, 2012). Overexpression of GPCRs and integrins in nodules suggests their role in hemocytes adhesion and aggregation associated with the formation of granuloma (Stavitsky, 2004; Williams and Williams, 1983).

Several enzymes involved in ROS production were also over-expressed during focal response (Table 3.3). These included a dual oxidase, which is a key component mediating host-microbe interactions in mucosa (Bae et al., 2010; Ha et al., 2005). Dual oxidase regulates oxidative burst and ROS production in the gill muscosa of the shrimp *Marsupenaeus japonicus*, favoring shrimp survivorship during viral infections (Inada et al., 2013). Interestingly, transcripts of dual oxidase were only expressed in nodules, suggesting this enzyme was induced upon direct clam-QPX interactions as a part of the mantle mucosa-related immune response. Other transcripts associated with oxidation-reduction processes also exhibited nodule-exclusive pattern, including the allene

oxide synthase-lipoxygenase (AOSL), lysyl oxidase-like protein (LOXL), ww domain-containing oxidoreductase (WWOX), c-terminal binding protein (CtBP), isocitrate dehydrogenase (ICD) and methylenetetrahydrofolate reductase (MTHFR). These molecules are important for maintaining the redox homeostasis of extracellular environment as they are key effectors for oxi-reduction reactions. Over-expression of these transcripts in nodules suggests the need for the host to promptly balance out excessive ROS and other toxic intermediates produced during interaction with QPX. In addition to regulation of redox process, many of these molecules also take part in the immune modulation indirectly. For example, AOSL play a role in coral immunity by controlling the production of the inflammation regulator arachidonic acid during apoptosis (Libro et al., 2013), and LOXL acts both as a scavenger receptor and regulator for extracellular matrix remodeling that initiate hemocyte migration and tissue regeneration (Bignon et al., 2011), while WWOX was shown to promote proliferation of immune cells through inhibition of their apoptosis (Chang et al., 2007; Yang et al., 2013). In addition, ICD, MTHFR and cytochrome p450 are major detoxification enzymes (Behrendt et al., 2010; Pan et al., 2011). In fact, immune cells and their secreted effectors require the proper redox state in the extracellular environments to exert their immune functions, which makes the maintenance of redox homeostasis essential for persistent and effective host defense (Lotze et al., 2007; Mone et al., 2014). This is particularly true in the case of QPX disease where the neutralization of parasites depends on extracellular killing pathways (Soudant et al., 2013).

Apoptosis is an essential host mechanism to remove damaged and infected cells without causing inflammatory destructions to surrounding tissues (Sokolova, 2009; Terahara and Takahashi, 2008). Interestingly, apoptosis seems to be largely inhibited during *M. mercenaria* focal response, as shown by the down-regulation of pro-apoptosis transcripts and the up-regulation of anti-apoptosis transcripts (Table 3.2). For example, the tumor necrosis factor (TNF)-like protein and 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase-like protein were significantly under-expressed in nodules. Similarly, the pro-apoptotic p53-induced protein and solute carrier family 25 member protein were also under-expressed in nodules. Meanwhile, inhibitor of apoptosis protein (IAP) was over-expressed in nodules. IAPs regulate immune cell expansion and survival in highly inflammatory environments in mammals (Gentle et al., 2014) and they may share similar function in clams by inhibiting hemocyte death during interaction with QPX. In fact, ROS production during parasite killing may trigger apoptotic cell death in molluscs (Terahara and Takahashi, 2008), and proper control of apoptosis mechanisms is required to maintain cellular homeostasis during immune response. This suspected inhibition of host apoptosis is supported by the above-mentioned over-expression of integrins, as these were shown to protect cells from apoptosis and induce anti-apoptotic pathways during cell adhesion and spreading in the snails *Lymnaea stagnalis* (Plows et al., 2006) and *B. glabrata* (Yoshino and Laursen, 1995).

Infection and tissue injury trigger host immune responses via immune signaling pathways (Newton and Dixit, 2012), by activating transcription factors and initiating the production of

immune effectors and regulators. Immune signaling pathways identified in mollusks include Toll, MAPK/JNK and JAK/STAT signaling pathways (Canesi et al., 2006; Moreira et al., 2012a; Philipp et al., 2012; Toubiana et al., 2014). During *M. mercenaria* focal response to QPX, a variety of transcripts encoding kinases and phosphatases were over-expressed (Table 3.2), suggesting the involvement of MAPK and other kinase-mediated cascades in regulating the focal inflammatory response (Theodosiou and Ashworth, 2002), whereas the under-expression of EF-hand domain containing protein and calmodulin may indicate the suppression of calcium-regulated pathways (Grabarek, 2011). Overexpression of E3 ubiquitin-protein ligase and its upstream regulator COP9 signalosome suggests the activation of the damage surveillance ubiquitin/proteasome pathway (Bech-Otschir et al., 2002; Liu et al., 2002; Nezames and Deng, 2012). In parallel, the over-expression of rho GTPase and rho kinase suggests the induction of anti-apoptotic Rho-mediated signaling pathway (Krijnen et al., 2010; Tosello-Trampont et al., 2003) and reinforce the idea that apoptotic inhibition is extensively initiated by *M. merceanria* to help fight QPX. However, a very limited number of DE transcripts was detected in relation to conventional signaling pathways of innate immunity, such as the complement pathway and the Toll/TLR pathway. Only 1 transcript encoding macrophage-expressed gene protein 1 (MPEG1), a putative member of the complement pathway (D'Angelo et al., 2012; He et al., 2011), was differentially expressed in QPX nodules. As for the Toll/TLR pathway, only a few receptors were identified (Table 3.2) but none of their downstream components.

Interestingly, components of the Notch and the Wnt signaling pathways were over-expressed during focal response (Table 3.2). These included two putative Notch family members, the neurogenic locus Notch protein and the mediator protein nicastrin, and the tyrosin-protein kinase RYK which belongs to the Wnt pathway. Wnt signaling pathway regulates many cellular immune processes and is evolutionarily conserved across taxa (Holstein, 2012; Staal et al., 2008a). Wnt signaling has been intensively exploited for its regulatory functions during wound healing and tissue regeneration (Fathke et al., 2006; Zhang et al., 2009b), so its over-expression may be related to wound healing to repair damage resulting from tissue digestion by the parasite or tissue necrosis. The Notch signaling network was scarcely explored in bivalves even though it has been reported to be associated with several aspects of immune response in mammals (Yuan et al., 2010), especially in regulating granulomatous reactions to foreign bodies (Rangel et al., 2014). As an evolutionarily conserved pathway involved in modulating intercellular signaling, the Notch pathway presumably shares an equally important role in *M. mercenaria* by modulating the formation of granuloma. Notch signaling triggers macrophage expression of genes involved in pro-inflammatory responses (Palaga et al., 2008), but can suppress inflammation responses triggered by canonical TLR cascade (Zhang et al., 2012b), in agreement with our observations. These tightly regulated mechanisms ensure tailored immune responses against different pathogens and are crucial for the host to achieve high immune competency while avoiding excessive immune activation and self-inflicted damages.

Several proteases (A.K.A proteinases, peptidases) were also differentially regulated during *M. mercenaria* focal response against QPX. These proteases mostly belong to the serine and metallo protease families and were generally over-expressed in QPX nodules (Table 3.3). Proteases serve as key immune modulators partially through their ability to digest and remodel the extracellular matrix and tissues associated with hemocyte activation (Manoury et al., 2011). Commonly associated with lysosomes and granules of inflammatory cells, serine proteases participate in immune regulation either directly by degradation of pathogens or indirectly through activation of cell surface receptors and signal molecules (Manoury et al., 2011; Meyer-Hoffert and Wiedow, 2011; Shpacovitch et al., 2008). The function of metalloproteases in immune regulation is even more diverse, acting as immune effectors, signal transducers, and mediators of immune cell development and migration (Khokha et al., 2013). Metalloproteases are also known to be involved in many pro-inflammatory pathways, particularly in the Notch pathway where they act as a type of downstream element to Notch (Murthy et al., 2012).

At the same time, immune effectors with known universal protease inhibitor activities, such as alpha2-macroglobulin (α 2M) and thioester-containing protein (TEP, a subfamily of α 2M), were also collectively overexpressed in QPX nodules (Table 3.3). The α 2M superfamily inhibits peptidases of diverse origins (Borth, 1992; Sottrup-Jensen, 1989). The simultaneous over-expression of proteases and protease inhibitors may reflect a finely adjusted defense response of *M. mercenaria* to maintain homeostasis and regulate self- and pathogen-derived proteases, as shown in other host-pathogen systems (Armstrong, 2006; Manoury et al., 2011), including bivalves (Allam et al., 2014). Proteases have been identified as major virulence factors of QPX (Anderson et al., 2003a; Rubin et al., 2014), and are thought to degrade host proteinaceous immune effectors and hydrolyze host tissues to fulfill nutritional requirements. Therefore, inhibition of pathogen proteases contribute to host protection, and was shown to represent a determinant factor for resistance against infectious diseases in bivalves (He et al., 2012; La Peyre et al., 2010; Xue et al., 2006; Yu et al., 2011). In parallel, TEPs have been extensively studied in mollusk immunity (Armstrong, 2006; Moné et al., 2010), and beside their function as protease inhibitors, they also play a role as PRRs or opsonins to facilitate microbial phagocytosis and encapsulation. Consistent over-expression of TEPs was noted in this study in agreement with findings following experimental infection with QPX (Perrigault et al., 2009c). These results support a critical role of TEPs in clam immune response against QPX either via the protease-inhibitor activity of these proteins, or by mediating parasite encapsulation, or both.

In addition, several metal ion transporters were over-expressed in nodules (Table 3.3), including the putative copper ion binding protein ceruloplasmin precursor, the transferrin enzyme ferric-chelate reductase and the divalent metal transporter (A.K.A natural resistance-associated macrophage protein; 328 fold increase). These molecules contribute to host defense by controlling the supply of essential micronutrients in the vicinity of infection sites thus reducing parasite survival (Ong et al., 2006; Stafford et al., 2013) and favoring the production of antimicrobial factors (Sun et al., 2014b). It is noteworthy to point out that some of the focally

over-expressed transcripts might be partly driven by the dramatic increase of hemocyte proportion within tissues in the vicinity of infection foci as compared to the surrounding host tissues (Soudant et al., 2013; Torreilles et al., 1996). This would be especially the case for transcripts known to be highly expressed in hemocytes, such as the cell surface PRRs, secreted humoral immune effectors, cell signal transducers and enzymes associated with ROS production.

3.3.5. Systemic response

Significant transcriptomic alterations were observed during *M. mercenaria* systemic immune response against QPX, with a total of 1,681 DE transcripts, which is about two times the number of focal DE transcripts (829). However, only about one third of the DE transcripts (513) were over-expressed in response to QPX infection, the larger remaining part (1,168) represented significantly under-expressed transcripts (Figure 3.4, Additional file 3.3), possibly due to the chronic stress imposed by the infection. Transcriptome-wide depression has been demonstrated in many marine invertebrates as the result of pathogenic or environmental stress (Libro et al., 2013; Moya et al., 2012; Pauletto et al., 2014; Sun et al., 2014a; Zhao et al., 2012). A considerable number of systemically under-expressed transcripts was related to metabolism and biomineralization, which could be the result of host resource allocation during on-going infection. The chronic inflammation induced by infection likely created extra energy demands, which require resources being allocated from other physiological processes, such as growth and reproduction, to immune processes which are critical for the survival of the host. In fact, slow growth and lower tissue condition are frequently observed in QPX-infected *M. mercenaria* (Smolowitz et al., 1998), and similar energy trade-offs were also observed in other organisms between immune defense and other energy expenditure pathways (Hegemann et al., 2012; Lourenco et al., 2009; Rauw, 2012; Simmons, 2011). Interestingly, a suite of transcripts over-expressed during focal response was significantly under-expressed in non-nodule tissues as compared to healthy clams (Additional file 3.3), which included several immune effectors and mediators associated with nodule formation and focal inflammation (e.g. integrins, notch proteins and peroxidases). In fact, maintaining high levels of these focally-induced molecules could be costly and dangerous as some are toxic to both the parasite and the host, so their production must be restrained within areas where they can directly exert defense function, and reduced outside the infection foci to minimize risks of undesirable effects on the host (Hegemann et al., 2012; Rauw, 2012).

On the other hand, systemically over-expressed transcripts included stress proteins and other soluble immune factors such as lysozyme (c-type lysozyme 2), lectins (C1q domain containing protein, macrophage mannose receptor 1, low affinity immunoglobulin epsilon fc receptor), AMP (hemocyte defensin), proteases (cathepsin K, calpain 11, isoaspartyl peptidase/L-asparaginase, ASRGL, counting factor associated protein d) and ferric-chelate reductase (Table 3.4, Additional file 3.3). Over-expression of host stress proteins, such as heat shock proteins (HSP 70, HSP 90) and universal stress protein (USP) was also noted, in agreement with

observations made during infection in other bivalve species (Fabbri et al., 2008; Genard et al., 2013; Moreira et al., 2012b). Increased levels of stress proteins provide host cells with protection against incorrect protein folding caused by infection, inflammation, oxidative stress and other destructive events (Osterloh and Breloer, 2008; Zugel and Kaufmann, 1999). The systemic over-expression of soluble immune effectors (e.g. humoral proteins) may help maintain comparatively high immune capacity to prevent the spread of QPX (or secondary pathogens) throughout the host. In addition, transcripts of anti-apoptotic factors (IL17, deoxyguanosine, baculoviral map repeat-containing proteins) were also over-expressed during the systemic response, indicating that anti-apoptotic processes noted during focal response are not limited to the infection foci.

3.3.6. Pathway alterations during *M. mercenaria's* response to QPX

Transcriptomic alterations during both focal and systemic response discussed above were also highlighted in the pathway enrichment analysis of the DE transcripts. This analysis aims at extracting an overview of phenotypic changes on the underlying functional level, to reduce the complexity of biological information given by the long lists of DE genes/transcripts (Ouzounis et al., 2012). The KEGG pathways of focal adhesion (04510), ECM-receptor interaction (04512), Notch signaling pathway (04330) and apoptosis (0421) were significantly over-represented during both focal and systemic response (Figure 3.5), even though fold enrichment were generally higher during the focal response. Other immune-related pathways particularly enriched during the focal response included regulation of actin cytoskeleton (04810), cell adhesion molecules (CAMs, 04514), the leukocyte transendothelial migration (04670), complement and coagulation cascade (04610) and Wnt signaling pathway (04310). These pathways are involved in immune cell activation during migration, attachment and parasite encapsulation, which serve as the underlying mechanisms for nodule formation and QPX killing. On the other hand, basic metabolic pathways such as the citrate cycle (TCA) and pyruvate metabolism were specially enriched during the systemic response (Figure 3.5). These alterations were largely in accordance with the under-expression of metabolism-associated DE transcripts in infected tissue compared to the healthy tissue, possibly reflecting changes in the energy allocation strategy during infection as discussed above.

Interdependence of KEGG pathways was observed and most of these are interrelated with each other via shared components, forming a signaling network to allow for pathway crosstalk. To investigate these interactions, we extracted the DE transcripts shared by multiple enriched pathways and constructed a schematic pathways network that are significantly altered by QPX infection (Figure 3.6). In this framework, *M. mercenaria* response to QPX infection was initiated upon the sensing of danger signals via cell membrane receptors. The signals subsequently transmitted down through the MAPK, Wnt and Notch pathways and triggered the production of a series of host defense factors as the end results. In parallel, activation of pathways regulating actin cytoskeleton and leukocyte transendothelial migration facilitated the recruitment of hemocytes to the infection area to build a barrier of cellular defense against the parasite.

Recruited hemocytes then attached to and encapsulated QPX cells as suggested by the modulation of focal adhesion and ECM receptor interaction pathways. These cellular activities were performed under a tight regulation of the apoptosis pathway to determine cell fates, resulting in either the survival or death of *M. mercenaria* cells.

3.3.7. Distinctive transcriptomic pattern of healthy clams

A suite of transcripts (407, Figure 3.4, Table 3.5 and Additional file 3.4) exhibited higher transcription levels in healthy clams as compared to diseased clams (considering both nodule and non-nodule tissues). A considerable fraction of these transcripts were related to metabolic processes, nucleic acids binding and transcriptional regulation. The over-expressed immune-related transcripts identified in healthy clams are of particular interest as they may be involved in *M. mercenaria* resistance towards QPX. For example, an antimicrobial protein (aplysianin A (Iijima et al., 1995; Kamiya et al., 1986)) was exclusively identified in healthy clams with almost no detection in diseased clams. In addition, the highest expression levels of a serine protease inhibitor were also observed in healthy clams. A serine protease inhibitor has been linked to oyster (*Crassostrea virginica*) resistance against the protozoan parasite *Perkinsus marinus* (He et al., 2012; La Peyre et al., 2010; Oliver et al., 2000; Xue et al., 2006; Yu et al., 2011). Therefore, the high expression of the serine protease inhibitor in healthy clams supports its involvement in *M. mercenaria*'s resistance against QPX, likely by inhibiting the activity of parasite proteases. Moreover, a pathogen recognition protein (c-type lectin domain family 10 member A-like) was significantly over-expressed in healthy clams as compared to diseased animals, which may also contribute to clam resistance against QPX by promoting microbial recognition and encapsulation (Chernikov et al., 2013). Previous studies demonstrated that clam genetic background affects *M. mercenaria* resistance toward QPX (Dahl et al., 2008; Ford et al., 2002; Smolowitz et al., 2008), therefore immune-related transcripts specifically associated with healthy clams represent excellent candidates as molecular markers for further research on screening and breeding QPX-resistant *M. mercenaria* strains.

On the other hand, a subset of transcripts (126, Figure 3.4, Table 3.5 and Additional file 3.4) exhibited lowest expression levels in healthy clams. These include stress protein HSP 70 and USP, as well as the detoxification molecule cytochrome p450, which together highlight the stress experienced by clams as the result of QPX infection (Behrendt et al., 2010; Cardona et al., 2011; Metzger et al., 2012; Monari et al., 2011; Narusaka et al., 2004; Roberts et al., 2010). In addition, other immune related transcripts including a protease (isoaspartyl peptidase/L-asparaginase, ASRGL), a protease inhibitor (GTP-binding protein yptV4), a C-type lectin (MRC1), and molecules involved in tissue regeneration and cell signaling, were also under-expressed in healthy clams as compared to infected individuals, suggesting their role in fighting the infection.

3.4. Conclusions

This is one of the first studies contrasting focal and systemic immune responses to infections

in invertebrates using high-throughput sequencing. Resulting transcriptome represents a significant addition to the so far limited public genomic information available for *M. mercenaria*. The transcriptomic profiles of healthy and infected clams reflected complex interactions between the host immune system and the pathogen leading to molecular changes at both the infection foci and the systemic level. In general, the systemic responses of *M. mercenaria* reflected prevailing transcriptomic suppression accompanied with a contrasting over-expression of stress proteins and soluble antimicrobial effectors, whereas the focal response highlighted cell-cell interactions between hemocytes and the parasite that typically result in local inflammation, extracellular degradation, encapsulation, granuloma formation, and wound repair. What needs to be kept in mind is that the regulation of these genes can be the result of either an effective immune response or a symptom of a future death. In fact, several apoptotic and anti-apoptotic genes were regulated. This highlights a well-tailored defense mechanisms to adjust to the infection development (e.g. through the modulation of energy allocation, apoptotic and anti-apoptotic processes and mobilization of different signaling pathways). In addition, the identification of immune-related transcripts that were particularly associated with healthy clams offered new perspectives on the molecular features putatively involved in clam resistance against QPX.

Table 3.1. RNA samples for RNA-seq analysis. Each pool is made with equal amounts of RNA from 3 individual clams. Pools A and B were derived from the same clams.

	Library	Reads #
	A1	30,246,444
Nodule (QPX lesion from diseased clams)	A2	34,236,910
	A3	46,392,343
	B1	26,707,285
Non-nodule (“healthy” tissue from diseased clam)	B2	27,807,743
	B3	39,648,926
	C1	36,453,828
Healthy (healthy tissue from healthy clam)	C2	42,941,967
	C3	41,572,615

Table 3.2. Transcripts with annotated functions (GO terms) related to immune recognition, signaling and regulation that were differentially expressed during *M. mercenaria* focal response against QPX. Additional information on these transcripts is given in Additional file 3.2. "Inf" designates an infinite fold change calculated for focal response as the expression of that transcript in non-nodule tissue was equal to 0.

Transcripts ID	Annotation	Regul -ation	Fold change	Function/GOs
Pathogen recognition receptors (PRRS)				
Scavenger receptors				
comp186077_c0_seq3	somatomedin-b and thrombospondin type-1 domain-containing (RPE spondin)	Up	71.3	F:scavenger receptor activity; P:immune response; F:polysaccharide binding
comp179365_c0_seq8	insulin-related peptide receptor	Up	11.5	F:scavenger receptor activity; P:transmembrane receptor protein tyrosine kinase signaling pathway
comp169098_c0_seq1	hemicentin-1	Up	10.6	F:scavenger receptor activity; P:cell adhesion
comp169486_c0_seq1	lysyl oxidase-like protein 2	Up	22.5	F:scavenger receptor activity; F:copper ion binding
comp192413_c0_seq18	mam domain-containing glycosylphosphatidylinositol anchor protein 1	Up	Inf	F:polysaccharide binding; F:scavenger receptor activity; P:immune response
C-type lectin (CTL)				
comp176879_c0_seq8	c-type mannose receptor 2	Up	15.8	F:binding; F:carbohydrate binding
comp190222_c0_seq3	perlucin-like protein	Up	49.1	F:binding; F:carbohydrate binding;
Toll-like receptors (TLRs)				
comp189381_c0_seq1	toll-like receptor 1	Up	16.5	F:protein binding
comp190056_c0_seq8	toll-like receptor e precursor	Down	Inf	P:signal transduction; F:protein binding
comp188195_c0_seq7	toll-like receptor g precursor	Up	Inf	F:protein binding; P:signal transduction
comp189381_c1_seq2	cell surface receptor tollo (toll 8)	Up	10.9	P:signal transduction; F:protein binding
Integrin				
comp189082_c0_seq13	integrin alpha 4	Up	Inf	P:cell-cell adhesion; C:integrin complex
comp189082_c0_seq24	integrin alpha 4	Up	16.2	P:cell-cell adhesion; C:integrin complex
comp185789_c0_seq4	integrin alpha-ps	Up	13.7	P:cell adhesion; C:integrin complex
comp184975_c0_seq10	integrin beta-1-like	Up	12.4	F:protein binding; F:receptor activity;
Low-density lipoprotein (LDL)				
comp181432_c3_seq2	LDL receptor-related protein 12	Up	12.1	-
comp187193_c1_seq4	LDL receptor-related protein 12	Up	Inf	F:protein binding

G-protein coupled receptor				
comp177186_c1_seq2	G-protein coupled receptor partial	Up	13.0	P:cell surface receptor signaling pathway; F:transmembrane signaling receptor activity
comp191316_c2_seq1	G-protein coupled receptor 64-like	Up	28.1	-
comp177657_c2_seq5	guanine nucleotide-binding protein subunit	Up	Inf	P:G-protein coupled receptor signaling pathway; F:signal transducer activity
comp187628_c0_seq44	substance-k (neurokinin) receptor	Up	9.1	P:G-protein coupled receptor signaling pathway; F:G-protein coupled receptor activity;
comp192446_c0_seq4	orexin receptor type 2	Up	12.4	P:G-protein coupled receptor signaling pathway;
Immune signaling and cell communication				
Phosphatase and kinases				
comp192546_c5_seq4	immunoglobulin i-set domain protein	Up	13.2	F:protein serine/threonine kinase activity
comp165267_c0_seq3	von willebrand factor type egf and pentraxin domain-containing protein	Up	11.0	F:protein tyrosine kinase activity
comp191437_c1_seq4	focal adhesion kinase 1	Up	19.7	F:signal transducer activity;
comp188649_c0_seq3	calcium calmodulin-dependent protein kinase	Up	9.3	F:calmodulin-dependent protein kinase activity
comp190317_c0_seq7	neuronal cell adhesion	Down	-13.3	F:rhodopsin kinase activity
Rho signaling				
comp189234_c1_seq21	rho gtpase-activating protein 15-like isoform x3	Up	18.1	P:signal transduction
comp185122_c1_seq7	rho gtpase-activating protein 24	Up	27.1	P:signal transduction
comp187736_c1_seq25	rho-related protein raca	Up	Inf	F:protein binding
comp188711_c0_seq2	rho-associated protein kinase 2	Up	Inf	P:intracellular signal transduction
Ubiquitin pathway				
comp190451_c2_seq9	e3 ubiquitin-protein ligase hectd1	Up	164.9	F:ubiquitin-protein ligase activity; F:metal ion binding
comp184512_c0_seq2	e3 ubiquitin-protein ligase march6	Up	15.0	F:zinc ion binding
comp192081_c0_seq1	e3 ubiquitin-protein ligase ubr3	Up	8.7	-
comp179031_c0_seq4	cop9 signalosome complex subunit 5	Up	Inf	F:protein binding
comp179031_c0_seq2	cop9 signalosome complex subunit 5	Up	Inf	F:protein binding
Wnt and Notch pathway				
comp185090_c0_seq10	tyrosine-protein kinase ryk	Up	26.3	P:Wnt receptor signaling pathway
comp187449_c0_seq2	fizzy-like protein	Up	13.0	F:protein binding
comp175460_c0_seq1	neurogenic locus notch	Up	11.7	P:Notch signaling pathway;

comp182793_c0_seq5	neurogenic locus notch protein	Up	9.6	P:G-protein coupled receptor signaling pathway
comp192565_c0_seq10	nicastrin-like protein	Up	12.6	P:protein processing
Calcium mediated signal transduction				
comp183265_c0_seq1	calmodulin 3b (phosphorylase delta)	Down	-11.6	F:calcium ion binding
comp191993_c0_seq4	EF-hand Ca-binding domain-containing protein 5	Down	-10.9	-
comp191855_c0_seq2	EF-hand Ca-binding domain-containing protein 6	Down	-15.9	-
Complement pathway				
comp165285_c0_seq7	macrophage-expressed gene 1	Up	11.5	-
Signal transducer				
comp182953_c0_seq4	signal recognition particle receptor subunit alpha	Up	13.8	F:signal recognition particle binding
comp182953_c0_seq5	signal recognition particle receptor subunit alpha	Up	73.0	F:signal recognition particle binding
comp171563_c0_seq4	gtp-binding nuclear protein	Up	Inf	P:small GTPase mediated signal transduction
comp188686_c0_seq19	neuralized pats1	Down	-758.9	P:small GTPase mediated signal transduction
comp189853_c0_seq1	unc5c-like protein	Down	-12.5	P:signal transduction
Cell death regulation				
Apoptosis process				
comp175357_c1_seq16	solute carrier family 25 member 38-like isoform 1	Down	-25.0	P:transmembrane transport
comp191590_c0_seq3	p53-induced protein with a death domain isoform	Down	-39.1	F:protein binding; P:signal transduction
comp191147_c0_seq62	inhibitor of apoptosis	Up	24.6	-
comp191147_c0_seq70	inhibitor of apoptosis	Up	Inf	F:metal ion binding; F:zinc ion binding;
comp191055_c2_seq4	programmed cell death protein 10	Up	367.1	-
comp190690_c2_seq9	cell death abnormality protein 1-like	Up	Inf	F:binding; F:zinc ion binding
comp186101_c3_seq4	3-hydroxy-3-methylglutaryl-coenzyme A reductase	Down	-10.8	P:positive regulation of apoptotic process; P:negative regulation of wound healing; P:oxidation-reduction process;
Tumor necrosis factor (TNF)				
comp182922_c0_seq5	TNF ligand superfamily member 10-like	Up	8.5	-
comp176786_c2_seq3	TNF-like protein	Down	-26.3	F:tumor necrosis factor receptor binding; P:immune response
	TNF ligand superfamily member 10-like	Up	8.5	-

Table 3.3. Transcripts with putative functions (GO terms) related to immune effectors that were differentially expressed during *M. mercenaria* focal response against QPX. Additional information on these transcripts is given in Additional file 3.2. "Inf" designates an infinite fold change calculated for focal response as the expression of that transcript in non-nodule tissue was equal to 0.

Transcripts ID	Annotation	Regula-tion	Fold change	Function/GOs
Oxidation-reduction processes				
comp186178_c0_seq12	dual oxidase	Up	Inf	P:response to oxidative stress; P:oxidation-reduction process; F:peroxidase activity
comp185478_c0_seq4	allene oxide synthase-lipoxygenase protein	Up	10.0	P:oxidation-reduction process; F:oxidoreductase activity; F:metal ion binding;
comp186926_c0_seq7	c-terminal-binding protein	Up	Inf	P:oxidation-reduction process; F:NAD binding;
comp187462_c1_seq6	chorion peroxidase	Up	11.8	P:oxidation-reduction process; P:response to oxidative stress; F:peroxidase activity
comp157634_c0_seq8	cytochrome p450	Up	178.6	P:oxidation-reduction process; F:oxidoreductase activity; F:iron ion binding
comp183426_c1_seq2	dbh-like monooxygenase protein 1-like protein	Up	24.8	P:oxidation-reduction process; F:dopamine beta-monooxygenase activity; F:oxidoreductase activity
comp188723_c0_seq1	isocitrate dehydrogenase	Up	Inf	P:oxidation-reduction process; F:magnesium ion binding; F:NAD binding
comp169486_c0_seq1	lysyl oxidase-like protein 2	Up	22.5	F:oxidoreductase activity; P:oxidation-reduction process; F:scavenger receptor activity
comp192316_c0_seq4	methylenetetrahydrofolate reductase	Up	Inf	P:oxidation-reduction process; F:methylenetetrahydrofolate reductase (NADPH) activity;
comp189621_c0_seq4	procollagen-oxoglutarate 5-dioxygenase 3	Up	9.7	P:oxidation-reduction process; F:iron ion binding
comp185148_c0_seq6	ww domain-containing oxidoreductase	Up	Inf	F:oxidoreductase activity; P:metabolic process
Protease				
comp184786_c1_seq4	fur protein precursor	Up	Inf	F:serine-type endopeptidase activity
comp180950_c0_seq5	lysosomal protective protein precursor	Up	47.1	F:serine-type carboxypeptidase activity
comp180950_c0_seq2	lysosomal protective protein precursor	Up	26.9	F:serine-type carboxypeptidase activity
comp189961_c0_seq12	N-acetylated-alpha-linked acidic dipeptidase 2	Up	16.3	F:metallopeptidase activity;
comp178551_c1_seq1	membrane metallo-endopeptidase-like 1-like	Up	Inf	F:metalloendopeptidase activity;
comp184011_c0_seq4	blastula protease 10	Up	8.1	F:metalloendopeptidase activity;
comp191868_c1_seq1	matrix metalloproteinase-19	Up	59.1	-
comp174947_c0_seq3	isoaspartyl peptidase l-asparaginase-like	Up	Inf	F:hydrolase activity

comp183848_c0_seq3	kyphoscoliosis peptidase	Down	-8.7	P:microtubule-based movement
comp188831_c0_seq3	puromycin-sensitive aminopeptidase-like isoform	Down	-65.5	F:metallopeptidase activity;
comp191458_c3_seq5	aspartic protease with reverse transcriptase activity	Down	-245.4	F:aspartic-type endopeptidase activity;
Protease inhibitor				
comp189919_c1_seq4	alpha macroglobulin	Up	21.9	F:endopeptidase inhibitor activity
comp189919_c1_seq2	alpha macroglobulin	Up	12.4	F:endopeptidase inhibitor activity
comp181286_c4_seq1	thioester-containing protein	Up	10.2	F:endopeptidase inhibitor activity
comp191416_c1_seq2	thioester-containing protein-a	Up	14.8	F:endopeptidase inhibitor activity
comp191416_c1_seq1	thioester-containing protein-b	Up	9.5	F:endopeptidase inhibitor activity
comp192366_c0_seq1	thioester-containing protein-c	Up	32.5	F:endopeptidase inhibitor activity
comp191416_c0_seq5	thioester-containing protein-e	Up	12.9	F:endopeptidase inhibitor activity
comp191416_c0_seq4	thioester-containing protein-e	Up	22.4	F:endopeptidase inhibitor activity
Ion transporter and sequester				
comp190604_c1_seq1	ceruloplasmin precursor	Up	21.8	P:copper ion transport; P:cellular iron ion homeostasis; F:ferroxidase activity
comp182612_c1_seq1	ferric-chelate reductase 1	Up	16.7	Iron transfer
comp180332_c1_seq1	ferric-chelate reductase 1-like	Up	11.8	Iron transfer
comp177359_c0_seq1	selenium binding protein	Down	-11.0	P:protein transport; F:selenium binding
comp174164_c0_seq20	divalent metal transporter	Up	328.2	P:transport; F:transporter activity
Wound repair				
comp185425_c1_seq1	actin-related protein 2 3 complex subunit 5-like	Up	Inf	C:cytoskeleton; P:regulation of actin filament polymerization
comp93954_c0_seq1	extracellular matrix protein 2 isoform1	Up	25.9	F:protein binding
comp188753_c5_seq1	cartilage matrix protein	Up	25.8	F:chitin binding; P:chitin metabolic process; C:extracellular region
comp142858_c0_seq1	epidermal growth factor-like protein 8-like	Up	18.5	F:protein binding; F:calcium ion binding
comp192650_c1_seq4	multiple epidermal growth factor-like domains 6	Up	21.9	F:protein binding
comp186665_c0_seq1	thrombospondin- partial	Up	106.9	F:protein binding
comp186665_c0_seq4	thrombospondin- partial	Up	38.0	F:protein binding
comp184960_c0_seq4	septin-7-like isoform 8	Up	Inf	P:cell cycle; F:GTP binding; C:septin complex

comp184960_c0_seq42	septin-7-like isoform 6	Up	8.9	-

Table 3.4. Selected transcripts with annotated functions (GO terms) related to immune response that were up-regulated during *M. mercenaria* systemic response against QPX. Additional information on these transcripts is given in Additional file 3.3. "Inf" designates an infinite fold change calculated for focal response as the expression of that transcript in non-nodule tissue was equal to 0.

Transcripts ID	Annotation	Regul -ation	Fold change	Function/GOs
Stress protein				
comp39934_c1_seq1	heat shock 70 kda protein	Up	Inf	P:response to stress
comp181704_c3_seq49	heat shock 70 kda protein 12b	Up	452.9	-
comp68505_c0_seq1	heat shock protein 70	Up	37.8	P:response to stress
comp38810_c0_seq1	hsp90 family member	Up	64.7	P:response to stress
comp192296_c2_seq3	usp-like protein isoform 2	Up	46.2	P:response to stress
Immune effectors				
comp192209_c0_seq3	hemocyte defensin partial	Up	173.5	P:defense response
comp186386_c5_seq2	c-type lysozyme 2	Up	20.0	-
comp186386_c5_seq1	c-type lysozyme 2	Up	Inf	-
comp164821_c0_seq2	c1q domain containing protein	Up	8.3	Lectin
comp164821_c0_seq3	c1q domain containing protein	Up	11.9	Lectin
comp190576_c0_seq27	low affinity immunoglobulin epsilon fc receptor	Up	134.1	F:carbohydrate binding; Lectin
comp190576_c0_seq26	low affinity immunoglobulin epsilon fc receptor	Up	201.6	F:carbohydrate binding; Lectin
comp191987_c0_seq10	macrophage mannose receptor 1-like	Up	7.8	F:carbohydrate binding; Lectin
comp189507_c1_seq9	ferric-chelate reductase 1	Up	10.7	Iron transport
Peptidases				
comp156268_c1_seq1	cathepsin k-like	Up	9.5	-
comp184090_c0_seq6	calpain 11-like	Up	10.1	-
comp189096_c0_seq4	counting factor associated protein d-like	Up	36.1	F:cysteine-type peptidase activity
comp176418_c0_seq1	isoaspartyl peptidase	Up	8.0	F:hydrolase activity
Anti-apoptotic factors				
comp178822_c0_seq1	interleukin IL17-like	Up	11.6	P:inflammatory response; F:cytokine activity
comp176786_c2_seq3	deoxyguanosine mitochondrial	Up	29.5	F:tumor necrosis factor receptor binding; P:immune response;
comp192603_c1_seq8	baculoviral iap repeat-containing protein 7-a	Up	10.3	P:negative regulation of peptidase activity
comp192603_c1_seq7	baculoviral iap repeat-containing protein 7-a	Up	14.3	P:negative regulation of peptidase activity
comp191786_c0_seq3	baculoviral iap repeat-containing protein partial	Up	8.9	-

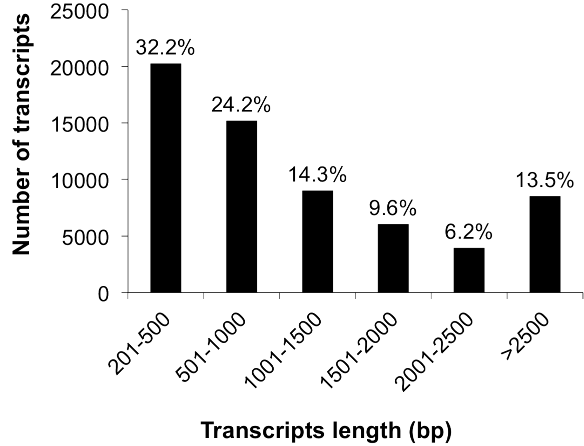
Table 3.5. Selected transcripts with annotated functions (GO terms) related to immune response that were differentially expressed in naïve *M. mercenaria* as compared to QPX infected individuals. Additional information on these transcripts is given in Additional file 3.4. "Inf" designates an infinite fold change calculated for focal response as the expression of that transcript in non-nodule tissue was equal to 0.

Transcripts ID	Annotation	Fold change	Function/GOs
Up-regulated			
comp168250_c0_seq2	macrophage expressed protein	84.0	-
comp160023_c0_seq1	serine protease inhibitor 1	18.1	F:protein binding
comp117137_c0_seq1	proline-rich transmembrane protein 1	148.6	P:response to biotic stimulus
comp134883_c0_seq1	c-type lectin domain family 10 member a-like	121.4	F:carbohydrate binding
comp180146_c3_seq31	aplysianin a precursor	Inf	P:defense response; F:oxidoreductase activity
comp179365_c0_seq7	insulin-related peptide receptor	20.0	F:scavenger receptor activity
comp187368_c2_seq9	apoptosis 1 inhibitor	16.2	P:apoptotic process;
comp193015_c0_seq1	baculoviral iap repeat-containing protein 4	391.0	P:negative regulation of apoptotic process; F:ubiquitin-protein ligase activity
Down-regulated			
comp39934_c1_seq1	heat shock 70 kda protein	Inf	P:response to stress
comp192296_c2_seq3	usp-like protein isoform 2	-46.2	P:response to stress
comp169961_c0_seq2	cytochrome p450 1a1	-75.1	-
comp176418_c0_seq1	isoaspartyl peptidase l-asparaginase-like	-8.0	F:hydrolase activity
comp191987_c0_seq10	macrophage mannose receptor 1-like	-7.8	F:carbohydrate binding
comp186563_c0_seq7	bile salt-activated lipase-like	-9.8	F:hydrolase activity

A.

***M. mercenaria* transcriptome sequencing, assembly and annotation overview**

Sequencing results		Assembly results		Annotation results	
Read bases (G)	65.70	Transcripts	62,980	Nr annotation	19,107
Read pairs	320,298,160	Average length of transcripts (bp)	1,298	GO mapped	13,584
Read length (bp)	100	Smallest transcripts (bp)	201	KO mapped	6,425
		Largest transcripts (bp)	23,103	InterPro hits	29,815



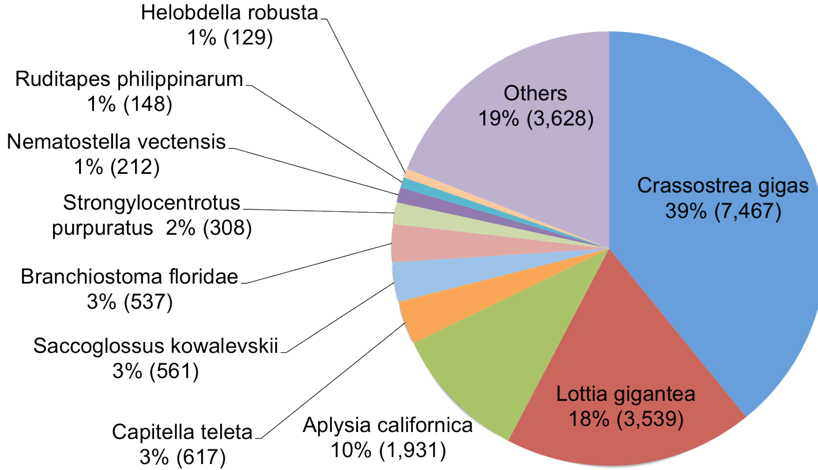


Figure 3.1. *M. mercenaria* de novo assembled transcriptome summary. A. Transcriptome sequencing, assembly and annotation overview. B. Assembled transcripts size distribution. C. Distribution of the top 10 species that *M. mercenaria* transcripts shown BLAST hits. Transcripts were searched using BLASTx against NCBI nr database with a cutoff value of E<10E-03.

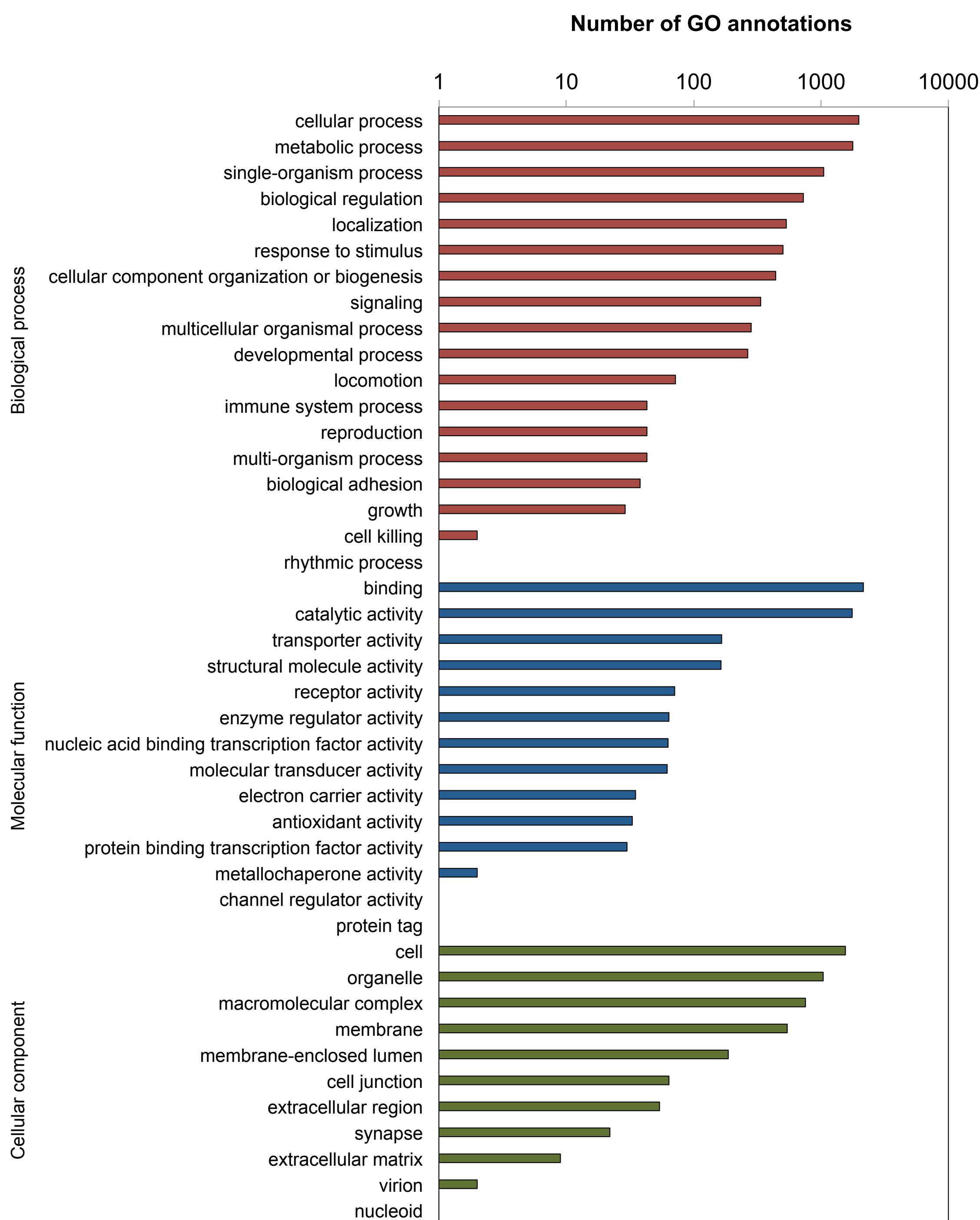
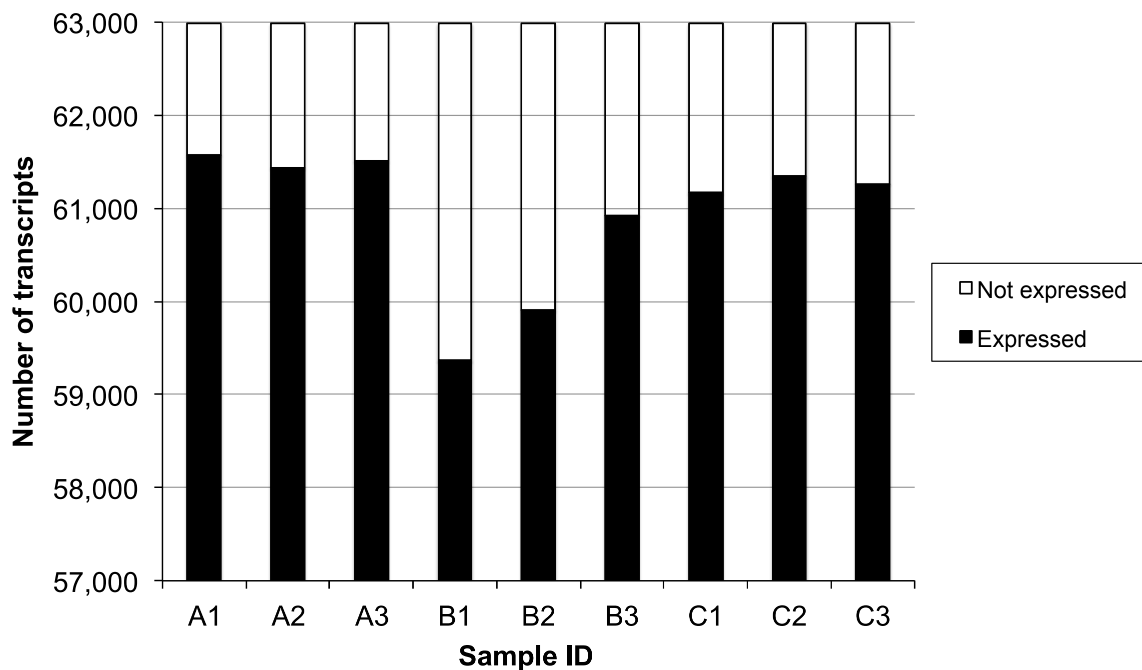


Figure 3.2. Gene Ontology (GO) annotations of the *M. mercenaria* transcriptome. GO terms were identified by Blast2GO and the results were summarized in three main GO categories: biological process (8,168 annotations), cellular component (4,600 annotations), molecular function (4,231 annotations) at level-2.

A.



B.

Transcripts expression and reads coverage of each sample library

		Nodule			Non-nodule			Healthy		
		A1	A2	A3	B1	B2	B3	C1	C2	C3
Reads coverage	Reads/transcript	383	440	607	363	379	525	458	546	523
	Average	477			422			509		

Figure 3.3. Number of transcripts expressed and reads coverage in each sample. A: The X-axis indicates the sample (A1-A3: nodule; B1-B3: non-nodule; C1-C3: healthy, refer to Table 3.1 for more details). The Y-axis indicates the number of transcripts expressed in the samples. B: Summary statistics of the reads coverage in each sample.

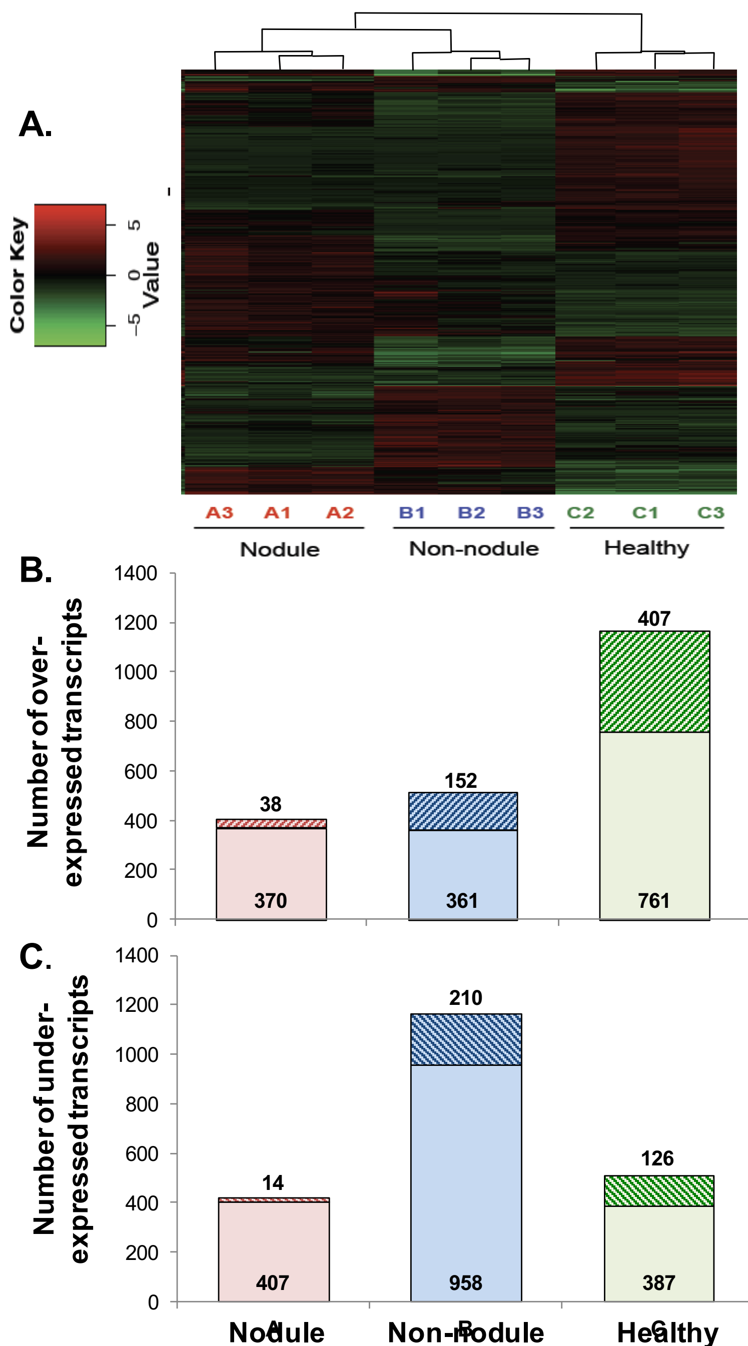


Figure 3.4. Heatmap (A) and number of differentially expressed transcripts (B and C) across all samples (FDR ≤0.001, and |log2 (fold change)| >2). Over-expressed transcripts are shown in red in A and are enumerated in B. Under-expressed transcripts are shown in green in A and are enumerated in C. Replicate biological samples are displayed in A (see Table 3.1 for more details). For B and C: the cross-shaded areas inside each bar represent the number of transcripts with highest (B) or lowest (C) expression levels in a condition as compared to the other two (e.g. the expression levels of 38 focally over-expressed transcripts in B were higher in nodule than both in non-nodule tissues and healthy clams).

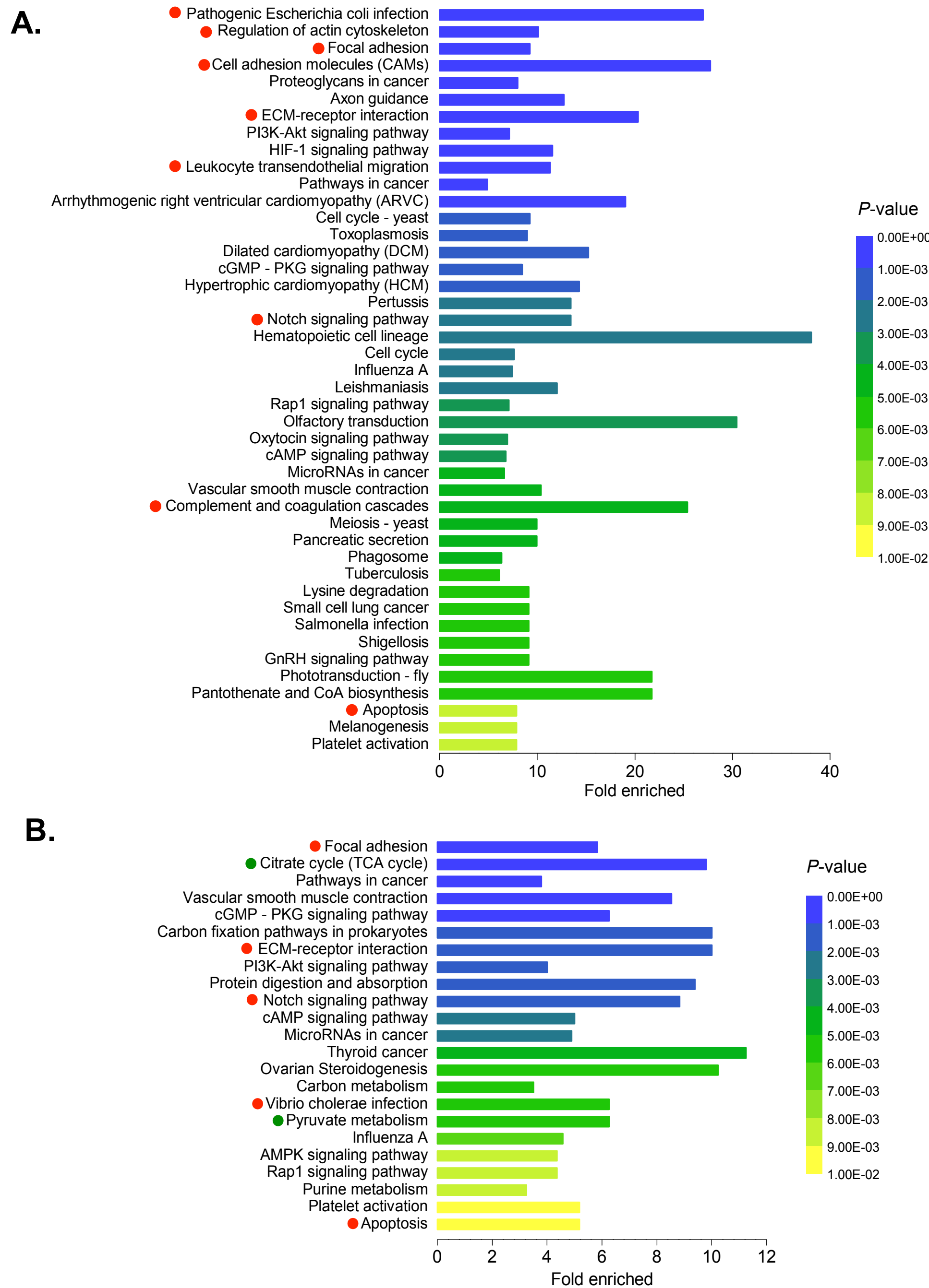
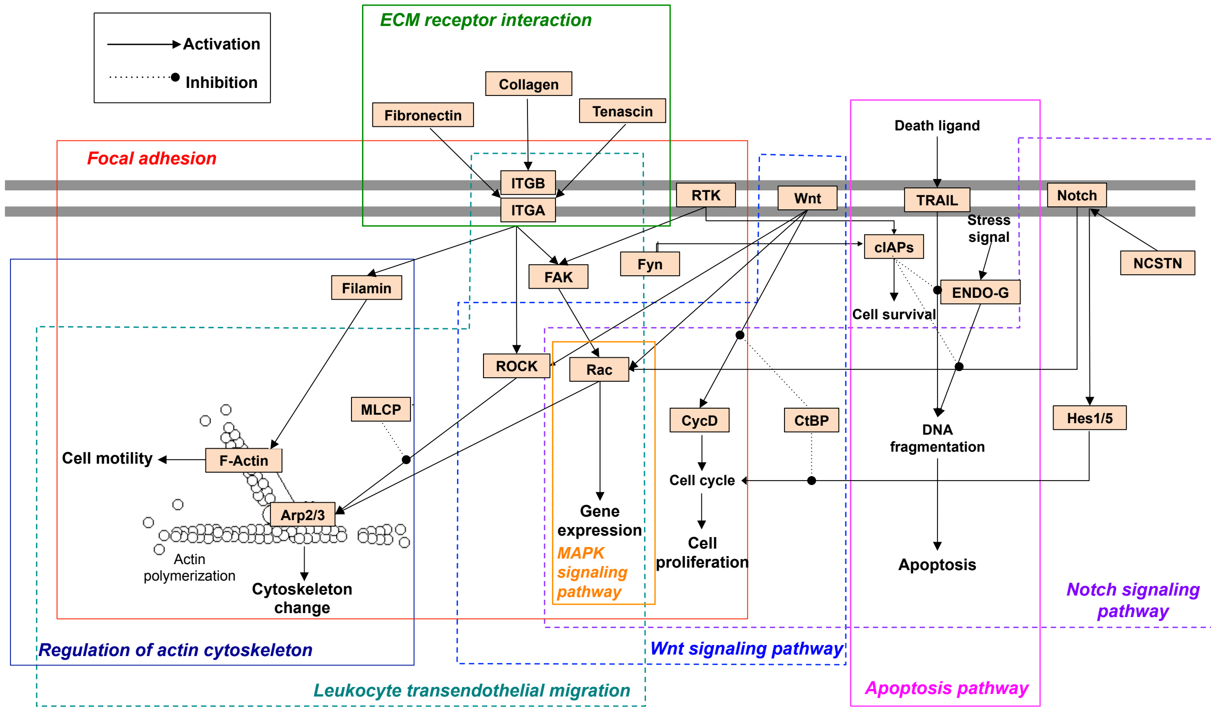


Figure 3.5. Significantly enriched KEGG pathways in *Mercenaria mercenaria* derived from the differentially expressed (DE) genes during focal (A) and systemic (B) response against QPX. The KEGG pathways having significant enrichment ($P < 0.01$) are presented, and the bar shows the x-fold enrichment of each KEGG pathway. Pathways involved in immune response are marked with red dots while metabolism-related pathways are marked with green dots.



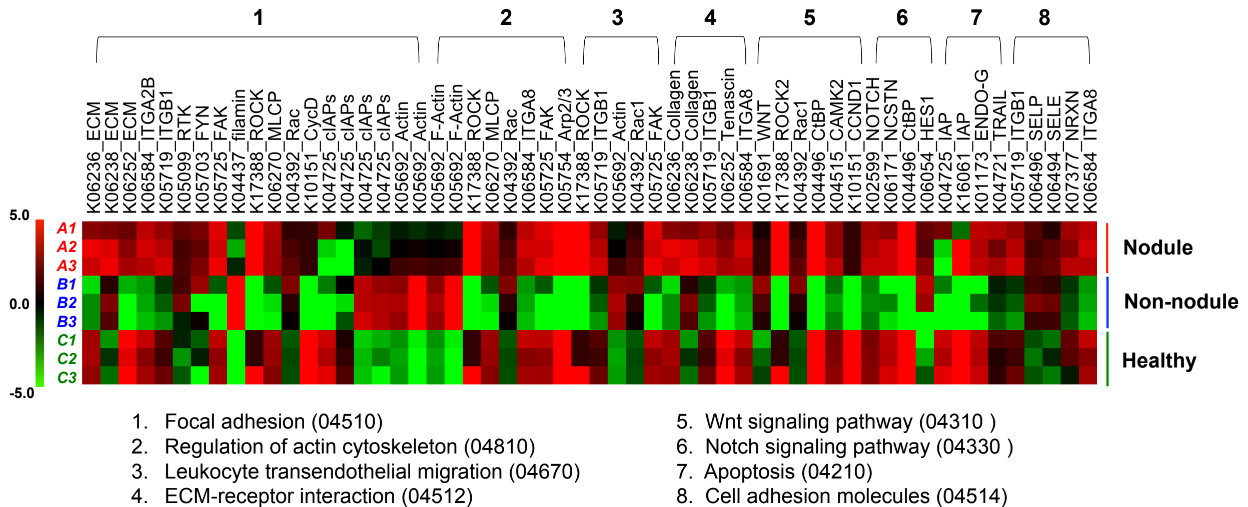


Figure 3.6. Overview of immune-related enriched pathways of differentially expressed (DE) transcripts during *Mercenaria mercenaria* response to QPX. A: Schematic diagram of enriched pathways and their interactions. Only pathway components encoded by DE transcripts (shown in text boxes) are presented within each enriched KEGG pathway (framed in orthogonal polygons). Arrows display possible interactions (e.g. activation, inhibition) between pathway components. B: Overview of DE transcripts expression associated with enriched pathways in nodule, non-nodule and healthy *M. mercenaria* tissues. The red and green heatmap values indicate $\log_2$ fold of relative expression levels for

individual transcripts. Arp2/3: actin related protein 2/3 complex; CtBP: C-terminal binding protein; CAMK2: calcium/calmodulin-dependent protein kinase (CaM kinase) II; CycD: cyclin D1(CCND1); ECM: von Willebrand factor; ENDO-G: endonuclease G; FAK: focal adhesion kinase; FYN: tyrosine-protein kinase; HES1: hairy and enhancer of split 1; IAP: baculoviral IAP repeat-containing protein, cIAPs; ITGA: intergrin alpha; ITGB: intergrin beta; MLCP: serine/threonine-protein phosphatase PP1 catalytic subunit; NRXN: neurexin; NCSTN: nicastrin; Rac: Ras-related C3 botulinum toxin substrate; ROCK: Rho-associated protein kinase; RTK: proto-oncogene tyrosine-protein kinase; SELE: selectin, endothelial cell; SELP: selectin, platelet; TRAIL: tumor necrosis factor ligand superfamily member 10); WNT: wingless-type MMTV integration site family.

Additional files:

Additional file 3.1. Sequence summary of *M. mercenaria de novo* assembled transcriptome. This is a Microsoft Excel worksheet that contains descriptions and annotation information of individual transcript sequence.

Additional file 3.2. Differentially expressed (DE) sequences associated with *M. mercenaria* focal response against QPX. This file is a Microsoft Excel worksheet that contains separate lists of up- and down-regulated transcripts with their expression levels and fold change values given.

Additional file 3.3. Differentially expressed (DE) sequences associated with *M. mercenaria* systemic response against QPX. This file is a Microsoft Excel worksheet that contains separate lists of up- and down-regulated transcripts with their expression levels and fold change values given.

Additional file 3.4. Transcripts that were differentially expressed in naïve *M. mercenaria* as compared to QPX infected individuals. This file is a Microsoft Excel worksheet that contains separate lists of up- and down-regulated transcripts with their expression levels and fold change values given.

Chapter 4 Alterations of the immune transcriptome in resistant and susceptible hard clams (*Mercenaria mercenaria*) in response to Quahog Parasite Unknown (QPX) and temperature

Manuscript published as:

Wang K, Pales Espinosa E, Tanguy A, Allam B. (2016). Alterations of the immune transcriptome in resistant and susceptible hard clams (Mercenaria mercenaria) in response to Quahog Parasite Unknown (QPX) and temperature. Fish and Shellfish Immunology 49: 163-176.

Abstract

Quahog Parasite Unknown (QPX) is a fatal protistan parasite that causes severe losses in the hard clam (*Mercenaria mercenaria*) fisheries along the northeastern coast of the US. Field and laboratory studies of QPX disease have demonstrated a major role for water temperature and *M. mercenaria* genetic origin in disease development. Infections are more likely to occur at cold temperatures, with clam stocks originating from southern states being more susceptible than clams from northern states where disease is enzootic. Even though the influence of temperature on QPX infection has been examined in susceptible and resistant *M. mercenaria* at physiological and cellular scales, the underlying molecular mechanisms associated with host-pathogen interactions remain largely unknown. This study was carried out to explore the gene expression changes in *M. mercenaria* in response to temperature and QPX infection on the transcriptomic level, and also to compare molecular responses between susceptible and resistant clam stocks. A *M. mercenaria* oligoarray (15K Agilent) platform was produced based on our previously generated transcriptomic data and was used to compare gene expression profiles in naive and QPX-infected susceptible (Florida stock) and resistant (Massachusetts) clams maintained at temperatures favoring disease development (13 °C) or clam healing (21 °C). In addition, transcriptomic changes reflecting focal (the site of infection, mantle) and systemic (circulating hemocytes) responses were also assessed using the oligoarray platform. Results revealed significant regulation of multiple biological pathways by temperature and QPX infection, mainly associated with immune recognition, microbial killing, protein synthesis, oxidative protection and metabolism. Alterations were widely systemic with the most changes in gene expression revealed in hemocytes, highlighting the role of circulating hemocytes as the first line of defense against pathogenic stress. A large number of complement-related recognition molecules with fibrinogen or C1q domains were induced following QPX challenge, and the expression of these molecules was significantly higher in resistant clams as compared to susceptible ones. These highly variable immune proteins may be candidate molecular markers for future study of *M. mercenaria* resistance against QPX. Beyond the specific case of clam response to QPX, this study also provides insights into the primitive complement-like system in the hard clam.

Keywords:

Hard clam; QPX; Microarray; Gene expression; Temperature; Immunity

4.1. Introduction

The hard clam *Mercernaria mercenaria* is among the most commercially important bivalves in the United States. It is widely exploited along the North American Atlantic coasts from the Maritime Provinces of Canada to Florida. *M. mercenaria* is often considered as a robust bivalve species and very few infectious agents have been reported to cause problems in wild and aquacultured stocks. Among these, the Quahog Parasite Unknown (QPX) is a protistan parasite known to cause lethal infections and substantial losses in the hard clam industry (Ford et al., 2002; Lyons et al., 2007; Maas et al., 1999; Ragan et al., 2000; Smolowitz et al., 1998; Stokes et al., 2002; Whyte et al., 1994). QPX is considered as an opportunistic pathogen and is widely present in coastal environments (Gast et al., 2008; Liu et al., 2009; Lyons et al., 2005), where it can be found associated with a variety of substrates such as seawater, sediment and marine aggregates (Liu et al., 2008; Liu et al., 2009; MacCallum and McGladdery, 2000; Ragone Calvo et al., 1998). Encounters between QPX and *M. mercenaria* are therefore considered frequent but usually do not result in disease outbreaks until other disease-favoring factors emerge (Gast et al., 2008; Liu et al., 2009; Lyons et al., 2005). Such factors may include the presence of highly virulent parasite variants, susceptible host strains and shifts of environmental conditions that either add to the pathogen abundance or infectivity, or lower the resistance of the host (Dahl and Allam, 2015; Dahl et al., 2011; Perrigault et al., 2011).

Previous studies of QPX disease have demonstrated a significant association between temperature and *M. mercenaria* genetic background and disease development. For instance, QPX infection is considered as a “cold-water disease” since the infection has never been detected in clams further south to Virginia where the water temperature is comparatively warmer. Laboratory infection trials also reported higher disease prevalence in clams held at 13 °C as compared to temperatures of 21 °C and higher, where parasite establishment was largely inhibited and signs of host healing were detected (Dahl and Allam, 2008; Dahl et al., 2011; Perrigault et al., 2011). In fact, environmental factors, such as temperature, salinity and dissolved oxygen, can substantially affect the physiology of aquatic animals. Temperature, of special importance, has been demonstrated to significantly modulate host immune performance and pathogen virulence, thus changing the pattern of host-pathogen interactions and affecting the development of disease (Chu and LaPeyre, 1993; Paillard et al., 2004; Perrigault et al., 2012; Perrigault et al., 2011). Additionally, marked differences in QPX susceptibility have been observed between clams from different genetic backgrounds. Generally, clams originating from the warm southern states (e.g. Florida and South Carolina) appear to be more sensitive to QPX infection than those from relatively cold northern states (e.g. Massachusetts, New York, New Jersey), suggesting the existence of genetically controlled factors that regulate *M. mercenaria* resistance against this parasite (Calvo et al., 2007; Dahl et al., 2008; Dahl et al., 2010; Ford et al., 2002; Smolowitz et al., 2008).

The ability of a pathogen to establish infection largely depends on the capacity of the host defense system to induce an effective immune response against the invasion and the ability of the pathogen to evade or overcome the host defense. Like other invertebrates, hard clams lack the adaptive immune system and rely solely on innate immune system to defend against infections. As a benthic filter-feeder, hard clams are exposed to an environment rich in microorganism, but very few are known to infect this species. This raises fundamental questions about how *M. mercenaria* is able to protect itself against this diverse microbial pool without the adaptive immune mechanisms. A recent study comparing *M. mercenaria* defense response against bacteria (*Vibrio alginolyticus*) and QPX stimuli identified specific response patterns associated with QPX challenge. Interestingly, the extent of the defense response also varied considerably between resistant and susceptible clam stocks (Perrigault and Allam, 2012).

A major characteristic of clam immune response to QPX infection is the presence of granulomatous inflammation and hemocyte encapsulation of parasite cells leading often to the formation of nodules in mantle tissues (Anderson et al., 2003b; Smolowitz et al., 1998). Dead QPX cells can sometimes be observed inside the infection nodules, suggesting effective defense reactions are mounted by clam under host-favoring conditions that result in parasite clearance and host healing (Calvo et al., 1998; Dahl and Allam, 2007; Dahl et al., 2010; Dove et al., 2004a). Interestingly, the presence of factors inhibiting QPX growth has been reported in clam plasma (Perrigault et al., 2008), and preliminary molecular investigations also demonstrated the modulation of several stress- and defense-related genes during QPX disease development (Perrigault et al., 2009c). However, the nature of these specific anti-QPX factors and the underlying mechanisms for immune response and host healing are largely unknown. Growing evidence has demonstrated that innate immune responses in invertebrates are in fact more sophisticated than previously thought (Buchmann, 2014; Degnan, 2015; Soderhall, 2010). The identification of genes associated with host-defense in *M. mercenaria* can provide insights into the diversity and evolution of innate immune mechanisms, and may also have practical implications to improve the disease resistance for this economically and ecologically important species.

In this study, we used a high-throughput genomic approach to generate an in-depth understanding of *M. mercenaria* defense system and to identify molecular pathways and effectors involved in hard clam immune response against QPX. High throughput gene expression techniques such as microarrays have been widely adopted as powerful tools for functional genomics investigations in non-model organisms. Micro (oligo)-array is an affordable, sensitive and reproducible high-throughput platform for analyzing the expression of tens of thousands of genes simultaneously. This approach has been used for probing host-pathogen interactions in several bivalve species significantly advancing our understanding of immunological regulatory pathways and providing physiological perspectives on the environmental facilitation of infection (Allam et al., 2014; Leite et al., 2013; Milan et al., 2011; Moreira et al., 2014; Nunez-Acuna et al., 2013; Rubin et al., 2014; Wang et al., 2010). Transcriptome profiling by microarray technique can directly

compare gene expression profiles between samples of different conditions or traits (e.g. healthy vs. diseased, susceptible vs. resistant), allowing for the identification of candidate genes and underlying mechanisms involved in interested features. Our study design assessed transcriptomic changes in hard clams during QPX infection. Hemocytes and mantle tissues were inspected for gene expression difference between healthy and diseased clams, providing insights into factors involved in systemic and focal immune responses, respectively. Gene expression in response to QPX infection was also compared between resistant (MA) and susceptible (FL) clam stocks, as well as in clams held at temperatures that promote (13 °C) or inhibit (21 °C) disease development. Our goal was to explore the molecular immune mechanisms used by hard clams to fight QPX and to identify immune genes or isoforms potentially involved in *M. mercenaria* resistance to QPX infection.

4.2. Materials and methods

4.2.1. Hard clams

Adult (50-55 mm in length) aquacultured *M. mercenaria* originating from Florida (FL) and Massachusetts (MA) were used in this study. QPX-free FL clams were obtained from a commercial source (Farm Raised Clams, St James City, FL) and MA clams presumably infected by QPX were collected from an enzootic clamming area in Wellfleet Harbor. Disease status of subsets from each batch was checked by a specific quantitative real-time PCR assay (Liu et al., 2009) and QPX prevalence was equal to 63% in the MA clams and 0% in the Florida stock (n = 30 clams/batch). Clams were acclimated at 21 ± 1 °C in 150-L tanks with re-circulating water (30 ppt) upon arrival. After 1-week acclimation, half of the FL clams were challenged with QPX as described previously (Dahl et al., 2011) by injecting *in vitro* cultured parasite cells into the pericardial cavity (5×10^4 cells/clam), and the other half received injection of sterile culture media as controls. Following injection, both control (FLc) and QPX challenged (FLq) FL clams, as well as the naturally infected MA clams were separately transferred to 40-L tanks and maintained at 13 and 21 °C. Temperature adjustment for the 13 °C treatment was performed within 8 days by decreasing the temperature by 1 °C per day as previously described (Dahl et al., 2011; Perrigault et al., 2011). For each clam group (MA, FLc, FLq), a total of 60 clams were randomly assigned into 3 replicate tanks (20 clams per tank) held at each temperature condition (13 and 21 °C). All tanks were individually equipped with re-circulating filtration systems and aerated continuously. Water quality, temperature, salinity (30 ppt) and ammonia level were monitored and adjusted weekly. Clams were fed daily with commercial algae (DT's Live Phytoplankton, Sycamore, IL) and monitored twice a day for mortality.

4.2.2. RNA samples

After 2-month incubation at targeted temperatures, three clams were randomly sampled from each replicate tank, totaling 9 clams per clam type and per temperature

condition. This timeframe was chosen because it was shown to be sufficient for QPX to initiate infection in susceptible clams or for clams to prompt healing under optimal conditions (Dahl and Allam, 2007; Dahl et al., 2011). Clams were individually processed to collect hemolymph and mantle tissues. Hemolymph (generally 1.2–1.8 ml) was withdrawn from the adductor muscle sinus with a 1-ml syringe and centrifuged (700g, 10 min, 4 °C) to pellet the hemocytes from the acellular fraction. Mantle biopsies and hemocyte pellets were flash-frozen in liquid nitrogen and immediately stored at -80 °C until processed for RNA extraction. Trizol reagent (MRC, Inc., Cincinnai, OH, USA) was used to isolate RNA from hemocytes and mantle tissues according to the manufacturer's protocol. RNA quality and quantity were assessed using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, WI, USA). RNA samples were pooled using the same RNA quantity from each individual to generate 3 pools per condition (tissue type, clam strain, temperature). A total of 30 RNA pools (only mantle RNA samples were assessed for MA clams) were generated and used for the downstream reactions and oligoarray hybridization (Table. 4.1).

4.2.3. Oligoarray design, construction and hybridization

Our *in situ* synthesized oligoarrays were produced based on sets of expressed sequence tags (ESTs) obtained from previous SSH libraries (Perrigault et al., 2009c) as well as a transcriptome library generated from 454 sequencing of RNA obtained from *M. mercenaria* mantle tissues, gills, digestive gland and hemocytes (*unpublished data*). Sequences were annotated using the Blast2GO software (http://www.blast2go.com/b2ghome) with blast search against NCBI non-redundant (nr) database (blastx, E-value cut off of 10 E^{-5}). A total of 3,092 curated annotated sequences and 11,166 non-annotated sequences were included with a focus on sequences generated from hemocytes (7,839 sequences) to emphasize the discovery of novel immune-related transcripts. All sequences were submitted to Agilent eArray application (https://earray.chem.agilent.com/earray/) for probe production, with 1 probe (60-mer) produced for each single submitted sequence. In parallel, a set of 595 probes designed based on annotated QPX ESTs (Rubin et al., 2011) was also incorporated into this array in an attempt to target potential molecular evidence of parasite response to the host in different clam broodstocks and temperature conditions. Probes were synthesized *in situ* (14,853) along with positive and negative controls (891) using 8×15K-feature Agilent format slides and a total of 15,744 probes were included on the oligoarray.

Cyanine dye (Cy3 or Cy5) labeled complementary RNA (cRNA) was synthesized from 150 ng of RNA purified from mantles and hemocytes of FLc, FLq and MA clams (as described in 2.2, Table 4.1) using the Two-Color Microarray-Based Gene Expression Analysis Protocol (Quick Amp Labeling) according to the manufacturer's manual. Labeled cRNA was purified using Illustra CyScribe GFX Purification Kit (GE Healthcare). cRNA quantity and quality (including dye incorporation) were determined by spectrophotometry (Nanodrop 1000 ND-1000 spectrophotometer, Thermo Scientific, Wilmington, WI, USA). Samples were considered satisfactory if cRNA concentration

and incorporation efficiency exceeded 300 ng/ul and 8 pmol Cy/ug cRNA, respectively. All arrays were hybridized following a balanced block design with the same amount of cRNA (300 ng of each Cy3- and Cy5-labeled cRNA). Arrays hybridization and washes were conducted according to the kit protocol and the arrays were scanned with a GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA, USA) using the suggested Agilent scan settings.

4.2.4. Oligoarray data analysis

Fluorescence intensities of each spot were extracted using GenePix software and the generated intensity data were normalized using the LIMMA package in R software to remove within-array (method: global lowess) and between-array (method: quantile) non-biological variation (Smyth and Speed, 2003). After normalization, probes with intensities less than two times of background intensities were eliminated from further analysis (applied to all biological replicates for the probes). The filtered data were then submitted to statistical analysis using the Multi Experiment Viewer (MeV) program (Saeed et al., 2003). Gene expression in hemocytes was compared between healthy (FLc) and QPX challenged (FLq) Florida clams at 13 °C and 21 °C (n=3 for each treatment) to examine the molecular response induced by QPX infection and temperature. Mantle samples were examined within and between MA and FL infected clams at temperatures of 13 and 21 °C, in an attempt to identify immune genes and pathways potentially associated with *M. mercenaria* resistance against QPX and their regulation patterns at different temperature. The data analysis was based on the relative gene expression levels among compared samples, which was calculated as the ratio of intensity for a transcript in each treatment against the mean intensity of that transcript in all treatments. The criteria for final determination of differentially expressed genes were the significance by statistical testing (p-value < 0.01, t-test or ANOVA) together with one and half fold increase or decrease from the mean (up- or down-regulation). K-means clustering and hierarchical clustering were then used to cluster those significantly differentially expressed genes with similar expression profiles (Soukas et al., 2000; Yeung et al., 2001). The complete dataset for *M. mercenaria* oligoarray can be found at the Gene Expression Omnibus public database (GEO) (http://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE76182.

4.3. Results

The QPX infection status in clams after 2 months was described in Dahl et al. (2011). Briefly, temperature significantly impacted disease development. Among FL clams experimentally-injected with QPX (FLq), disease development was maximal in clams maintained at 13 °C (73 % QPX disease prevalence) as opposed to those held at 21 °C (10% prevalence). Disease prevalence in naturally-infected (MA) clams was 33% and 20% in clams held at 13 and 21ºC, respectively. Furthermore, moderate to heavy infections constituted most of the positive clams maintained at 13 °C, while none of the

positive clams from the 21°C treatment showed heavy infection, and signs of healing were only noticeable in clams held at 21°C (Dahl et al., 2011).

4.3.1. Differential gene expression in FL clams

The modulatory effect of temperature and QPX infection on *M. mercenaria* gene expression was investigated in hemocyte and mantle samples of FL clams maintained at 13 and 21 °C. Based on stringent criteria for the identification of significant differentially expressed (DE) genes (one-way ANOVA $p<0.01$ in conjunction with relative fold change > 1.5), 887 and 311 DE genes were respectively identified in hemocyte and mantle samples in response to QPX challenge and temperature modulation (Supplementary file 4.2 and 4.3). The DE genes were clustered by K-means clustering (KMC) into groups based on the expression pattern similarities in order to gain further insight into their biological functions. Within each KMC group, DE genes were further clustered using hierarchical clustering (HCL) to explore the potential functional information of novel (unannotated) DE genes, since genes clustered closely usually have linked or co-regulated expression thus possibly share similar function or biological importance (Eisen et al., 1998).

The 887 DE genes in FL hemocyte samples were clustered into 8 groups based on the results of K-means clustering (Fig 4.1A, Supplementary file 4.2). Cluster 1 (CL1) contained 135 DE genes that showed lower expression in QPX infected clams (FLq) held at 13 °C as compared to those held at 21 °C. Several genes from this cluster are known to play a role in mollusk immunity such as the angiopoietin-related protein, cathepsin L2 cysteine protease, complement c1q-like protein (C1q), complement c1q tumor necrosis factor-related protein 2 (C1qTNF2) and vitelline membrane outer layer protein 1. Cluster 2 (CL2) contained 129 DE genes that exhibit maximal expression in naïve FL (FLc) clams held at 21 °C. Some immune-related genes featured in this cluster include beta-glucan recognition protein, complement factor b-like protein and galectins. Cluster 3 (CL3) consisted of 26 DE genes that were found to be over-expressed in FLq hemocytes at 13 °C but under-expressed at 21°C, with most of these being unannotated genes, except the neurogenic locus notch homolog protein 1-like, proteasome subunit beta type-6-like and ribosomal proteins. Cluster 4 (CL4) and cluster 7 (CL7) contained DE genes that were significantly up-regulated in QPX-injected clams held at either 13 °C or 21°C, or at both temperatures (Fig 4.1). Out of the 69 DE genes clustered in CL4, those encode molecules potentially involved in clam defense system were selected and presented in Fig 4.1B, including immune effector proteins (cathepsin k, endo-1,3-β-glucanase, ferritin and cytochrome peroxidase) and immune pattern recognition proteins (ARPs, C1qs, galectins, tenascin and fibrinogen domain containing proteins). CL7 contains 133 DE genes that presented the highest expression in FLq clams held at 21 °C (Fig 4.1C). About 70% (102) of the DE genes in CL7 are unannotated genes, while those annotated DE genes were mostly pathogen pattern recognition proteins (PRRs) such as C1qs, thioester-containing protein (TEP) and sialic acid-binding lectins (Fig 4.1C). Cluster 5 contains a list of 41 DE genes that were significantly down-regulated in FL clams after QPX challenge for both

temperatures. This collection featured genes encoding structural proteins associated with cytoskeleton and ribosome (e.g. ribosomal proteins, actins, tubulins and ATP synthases), and one immune protein (lipopolysaccharide-induced tumor necrosis factor-alpha factor-like protein). Cluster 6 (CL6) contains 209 DE genes that were highly expressed at 13 °C as compared to 21 °C. Many DE genes from this cluster represent stress-related proteins, such as heat-shock proteins, cytochrome oxidase, metallothionein, and omega glutathione s-transferase. Cluster 8 (CL8) was characterized by DE genes (147) that are under-expressed in hemocytes from the low temperature treatment (13 °C) of both naïve and infected FL clams. Genes clustered in this group were typically those associated with growth and metabolism (eg. ribosomal proteins, latent-transforming growth factor beta-binding protein 4, short-chain collagen partial), except a few proteins involved in defense (eg. transmembrane serine 6, cytochrome p450 1a1, complement c1q subcomponent subunit b and caprin-2).

As for the mantle tissues of FL clams, a total of 311 DE genes has shown significant modulation in response to temperature and QPX infection and were clustered into 6 expression pattern groups by KMC (Fig. 4.2 and Supplementary file 4.3). Cluster 1 contained 62 DE genes that were down-regulated in mantle tissues from clams held at 13 °C as compared to 21°C (Fig. 4.2). These included some structural and metabolism-related proteins (e.g. ribosomal proteins, actins, tubulins, acyl-CoA-dehydrogenase and d-beta-hydroxybutyrate mitochondrial), as well as several immune-related proteins, such as antioxidant cytochrome c, the tumor suppressor protein inhibitor of growth protein 3-like, the immune signal transducer serine threonine-protein kinase ctr1 and chitin deacetylase-like protein (Supplementary file 4.3, Fig. S4.1). In contrast, Cluster 3 contained 84 genes and highlighted DE genes that were highly expressed at 13 °C (Supplementary file 4.3, Fig. S4.1), which included acyl-CoA desaturase (lipid metabolism), mucin-associated protein (biomineralization), NADH dehydrogenase subunit and heavy metal-binding protein hip (detoxification). Cluster 2 and Cluster 5 respectively included 56 and22 DE genes that were highly expressed in FLq clams at 21 and 13 °C, respectively (Fig. 4.2 and 4.3). The DE genes in Cluster 2 (Fig. 4.3A) belonged to category of immune-related proteins, such as the tandem repeat galectin, lysozyme, polyubiquitin-c-like isoform1, C1q, and CD209 antigen-like protein. Meanwhile, only genes of the PRR C1qDCs were found in Cluster 5, whereas two QPX genes, the pyruvate dehydrogenase component x and 60s ribosomal protein, were amid the DE genes over-expressed following QPX injection at 13 °C, which could be linked to ongoing active infection in this batch. The remaining two clusters (Cluster 4 and Cluster 6, Fig. 4.3) included DE genes that were either down-regulated (Cluster 4) or up-regulated (Cluster 6) in infected FL clams as compared to control FL clams. Fifty-six DE genes were present in Cluster 4, of which the annotated genes were mostly associated with cell structural components (cytoskeleton, collagen and actin cytoskeleton), protein synthesis and processing (nucleotide binding, translation elongation factor, mRNA splicing), as well as several genes involved in apoptosis (Apoptosis 2 inhibitor, C1q-tumor necrosis factor 3) and detoxification processes (cytochrome p450, small heat shock protein). Out of the 28 DE genes revealed

by Cluster 6, only 5 were functionally annotated, which interestingly included one QPX gene involved in polysaccharide biosynthesis and one *M. mercenaria* tandem repeat galectin gene.

4.3.2. Differential gene expression in MA clams

The modulation of gene expression in mantle tissues from infected MA clams in response to temperature was evaluated. A total of 563 genes were differentially expressed between 13 and 21 °C (*t*-test $p < 0.01$ with at least 1.5 fold change, Supplementary file 4.4). Among these, 217 DE genes exhibited higher expression at 13 °C, and the remaining 346 were comparatively higher in the 21 °C samples. The DE genes from this comparison were categorized by their putative functions and are summarized in Fig. 4.4 and Supplementary file 4.4. DE genes that were highly expressed at 13 °C were dominated by those related to stress response and immune recognition (Fig. 4.4), which included HSP70, universal stress protein, metallothionein, angiopoietin-related proteins and fibrinogen domain-containing proteins (FREP). In addition, several structural proteins were also overexpressed in mantle tissues from MA clams held at 13 °C (Fig. 4.4, Supplementary file 4.4), particularly the tubulins which are known to associated with macrophage activity (Robinson and Vandre, 1995).

On the other hand, structural proteins overexpressed at 21 °C were mostly ribosomal proteins, with functions pertaining to mitochondria or ribosome biogenesis processes (Supplementary file 4.4). In parallel, genes associated with metabolic processes, signal transduction and protein synthesis were also overexpressed at 21 °C (Fig. 4.4), reflecting a higher metabolic rate in these clams as compared to those held at 13 °C. Interestingly, DE genes with functions related to apoptotic processes were exclusively overexpressed at 21 °C, implying a general underexpression of these genes at 13 °C (Fig. 4.4, Supplementary file 4.4).

4.3.3. Comparison of gene expression profiles between FL and MA clams

Gene regulation associated with QPX infection was also compared between the relatively susceptible FL and resistant MA clams (Dahl et al., 2008) in order to get further insights into genes potentially related to the *M. mercenaria* resistance to QPX. Comparisons were made among mantle samples of MA and FLq clams held at either 13 or 21 °C, and DE genes were identified based on the same criteria as above (p <0.01 by ANOVA in conjunction with over 1.5 fold change). A total of 1,569 DE genes were revealed from this comparison (Supplementary file 4.5). K-means and hierarchical clustering were applied to categorize expression patterns among annotated DE genes (Supplementary file 4.5). A total of 227 annotated DE genes were assigned into 6 K-means clusters based on the similarity of the expression patterns across samples (Supplementary file 4.5, Fig. 4.5). The first three clusters (CL1 to 3, Fig. 4.5) featured DE genes that displayed higher expression in MA clams than in FL clams. These 3 clusters contained DE genes with known function involved in pathogen recognition, such

as the C1q domain containing proteins, fibrinogen related proteins and angiopoietin-related proteins (both contain fibrinogen related domains, FReD, Fig. 4.5 and Supplementary file 4.5). These 3 main PRRs families were remarkably overexpressed in MA clams as compared to FL clams. Interestingly, higher expression of these PRRs was found at 13 °C in MA clams than 21 °C, suggesting a link between these immune genes and active host-pathogen interactions (Fig.4.6).

On the other hand, the remaining 3 clusters (CL 4 to 6, Fig. 4.5) highlighted DE genes that were overexpressed at 13 °C (Cluster 4) and 21 °C (Cluster 5) in both MA and FL clams, and those only up-regulated in FL clams at both temperatures (Cluster 6). DE genes up-regulated at 13 °C (Cluster 4) in both clam strains were enriched with functions related to immune and stress response. For example, sialic acid binding lectin, which is a pathogen binding protein, clathrins and tubulins, which are known to regulate macrophage activity, stress protein HSP70, immune enzyme serine protease and kazal-type proteinase inhibitor, as well as cytochrome b, which is involved in ROS detoxification, were concomitantly up-regulated, suggesting stress and active host-pathogen interactions are taking place under this condition. On the other hand, DE genes involved in other biological processes were largely under-expressed at 13 °C as compared to 21 °C (Cluster 5), such as those associated with protein biosynthesis processes (ribosome biogenesis protein, eukaryotic translation initiation factor 3, transcription factor containing protein, translation elongation factor hbs1-like protein, protein folding chaperon heat shock proteins) and metabolic processes (cytochrome oxidase, ATPase inhibitor, ATP-binding protein, cytochrome c, beta-1,4-galactosyltransferase; Cluster 5, Supplementary file 4.5). These were very similar to what observed in FL hemocytes from clams held at 13ºC (described above), reflecting a systemic modulatory effect of temperature and QPX infection on clams regardless of strain and tissue difference. In addition, a large fraction of DE genes that are overexpressed in FL clams as compared to MA clams were related to metabolic and protein biosynthesis processes (Cluster 6, Supplementary file 4.5), such as ribosomal proteins, histone ribonucleoproteins, and ATP synthase. Interestingly, in term of immune-related genes, none of the PRRs that were induced upon QPX infection in MA clams (e.g. FREPs, C1q and angiopoietin) was overexpressed in infected FL clams. In contrast, expression levels of some other defense-related genes were higher in FL clams than in MA, such as the LPS-induced TNF factor, peptidoglycan recognition protein, galactose-specific c-type lectin, proteasome subunit and several hydrolases (cathepsin 1, lipase, lysosomal cholesterol esterase), many of which were especially higher at 21 °C as compared to 13 °C. Finally, higher expression of stress proteins (superoxide dismutase, universal stress protein, omega glutathione s-transferase) was noticed in FL clams injected with QPX, especially when exposed to 13ºC as compared to their MA counterparts suggesting that these conditions are more stressful to the former clam stock (Cluster 6, Supplementary file 4.5).

4.4. Discussion

Successful management and control of QPX disease is of great importance for the hard clam industry, since mortalities caused by this parasite have resulted in significant economic losses (Calvo et al., 1998; Ford et al., 2002; Smolowitz et al., 1998). Thus, understanding the pathobiology of QPX disease and host-pathogen interactions is urgently needed to set forth disease mitigation strategies and develop resistant clam stocks. Currently, our knowledge about QPX disease mostly focused on clam physiology and baseline immune processes. Information on the molecular aspects of clam response to QPX is still very limited, with a narrow collection of common immune-related genes thought to be involved in host-pathogen interactions (Perrigault and Allam, 2008; Perrigault and Allam, 2009; Perrigault and Allam, 2012; Perrigault et al., 2011; Perrigault et al., 2009c). These investigations have described different clam defense strategies mounted against bacterial and QPX infections (Perrigault and Allam, 2012), and the interwoven genetic and environmental determinants associated with clam resistance (Calvo et al., 2007; Dahl et al., 2008; Ford et al., 2002). Our study used high-throughput genomic tools to comprehensively assess clam response to QPX at the transcript level. The comparisons between healthy and infected clams, and between relatively resistant and susceptible stocks provided insights into mechanisms involved in disease development and host-QPX interactions. At the same time, the evaluation of QPX-associated responses in mantle and hemocytes allowed for the understanding of tissue-specific defense strategies as mantle represents the main infection site (Smolowitz et al., 1998) while hemocytes reflect an overall systemic clam response toward the invader (Perrigault et al., 2008).

4.4.1. Modulatory effects of temperature on gene expression in hemocytes

We have previously shown that temperature significantly regulates *M. mercenaria* immunity and QPX disease dynamics (Dahl et al., 2011; Perrigault et al., 2011). In particular, cold temperatures (13 °C) were shown to dampen cellular immunity and significantly promote the establishment of QPX disease, while warmer temperatures (21 and 27°C) prohibit infection development and favor healing of pre-existing lesions (Dahl et al., 2011; Perrigault et al., 2011). Results from the current study show that temperatures altered both constitutive and QPX-induced gene expression in FL clams. In parallel, this study shows that QPX challenge modulated the expression of several immune genes (at either 13 °C or 21 °C, or both; clusters CL4 and CL7, Fig. 4.1 and Supplementary file 4.2. Among these, several DE genes belonged to the fibrinogen-related protein families (FREPs) and the complement C1q domain containing proteins (C1qDCs).

The FREPs are a group of proteins that contain fibrinogen or fibrinogen-like domains. Members of the FREPs family were largely found in CL4, and included the angiopoietin-related proteins, fibrinogen c domain-containing 1-like proteins, fibrinogen-like proteins, ficolin-1-like isoform and tenascin (Fig. 4.1B). As CL4 clustered those DE genes induced

by QPX challenge especially at 13 °C, one can expect that FREPs contribute to clam defense response against ongoing QPX infections. On the other hand, C1qDCs proteins were particularly regulated in challenged clams held at 21 °C (Fig. 4.1C), implying the possible participation of these proteins in neutralizing QPX and preventing infection. Interestingly, levels of C1qDCs were constitutively higher in FLc clams held at 21 °C as compared to 13 °C (cluster CL7, Fig. 4.1C), suggesting a fundamental role of these proteins in *M. mercenaria*'s resistance to QPX.

It should be stated, however, that the exact functions of FREPs and C1qDCs have not been clearly characterized in bivalves. Accumulating evidence supports their important roles in innate immunity as pattern recognition receptors that mediate non-self recognition (Gerdol et al., 2011; Hertel et al., 2005; Zhang et al., 2008; Zhang et al., 2009a). In fact, both FREPs and C1qDCs are known to activate the complement pathway of the innate immune system in vertebrates, however their modes of action are slightly different with C1qDCs triggering the classical pathway, while FREPs activate the lectin pathway (Dodds and Matsushita, 2007; Fujita et al., 2004). Up-regulation of both groups in hard clams upon QPX challenge may indicate the activation and involvement of complement proteins in *M. mercenaria* defense mechanisms. It can be further speculated that an ancestral complement system existed and may be actively involved in the neutralization of QPX in *M. mercenaria*, possibly via parasite recognition, immobilization, and tissue damage repair, which leads the way to ultimate healing. Interestingly, another complement-related protein, the thioester-containing protein (TEP), was also found overexpressed after QPX infection, particularly at 21 °C (Fig. 4.1C). TEP is a functional homologue to vertebrate C3, which is the central component of the complement system, and is widely recognized as an essential player in the anti-parasite defense mechanisms of insects (Blandin et al., 2004; Blandin et al., 2008). Finally, other immune genes found in cluster CL4 and CL7 suggest the mobilization of diverse immune mechanisms and pathways for the purpose of parasite neutralization, with the active involvement of hydrolases (e.g. cathespsin K, endo-1,3- β-glucanase, and hydrolase), lectins (e.g. tandem repeat galectin, sialic acid-binding lectins), metal transporters (e.g. ferritin, heavy metal-binding protein hip) and reduction/oxidation (redox) enzymes (e.g. cytochrome b peroxidase, cytochrome oxidase, eosinophil peroxidase).

The constitutive expression of the above immune-related genes was higher in control clams held at 21°C as compared to those maintained at 13 °C, potentially linking host resistance at the former temperature to immune fitness of the host. In addition, other biological pathways such as metabolic activities, protein synthesis and anti-oxidative processes were also significantly influenced by temperature constitutively. For example, generally higher expressions of ribosomal proteins and fatty acid-binding proteins were found in FLc clams at 21 °C (CL2 and CL8, Supplementary file 4.1), indicating more robust protein synthesis and growth at the higher temperature. On the other hand, several proteins involved in the oxidative stress response were significantly induced at 13 °C, such as the omega-glutathione s-transferase, NADH-dehydrogenase subunit 3 and metallothionein proteins (CL1 and CL6, Supplementary file 4.1). This suggests the

activation of oxidative defense mechanisms to protect against damaging reactive oxygen species (ROS) produced in excess during exposure to sub-optimal temperatures, which is known to induce hypoxia stress (Heise et al., 2006). As reported in many marine ectothermal animals, the reduction of blood circulation and gas exchange at temperatures below an animal optimal thermal range can significantly lower the oxygen supply, sometimes below tissue demand, resulting in functional hypoxia and oxidative stress (Heise et al., 2007; Pörtner, 2002; Pörtner et al., 2001). Thus, for *M. mercenaria* held at 13 °C, higher levels of oxidative defense activities need to be maintained in order to avoid oxidative damage, which may consume a considerable portion of energy that otherwise could have been used for purposes such as growth and immunity. This might partially account for the overall lower host defense levels at 13 °C as compared to 21 °C, and may also explain the dubbing of QPX disease as a "cold water disease" (Dahl et al., 2011; Perrigault et al., 2011), particularly since parasite growth *in vitro* is in fact optimal at 21ºC (Perrigault et al., 2009b), which makes disease onsets largely depend on clam immune performance (Perrigault et al., 2011).

4.4.2. Modulatory effects of temperature on gene expression in mantle tissue of FL clams

QPX lesions are most often found inside clam mantle tissues, sometimes leading to the development of nodules. The formation of nodules reveals intensive interactions between host defense system and the invading parasite. In this context, mantle-related host factors likely contribute to the inflammatory response and encapsulation of QPX cells at the lesion sites (Anderson et al., 2003b; Smolowitz et al., 1998). Tissue extracts from *M. mercenaria* mantle were shown to significantly inhibit QPX growth *in vitro*, even though the nature of these antimicrobial factors remains unknown (Perrigault et al., 2009a). Gene expression profiles of mantle tissues were determined in FL clams in response to temperature and QPX challenge and allowed the identification of candidate anti-QPX factors (Fig. 4.2). For example, similar to the observations in FL clam hemocytes, the overexpressed DE genes induced by QPX challenge mainly included complement C1q like proteins, tandem repeat galectin and galectin-3, all of which are PRRs involved in pathogen recognition and neutralization (Kim et al., 2008; Tasumi and Vasta, 2007; Wang et al., 2012a; Xu et al., 2012). The induction of PRRs in mantle suggests that parasite detection and targeting might be a key process for QPX neutralization. The induction of complement C1qDCs in both mantle and hemocytes following QPX stimulation corroborates the idea that the complement-like system may play a central role in bivalve defense against protozoan parasites, despite the fact that the functionality of complement pathway has not been extensively characterized in bivalve species (Leite et al., 2013; Venier et al., 2011a). A crucial role of galectins has been recently confirmed in bivalves as these were shown to mediate hemocyte recognition and binding of the protozoan parasites *Perkinsus marinus* in the oyster *Crassostrea virginica* and *Perkinsus olseni* in Manila clam *Ruditapes philippinarum* (Kim et al., 2008; Tasumi and Vasta, 2007). In this study, the up-regulation of multiple galectins and tandem-repeated

glaectins upon QPX infection also implies their contribution to protection against protozoan infections, possibly facilitating parasite recognition. Not surprisingly, higher induction levels of these galectins were also noticed at 21°C as compared to 13°C (cluster 2), which again suggests a positive relationship between suitable environmental conditions and better immune performance in *M. mercenaria.* Interestingly, one QPX gene involved in polysaccharide biosynthesis showed up as DE gene in infected FL clam mantle tissue (cluster 6), which could reflect the intensity of the parasite proliferation at the temperature that favored disease establishment.

In addition, FL mantle samples constitutively exhibited expression profiles very similar to those observed in hemocytes (Fig. S4.1). For example, the DE genes up-regulated at 21°C have annotated functions generally related to protein synthesis (e.g. ribosomal proteins, small heat shock protein, alpha-B crystallin), cellular components (β-actins, laminin receptor) and metabolism (chitin deacetylase-like, cytochrome, protein kinases). Whereas at 13 °C, the up-regulated DE genes were largely associated with cell respiration (e.g. nadh dehydrogenase subunit 1 and 3) and fatty acid desaturation (e.g. acyl-desaturase, such as, acyl delta desaturase), suggesting that these processes are involved in the biological adjustments of *M. mercenaria* in response to cold environment.

The total number of DE genes regulated by temperature and QPX infection in FL clam mantle is 311 (Fig. 4.2), which is a considerably smaller than that of hemocyte (877). This directly reflects the fact that hemocytes are more sensitive and responsive to stimuli, possibly because they play a central role as sentinels that monitor and react immediately to changes and danger signals. Hemocytes are the primary immune cells in invertebrates, and alterations of host immune response are readily reflected by gene expression in hemocytes. Moreover, bivalve hemocytes also function in many other processes besides immune protection, such as tissue repair, shell production and nutrition (Donaghy et al., 2009), so temperature and QPX impacts on all other processes can be reflected by hemocytes. On the other hand, the makeup and functions of clam mantle tissues may be more stable as compared to hemocytes, and this property can also be reflected in gene expression data. However, given the fact that bivalves have open circulatory system with hemocytes circulate in all tissues, gene expression changes monitored in mantle could also include the response of circulating hemocytes although the activity may be diluted due to the small fraction. Nevertheless, considering the typical localization of QPX lesions in *M. mercenaria*, changes induced inside mantle tissues provide insights into the molecular responses of *in situ* host-pathogen interactions, regardless of their contributors.

4.4.3. Comparisons of gene expressions in FL and MA clams in response to temperature and QPX infection

Variations in resistance to QPX disease have been broadly reported in hard clams from different geographic and genetic origins. In general, better QPX resistance and host survival are associated with *M. mercenaria* stocks originating from northern states (Massachusetts, New York and New Jersey), while higher susceptibility and mortality are

found in southern stocks (South Carolina, Florida) (Calvo et al., 2007; Dahl et al., 2010; Ford et al., 2002; Kraeuter et al., 2011; Smolowitz et al., 2008). The reasons leading to the observed difference in resistance have been previously ascribed to 1) the poor adaptation of southern strains to the enzootic northern cold waters and 2) variations in genetic background among clam stocks that result in different abilities of the immune system to mount effective defense against the invading parasite (Calvo et al., 2007; Ford et al., 2002). However, results from the field and laboratory observations of Dahl et al. (2008; 2010) have played down the cause of "poor acclimation" from being a main disease-aggravating factor in southern clam strains, leaving the factor of genetic variations, likely driven by the selective mortality caused by enzootic QPX in northern locales, to act as the most important determinant for hard clam susceptibility to QPX disease. In our study, significant difference in molecular response to QPX infection was observed between the susceptible (FL) and resistant (MA) clam strains, providing additional support to the argument that clams ability to resist QPX infection is closely associated with their genetic makeup. The comparison of expression profiles among defense-related genes in response to QPX induction between the two strains give further insights into gene candidates potentially regulating this trait.

A total of 1,569 DE genes were found between challenged clams from both stocks held at both experimental temperatures (Supplementary file 4.5), highlighting a divergence in molecular responses between FL and MA clams to QPX infection. This dissimilarity in gene regulation might be related to myriad biological processes, including pathways regulating both immune response and metabolism, and the observed difference in QPX resistance between the host strains might result from the collective effect of all processes involved. Interestingly, a large portion of the DE genes were up-regulated in MA clams when compared to FL clams (Fig. 4.5 and Supplementary file 4.5), especially those with putative functions related to immune response (e.g. PRRs, C1q and FReDs containing proteins, Fig. 4.5 and Supplementary file 4.5). This suggests that the rather resistant MA clams can react to the parasite infection more strongly than susceptible FL clams. Furthermore, higher expression levels of these DE genes were observed in MA clams held at 13 °C, where more QPX infections were developed (Dahl et al., 2011). Therefore, it seems that MA clams have better adaptation to cold temperature and maintain higher expression of immune-related genes than FL clams. On the other hand, FL clams overexpressed DE genes that were related to metabolic and protein biosynthesis (Cluster 6, Supplementary file 4.5), which may be related to fast growth typically observed in southern aquacultured strains. Interestingly, the defense-related genes up-regulated in FL clams upon infection did not overlap with those over-expressed in MA clams, which may be linked to the susceptibility of the former clams. This may also suggest the attempt of FL clams to possibly compensate for their inability to produce effective anti-QPX factors by triggering the up-regulation of universal defense-related proteins, and this compensatory overexpression of immune molecules was more pronounced at their optimal temperature (21 °C). Such strategy is energetically savvy and might be considered as evidence that resistant MA clams have gone through selection

processes that endowed them with better adaptations to survive in QPX enzootic areas (Gast et al., 2008; Lyons et al., 2005; Ragone Calvo et al., 1998). The fact that QPX is widely distributed in northern cold waters has led to speculation that certain levels of selective mortality have taken place in the local clam stocks due to frequent QPX encounter, which may have increased the overall resistance in surviving animals (Calvo et al., 1998; Gast et al., 2008). Whereas the southern clam stocks may never have been exposed to the selection pressure imposed by this parasite since QPX has never been reported in waters further south than Virginia (Gast et al., 2008).

The over-expressed immune genes associated with MA clams largely included those specifically induced by QPX as shown from the comparison between FLc and FLq clams, such as the FReD and C1q domain containing proteins (Fig. 4.6). Efficient targeting of foreign invaders is a key step for the activation of anti-parasite defense mechanisms and often result in the prompt neutralization of the invader. Higher resistance to QPX observed in MA clams could partly result from higher expression of these QPX-responsive PRRs, which were speculated to be the primary recognition proteins for QPX-specific molecular structures (see above). The broad under-expression of these proteins in FL clams suggests apparent incompetency of susceptible clams to mount sufficient defense response against QPX. In addition, expression level for these DE PRR genes appears to be correlated with infection intensity. For instance, the highest over-expression was generally measured in MA clams held at 13 °C, the temperature that causes the most severe QPX infections; and the up-regulation was not uniform in MA samples at 21 °C (Fig. 4.5). This last observation may come from the fact that MA clams held at 21ºC displayed a range of infection severities with some clams displaying complete signs of healing (Dahl et al., 2011).

A great number of DE sequences containing C1q domain and FReD have been noticed (Fig. 4.6), possibly reflecting the high sequence diversification in these two gene families (as discussed in Chapter 2). In fact, beside *M. mercenaria*, members of these two gene families have been shown to display extensive sequence diversity in many molluscan species (Ghai et al., 2007; Hanington et al., 2012; Leite et al., 2013; Mone et al., 2010; Venier et al., 2011a; Zhang et al., 2009a; Zhang et al., 2013a). The broad sequence variability in these genes usually translates into immune molecules with highly diversified pathogen recognition domain structures (Cerenius and Soderhall, 2013; Mone et al., 2010; Zhang and Loker, 2004). Mechanisms leading to this diversification may include alternative splicing, recombinatorial and somatic diversification through gene conversions and point mutations (Hanington et al., 2012; Zhang and Loker, 2004). The polymorphism and diversification of these putative immune receptor sequences is important for the host to maintain a dynamic and rich repertoire of putative recognition molecules so that response against a variety of pathogen epitopes could be promptly mounted. In this study, the differential expression of diverse forms of C1qDCs and FREPs between MA and FL clams might suggest high responsiveness of these molecules against QPX, particularly when taking into consideration that the overexpression was mostly associated with the more resistant clam stocks held at the condition favoring

disease development (MA clams, 13 °C). The regulation of these genes likely help MA clams fight QPX, but can also contribute to the elimination of putative secondary pathogens.

It is widely recognized that invertebrates do not have acquired immunity, and their innate immune system exhibits less diversity in receptor repertoire leading to lower specificity. Their ability to detect parasites exclusively relies on invariable germline-encoded immune receptors and effectors that interact with universal microbial antigens (Medzhitov and Janeway, 1997). However, increasing evidence suggests the presence of sophisticated recognition systems in some invertebrate species, including echinoderms (sea urchin), insects (*Drosophila melanogaster* and *Anopheles gambiae*), crustaceans and mollusks (*Biomphalaria glabrata*) (Brites et al., 2008; Dong et al., 2006; Pancer, 2000; Watson et al., 2005; Zhang, 2004). In addition, remarkably different immune responses were observed in *M. mercenaria* following QPX and bacterial challenge, suggesting the involvement of different immune pathways for the discrimination and elimination of different pathogen types (Perrigault and Allam, 2012). Together, these observations imply the existence of a form of specific immunity in invertebrates, which has been suggested to be linked to those highly diversified immune molecules (Mone et al., 2010). This broad reservoir of recognition molecules also serves as the source for the development of host adaptation to parasite-driven selective pressures (Cerenius and Soderhall, 2013; Dheilly et al., 2015; Zhang et al., 2013a; Zhang and Loker, 2003). For example, the *B. glabrata* FREPs are a group of highly variable receptors that precipitate variable antigens of trematode parasites (Mone et al., 2010). Exceptional diversifications exist among these molecules, creating quite individualized FREP pools that vary from one snail to another. Therefore, the recognition capacity has been dramatically enlarged in a random way, however individuals possessing the receptor variants that are capable of recognizing specific antigens would be favored in an environment where they are exposed to corresponding pathogens, allowing these snails to survive the selective pressure (Mone et al., 2010). The higher expression levels of diversified C1qDCs and FREPs associated with resistant MA clams are very likely the result of QPX-derived selection process, however via unknown mechanisms.

Overall, more and more studies advocate that invertebrate innate immunity has considerable specificity and is capable of discriminating between pathogens. Recent identification of several components of the lectin-based complement pathway from ascidians reveals that the primitive complement system is one of the most highly organized innate immune systems in invertebrates (Fujita, 2002; Fujita et al., 2004). In fact, the complement system plays a pivotal role in innate immunity before the evolution of an adaptive immune system in vertebrates and is widely thought to act as an evolutionarily transitional mechanism that links innate immunity to acquired immunity (Fujita, 2002; Sekine et al., 2001). Since both the FREPs and C1q proteins are important components initiating the complement system, a primitive complement-like system capable of providing tailored immune protection against various pathogens is speculated to also exist in *M. mercenaria*.

Although the expression of *M. mercenaria* C1qDCs and FREPs increases in response to QPX challenge, the nature of the QPX ligands that these receptors recognize is unknown. In addition, the specific roles of these molecules in *M. mercenaria* immunity remain mysterious. Future studies should focus on addressing the specific role of these molecules in hard clam immunity against QPX infection, and their mechanisms of interaction with various parasite antigens. In-depth understanding of these questions should shed light on the properties of anti-QPX factors present in *M. mercenaria*. It may lead to the discovery of promising molecular candidates for marker-assisted selection of disease resistant hard clam broodstocks to better control QPX disease and minimize losses caused by this parasite.

4.5. Conclusions

This study represents the first attempt to investigate the molecular immune response of the hard clam *M. mercenaria* using high-throughput techniques. The first *M. mercenaria* oligoarray was designed and used to explore transcriptomic changes in clams during QPX infection. Gene expression profiles were compared between naïve and QPX-challenged clams at temperatures known to affect infection establishment in order to gain an understanding of molecular mechanisms of host response at conditions known to favor disease establishment or healing. A large set of defense-related genes was regulated in infected clams, including genes involved in microbe recognition, pathogen killing, metabolism and stress response. The results suggest that the modulation of disease development by temperature is mainly through alteration of constitutive and QPX-inducible immune responses. Comparison of gene expression profiles between susceptible and resistant clam broodstocks identified molecular candidates that could mediate clam resistance against QPX. Special interest was placed upon the key families of highly diversified recognition molecules, such as C1qDCs and FREPs, which not only significantly induced after the parasite challenge but also displayed higher expression in resistant clams as compared to the susceptible stock. The findings underscore the role of these receptors in QPX recognition and possibly mediation of subsequent parasite elimination via the initiation of a primitive complement-like system. However, further investigations are needed to characterize the nature of these molecular components and probe their specific role during *M. mercenaria*-QPX interactions, with perspectives on their molecular functions, diversification mechanisms and interactions with various pathogen epitopes.

Table 4.1. Biological samples analyzed using the *M. mercenaria* oligoarray (the number of pooled RNA samples is given, each pool is made with equal quantities of RNA from 3 individual clams). NA: not assessed.

		13 °C		**21 °C**	
Clam source and disease status		Hemocytes	Mantle	Hemocytes	Mantle
FLc	Florida Naive	3	3	3	3
FLq	Florida Injected	3	3	3	3
MA	Massachusetts Naturally-infected	NA	3	NA	3

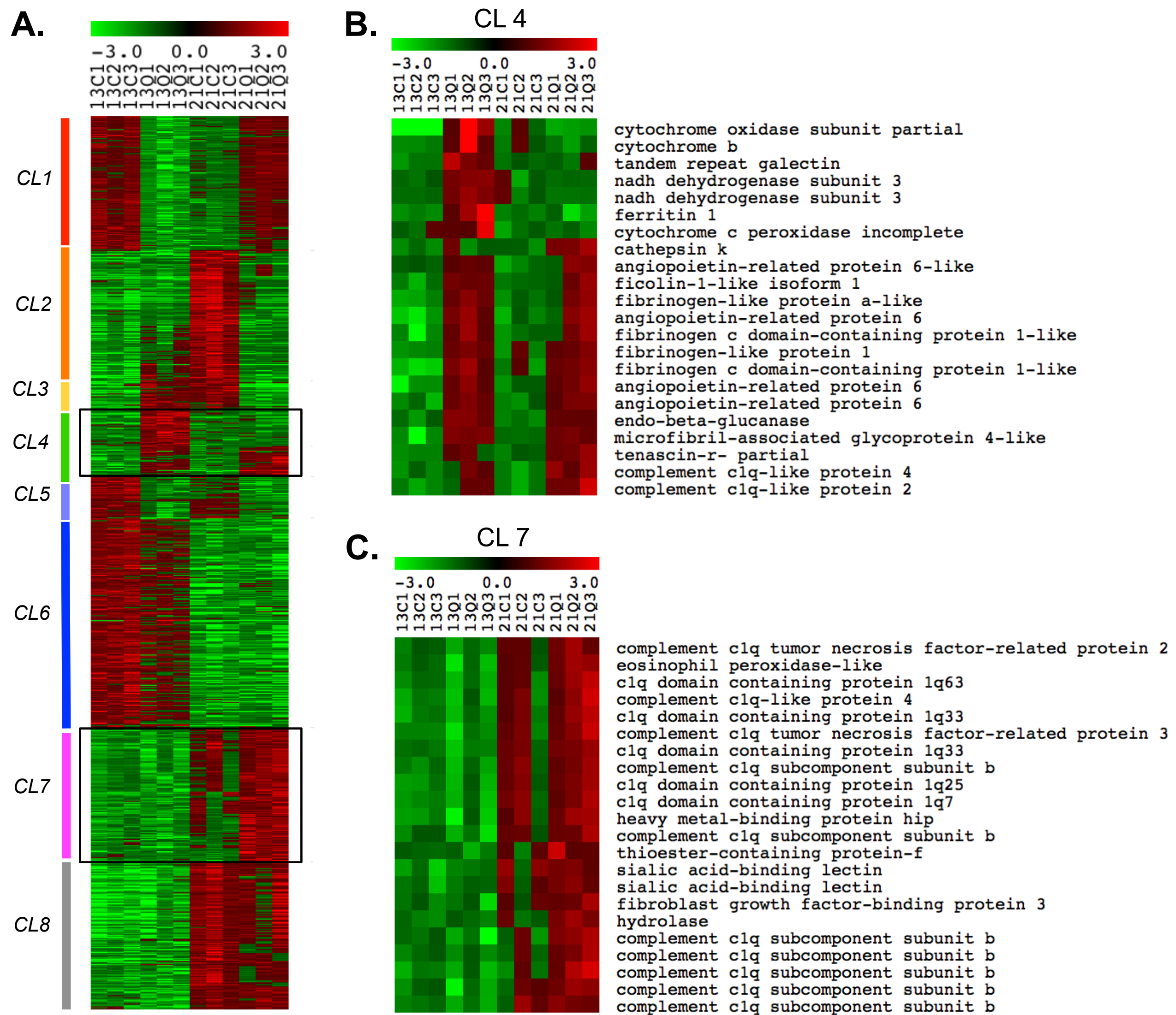


Figure 4.1. Gene clusters generated by K-means clustering of the 887 differentially expressed genes in FL clam hemocytes. (A) Overview of all 8 clusters. (B) Immune related transcripts identified in cluster 4 (CL4). (C) Immune related transcripts identified in cluster 7 (CL7). Gene expression heat maps reflect relative gene expression fold changes where a gradient of red to green represents a 3-fold or greater increase in gene expression to a 3-fold or greater decrease in gene expression. Columns represent the gene expression of individual samples from each treatment with each gene displayed horizontally. Sample names give temperature treatment (13 or 21ºC), challenge status (C: control, Q: challenged with QPX), and replicate number (1 to 3).

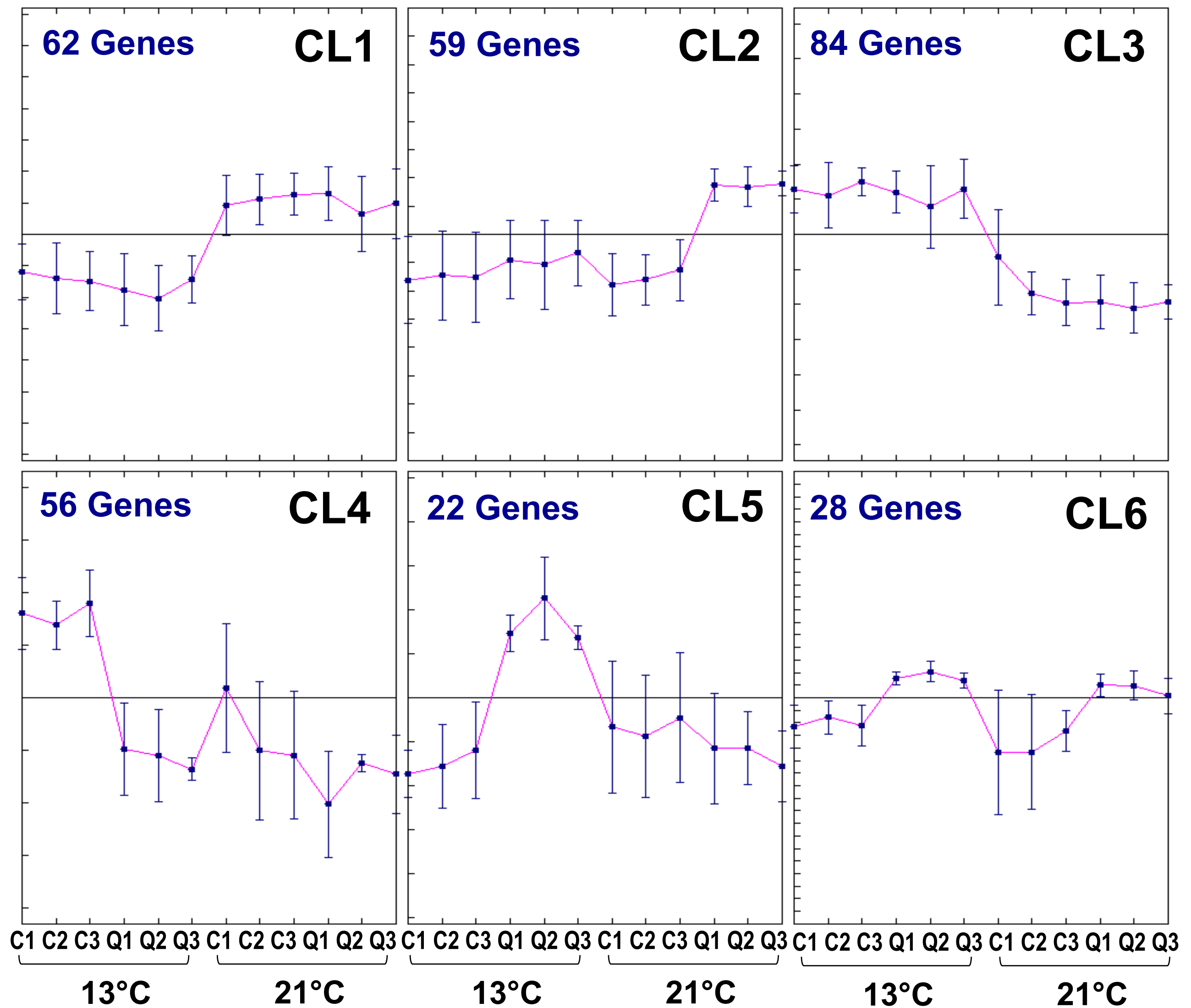


Figure 4.2. Gene clusters generated by K-means clustering of the 311 differentially expressed genes in FL clam mantle samples. Gene clusters are generated based on the similarity of their gene expression profiles in the different treatments. The relative fold change of the DE gene expression within each cluster can be directly compared across samples/treatments based on their value on the Y-axis (mean ± S.D.). Sample names are similar to those in Figure 4.1.

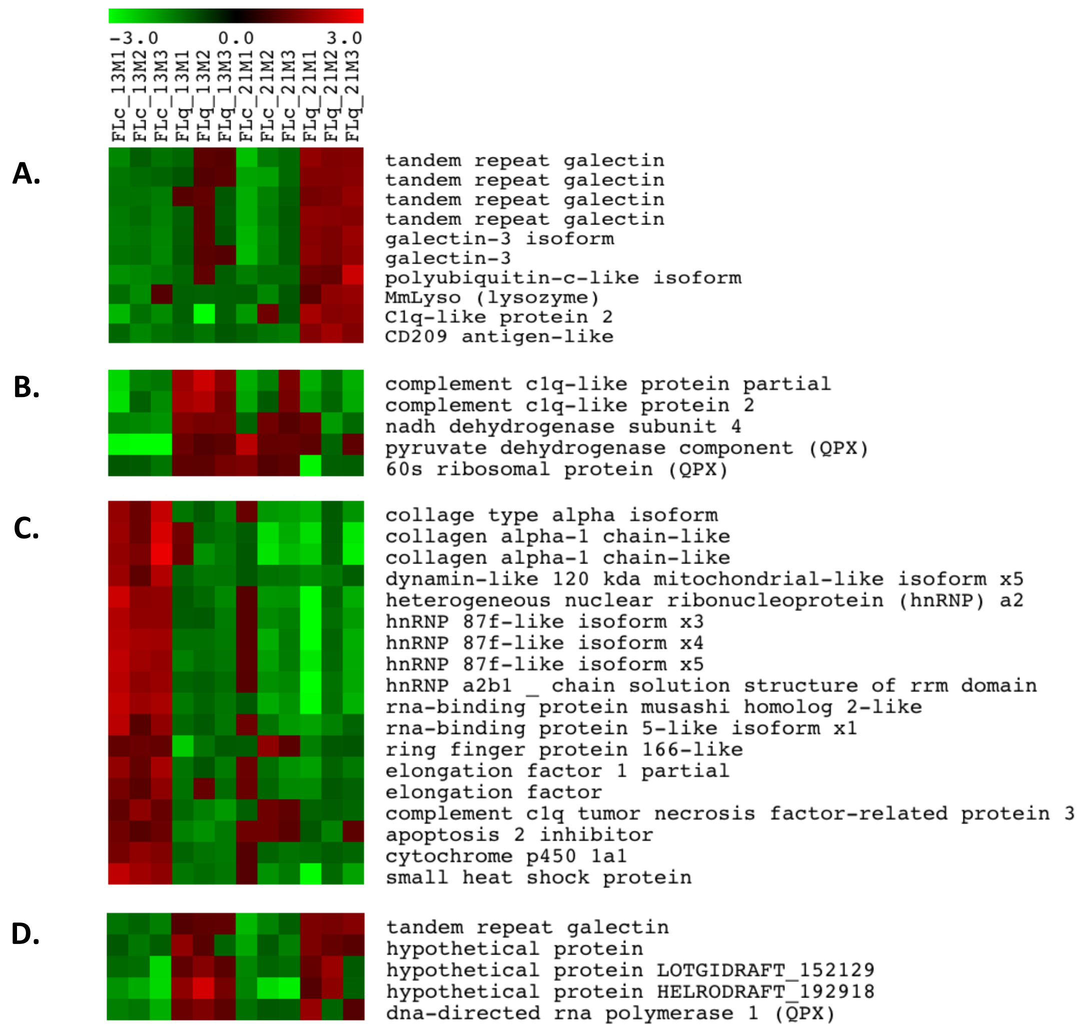


Figure 4.3. Selected differentially expressed transcripts in FL clam mantles from K-means cluster 2 (A), cluster 5 (B), cluster 4 (C), cluster 6 (D). Expression heat maps reflect relative gene expression fold changes where a gradient of red to green represents a 3-fold or greater increase in gene expression to a 3-fold or greater decrease in gene expression. Columns represent the gene expression of individual samples from each treatment with each gene displayed horizontally.

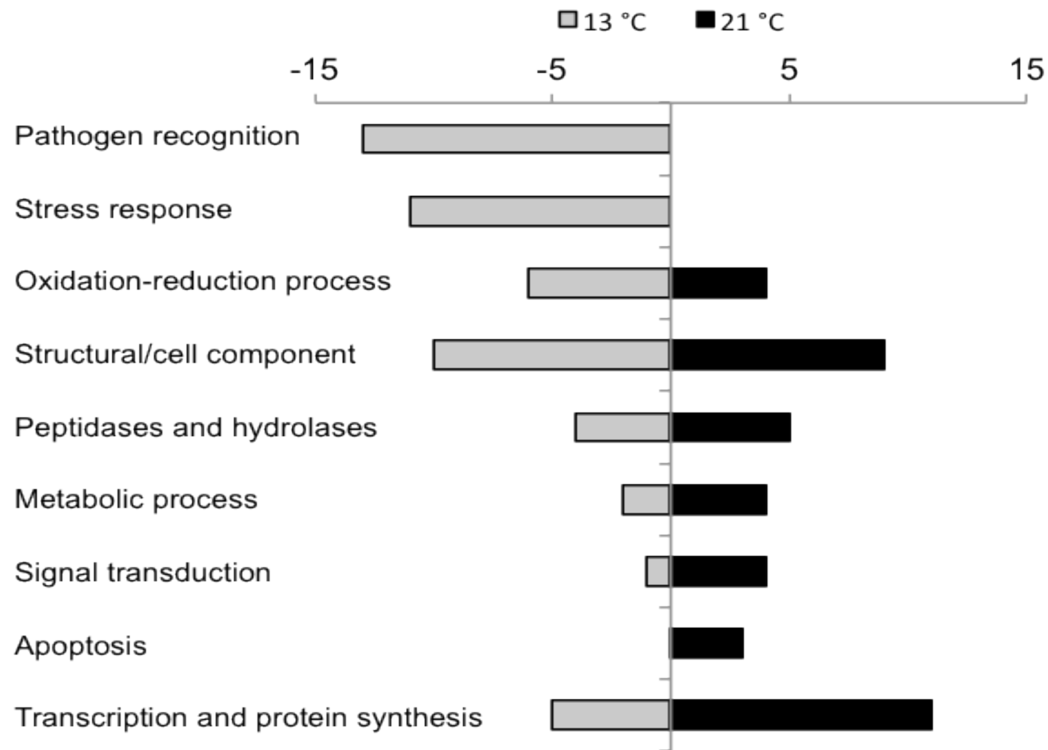


Figure 4.4. Overview of annotated differentially expressed (DE) gene functions in naturally infected (MA) hard clam mantle as modulated by temperature (13 and 21 °C). The number of DE genes grouped into each functional annotation category are indicated on the horizontal axis (negative values represent for DE genes overexpressed at 13 °C and positive values represent those overexpressed at 21 °C).

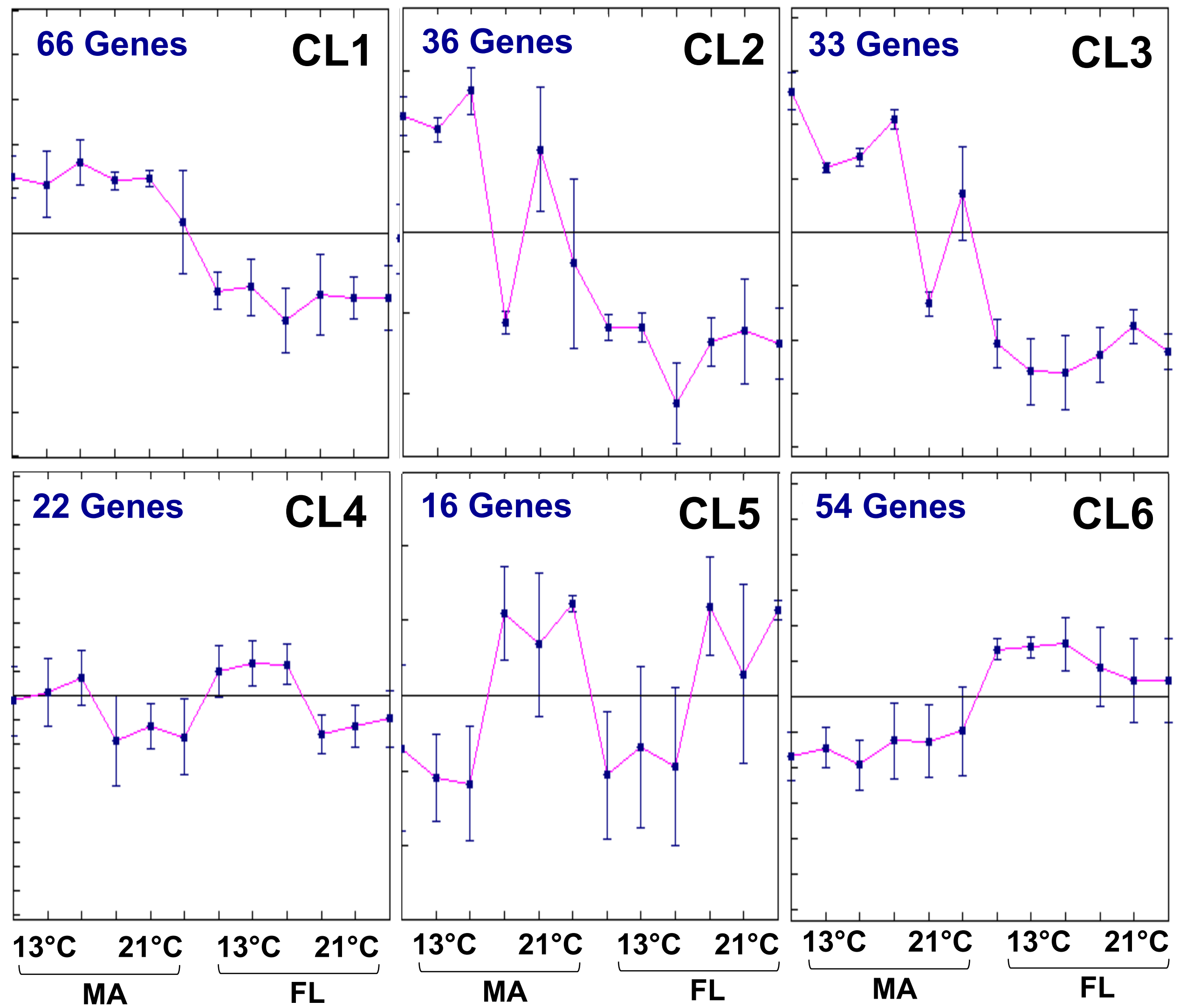


Figure 4.5. Gene clusters generated by K-means clustering of the annotated genes differentially expressed in mantle tissues between QPX infected FL and MA clams at 13 °C or 21°C. Gene clusters are generated based on the similarity of their gene expression profiles in the different treatments. The relative fold change of the DE gene expression within each cluster can be directly compared across samples/treatments based on their value on the Y-axis (mean ± S.D.).

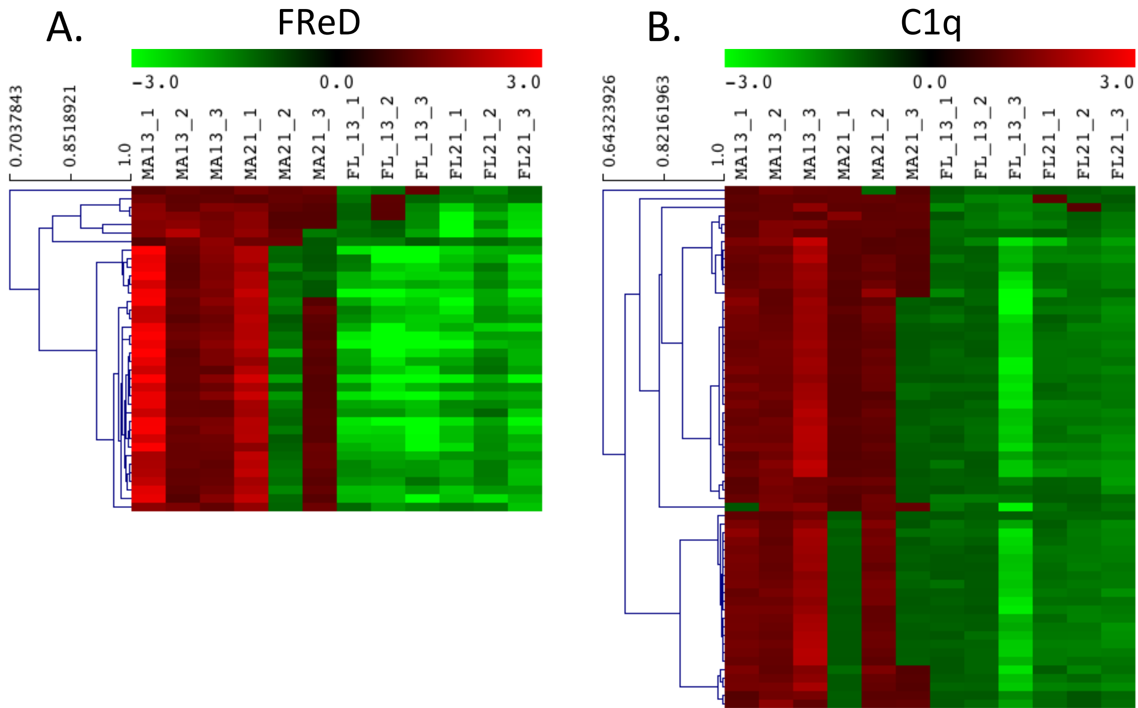


Figure 4.6. Subsets of differentially expressed genes identified between QPX infected FL and MA clam mantles containing (A) fibrinogen-related domains (FReD) and (B) complement C1q domains. Gene expression heat maps reflect relative gene expression fold changes where a gradient of red to green represents a 3-fold or greater increase in gene expression to a 3-fold or greater decrease in gene expression. Columns represent the gene expression of individual samples from each treatment with each gene displayed horizontally.

Supplementary files:

Fig. S4.1. Hierarchical clustering of K-means gene clusters of differentially expressed genes in FL clam mantles. (A) cluster 1 (CL1), (B) cluster 3 (CL3). Gene expression heat maps reflect relative gene expression fold changes where a gradient of red to green represents a 3-fold or greater increase in gene expression to a 3-fold or greater decrease in gene expression. Columns represent the gene expression of individual samples from each treatment with each gene displayed horizontally.

Supplementary file 4.1. Probes information of *M. mercenaria* oligoarray. This is a Microsoft Excel worksheet that contains information on sequences used to design the oligonucleotide probes. The table contains the top BLASTx match accession number and E value, Gene Ontology terms and InterPro domain numbers associated with each sequence.

Supplementary file 4.2. A list of differentially expressed (DE) genes revealed in FL hemocyte samples in response to temperature change and QPX infection. This file is a Microsoft Excel worksheet that contains a list of 887 DE genes and the relative fold change values of individual genes at 13 and 21 °C arranged into the 8 K-means clustering groups.

Supplementary file 4.3. A list of differentially expressed (DE) genes revealed in FL mantle samples in response to temperature change and QPX infection. This file is a Microsoft Excel worksheet that contains a list of 311 DE genes and the relative fold change values for individual genes at 13 and 21 °C arranged into the 6 K-means clustering groups.

Supplementary file 4.4. List of differentially expressed (DE) genes in MA clams exposed to 13 and 21 °C. This file is a Microsoft Excel worksheet that contains a list of 563 DE genes with the relative fold change values for each gene. DE genes associated with each temperature were categorized into different biological function groups according to the putative GO annotations.

Supplementary file 4.5. List of differentially expressed (DE) genes in mantle samples from MA and QPX challenged FL (FLq) clams maintained at 13 and 21 °C. This file is a Microsoft Excel worksheet that includes the list and the relative fold change values of the 1569 DE genes and the annotated 227 DE genes, which are arranged into 6 K-means clustering groups.

Chapter 5 Effect of "heat shock" treatments on QPX disease and stress response in the hard clam, *Mercenaria mercenaria*

Manuscript in review:

Wang, K., Pales Espinosa, E., Allam, B. (2016). Effect of "heat shock" treatments on QPX disease in the hard clam, Mercenaria mercenaria. Journal of Invertebrate Pathology

Abstract

The hard clam, *Mercenaria mercenaria*, is one of the most valuable commercial mollusk species along the eastern coast of the United States. Throughout the past 2 decades, the hard clam industry in the Northeast was significantly impacted by disease outbreaks caused by a lethal protistan parasite known as Quahog Parasite Unknown (QPX). QPX is an opportunistic pathogen and the infection has been shown to be a cold water disease, where warmer conditions (above 21°C) lead to disease reduction and clam healing. *In vitro* studies also showed a sharp reduction in parasite growth and survivorship at temperatures exceeding 27°C. In this study, we evaluated the effect of short-term exposures to high temperatures on QPX disease dynamic and clam recovery. Infected clams were collected from an enzootic site and subsequently submitted to one of ten "heat shock" treatments involving a gradient of temperatures and exposure times. QPX prevalence was compared before and 10 weeks after heat shock to assess the effect of each treatment on disease progress. Expression of several stress-related genes was measured 1 and 7 days after heat shock using qPCR to evaluate the effect of each treatment on clam physiology. Anti-QPX activity in clam plasma was also measured in an attempt to link changes in defense factors to thermal stress and disease progress. Our results suggest that brief exposures to moderate high temperatures promote the greatest remission while imposing the mildest stress to clams. These results are discussed with the aim of providing the industry with possible strategies to mitigate QPX disease.

Keywords: Hard clam; QPX; Heat shock; Immunity; Stress; Hypoxia

5.1. Introduction

The hard clam, *Mercernaria mercenaria*, is a bivalve species native to the North American Atlantic coasts and its distribution ranges from the Maritime Provinces of Canada to Florida. Hard clams, also known as northern hard clams or quahogs, are of great ecological and commercial significance representing the most important marine resource in dockside value in several northeastern states. It is a relatively sturdy bivalve species and its only notorious infectious agent is the protistan parasite QPX (Quahog Parasite Unknown), which has been reported to cause severe mortality episodes among both wild and cultured clams (Ford et al., 2002; Lyons et al., 2007; Maas et al., 1999; Ragan et al., 2000; Smolowitz et al., 1998; Stokes et al., 2002). QPX disease outbreaks have posed great threats to the clam industry during the past few decades ever since the first reported mortality event in 1959 in New Brunswick (Drinnan, 1963). QPX is an opportunistic pathogen that has been detected in a wide variety of substrates, and is thought to be ubiquitous in the coastal environments where it can frequently interact with hard clams without causing disease (Gast et al., 2008; Liu et al., 2009; Lyons et al., 2005). Previous surveys have shown a wide distribution of QPX in both epizootic and non-epizootic waters, sometimes being present at low prevalence in clam populations that appear to be healthy (Liu et al., 2008; Liu et al., 2009; MacCallum and McGladdery, 2000; Ragone Calvo et al., 1998). This seems to suggest that even though the parasite has a broad distribution and regularly interacts with the clam host, it does not initiate epizootic events until other determinant factors, such as increased host susceptibility and favorable environmental conditions, are involved. In addition, it is noteworthy that clams are able, under certain conditions, to mount an effective defense response against the infection leading to complete healing and recovery, as observed by histological examination showing evidence of dead QPX cells inside old lesions (Calvo et al., 1998; Dahl and Allam, 2007; Dahl et al., 2010; Dove et al., 2004a).

Previous studies reported that the ability of clams to resist QPX infection is largely influenced by environmental factors, such as temperature and salinity, which significantly alter the host-parasite interaction by affecting the host immune performance and the virulence of the parasite (Perrigault et al., 2010; Perrigault et al., 2012; Perrigault et al., 2011). Among all the investigated environmental factors, temperature seems to play a predominant role in regulating the development of QPX disease (Dahl et al., 2011). In the field, QPX disease has never been detected in clams south of Virginia where water temperature is comparatively warmer despite the fact that clam broodstocks originating from southern states (Florida and South Carolina) are more susceptible to QPX than northern stocks (Calvo et al., 2007; Dahl et al., 2008; Dahl et al., 2010; Ford et al., 2002). In addition, results of previous laboratory investigations showed that naturally-infected clams exposed to 13 °C exhibit significantly higher disease-related mortality than their counterparts exposed to 21 or 27 °C, whereas the clams submitted to the latter two conditions displayed signs of healing and recovery from QPX infection (Dahl and Allam, 2007;

Dahl et al., 2011; Perrigault et al., 2011). Further, this healing process observed at or above 21 °C was associated with higher clam hemocyte resistance to cytotoxic QPX extracellular products, higher plasma anti-QPX activity and higher expression of host immune factors as compared to 13 °C (Perrigault et al., 2011; Wang et al., 2016b). Finally, *in vitro* studies showed significant decrease in QPX growth and survival at temperatures exceeding 23 ºC (Perrigault et al., 2010). These observations underline a major effect of temperature on disease dynamic and support the categorization of QPX infection as a "cold water disease" (Perrigault et al., 2011).

Beyond the specific case of clam-QPX interactions, temperature is one of the main factors affecting the wellbeing of ectothermic aquatic species. Temperature can significantly modulate the growth and virulence of marine microbes as well as host immune competency as demonstrated in several cases of bivalve infectious diseases. A good example is the Dermo disease in the eastern oyster *Crassostrea virginica* caused by the protozoan parasite *Perkinsus marinus*. *P. marinus* is well adapted to warm waters and the disease can be mitigated in cold-water environments (Chu, 1996; Chu and LaPeyre, 1993). On the other hand, the “brown ring disease” of Manila clam (*Ruditapes philippinarum*), a bacterial disease caused by *Vibrio tapetis,* has been described as a “cold water disease” with outbreaks often found where the water temperature is low (8 to 13 °C) (Paillard et al., 2004). The compromised host defense mechanisms seem to be, at least partially, responsible for the outbreaks of major cold-water diseases of bivalves, as host immune factors fail to neutralize invading microbes (Allam et al., 2001; Allam et al., 2002; Paillard et al., 2004).

Every living organism has a specific optimal temperature range that is most suitable for various physiological functions; temperatures outside this range lead to thermal stress that can compromise the species’ growth, immune functions or even survival. The heat shock proteins (HSPs) are known to protect organisms subjected to a wide range of stressors, especially thermal stress. HSPs are molecular chaperones that play a fundamental role in the stability of thermo-labile proteins, ensuring correct folding of damaged proteins. HSPs are highly conserved with molecular weights ranging from 12 to 100 kDa. When exposed to stress, up-regulation of HSPs is observed universally in most taxa, however, this response is not restricted to thermal stress since other stressors such as exposure to chemical contaminants or to pathogens as well as wounds and tissue damage also leads to HSPs up-regulation (Roberts et al., 2010). Although HSPs do not directly participate in stress response, they contribute to the maintenance of cellular homeostasis and their levels are generally correlated with the resistance of the organism to stress. For example, Pan et al. (2000) showed that thermal shock of Atlantic salmon results in a significant rise of HSP 70 levels that dramatically improved fish survival rate following transfer to high salinity water as compared to control populations not submitted to thermal shocks. Another study in brine shrimp by Sung et al. (2007) also demonstrated that a non-lethal heat shock significantly increased the expression of HSP 70, leading to higher survival in shrimp

larvae subsequently challenged with pathogenic bacteria. The mechanism of HSPs induction leading to improved resistance against infection is not thoroughly understood, although it has been demonstrated that HSPs contribute to the host immune response, serving as signaling molecules that initiate the inflammatory cascade or binding and forming complexes with non-self proteins to enhance the recognition and opsonization of foreign entities (Roberts et al., 2010).

The main objective of this study is to evaluate the effect of "heat shock" treatments (acute short-term exposure to high temperature) on the dynamic of pre-established QPX infections. The design of this experiment is based on previous findings that high temperature reduces the establishment of QPX infection and promotes the host-healing process. We hypothesize that heat shock treatment would have the potential to limit the proliferation of the parasite and stimulate the host immunity to resist and recover from QPX infection. This work also serves the aim of developing field-applicable strategies for QPX disease control and reducing its impact on the hard clam fishery.

5.2. Materials and Methods

5.2.1. Hard clams

Adult hard clams (51 ± 5 mm in length, mean ± sd) naturally infected with QPX were collected from a QPX enzootic clamming area in Massachusetts (MA) in early February 2012 (4 to 5ºC, 31 ppt salinity). Clams were transported overnight to the laboratory and submitted immediately to a 2-week acclimation period in 150-L tanks with re-circulating seawater (28-30 ppt) at 18 °C. During the acclimation, clams were fed daily with commercial algae (DT's Live Phytoplankton, Sycamore, Illinois, USA). At the end of acclimation, a total of 70 clams were randomly sampled, dissected and processed to determine the initial QPX prevalence (36.6 %) before submission of the remaining clams to the heat shock treatments.

5.2.2. Heat shock treatments

Following the 2-week acclimation, clams were randomly assigned to one of 10 treatments (Table 5.1). The combination of different exposure times and temperatures employed during the treatments was intended to help determine the minimal exposure temperature and duration needed to significantly reduce QPX infection and enhance the host healing process. During the treatments, clams were taken out of the water and were maintained either in incubators (21, 27, 32 and 37 °C) or at room temperature (18 °C) to achieve the targeted temperatures. For accurately measuring of temperature during the heat shock treatment, the internal temperatures of clams (the actual temperature of clam meat inside the shells) were measured and recorded using hypodermic thermocouple probes (HYP-2 probes connected with HH147U electronic data loggers (Omega Engineering, Stamford, Connecticut, USA) that were carefully inserted inside a clam from each temperature treatment. Timing for each heat shock period started immediately

after the monitored temperatures of clam internal tissues reached the target temperatures. An additional undisturbed control group was included where clams were continuously submerged in seawater maintained at 18 °C. After heat shock, clams from each treatment were transferred to separate 40-L re-circulating tanks with seawater maintained at 18 °C and were feed daily with commercial algae (DT's Live Phytoplankton, Sycamore, IL). Two replicate tanks (20 clams/tank) were made for each treatment.

At Day 1 and Day 7 post heat-shock, 3 and 4 clams respectively were collected from each tank to assess the effect of heat shock treatments on clam physiology. The expression of a selection of stress related genes was assessed and the anti-QPX activity of clam plasma was also tested (see below). The remaining clams in each treatment were kept at 18 °C for 10 weeks to allow for disease progress. Mortality of clams from each tank was checked twice daily and moribund individuals were removed once detected. After the 10-week incubation, all remaining clams were dissected and processed for QPX diagnosis.

5.2.3. Anti-QPX activity

Hemolymph of clams sampled at Day 1 and Day 7 post heat-shock was withdrawn from the adductor muscle with a 1 ml-syringe. Plasma was recovered by centrifugation of the hemolymph at 700 x g, 10 min, 4 °C and the supernatant (plasma) was sterilized by filtration (0.22 μm), aliquoted and preserved at -80 °C for the assessment of anti-QPX activity. The measurement of plasma anti-QPX activity was adapted from the previously described *in vitro* growth inhibition assay (Perrigault et al., 2011; Perrigault et al., 2009b) with modifications, as the fluorescein di-acetate substrate was replaced with a commercial adenosine tri-phosphate (ATP) content-based assay for the assessment of the QPX biovolume. Briefly, exponentially-growing QPX cells were harvested and washed with filtered artificial seawater (FASW, 31 ppt), and then resuspended in Minimal Essential Medium (MEM). A volume of 50 μl of this QPX suspension containing 1×10^3 cells were added to 50 μl undiluted clam plasma in a black 96-well plate. QPX growth inhibition assays were performed in duplicate wells and an additional replicate without QPX cells was used to quantify the background luminescence signal generated by plasma sample. For no inhibition controls, FASW was substituted for plasma to monitor QPX growth. After 4 days of incubation at 23 °C, QPX biovolume in each well was measured using the ATPlite assay kit following the manufacturer's protocol (PerkinElmer, Boston, Massachusetts, USA). The ATPlite assay measures the total ATP content in live QPX cells, which was shown to linearly correlate with QPX biovolume during preliminary assays. The assay is based on the production of bioluminescence caused by the reaction of ATP with the firefly luciferase and D-luciferin included in the kit and the emitted light is proportional to the ATP concentration. Anti-QPX activity was expressed as the percentage of luminescence intensity in presence of plasma compared to the FASW controls ($[I_{QPX\ in\ plasma} - I_{plasma}]/[I_{QPX\ in\ FASW} - I_{FASW}] \times 100\%$).

5.2.4. Total RNA isolation and cDNA synthesis

Following hemolymph sampling, clams were individually dissected and biopsies of gill and mantle from each clam were immediately flash frozen in liquid nitrogen and stored at -80 °C until processing. Total RNA extraction using TRI ® Reagent (Invitrogen, Carlsbad, California, USA) was performed on gill biopsies of clams sampled at Day 1 and Day 7 from each heat shock treatment. Further RNA clean-up and on-column DNase digestion were performed with RNeasy Mini Kit (Qiagen Ltd., Crawley, UK) according to the manufacturer's guidelines. RNA quantity and quality were assessed on a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA) and 5 µg of total RNA were subjected to reverse transcription using oligo dT18 and Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, Madison, Wisconsin, USA) for the first strand cDNA synthesis. The cDNA samples were then used for gene expression studies.

5.2.5. Real-time PCR analysis of relative expression of stress-related genes

Relative expression of selected stress-related genes was assayed by quantitative real-time PCR (qPCR), for the evaluation of the impact of heat shock treatments on clam physiology. The tested genes included HSP 25, HSP 70, Glutathione Peroxidase (GPx) and Universal Stress-related Protein (USP). These genes have been reported to reflect general thermal and/or hypoxia stress in many invertebrate species (Bao et al., 2011; Monari et al., 2011; Park et al., 2007; Roberts et al., 2010; Wang et al., 2011a). Primers used for the amplification of these genes were designed based on recently-generated RNASeq datasets (Wang et al., 2016a) and are presented in Table 5.2. PCR efficiency was assessed for each primer pair. The qPCR assay was performed with Mastercycler ep realplex (Eppendorf, Hauppauge, New York, USA) in a 10 µl reaction volume containing 10 ng cDNA template, 100 nM of each primer and 5 µl 2×Brilliant SYBR® Green QPCR master mix (Agilent, Santa Clara, California, USA). The reactions were programed as 10 min at 95 °C for activation of the SureStart® DNA polymerase, 40 cycles of amplification of target cDNA (denaturation at 95 °C for 30 sec, annealing and extension at 60 °C for 1 min), with fluorescence signals collected at the end of each cycle. A melting curve was generated at the end of thermal cycling. The comparative CT method ($2^{-\Delta\Delta Ct}$ method) was used to calculate the relative expression levels of all selected stress related genes (Livak and Schmittgen, 2001). Transcription elongation factor 1 alpha (EF1a) was used as the reference gene (Perrigault and Allam, 2012; Perrigault et al., 2009c).

5.2.6. QPX diagnosis

QPX disease status of individual clams was determined in mantle tissues using the standard qPCR diagnosis protocol (Liu et al., 2009). Changes in QPX prevalence and intensity following heat shock treatments were compared to evaluate the effect of each treatment on QPX infection

and disease development. QPX prevalence was calculated as the percentage of QPX positive clams in all sampled clams in each treatment and the QPX intensity was assessed as the number of QPX cells per gram clam tissue and was categorized into the following ranks: light (≤ 10 QPX cells per gram of clam tissue), moderate (11-100 QPX cells), heavy (101-1,000 QPX cells), or severe (> 1,000 QPX cells).

5.2.7. Statistics

Disease prevalence of clams at the end of the experiment (10 weeks) was separately compared with the initial prevalence (36%) using the exact binomial test (Sokal and Rohlf, 1995) to determine the significance of treatment effect on QPX infection. Counts of QPX-infected and uninfected individuals from the two replicate tanks of the same treatment were used to calculate the prevalence. The statistical analysis was performed through VarsaStats online platform (http://vassarstats.net/binomialX.html). Statistical analysis of plasma anti-QPX activity and relative gene expression was performed using IBM SPSS 20 software package. Comparisons between Day 1 and Day 7 were made using Student's t-test. One-way ANOVA were conducted to evaluate the effect of different temperature and treating duration combinations among all heat shock treatments on anti-QPX activity and relative transcription of stress related genes. Treatments that showed significant differences were further subjected to a Holm-Sidak post-hoc test. Data were log10 or arcsin transformed whenever the variance was large but results are presented as non-transformed values. All results were considered significant at an overall level of $P<0.05$.

5.3. Results

5.3.1. Disease prevalence

In general, heat shock treatments with different temperatures and exposure times resulted in various impacts on QPX prevalence. Compared to the initial QPX prevalence (36.6%) at the beginning of the experiment, disease prevalence remained relatively unchanged in untreated control clams kept in seawater at 18ºC (36%) while a general decrease in disease prevalence was found in most of the heat shock treatments, except in the 27 °C 8h and 32°C 4h treatments, where disease prevalence showed a slight yet non-significant increase to 38.1% and 40.0%, respectively (Fig. 5.1A). The only treatment that displayed a significant decrease in disease prevalence was the 27°C 2h treatment (Binomial exact test, $P<0.01$), which exhibited 10% prevalence after the 10 weeks recovery (a 72% reduction as compared to untreated controls). Disease reduction was also seen in the 32°C 2h treatment (23% prevalence or 36% reduction), although this change was not statistically significant. Among clams subjected to the same temperature, various exposure durations resulted in different recovery performances. For instance, in clams treated with 27 °C, the most effective heat shock exposure time was 2 hours

and extending the exposure time to 4, 8 and 18 h did not further decrease the disease prevalence but had no or even adverse effects that resulted in a slight increase in prevalence. Similarly, in the 32 °C treatments, the short exposure (2 h) also led to more noticeable disease reduction than its longer duration counterpart (4 h). The 2-hour treatment at 37 °C seems to be the least effective in terms of disease reduction among the three 2-hour treatments (27, 32, 37 °C) where the higher the treating temperature, the less disease mitigation effect resulted. On the other hand, for the clams subjected to 21°C treatments, longer exposure time (18 h, 27% prevalence) seems to be more effective in decreasing QPX infection than the shorter one (8 h, 35% prevalence). Compared to the untreated control group, the 18 h of air exposure at 18 °C resulted in very minor change in QPX prevalence.

5.3.2. Disease intensity

QPX disease intensity, expressed as average parasite cell counts per gram of clam tissues in each treatment, is shown in Fig. 5.1A. In general, changes in disease intensity 10 weeks after heat shock treatments followed similar trends as disease prevalence with the lowest QPX infection intensity found in the 27 °C for 2h treatment however the difference was not statistically significant and therefore no clear trends can be drawn. For other treatments at 27 °C but with longer duration, the QPX intensity tend to increase with the exposure time. The highest QPX intensity among all treatments was found in the 27 °C 18h treatment, suggesting an adverse effect of excess heat exposure in air on disease control. Among the two 32 °C treatments, a 2 hours exposure resulted in slightly lower QPX cell counts as compared to the 4 hours treatment, in agreement with the prevalence data. Disease intensity was also relatively low in the 37 °C 2h treatment, however noticeably high clam mortality were resulted in this treatment condition (discussed below; Fig. 5.1B).

5.3.3 Mortality

Throughout the 10-week period, dead and moribund clams were collected and analyzed for disease status by qPCR (Fig. 5.1B). Mortality was generally low in most treatments (2 to 4 clams or 5 to 10% mortality) and tended to increase with higher temperatures and/or extended exposure times, reaching 50% (20 dead clams) in the 37ºC 2h treatment. Diagnostic results indicated that not all cases of mortality were associated with QPX infection, suggesting that some mortality may have resulted from stressful experimental conditions. Overall, dead clams from the low temperature or short exposure time treatments displayed moderate to severe QPX infections. This was the case for both of the 18 °C treatments, for 21 °C 8 h treatment, as well as the 2-hour exposures at 27 °C and 32 °C. On the other hand, an increasing proportion of uninfected clam was detected among dead and moribund clams from treatments using high temperatures or longer exposure times or a combination of both. For example, higher percentage of uninfected clams was found among dead clams from the 27 °C 18 h treatment as compared to the 4 and 8h

treatments at the same temperature, and the percentage increased at higher temperature (32 °C 4h vs. 27 °C 4h). At 37 °C, half of the dead clams were negative for QPX, and light infections dominated the positive clams.

5.3.4. Expression of stress-related genes

To evaluate the stress level experienced by clams subjected to different heat shock conditions, the relative expressions of 4 stress-related genes were assessed following treatment (Fig. 5.2). In general, the expression of HSP 70 was induced in clams exposed to elevated temperatures at Day 1 following heat shock treatments (Fig. 5.2A). This induction was especially prominent in clams from the 27 °C 2 h, 32 °C 2h, 32 °C 4 h and 37 °C 2 h treatments, where expression values were significantly higher as compared to untreated control clams (represented by the *x*-axis). In clams subjected to 27 °C and 32 °C, highest expression of HSP70 was found in the 2 h treatments with induction of HPS 70 slightly declining as the treating time was extended, even though the expression of other stress proteins (HSP 25 and GPx, Fig. 5.2B and C) increased with longer treatments. The thermal stress among clams exposed to 37 °C seemed to be long-lasting and very difficult to overcome, as the level of HSP 70 expression in this batch remained significantly higher than that in controls at Day 7, whereas the induced HSP 70 had dropped back to control levels in all other treatments.

The induction of HSP 25 in heat-shocked clams was consistent with both the temperature level and the duration of thermal exposure at Day 1 (Fig. 5.2B). As the temperature increased, the expression level of HSP 25 rose dramatically. For example, the fold change of HSP 25 expression in clams submitted to 27 °C ranged from about 2 to 6 times compared to controls and increased to about 50 times when temperature reached 32 °C and 400 times at 37 °C. In addition, longer exposure times also induced higher levels of HSP 25 among treatments submitted to the same temperature. For example, clams subjected to 27 °C for 18 hours exhibited significantly higher expression of HSP 25 than those held at the same temperature for 2 and 4 hours. After 7 days, stress caused by heat shock seemed to be greatly recovered in clams subjected to most treatments, as indicated by the restoration of HSP 25 expression to a basal level as in control clams. The exceptions were found in most extreme conditions (32 °C 4h and 37 °C 2h treatments) where the HSP 25 levels remained significantly higher than control levels.

The expression of the antioxidant GPx seems to be generally associated with the duration of air exposure rather than the temperature level of each treatment. For instance, the expression of GPx was significantly up-regulated at Day 1 in clams submitted to 18 ºC air exposure for 18 h as compared to controls which remained submerged in seawater (Fig. 5.2C). Shorter exposure times (2 h, 4 h and 8 h) did not trigger substantial modulation of GPx in most of the temperature treatments (except the most extreme high temperature treatment 37 °C 2h). However, as the exposure time extended, GPx expression considerably increased. Higher expression of GPx was

also associated with higher temperatures in clams submitted to the same long-term exposure (18 h). The expression of GPx dropped to normal levels after 7 days of recovery in treatments using temperatures at or below 27 °C. However, GPx remained significantly highly expressed in the 32 °C 2 h and 37 °C 2h treatments.

The expression of the *M. mercenaria* USP gene generally reflected the compounded stress sourced from both heat shock and extended air exposure (Fig. 5.2D). USP was generally up regulated in all experimental treatments as compared to the control treatment, although the overall extent of this modulation was not as high as other stress-related genes. On Day 1, clams from the 21°C 18 h, 27 °C 8 h and 27 °C 18 h treatments were found to significantly overexpress USP, however, the 18-hour air exposure at 18 °C and shorter treatments at higher temperatures did not induce significant regulation of USP. At Day 7, the expression of USP in most treatment groups regained a level that is slightly higher than that of controls but without significant difference except in the 18 °C 18 h air exposure and 37 °C 2h heat shock treatments.

Discriminant analysis (DA) using expression levels of all tested stress-related genes was performed to provide an integrative assessment of the stress response following each heat shock treatment (Fig. 5.3). Results show significant impact of heat shock treatments on overall stress, which varied with treatment conditions and post treatment recovery time. At day 1 (Fig. 5.3A), treatment effect on the expression of stress genes was clearly discriminated on function 1 (91.3% total variance explained, Eigenvalue = 10.874, Wilks Lambda = 0.036, $p < 0.001$), with the most pronounced separation found in treatments with most extreme conditions. For example, centroid of 37 °C 2h treatment was remarkably separated from all other treatments and positioned furthest from the control centroid on the DA scatter plot. Centroids of the 32 °C treatments (2h and 4h) also exhibited marked separation on function 1, however with less distance from controls as compared to the 37 °C treatment. The treatment of 27 °C 18h had its group centroid modestly yet clearly separated, whereas the control and remaining treatments were tightly clustered together. On the other hand, group centroids were less separated at day 7 (Fig. 5.3B) as compared to day 1 by the discriminant functions (function 1 explained 77.5 % total variance, Eigenvalue = 0.997, Wilks Lambda = 0.383, $p < 0.001$). Only the 37 °C treatment was noticeably separated, and the 2 32 °C treatments were only slightly divergent from the cluster formed by the control and all other treatments. This shift of patterns indicated a recovery of stress from day 1 to day 7 for most treatments (excluding the most extreme temperature).

5.3.5. Anti-QPX activity

The anti-QPX activity (AQA) of clam plasma after 1 and 7 days post heat shock are shown in Fig. 5.4. The data are expressed as percentage of suppression of QPX growth in tested plasma as compared to control cultures (plasma substituted with QPX growth in FSW). In general, the AQA across all experimental treatments at Day 1 (Fig. 5.4A) were comparable or slightly lower

than that of the control treatment clams, suggesting a decrease in plasma ability to neutralize the parasite caused by possible stress in clams exposed to high temperature and/or hypoxia due to air exposure. At Day 7 (Fig. 5.4B), the AQA level generally recovered in most of the heat shock treatment groups to a level equaling to or slightly higher than the control, whereas the AQA of controls remained almost unchanged as compared to Day 1. However, no significant difference in AQA was found between experimental groups and control clams. Incidentally, the highest AQA was found in the 27 °C 2 and 4 h treatments, which was respectively 9% and 12% higher than that measured in controls, corresponding well with the lower QPX prevalence observed in the first and with lower disease intensity in both treatments (Fig. 5.1). Among treatments at each temperature level, longer exposure time led to a slight decrease in AQA, even though the difference was not significant.

5.4. Discussion

Previous studies suggested that QPX disease is a “cold water disease” and that the exposure of clams to relatively warmer environment for extended periods of time (e.g. 21 and 27ºC for 2 to 4 months) favors clam resistance to QPX thus impeding the propagation of the parasite among host populations and promoting the healing of infected individuals (Dahl et al., 2011; Perrigault et al., 2011). The current research was designed to evaluate the effect of short-term (2 h to 18 h) exposure to warm environment on disease dynamics in infected clams. The temperature conditions ranged from temperatures optimal for clams (18 and 21 °C) to high sub-lethal temperature extremes (27 to 37 °C), which represent heat-shock situations. Results showed significant disease remission in clams subjected to 27 °C for 2 hours, which resulted in reduction of disease prevalence and intensity. QPX reduction in this treatment was concomitant with an acute increase in the expression of heat-shock proteins (HSP 25 and HSP 70).

In this context, disease reduction could be the result of “cross-tolerance” induced by the thermal stress. Cross-tolerance, also referred to as “cross-protection”, is when exposure to sub-lethal stress would result in enhanced tolerance or protection against other stressors (Sung, 2011). Observations of cross-tolerance have been described in many aquatic organisms, including fish, crustaceans and bivalves. For example, heat exposure was able to increase the resistance of flounder cells against exposure to toxic chemicals (Brown et al., 1992). Similarly, thermal shocks (15 min at 26 °C) conferred protection that allowed for higher survival against subsequent osmotic shocks in salmon smolts (DuBeau et al., 1998). Heat shocks followed by 4 to 48 h recovery enhanced the capability of tide pool sculpin to cope with both osmotic and hypoxic stress and significantly increased their survival rate (Todgham et al., 2005). In crustaceans, it has been reported that a sub-lethal heat shock at 40 °C for 1 h provided brine shrimp larvae with higher thermotolerance for extended heat exposures the following days (Miller and McLennan, 1988). Thermal stress also enhanced the resistance of brine shrimp larvae to *Vibrio campbellii* and *Vibrio proteolyticus* infections, significantly increasing their survival in the presence of

pathogenic bacteria as compared to non-stressed animals (Sung et al., 2008; Yik Sung et al., 2007). Pacific oysters submitted to 1 h thermal stress at 37 °C acquired thermotolerance to survive a subsequent lethal high temperature exposure at 44 °C (Clegg et al., 1998). Similarly, exposure to a 3 h sublethal heat shock conferred tolerance to subsequent lethal heat treatment (35°C) in juvenile northern bay scallops, and this thermotolerance persisted for at least 7 days (Brun et al., 2009).

Previous investigations provide hints to the molecular processes associated with cross-tolerance. HSPs are the major group of stress proteins and their expression can be dramatically modulated as the key part of stress response. It is widely recognized that HSPs overproduction in response to physiological perturbations during thermal stress is critical for the acquired cross-tolerance against other environmental and biotic stressors in aquatic organisms (Rahman et al., 2004; Sun et al., 2002; Todgham et al., 2005). For example, the accretion of HSP 70 after short-term hyperthermic stress correlates with the attenuation of gill-associated virus (GAV) replication in the black tiger prawn (de la Vega et al., 2006). Similarly, the enhanced resistance of gnotobiotic brine shrimp larvae to *V. campbellii* and *V. proteolyticus* following thermal stress (discussed above) was associated with HSP 70 accumulation (Sung et al., 2008; Sung et al., 2007). Moreover, reduced mortality and lower bacterial loads after *V. campbellii* challenge were only observed among shrimp larvae with enriched HSP 70 levels (Sung et al., 2008). In addition, intracoelomic injection with proteins analog to mammalian HSP 70 and HSP 60, combined with a non-lethal heat shock, was shown to protect the fish *Xiphophorus maculatus* from mortality caused by the pathogenic bacteria *Yersinia ruckeri* (Ryckaert et al., 2010). The involvement of HSPs in the resistance to pathogenic challenge suggests a role for these proteins in immunity of aquatic organisms and supports our findings of better disease remission in clams displaying significantly up-regulated HSPs levels.

Although the exact mechanisms behind the cross-tolerance between heat shock and pathogen resistance have not been described, several possible explanations were proposed. Sung (2011) proposed that high HSPs production (particularly HSP 70) as a result of non-lethal thermal stress may stabilize cells against injury due to pathogen infestation, promote the proper folding of host immune proteins, re-fold proteins damaged by pathogens and stimulate the innate immune response. Additionally, heat shock may also induce the expression of a collection of immune-related genes resulting in the activation of immune pathways. For example, the prophenoloxidase cascade system was shown to be stimulated by heat shock in the shrimp *Litopenaeus vannamei* leading to an increase in host cell adhesion, encapsulation and phagocytosis of invading microbes (Loc et al., 2013; Pan et al., 2008).

While well-dosed heat shock presents great potential to enhance host resistance against infections, extreme heat exposure may result in adverse effects. Severe hyperthermic stress is known to disrupt normal physiological processes, decrease feeding, retard growth and molting,

and weaken the immune response, sometimes leading to mortality under severe conditions (Roberts et al., 2010). This was the case in clams submitted to 37 °C where high mortality (50%) was recorded among uninfected or lightly infected clams, suggesting that the heat stress itself was extreme enough to induce mortality. Interestingly, clam exposure to 32 and 37 °C for 2h did not reduce QPX disease as much as 27 °C. Moreover, longer exposure periods for the same heat shock temperatures seem to undermine the effectiveness of the cross-tolerance against QPX. Extended air exposure leads to hypoxia, which can significantly constrain the innate immune responses by reducing the activity of immune cells and suppressing or delaying the expression of important immune-related genes, thus increasing the susceptibility to infectious diseases (Cheng et al., 2002; Kvamme et al., 2013; Le Moullac and Haffner, 2000). Increased metabolic rate under high temperatures may create enhanced oxygen demand in hard clams, making the oxygen deficiency more severe and reducing the effectiveness of the heat treatment on QPX disease.

The impact of combined thermal and hypoxia stress is well reflected by the expression of the stress-related genes GPx and USP (Fig. 5.2 C and D). The expression of GPx peaked in treatments with the longest (18 h) air exposures and or the highest temperature (37 °C), and the overall induction of USP at Day 1 dissipated at Day 7 in most treatments except the 18 °C 18 h air exposure and 37 °C 2h heat shock. Hypoxia is often associated with the generation of large amounts of reactive oxygen species (ROS) (Duranteau et al., 1998) and the afterward re-oxygenation during recovery is known to cause excessive oxidants production in hypoxic tissues leading to oxidative stress (Askew, 2002). GPx is considered one of most readily mobilizable antioxidants that protect cells by buffering against a sudden increase in the generation of radical oxygen species (Rousseau et al., 2006), so the GPx expression has indicated severe hypoxic stress associated with each treatment. Additionally, USP responds to a variety of stressors, including heat, starvation, infections and oxidative stress (Kvint et al., 2003). The expression patterns of USP in response to the heat shock treatment could be linked to the compounded stress deriving from both heat shock and oxygen deprivation (Fig. 5.2D).

Members of the HSP 70 family are widely used as stress indicators, especially for thermal stress. The temperature change and heat exposure time required to induce heat shock and modulate HSP synthesis are known to be affected by the acclimation temperature, the heat tolerance of the organisms and the environmental conditions under which the organisms normally grow (Sung, 2014). Thermal shock at 32 °C or above was shown to effectively induce HSP 70 production in several oyster species that were previously acclimated at 12 to 18 °C (Clegg et al., 1998; Vincent and Chu, 2005). For example, in the European flat oyster, heat exposure stimulates HSP 70 synthesis with maximum levels observed in the gills between 2-3 h of post-stress recovery at 18 °C (Piano et al., 2005; Piano et al., 2004). A sub-lethal heat shock for 3 h stimulates HSP 70 and HSP 40 production in bay scallops, with the HSP 40 response being less vigorous and decreasing to pre-stress values by 8 days, whereas HSP 70 was

maintained for 14 days (Brun et al., 2009). In our study, clams responded promptly to temperature elevation resulting in over two-fold up-regulation of HPS 70 in the gills at or above 27 °C, with the highest level found in the 2 h treatments at each target temperature and the induction level decreased with longer exposure times. However, HSP 70 level decreased to baseline values after 7 days of recovery at 18 °C. This could reflect that the normal cell activity was gradually restored and suggests that the overproduction of HSP 70 proteins, which is energetically costly (Hoekstra and Montooth, 2013; Krebs and Loeschcke, 1994), is not required anymore to provide protection. This HSP 70 regulatory pattern is in agreement with the remarkable ability of hard clams to tolerate a wide range of environmental conditions (Grizzle et al., 2001), in particular a wide thermal range. For instance, hard clams populate both intertidal and subtidal habitats from Canada to Florida. The reported temperature tolerance range for the species is 1 to 34 °C with the optimal range from 16 to 27 °C (Malouf and Bricelj, 1989). In addition, hard clams have greater tolerance to low dissolved oxygen (DO) as compared to other bivalves, as they fare well with DO level as low as 0.9 mg/L at 16 to 19 °C (Malouf and Bricelj, 1989). The timely modulation of stress-related proteins, especially HSPs, may comprise a significant part of the mechanisms that allow hard clams to successfully counterbalance detrimental stimuli and gain adaptability to a variety of environmental conditions.

It is not too surprising that the best reduction in QPX was observed in the clams from the 27 °C 2h treatment, a condition that appears much milder than what have been reported to induce cross-protection in other aquatic animals. The 27 °C is the upper limit of the optimum temperature range for hard clams and different levels of physiological impairments result beyond this limit. For example, hard clams cease pumping and feeding at temperatures above 31 °C (Malouf and Bricelj, 1989). Hard clams used in this study were sampled in the field during winter (4 to 5ºC) and were acclimated for 2 weeks at 18 °C after collection. This initial acclimation may have primed the heat-shock response in clams. Therefore, the subsequent exposure to 27 °C represented an additional temperature rise of 10 °C, which appears adequate to stimulate significant up-regulation of HSP 70 and other stress response genes due to the high responsiveness of hard clams to changing conditions. Although certain thermotolerance could be attained progressively with prolonged heat shock time (as reflected by expressions of HSP 70), the compounded stress resulting from both heat and oxygen deprivation during the thermal incubation may have induced synergistic effects between both stressors that escalated the overall stress to higher levels. The overall stress levels were comprehensively reflected by the DA scatter plot (Fig. 5.3) which highlighted increased level of stress as a synergistic result from both heat level and exposure time. Severe stress may compromise the possible beneficial effect of mild heat shock treatments on disease recovery. From an energy expenditure perspective, stress response may undermine immune competency in clams exposed to very high temperatures, in agreement with the energy trade-off concept described in the stress model developed by Moberg (2000). Under stressful conditions, animals must coordinate their competing energy demands for

combating stress and maintaining other functions. In this context, response to mild stress requires little energy that can be easily met by reserves, resulting in minimal impact on other physiological processes. However, increasing stress severity and/or duration requires higher energy demands that are hardly met by reserves alone. Under this situation, extra resources must be allocated to stress response causing a reduction in energy available to other biological processes such as growth, reproduction and immunity, ultimately increasing the chances of infection and mortality (Segerstrom, 2007). In the current study, a heat shock treatment at 27 °C for 2 h appears to provide adequate induction of stress-related proteins to shield off clams from damage, without causing overwhelming stress to impair immune functions, resulting in the most significant cross-protection against QPX disease.

Nevertheless, we did not clearly observe any direct relationship between the heat shock treatments and clam immune competency as measured by plasma anti-QPX activity (AQA), even though AQA at Day 1 tended to decrease in treated clams suggesting a competition for resource allocation between stress tolerance and antimicrobial activity. This reduction was more marked at extended periods (18h) of air exposure. However, after 7 days, AQA was almost equal across all treatments, with slightly yet not significantly higher activities in the 27 °C 2h treatment (which resulted in the lowest QPX prevalence after 10 weeks) as compared to controls. Nonetheless, AQA did not represent a good proxy for QPX reduction in this study. It is possible that the sampling times used in this study (1 and 7 days) do not represent the best time points for the assessment of the beneficial effect of heat shocks on the clam immune system. Hooper et al. (2014) reported that abalones subjected to non-lethal heat shocks exhibited increased immune competency mostly on the cellular level, such as elevation in total hemocyte counts and phagocytic rate, while the humoral immune parameters such as the antibacterial activity and phenoloxidase, peptidase and acid phosphatase activities slightly declined or were not affected by the heat stress. This indicates that immunological changes caused by heat shock are only reflected in some, but not all hemolymph parameters; and the influence is more likely observed in cellular than in humoral defense parameters, which may explain the lack of major changes in plasma AQA in heat-shocked clams.

It is noteworthy to mention, however, that our evaluation of heat shock effect was mainly focused on clam parameters, whereas its impact on the physiology of QPX cells present in clam tissues was not investigated. It is likely that our heat shock treatments also caused stress in QPX cells since the optimum temperature for QPX proliferation is between 20 and 23 °C and higher temperatures reduce the viability and growth of parasite cells *in vitro* (Perrigault et al., 2010). On the other hand, it is also possible that the increasing levels (intensity and/or duration) of thermal exposure resulted in a differentially stressful condition to both the host and the parasite, possibly providing an advantageous opportunity for QPX to thrive. Such a scenario supports our

observations of limited reduction of QPX prevalence in clams submitted to 32 and 37 °C for 2 h as compared to the 27 °C treatments.

Overall, findings from our current study could have implications for the improvement of aquaculture operations and QPX disease management in hard clams. The development of non-traumatic methods for enhancing disease resistance in aquaculture has been increasingly focused on boosting HSP levels in economically-important crops. Methods that have been suggested to increase HSP levels in fish and shrimp include heat exposure, exogenous HSPs supplement, and oral or water administration of HSP stimulants as reviewed by Sung (2014) and Roberts et al. (2010). Given the fact that QPX disease development is largely suppressed by warm temperatures (Dahl et al., 2011; Perrigault et al., 2011) and our observation that brief heat shock exposures can potentially reduce the disease, we propose that some easily achievable heat shock procedures could be designed and incorporated into the current QPX disease management practices to enhance clam resistance to the infection, promote the healing process and minimize the risk of loss due to disease outbreaks. Such strategies naturally lend themselves to fisheries that involve clam handling such as the economically-important hard clam transplant fishery in the state of New York where clams are transported from production areas to depuration field sites in non-refrigerated vehicles during summer. More research is needed to further explore these promising strategies and to better understand the mechanisms favoring disease reduction with the aim of developing guidelines for applying the most appropriate heat shock treatments (both in exposure temperatures and periods) as a complementary measure for QPX disease control in hard clams.

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Table 5.1 Experimental design for laboratory heat shock treatments. All exposures were made in air excluding a control treatment where clams remained in seawater at 18 ºC (not shown). A " ✓" indicates that this treatment was implemented. Two replicate tanks (20 clams/tank) were made for each treatment.

	18 ºC (bench)	21 ºC (incubator)	27 ºC (incubator)	32 ºC (incubator)	37 ºC (incubator)
18 hours					
8 hours					
4 hours					
2 hours					

Table 5.2 Primer sequences for the tested genes

	Forward (5'-3')	**Reverse (5'-3')**
HSP 25	GTC GAT CCG AAG AAG CTG AAG TC	TTA CTT TGG GTC CGT CAA CAG C
HSP 70	GAG CTC CAC CAG CTT GAT AGA GT	GGC TGC TAA GGA CGA GTA TGA AC
GSH-Px	GAA TGT TGC ACG TCT GAA ACG C	CCC GAA GTT GAT CAT ATG GAC GC
USP	GAG GAA TGG GGA CAA TTA GAC GC	ATG ATG TTG ATG GTC GCT CTC G
EF1a	AGT CGG TCG AGT TGA AAC TGG TGT	TCA GGA AGA GAC TCG TGG TGC ATT

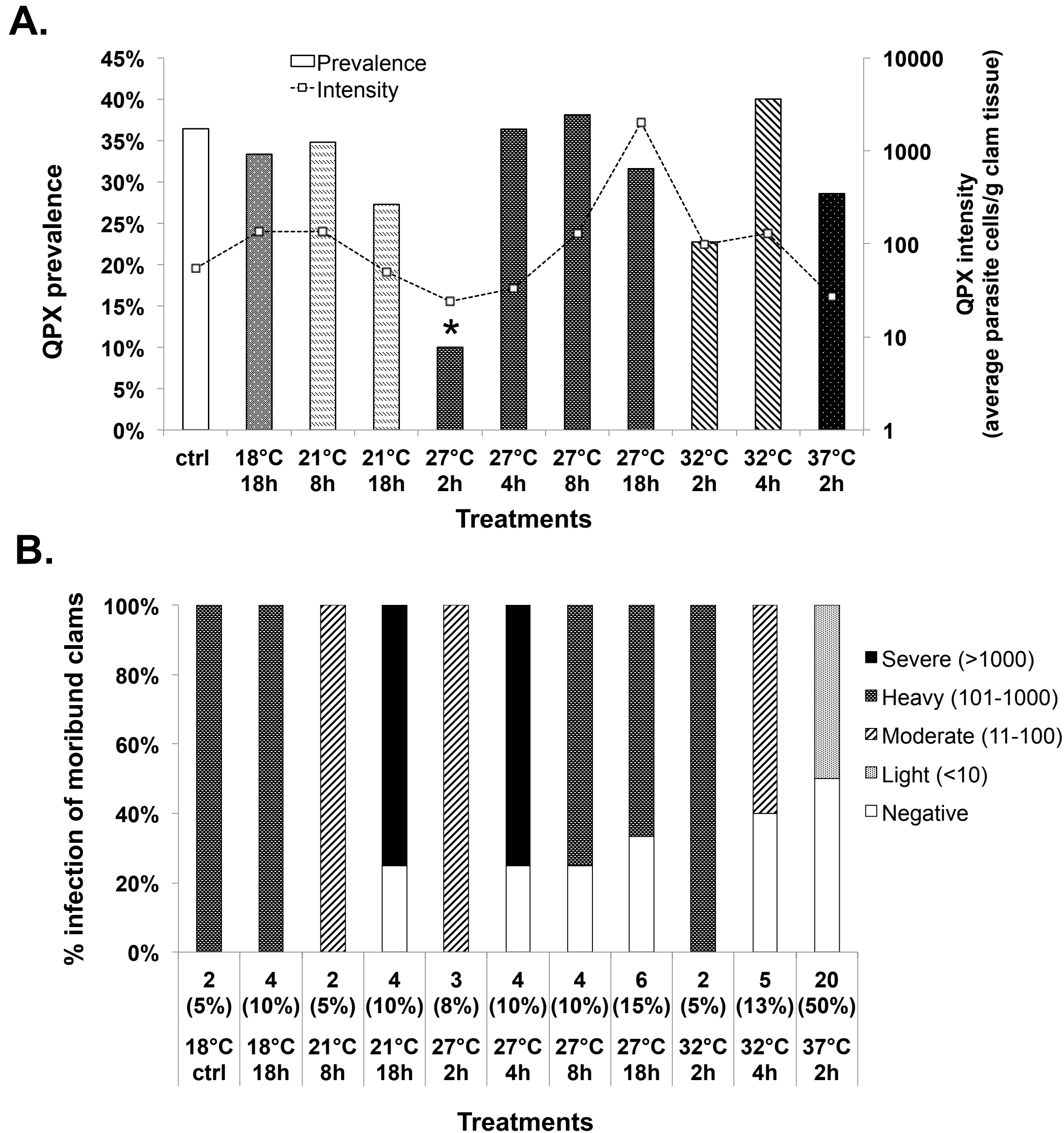


Figure 5.1. QPX disease status in experimental clams. (A) QPX prevalence (bars, left *y*-axis) and intensity (line, right *y*-axis) after 10-week incubation at 18 °C following the heat shock treatments. "*" denotes significant reduction in QPX prevalence as compared to untreated controls (Binomial exact test, P<0.01). (B) Proportions of QPX infection stages in moribund clams from each treatment collected throughout the 10-week experiment. Numbers of moribund clams (and percent mortality) are indicated along the *x*-axis.

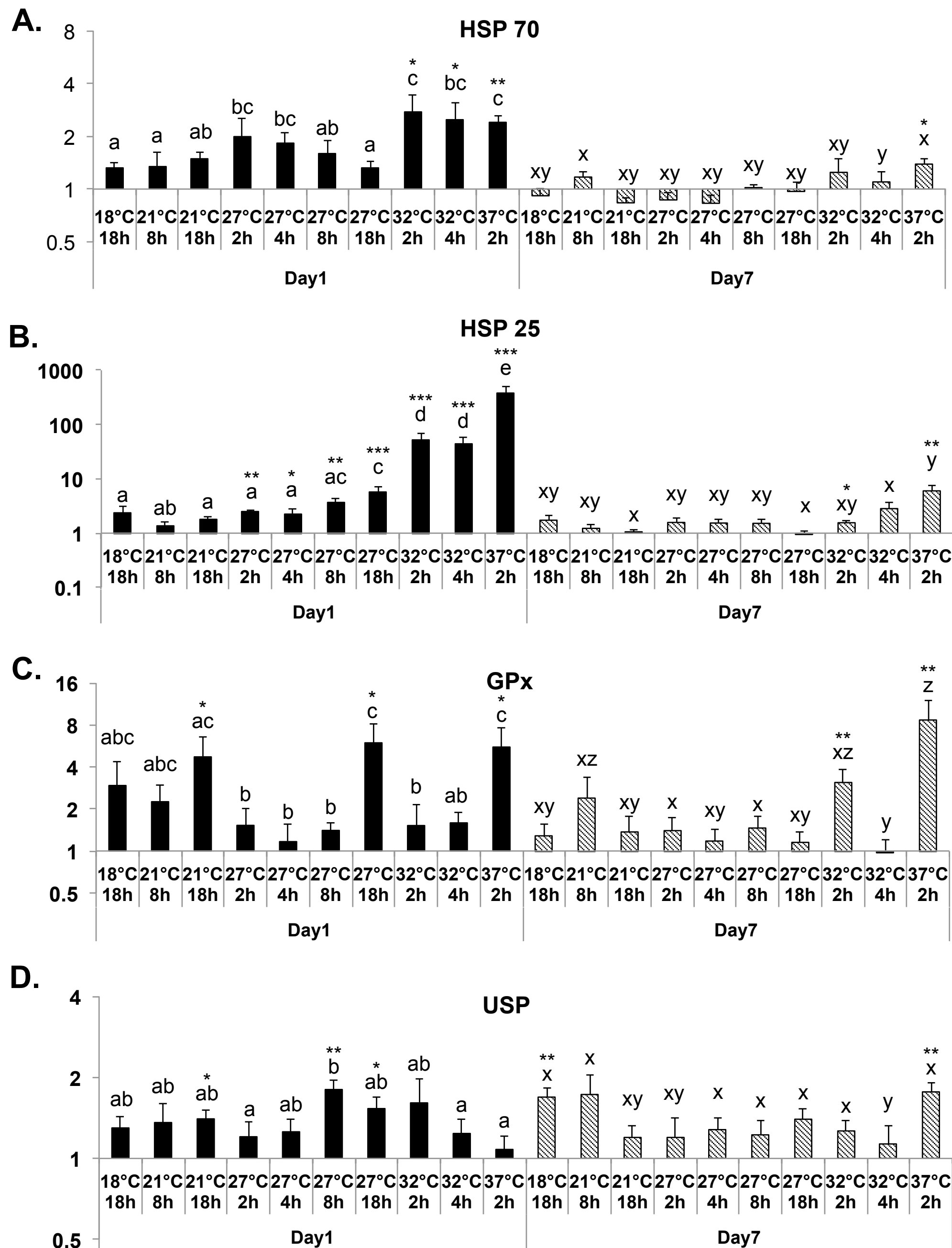
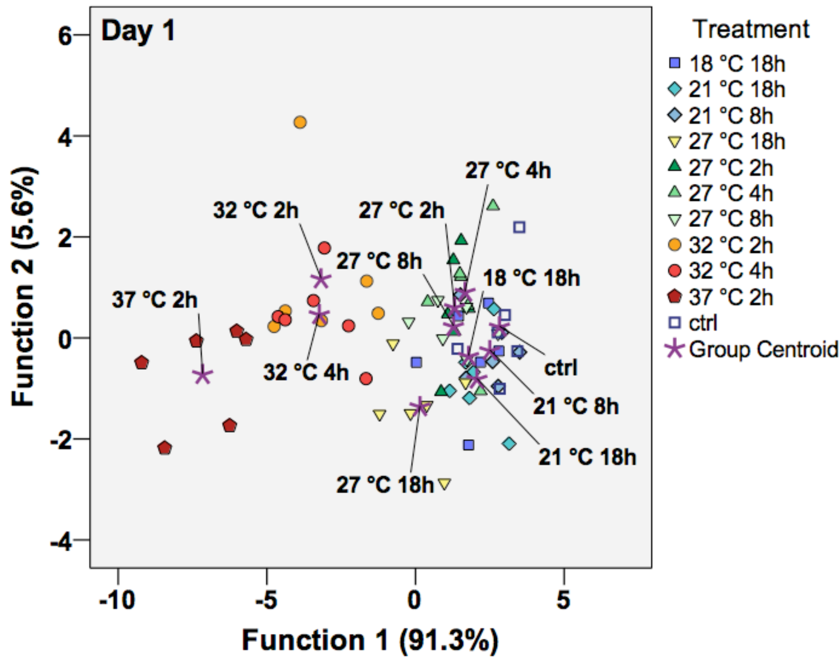


Figure 5.2. Expression of stress-related genes in clams sampled 1 and 7 days after heat shock. (A) heat shock protein 70 (HSP 70), (B) HSP 25, (C) glutathione peroxidase (GPx) and (D) universal stress protein (USP). Mean (and standard error) fold changes are shown (n = 6 for Day 1 and n = 8 for Day 7). Different letters indicate significant difference across heat shock treatments for Day 1 (a, b, c, d, e) and Day 7 (x, y, z) (ANOVA, $P < 0.05$). "*", "**" and "***" denote significant difference compared to controls (represented by the *x*-axis) at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively (*t*-test).

A.



B.

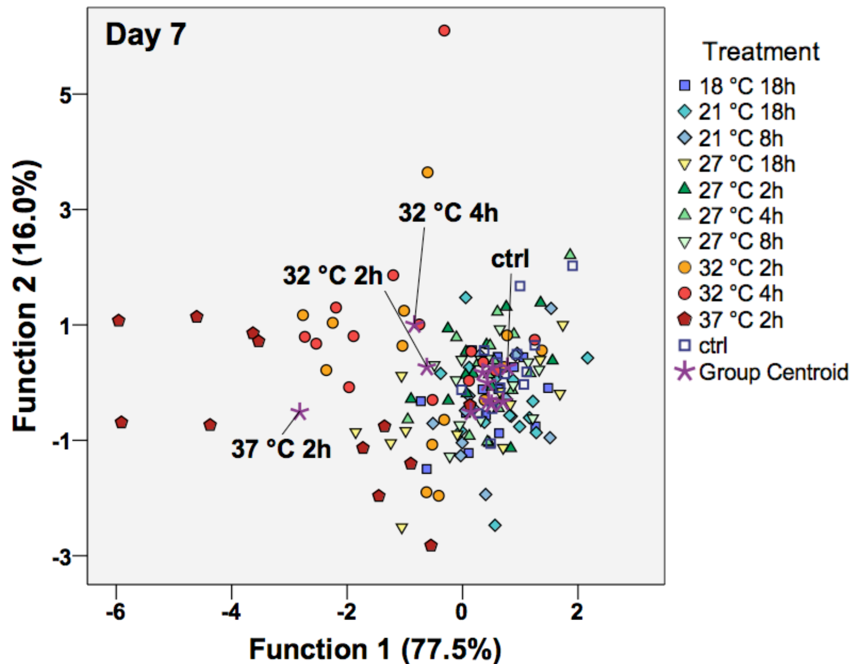


Figure 5.3. Discriminant analysis of all stress-related genes expression at day 1 (A) and day 7 (B) after heat shock treatments. Different treatment groups are indicated by different symbols and positions of group centroids for each treatment are indicated by the star symbol.

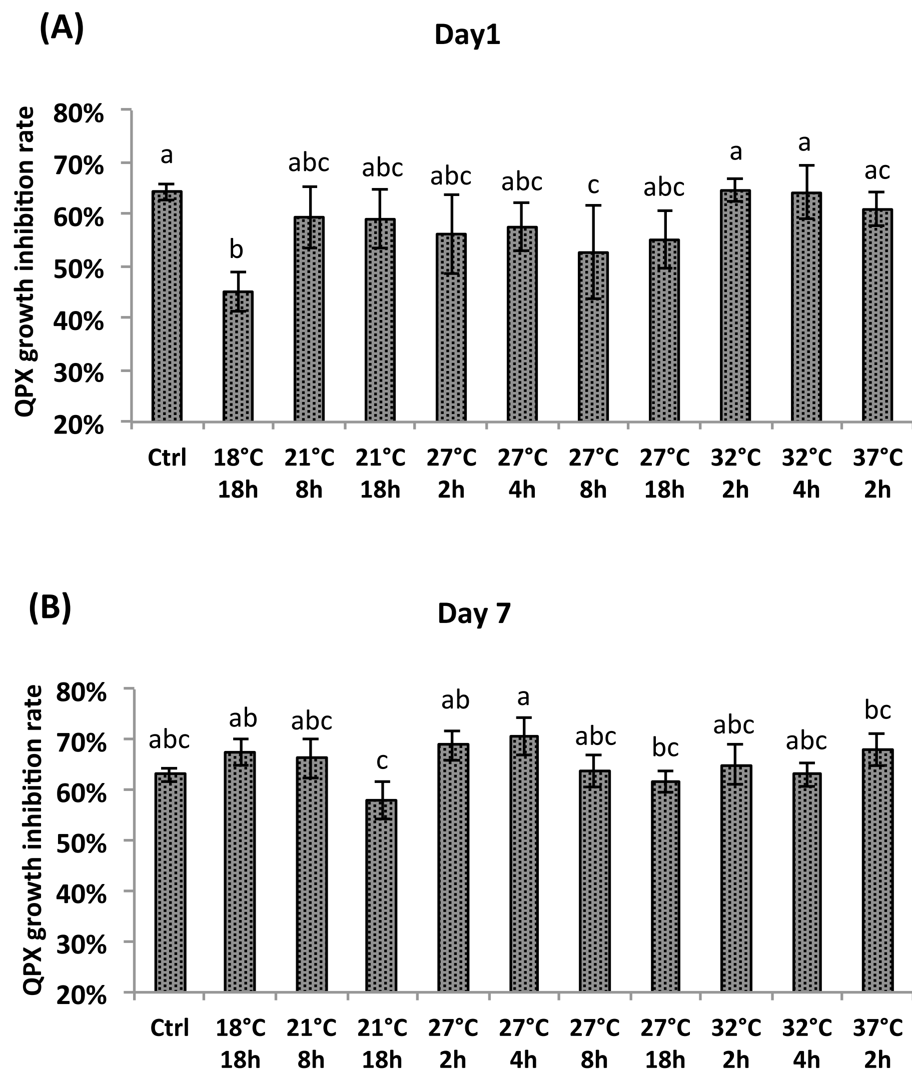


Figure 5.4. Anti-QPX activity in plasma expressed as percent growth inhibition rate (mean ± SE) from clams sampled at Day 1 (A) and Day 7 (B). Different letters indicate significant difference across treatments (ANOVA, $P < 0.05$).

Chapter 6 Conclusions and summary of major findings

Overview

QPX infection induces significant disease losses to the hard clam industry in the Northeastern U.S. The effective control of QPX disease is a critical need for the success and sustainability of the hard clam industry. In order to develop disease control strategies, a thorough understanding of disease mechanisms and host defense responses is essential. It is well recognized that the development and severity of an infectious disease is dynamically determined by the complex interactions between the host, the pathogen and the environment. Factors influencing this process include host immunity, genetic background, and physiological conditions; pathogen virulence and infectivity; and environment alterations that can favor either partner, causing disease outbreaks or infection failure.

Previous field observations and laboratory transmission studies have linked QPX disease development to two primary factors, temperature and host genetic background (Anderson et al., 2003a; Calvo et al., 2007; Dahl et al., 2008; Dahl et al., 2011; Dahl et al., 2010; Ford et al., 2002; Kraeuter et al., 2011; Perrigault et al., 2010; Perrigault et al., 2011; Smolowitz et al., 2008). Significant evidence has been provided that QPX is a cold-water disease and that southern hard clam strains are highly susceptible to QPX. In this dissertation, the two key disease-influencing factors, temperature and host origin, were examined under the lens of clam gene expression and molecular immune responses, to better understand QPX disease development and resistance from a standpoint of host immunity. Studies described in this dissertation have contributed to several critical aspects of host, pathogen and environment interactions surrounding QPX disease, with contents covering the host immunity, host genetic variability, and their interactions with temperature and QPX (Fig. 6.1). Results of this dissertation have provided important additions and molecular evidence to the current knowledge of QPX disease. These findings provide guidelines for future development of QPX disease control strategies in support of the hard clam industry. Major findings of this dissertation are reviewed and summarized in this chapter in light of previous knowledge on QPX disease.

Temperature and QPX disease development

QPX disease is largely considered as a cold-water infection. Disease outbreaks were only reported in the cold northern states (e.g. MA, NY, NJ and VA), but never south of VA. QPX infections caused greater losses in MA and were less severe in NJ and VA (e.g. MA, NY, NJ and VA), where clam mortalities associated QPX infections were frequently observed, but the disease has never been reported south of VA. The QPX infections caused greater losses in MA and were less severe in NJ and VA (Kraeuter et al., 2011). Moreover, the parasite QPX was ubiquitously detected in MA in association with a wide range of environmental samples including seawater, sediments, macroalgae, sea grasses and invertebrates, but only in few substrates in VA (algae, sediments and invertebrates) and never found in warm waters south of Virginia (Gast et al., 2008). Laboratory-based evidence further confirmed that QPX disease is primarily a cold-water infection because the disease developed (prevalence over 70%) in cold conditions (≤ 13°C) during laboratory transmission studies as compared to warmer conditions (≥ 21°C) where lower infection levels (<10% prevalence) were observed (Dahl et al., 2011; Perrigault et al., 2011). Perrigault et al. (2011) reported strong influence of temperature on clam

humoral and cellular defense parameters, which were correlated with QPX disease development. The current study highlights the regulatory effects of temperature on the clam immune transcriptome (Chapter 4), and results show a link between clam gene expression alterations and observed changes in clam defense and QPX development. In clams submitted to cold temperature (13°C), genes related to cold acclimation, stress and detoxification processes were significantly induced, whereas immune genes involved in major defense processes, such as pathogen recognition, hemocyte activation and ROS production, were largely suppressed. During QPX challenge, the parasite significantly induced the expression of some immune receptor genes (e.g. FREPs and C1qDCs) after QPX injection, and higher levels of these immune genes were found expressed at 21°C than 13°C. These observations support that cold conditions can lead to host immune suppression, possibly due to the cold stress and physiological adjustments to cold environment, which limited host ability to effectively respond to pathogen invasion thus increasing susceptibility and speeding up the infection establishment. In contrast, warm conditions (21~27°C) appeared to be more favorable for the host to maintain higher levels of immune factors, thus promoting host resistance to QPX infection.

Evidence of QPX disease reduction and host healing during long-term incubation at warm temperatures has been shown from previous studies (Dahl et al., 2011; Perrigault et al., 2009b). Significant reduction of QPX intensity was found in infected clams held at 21 °C for 9 months and a complete healing was observed after 2 months at 27 °C. Results of this dissertation showed that remission from QPX disease could be alternatively achieved by short-term high-temperature air exposures (Chapter 5). Significant decrease of both QPX disease prevalence and intensity was found in clams exposed to a moderately high temperature with short treatment time (2 h at 27°C). However, prolonged air exposure time (18 h) and extremely high temperature (37°C) caused severe stress (remarkable up-regulation of stress genes) and high clam mortality (50% died immediately following the 37°C treatment), while they did not result in any disease remission in clams. Relationship between heat exposure and host immunity was not clearly established by plasma anti-QPX activity. However, the significant induction of HSPs following heat shock treatments could favor clam healing, as they might provide cross-protection to the host. In fact, boosting host HSP levels has been increasingly used in several aquacultured species as a method to enhance disease resistance (Roberts et al., 2010; Sung, 2014). In parallel, heat exposure may disfavor the parasite, since QPX *in vitro* growth slows down significantly above 23°C and the parasite dies above 30°C (Brothers et al., 2000; Perrigault et al., 2010). The non-traumatic effect and simple nature of mild heat shock treatments have great potential for filed applications. This QPX mitigation method lends itself to fisheries that involve clam handling, such as the hard clam transplant program run by the New York State that allows the transport of clams from production areas to depuration sites in non-refrigerated vehicles during summer, where the heat exposure time and temperature conditions (27~32°C for 2~4h) can be readily achieved during the transportation. Future research could focus on the optimization and scaling up of this procedure.

Clam genetic variability of QPX susceptibility

In addition, previous field and laboratory studies have provided significant evidence that QPX susceptibility is closely associated with particular strains of hard clams, likely resulting

from genetic variability or unfavorable host-environment interactions (Calvo et al., 2007; Dahl and Allam, 2007; Dahl et al., 2010; Ford et al., 2002; Kraeuter et al., 2011). In general, clam strains originating from the south (e.g. SC and FL) showed high susceptibility to QPX when planted in northern sites, while local clams originating from the north performed better and exhibited higher level of resistance to QPX. The observations seemed to provide confounded evidence for both environmental and genetic factors that could influence clam susceptibility. Questions have been therefore raised about the major cause of susceptibility in southern strains, which could be either genetically derived or driven by environmental stress (mainly temperature) related to poor acclimation to northern locations. A study by Dahl et al. (2010) reported that FL clams acquired QPX infection soon (within 6 weeks) after planted in NY during the hottest summer months, which partially downplays the role of temperature and provides strong support to the link between clam genetic factors and QPX susceptibility. The current study (Chapter 4) compared gene expression profiles between susceptible (FL) and resistant (MA) clams in response to QPX infection and temperature (Wang et al., 2016b). The comparisons revealed fundamental transcriptional differences between the two strains, which exhibited interesting relationship with disease development and clam performance, highlighting major influence of genetically-derived factors on QPX resistance. For example, MA clams expressed much higher levels of defense-related genes than susceptible clams in response to QPX infection under both temperature conditions, which suggested an overall better immune competency in MA clams against QPX than FL clams. In particular, the QPX-induced immune receptors FREPs and C1qDCs were all found to have significantly higher expression level in MA clams. For a few variants of FREPs and C1qDCs that were highly expressed by MA clams, the transcripts were not detected in FL clams, likely indicating that they might not even exist in FL clams. This clear contrast could be linked to the susceptibility of FL clams, since inadequate recognition of the parasite might result from the lack of QPX-responsive PRRs. The presence and high expression levels of these PRRs measured upon QPX infection in MA clams may reflect the adaptation of northern strains to the ubiquitous nature of QPX in the local environment. In this context, the widespread presence of the parasite in the northeast (Liu, 2011; Lyons et al., 2005) may exert a selective pressure to drive the development of resistance in local clam populations via selective mortality. In contrast, QPX is absent in FL environment, and this lack of exposure eliminates the need for immune receptors with high QPX responsiveness in FL clams.

Results from this study (Chapter 4) also showed that MA clams exhibited higher expression levels of immune genes, in particular the pathogen recognition genes, under cold temperature (13°C), in correlation with higher QPX prevalence (23%) as compared to clams held at 21°C (8%). While in FL clams, most of these immune genes were induced by QPX only under the warm condition (21°C), where less QPX infections were found (10% disease prevalence compared to 73% at 13°C). This difference between northern and southern strains may be related to their adaptations to different water temperature ranges in their native environments. MA clams generally have better tolerance to low temperatures due to their adaptation to colder environment in the north (2~20°C yearly water temperature fluctuation along MA beaches, NOAA). The performance and immune response levels would therefore not be significantly affected by low temperature (13°C) and higher expression levels of immune receptors, commensurate with

higher QPX infection level, were induced in MA clams under cold condition. The FL clams might not perform as well at 13°C as northern clams because it is close to the lower limit of temperatures they encounter in this tropical environment (13~29°C yearly fluctuation in FL, NOAA). This was reflected in a low expression of immune genes, which could provide a window for QPX to flourish and establish infection (73% prevalence). On the other hand, adaptation to warm conditions have provided FL clams with enhanced immune protection at 21°C, which contributed to better resistance against QPX. Nevertheless, the expression levels of immune genes induced under warm conditions were still significantly lower in FL clams than in MA clams.

These observations confirm the close linkage between QPX susceptibility and host genetic background, and clearly demonstrate that unfavorable genotype-environment interactions play key roles in QPX disease development. The findings also provided support for previous field observations (Calvo et al., 2007; Dahl et al., 2010; Ford et al., 2002; Kraeuter et al., 2011) with evidence on the molecular level. Moreover, QPX-induced immune genes, especially those overexpressed by MA clams, could be particularly associated with QPX resistance (e.g. FREPs and C1qDCS).

Molecular basis for clam resistance to QPX

The immune receptors overexpressed in infected MA clams are key immune factors potentially associated with QPX resistance. These receptors included lectins, fibrinogen-related proteins (FREPs) and the complement component C1q domain containing proteins (C1qDCs) (Chapter 4). High sequence diversity of these PRRs was observed in *M. mercenaria* derived from different geographic locations (NY, MA and FL) (Chapter 2, 3 and 4). High PRRs sequence diversity, especially for FREPs and C1qDCs, has been widely reported in other mollusks (Dheilly et al., 2015; Gerdol et al., 2011; Hanington et al., 2012; Huang et al., 2015; Romero et al., 2011), and is thought to support a commensurate level of antigen-recognition diversity, by increasing the recognizable antigen pool and/or enhancing the binding capacity of specific types of antigens. Sequence diversity for hard clam FREPs has been examined in this study (Chapter 2) and discussed in light of the known role of FREPs in parasite recognition in other mollusks (Hanington et al., 2012; Hanington and Zhang, 2011; Huang et al., 2015; Romero et al., 2011). The expansion patterns of hard clam FREP sequences were similar to those found in other mollusks, suggesting shared mechanisms for sequence diversification (Chapter 2). The diversification of FREPs in mollusks occurs via alternative splicing, exon loss, and somatic mutations (Adema, 2015), and host FREPs repertoire can vary correspondingly with antigen changes. In hard clams, a group of FREP genes was significantly induced during QPX infection, with a subset only expressed in MA clams but absent in susceptible FL clams (Chapter 4). This subset of FREPs may be directly related to clam resistance against QPX and may present higher recognition capacities for QPX antigens. Thr exclusive presence of this subset in MA clams suggests selection by persisting QPX exposure. QPX-responsive variants of FREPs may be retained through selective mortality and genetically enriched subsequently to build up resistance. For variants generated somatically, higher somatic levels and/or specific somatic mechanisms may be selected among individuals by mortality, which prepares the host with anticipative, rather

than adaptive, specificity against QPX thus also contributing to resistance. The diversification of PRR sequences suggests an evolutionary strategy for obtaining immune specificity in clams to compensate for the lacking of adaptive immunity, which offers a simplified and less costly way to maintain a large pool of pathogen recognition proteins. These findings are promising for the development of disease mitigation strategies since the identified molecular features associated with QPX resistance could be used as potential molecular markers for future research on selective breeding of QPX-resistant clam families.

In addition, an extraordinary complexity of hard clam immune system was identified, supported by a rich set of immune genes associated with multiple immune processes and signaling pathways (Chapter 2). Components of the complement system, apoptosis pathway, Toll-like receptor (TLR), MAPK, NF-κB, Notch and Wnt signaling pathways were broadly identified in the hard clam transcriptome (Chapter 2). These pathways have been extensively investigated in vertebrates where they were shown to regulate innate immune mechanisms. The identification of these pathways, in notably high coverage, paralleled with the remarkably diversified PRRs pool in *M. mercenaria*, supported the presence of a sophisticated defense system that allows the elimination of a large variety of pathogens via different recognition receptors and killing mechanisms.

This sophistication in clam defense mechanisms was reflected by the well-orchestrated regulation of focal and systemic immune events during QPX infection (Chapter 3). Clam transcriptomic response to QPX was characterized using RNA-Seq techniques and contrasted between QPX lesion tissues (infection nodules, focal response) and unaffected tissues (anatomically symmetric region to nodules, systemic response) from diseased clams (Wang et al., 2016a). At infection lesions, over-expression of immune receptors, immune signaling pathways components, cell adhesion factors, wound repair genes and ROS production genes were noted. These results suggest that hard clams have activated the mechanisms of pathogen recognition, hemocyte adhesion, wound repair processes and extracellular killing mechanisms to fight QPX infection during direct host-parasite interactions. Meanwhile, the apoptosis processes were inhibited at the lesion sites to enhance survival of nodule-associated host immune cells for extended parasite killing, as reflected by the simultaneous up-regulation of anti-apoptosis genes and down-regulation of pro-apoptosis genes. On the systemic level, QPX infection caused an overall suppression of most immune and metabolic processes, however, the expression of stress proteins, some humoral immune effectors, anti-apoptosis factors and mediators regulating hemocyte mobility was specially induced. These findings reflected an overall systemic stress that affected various physiological processes. However, infected clams still managed to maintain humoral defense, hemocyte migration and proliferation to support immune cell recruitment and prevention of potential QPX spread and secondary infections.

Conclusions

In summary, this dissertation characterized hard clam molecular immune mechanisms in the QPX disease model, and the interactions with the environment (Fig 6.2). Findings allowed the characterization of clam immune factors correlated with QPX resistance, and provided molecular insights into host genetic susceptibility and the regulatory effect of temperature on QPX disease

development. During an infection event, host response initiates upon the sensing and hemocyte attachment of QPX cells/products via highly diversified immune receptors featured by FREPs and C1qDCs. The hemocyte-QPX interactions generate a series of danger signals and are subsequently transmitted inside immune cells via the signaling cascades (such as the MAPK, Wnt and Notch pathways) to trigger the production of various host immune effectors. Pathways regulating hemocyte proliferation and migration are systemically activated, thus facilitating the recruitment and infiltration of hemocytes to the infection area. At the lesion sites, hemocyte adhesion is initiated via focal adhesion and ECM receptor interaction pathways to build up effective cellular defense barriers by encapsulation and nodule formation, in order to isolate the QPX within infection foci. In parallel, apoptosis pathways are overall inhibited, in order to promote the survival of nodule-associated immune cells, thus enhancing the killing and neutralization of the parasite. Compared to susceptible clams from southern origins, clams from northern states express higher levels of putative QPX-recognition receptors upon infection (e.g. FREPs and C1qDCs) and appear to have some special variants of PRRs that were highly responsive to QPX, which may support higher resistance in northern clam strains. In addition, results also showed that temperature regulates clam immune responses and affects resistance against QPX. Cold temperature largely suppressed host immune system and down-regulated immune-related genes, thus exacerbating host susceptibility and promoting infection development. The adverse impact of low temperature on QPX disease resistance was more obvious in southern clam strains, as cold conditions resulted in higher levels of stress among these tropically-acclimated clams. Higher resistance observed in the northern strains may be linked to their adaption to a colder environment where QPX is prevalent. Warm conditions bolstered clam resistance and resulted in an overall activation of QPX-responsive immune factors and created situations favoring host defense and healing. Finally, heat shock treatments involving brief air exposure to moderate high temperatures were effective in reducing QPX disease, however, extreme treatment conditions (high temperature, long air exposure) caused overwhelming stress leading to host exhaustion and mortality. Collectively, findings from this dissertation represent valuable information for QPX disease management and future development of QPX disease control procedures to mitigate the impact of QPX on the hard clam fishing and aquaculture industries.

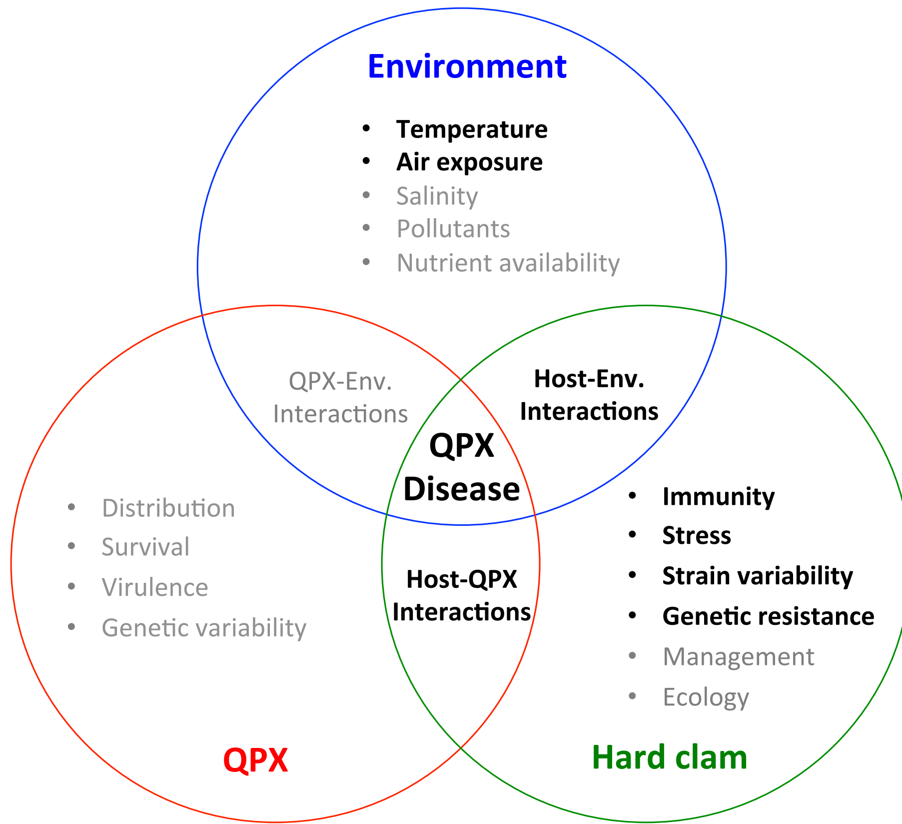


Figure 6.1. Contribution of this dissertation to the understanding of QPX disease development in the hard clam. Host and environmental factors investigated in this dissertation are shown in black bold letters. Other factors affecting QPX disease development but not covered by this study are shown in grey.

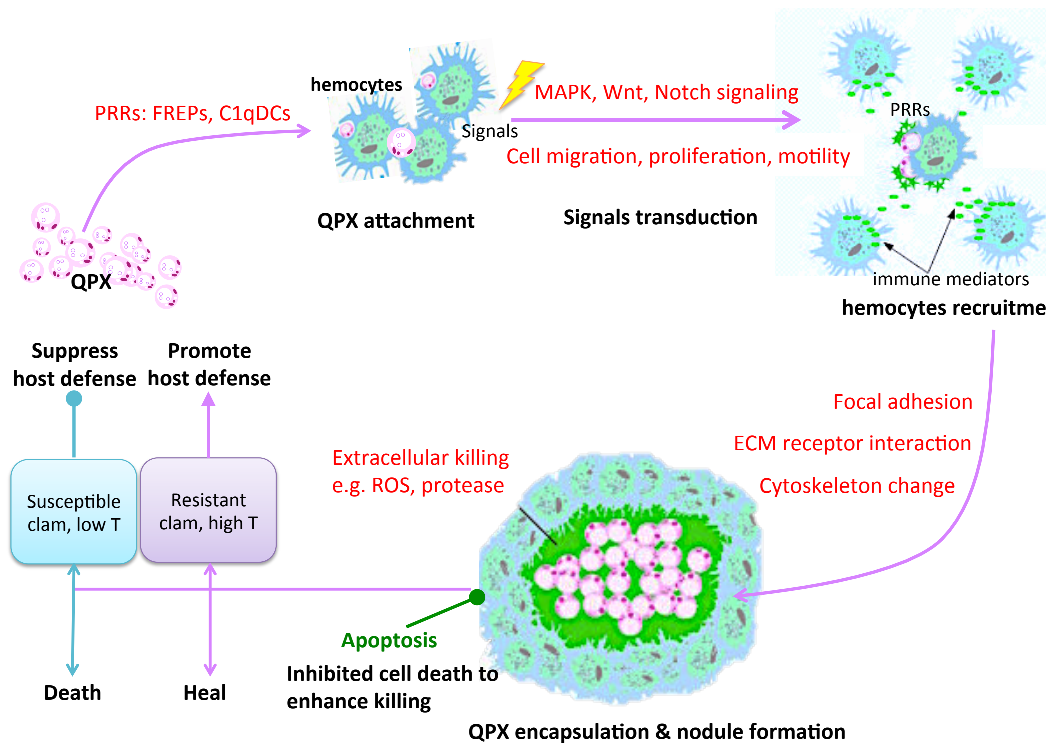


Figure 6.2. Schematic representation of clam immune response induced by QPX infection. *M. mercenaria* immune factors and signaling pathways activated during QPX infection are shown in red letters, and the process inhibited during clam-QPX interaction (apoptosis) is marked in green.

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