Investigation of factors involved in the interaction between Quahog Parasite Unknown (QPX) and its host, the hard clam *Mercenaria mercenaria*

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Mickael Perrigault

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Abstract of the Dissertation

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QPX (Quahog parasite unknown) is a protistan parasite affecting the hard clam *Mercenaria mercenaria* along the Northeastern coast of the United States. The geographic distribution and occurrence of disease epizootics suggests the involvement of environmental parameters and host genotype in disease development. Field investigations and laboratory transmission studies revealed some variations in the susceptibility of different hard clam stocks to QPX infection. Histological observations demonstrated that clams mount marked inflammatory responses against the infection that can sometimes lead to the healing of infected individuals. This study investigated host-pathogen interactions in QPX disease by studying individual components of the tripartite interaction: The pathogen, the host and the environment. Methods were developed to investigate virulence factors of the parasite QPX. Constitutive defense factors and immune response to QPX challenge in hard clams exhibiting different susceptibility to the parasite were also assessed using cellular, biochemical and molecular approaches. Finally, integrative approaches were used to determine the effect of environmental parameters (temperature, salinity, dissolved oxygen) on constitutive and QPX-induced clam defenses as well as on QPX itself. Results demonstrated that QPX produces virulence factors that are cytotoxic to *M. mercenaria* hemocytes. QPX cytotoxicity appears to be induced by clam factors and QPX extracellular products modulated host response against the parasite. *In vitro* growth of QPX was significantly reduced at temperatures above and below optimal levels ranging from 20 °C to 23 °C. QPX appeared to tolerate a wide range of salinities (22 to 37 psu) and parasite growth was only significantly affected at very low salinity (15 psu). Different QPX isolates displayed various levels of cytotoxicity against clam hemocytes and had different temperature optima supporting previous studies suggesting the existence of different QPX strains. This study also demonstrated the presence of constitutive compounds in clam tissues that significantly inhibited (mantle, gills) or stimulated (adductor muscle) QPX growth *in vitro*.

Difference in anti-QPX activity was also observed in plasma from different clam populations and was associated to their respective susceptibility to QPX. Molecular investigations of clam response to QPX led to the identification of several stress- and defense-related genes such as ferritin, metallothionein, defensins, lectins and a thioester-containing protein (or TEP). Components of signaling pathways (such as NF-kB) were also characterized and their transcriptional regulation was correlated to the abundance of some humoral transcripts. Further investigation of the modulation of hemolymph parameters and transcriptional regulation of ferritin and metallothionein during bacterial and QPX challenge indicated difference in clam response according to the challenge. Similarly, comparison of host response among QPX-resistant and -susceptible clam broodstocks exhibited significant difference. Effective response of resistant clams against QPX led to the elimination of the parasite and the restoration of constitutive defense status whereas susceptible clams exhibited a strong modulation characterized by an acute phase response but appeared less effective to eliminate QPX after 28 days. Finally, results demonstrated significant effect of temperature and salinity on clam constitutive defenses and also on host – pathogen interaction and QPX disease dynamics. For instance, low temperature (13 °C) impeded clam defenses causing QPX disease development whereas changes in hemolymph parameters among QPX-infected clams maintained at 21 °C were related to significant healing processes. Higher QPX-associated mortality was also observed in infected clams maintained at high salinity (30 psu) compared to 15 psu. Overall, this study provided essential insights on QPX virulence, *M. mercenaria* defenses and effects of environmental parameters on host – pathogen interactions and provided perspectives for the remediation of QPX disease. The results not only improved our knowledge of cellular and molecular pathways involved during QPX disease, but also generated important information on *M. mercenaria* defenses, and enhanced our understanding of invertebrate immunity in general. Generated molecular information significantly enhanced public databases and allowed the development of new tools for the investigation of *M. mercenaria* transcriptome.

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List of Abbreviations

AASH	Anti-Aggregant Solution
AMP	Anti-Microbial Peptide
APP	Acute Phase Protein
APR	Acute Phase Response
AU	Arbitrary Units
CEW	Chicken Egg White
CFU	Colony Forming Unit
DA	Discriminant Analysis
DCFH-DA	2',7'-Dichlorofluorescein-Diacetate
ECP	Extra Cellular Products
EST	Expressed Sequence Tag
FASW	Filtered Artificial Seawater
FDA	Fluorescein Diacetate
FITC	Fluorescein Isothiocyanate
FSSW	Filtered Sterile Seawater
GO	Gene Ontology
HSP	Heat Shock Protein
MEM	Minimal Essential Medium Eagle
M-MLV	Moloney Murine Leukaemia Virus
MT	Metallothionein
MTH	Muscle Tissue Homogenate
NCBI	National Center for Biotechnology Information
NOAA	National Oceanic and Atmospheric Administration
NR	Neutral Red
NRU	Neutral Red Uptake
OD	Optical Density
ORF	Open Reading Frame
PAMP	Pathogen-Associated Molecular Pattern
PC1	Principal Component 1
PCA	Principal Component Analysis
PDC	Percentage of Dead Cells
PKC	Protein Kinase C
PMA	Phorbol Myristate Acetate
PRR	Pattern Recognition Receptor
QPX	Quahog Parasite Unknown
RACE	Rapid Amplification of cDNA Ends
RACK	Receptor for Activated Protein Kinase C
RFU	Relative Fluorescence Unit
ROS	Reactive Oxygen Species
RT	Room Temperature
SSH	Suppression Subtractive Hybridization
TEP	Thioester-containing Protein
THC	Total Hemocyte Count
TLR	Toll Like Receptor
TRAF	Tumor Necrosis Factor Receptor-Associated Factor

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Part I

Introduction

1. QPX Disease in *Mercenaria mercenaria*

The northern quahog or hard clam *Mercenaria mercenaria* is exploited along the eastern coast of North America (from Maritime Canada to the Gulf of Mexico). This species is among the most commercially important bivalves in the United States. In 2008, hard clam harvests on the US coasts were 4,309 metric tons and had a value of $ 56.9 million. This clam species is well suited for aquaculture as it is characterized by relatively fast growth and high resistance to pathogens (Ford, 2001). However, severe mortality episodes among wild and cultured hard clams have been associated to a disease caused by the protistan parasite QPX (Quahog Parasite Unknown). QPX was first observed in tissues of clam populations affected by unusual mortalities in New Brunswick, Canada in 1954 (Whyte et al., 1994). In 1989, it accounted for almost 100% of mortalities among cultured clams at Prince Edward Island (Whyte et al., 1994). QPX was thereafter identified in other locations further south: New Jersey in 1977 (Ford et al., 2002b), Massachusetts in 1995 (Smolowitz et al., 1998), Virginia in 1996 (Ragone Calvo et al., 1998), and New York in 2002 (Dove et al., 2004). But QPX disease has never been detected south of Virginia (Figure 1.1).

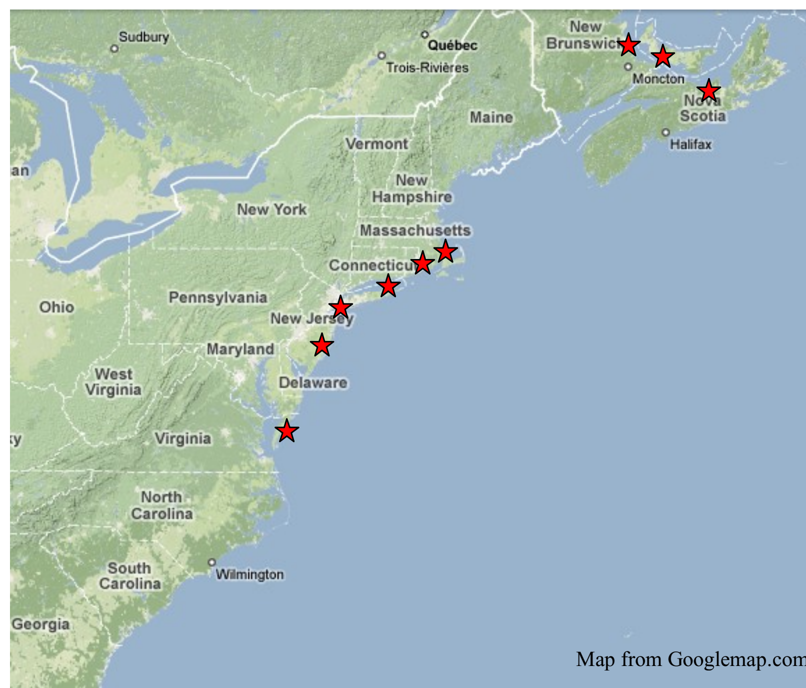


Fig. 1.1. Observation of QPX disease indicated by (★) along the northeastern coast of United States and Canada since 1994.

Typical QPX infection is characterized by a massive inflammatory response to the parasite that results in swellings and nodules formation in clam tissues (Figure 1.2). QPX infections are mostly observed in mantle and gill tissues but also can occur in the visceral mass (MacCallum and McGladdery, 2000; Ragone Calvo et al., 1998). In addition, Smolowitz et al. (1998) reported that QPX-infected clams presented swollen and retracted mantle edges, grew more slowly and had a lower condition index than uninfected *M. mercenaria.*

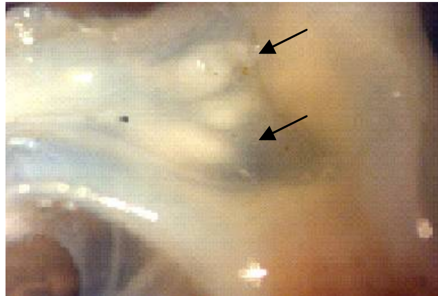


Fig. 1.2. Gross observation of QPX disease in *M. mercenaria* characterized by the presence of QPX-containing nodules at the base of siphon/mantle tissues (arrow).

Beside gross observations, the diagnosis of QPX-infected clams has been based mainly on histological methods. However, QPX infections are rarely systemic; they are mostly characterized by infectious foci and can be difficult to detect by histology. Molecular tools, based on primers specific to QPX, have also been developed. PCR was sufficiently sensitive to detect 20 fg. QPX genomic DNA and 1 fg. cloned QPX SSU rDNA. Field validation indicated that the PCR assay was equivalent to histological diagnosis if initially negative PCR products were reamplified (Stokes et al., 2001; Stokes et al., 2002). More recently, quantitative PCR techniques have been developed in an attempt to provide a diagnostic tool superior to that of histology (Liu et al., 2009; Lyons et al., 2006). However, histology is still the standard technique for the diagnostic of QPX disease because of the limitations of PCR technique such as the inability of PCR methods to distinguish between active QPX infections and the presence of dead cells (Burreson, 2008).

Histological observations of QPX infections in hard clams indicate that *M. mercenaria* is able to mount a defense reaction against QPX (Figure 1.3). In some instances, presence of dead and necrotic QPX cells indicates that hard clams are able to kill the parasite (Dove et al., 2004; Ragone Calvo et al., 1998; Smolowitz et al.,

1998). Previous studies have demonstrated different levels of susceptibility toward QPX among various clam broodstocks. For instance, hard clams originating from northern states are more resistant to QPX than clams from southern states (Dahl et al., 2008; Ragone Calvo et al., 2007). Interestingly, QPX disease was never observed in Florida despite the higher susceptibility of Florida clams compared to Northern broodstocks. These observations led Ragone Calvo et al. (2007) to suggest a genetic and environmental origin of QPX disease outbreaks. The effect of environmental factors was further suggested from results of QPX disease monitoring indicating higher QPX prevalence and associated clam mortalities during summer in Canada, Massachusetts and New York (Dove et al., 2004; MacCallum and McGladdery, 2000; Smolowitz et al., 1998).

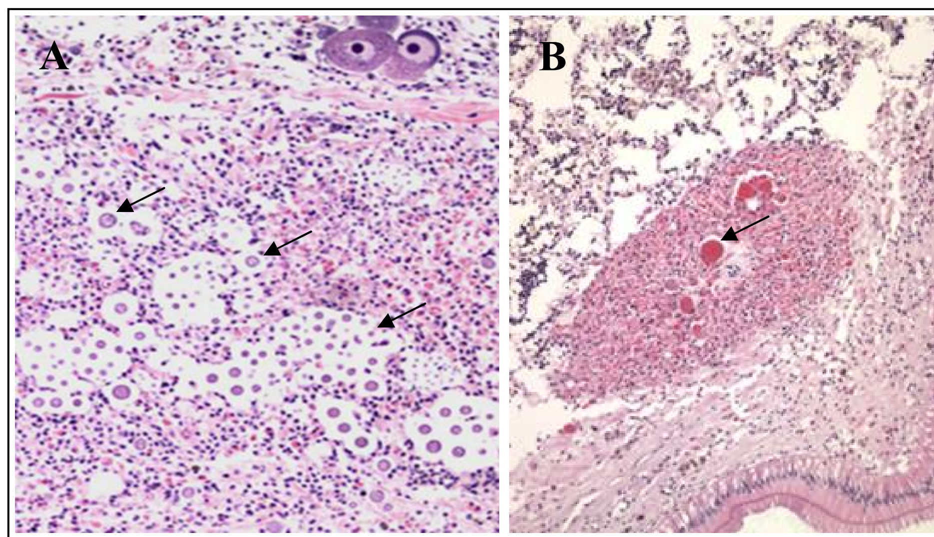


Fig. 1.3. Histological observations of infected clam tissues presenting [A] active QPX lesions and [B] dead QPX cells resulting from effective host response. Arrows indicate QPX cells

Infectious diseases are often the result of interactions between hosts, pathogens and their environment as represented in Figure 1.4. Pathogens possess a panel of virulence factors (mechanisms of avoidance, proteases, cytotoxic compounds) which interact with host defenses. Environmental parameters such as temperature, salinity and pollutants modulate host defenses and pathogen virulences by stimulating or depressing host defenses and / or virulence of pathogens. Therefore, disease occurs when host interacts with virulent pathogen under favorable environmental conditions for the development of the disease as symbolized by the

black area in Figure 1.4. However, our actual knowledge on QPX virulence factors, hard clam defenses and environmental factors involved in QPX disease is sparse.

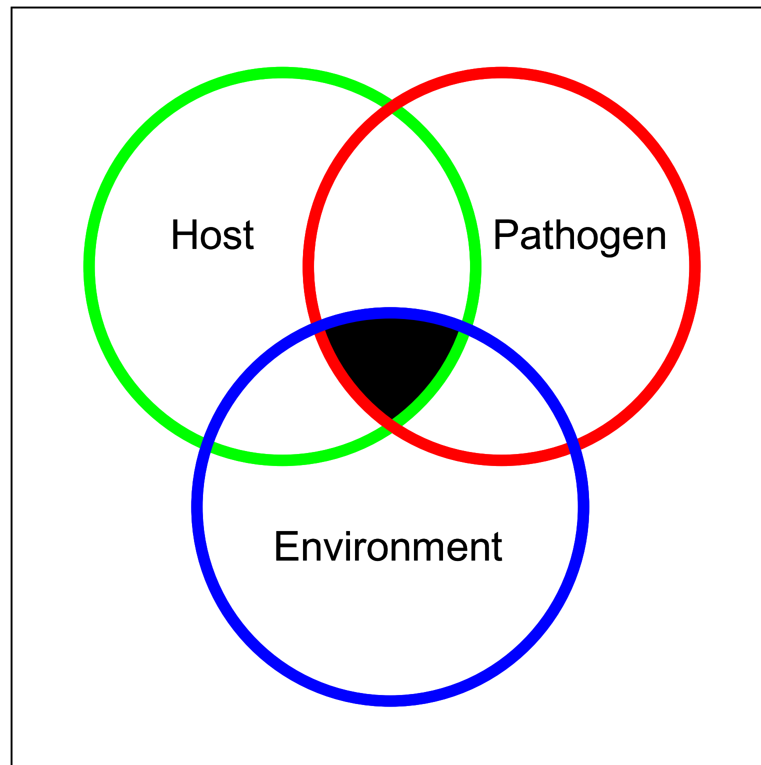


Fig. 1.4. Tripartite interactions between the host, the pathogen and the environment. Disease occurs at the intersection of all circles, symbolized by the black area

2. QPX and related organisms

2.1. *Labyrinthulomycota*

Phylum of Labyrinthulomycota is composed of unicellular eukaryotes that are ubiquitous in ocean. They are found in the water column, sediment and in association with organic detritus. They grow on a variety of substrates and under a large range of temperatures and salinities. These organisms are poorly studied but several authors have suggested that Labyrinthulomycota could be involved in remineralization of organic matter (Bongiorni et al., 2005; Raghukumar, 2002).

Species belonging to this phylum were originally classified in the division Mastigomycotina among Fungi, but certain characteristics have placed them in the division of Stramenopiles (Leander and Porter, 2001). These characteristics are mainly an ectoplasmic net (except the genus *Althornia*) associated with a sagenosome

at the periphery of the cell. Moreover, these organisms are characterized by asexual reproduction and the presence of biflagellate zoospores (Raghukumar, 2002).

2.2. *Labyrinthulids and Thraustochytrids*

Phylum of Labyrinthulomycota comprises two distinct lineages: Labyrinthulids and Thraustochytrids (PubMed). It is important to underline that the systematics of these groups is still evolving as many authors (Honda et al., 1999; Leander and Porter, 2001) have proposed modifications based on morphological and molecular comparisons.

According to Raghukumar (2002), labyrinthulids are defined by binary / mitotic growth and are always associated with living plants as mutualists, commensalists or parasites, such as *Labyrinthula sp.* that causes the wasting disease of *Zostera marina* along the North American coast (Muehlstein et al., 1988). Contrary to labyrinthulids, Thraustochytrid reproduction is characterized by direct division of the sporangium and successive bipartitions of the vegetative cells which develop into sporangia. Moreover, Thraustochytrids are rarely found with living organisms but are more typically characterized as saprophytes, associated with decomposing materials and organic detritus. In fact, most Thraustochytrids cannot grow on living algae / plants or during the early stages of decomposition (Raghukumar et al., 1992). This incapacity appears related to the presence of antimicrobial substances such as flavone glycosides produced by plants (Jensen et al., 1998). Thraustochytrids are defined by osmoheterotrophic nutrition but Raghukumar (1992) described some phagocytic capacities (amoebae formation) of these organisms to feed on bacteria. For instance, Sakata et al. (2000) developed cultures of Labyrinthulomycota on media composed of marine bacteria and microalgae. Thraustochytrids can subsist on very simple and diverse substrates by producing numerous enzymes to colonize the most varied and recalcitrant substrates (Bongiorni et al., 2005).

2.3. *Associations of Thraustochytrids with animals*

Associations between Labyrinthulomycota and animals have been rarely observed and involved mainly Thraustochytrid members such as *Aplanochytrium yorkensis* isolated from the pallial cavity of *Crassostrea virginica* (Perkins, 1973a). Thraustochytrid-like organisms have been identified in gill epithelia of the clam *Ruditapes decussatus* infected by *Perkinsus atlanticus* (Azevedo and Corral, 1997). Miller and Fleming (1983) observed different Thraustochytrids, including *Schizochytrium sp.,* in gills and eggs from lobsters infected by the nemertan *Pseudocarcinemertes homari*. Presence of this Thraustochytrid was considered as a secondary infection. Polglase (1980) identified *Ulkenia amoeboida* as the cause of ulcerations and edema of the skin in the lesser octopus *Eledone cirrhosa*. These symptoms were followed by octopus death after two to three days. McLean & Porter (1982) suggested that this Thraustochytrid could be a secondary pathogen. The same authors described epidermal alterations of the nudibranch *Tritonia diomedea* by a protistan (yellow spot disease). In that case, the host was able to respond by isolating the parasite into cysts and initiating active phagocytosis. But, five years later, McLean & Porter (1987) observed different symptoms. Lesions of the nudibranch were no longer yellow (characteristic of the yellow spot disease) but brown. Moreover, the nudibranch was no longer able to control the disease. Mucus produced by the Thrautochytrid was associated to considerable tissue damage. Although hemocytes actively phagocytosed parasites, host defense mechanisms were evidently insufficient. Encystment was unsuccessful in inhibiting parasitic activity. The authors attributed the differences between the two cases to some environmental actions on host defenses (pollutants) or a different Thraustochytrid infection. Finally, Jones and O’Dor (1983) described the case of the squid *Illex illecebrosus lesueur* maintained in captivity and infected by a Thraustochytrid. The protist grew in the gills, producing copious mucus and causing disruptions of adjacent tissues. No host response (phagocytosis, infiltration or attempt to encyst parasites) was observed. All these observations were reported sporadically without successful isolation of the parasite, and none of them constituted a durable host – pathogen interaction. For instance, interactions of these Thraustochytrid members with their host were characterized as secondary infection and resulted in the complete elimination of the parasite or in a rapid death of the host.

However, Bower (1987b) described a protistan parasite associated to mass mortality of juvenile abalones in Canada. *Aplanochytrium haliotidis* was first described as a Labyrinthulid, but subsequent studies defined it as a member of Thraustochytrids (Honda et al., 1999; Maas et al., 1999; Ragan et al., 2000; Stokes et al., 2002). *A. haliotidis* infects juvenile abalones (less than 190 days old), producing mortalities 4 days post infection. It is observed in the muscle, nervous system, foot and the head of abalones. The parasite produces an ectoplasmic net from a sagenosome, and amoeboid forms have been reported. *A. haliotidis* was never seen in host cells and parasite proteases can disrupt tissues (Bower, 1987d). Some studies focusing on its life cycle and growth were performed by Bower (1987a; 1987c) and demonstrated the inability of *A. haliotidis* to grow on simple media (pine pollen) used specifically for Labyrinthulomycota. However, the parasite is able to grow on many other substrates over a wide range of environmental conditions (temperature from 5 °C to 24 °C, salinity from 15 ppt to 45 ppt). Its life cycle is composed of a vegetative development (binary division) in host tissues. During abalone death, parasite cells are released in seawater, where many internal divisions are followed by the production of biflagellate zoospores. These flagellate stages can infect new abalones (Bower et al., 1989).

2.4. *Quahog Parasite Unknown (QPX)*

2.4.1. *Phylogeny and life cycle*

QPX is a unicellular protist, pathogen of the hard clam *M. mercenaria*, infecting both cultured and wild populations. It is a member of the phylum Labyrinthulomycota and several authors include QPX within the Thraustochytrid family (Honda et al., 1999; Leander and Porter, 2001; Maas et al., 1999; Ragan et al., 2000; Stokes et al., 2002) on the basis of its rRNA 18S sequence. QPX life cycle is similar to that described for the genus *Schizochytrium* (Raghukumar, 2002). The dominant trophic forms, thalli, grow and develop into sporangia, in which successive divisions produce many endospores. Sporangia eventually burst and release endospores, which mature into thalli, completing the vegetative life cycle (Figure 1.5, Kleinschuster et al., 1998).

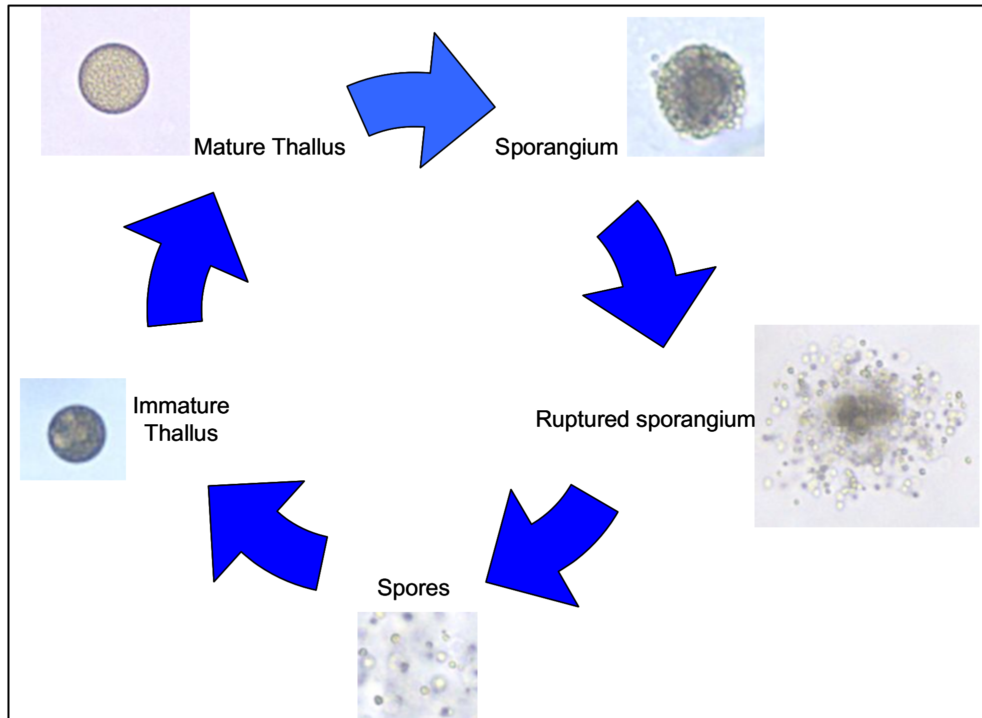


Fig. 1.5. QPX life cycle observed *in vitro*

2.4.2. In vitro culture

Culture medium was developed for *in vitro* QPX growth by Kleinschuster et al. (1998). This medium is composed of Minimal Essential Medium (MEM), supplemented with 10% fetal bovine serum (FBS) and is similar to media developed for other pathogenic protists such as *Perkinsus sp*. (Gauthier and Vasta, 1995; La Peyre, 1996). As other Thraustochytrids, QPX is also able to grow on very simple and diverse substrates. Prior studies reported QPX's capacity to grow in media composed of specific macroalgae species and to be associated with marine snow (Buggé and Allam, 2007; Lyons et al., 2005). Additionally, Gast et al. (2008) detected QPX by PCR in environmental samples of water, sediment, seaweeds, seagrass and various invertebrates. However, ecology of QPX and its potential reservoir in the environment are still understudied.

Culture media may have major effects on pathogen metabolism. For example, culture media developed for *Perkinsus sp.* were associated with alterations of protistan morphology and pathogenicity (Ford et al., 2002a; La Peyre et al., 1993). Earnhart et al. (2004) stimulated *Perkinsus* pathogenicity by adding host tissue

homogenates to the culture medium. The addition of oyster tissue homogenates to defined media affected morphology, proliferation, and protease production in *P. marinus* (Brown et al., 2005; MacIntyre et al., 2003). Similarly, Anderson et al. (2003a) showed enhancement of QPX growth after supplementation of media with low levels of *M. mercenaria* plasma, whereas higher plasma concentrations produced dose-dependent inhibition.

2.4.3. QPX extra cellular products

QPX produces copious mucus in MEM-FBS medium. Mucoid secretions have been investigated in other Thraustochytrids and are composed of 45% sugars, 25% proteins, 10 % sulfates and 3 % uronic acids (Jain et al., 2005). Mucus appears to be involved in many processes including nutrition, adhesion and protection (against desiccation) (Raghukumar, 2002). Histological observation of infected clam tissues reported lucent areas surrounding QPX cells (Figure 1.3), suggesting production of a mucofilamentous net that physically produces a barrier against migration of hemocytes to the parasite and thus, inhibits phagocytosis and also protects the parasite from humoral defenses (Anderson et al., 2003a; Smolowitz et al., 1998). Anderson et al. (2006) identified several gelatin-degrading proteases from QPX, suggesting its capacity to degrade extracellular matrices (ECM) in tissues. These findings were confirmed by histological observations of tissue disruptions in QPX-infected clams (Dove et al., 2004; Smolowitz et al., 1998). Interestingly, investigations of *Perkinsus* virulence factors also reported gelatin- and casein-degrading proteases (La Peyre and Faisal, 1995; La Peyre et al., 1995b; Ordás et al., 2001). Some of these proteases were thereafter characterized as serine proteases and were isolated (Brown and Reece, 2003; Faisal et al., 1999b). Alteration of bivalve defenses by extracellular products (ECP) from protistan pathogens was documented. For example, *Perkinsus* ECP modulates host defenses by inhibiting reactive oxygen species (ROS) production, reducing hemocyte migration and phagocytosis in oyster (Anderson, 1996; Anderson, 1999a; Anderson, 1999b; Garreis et al., 1996; Oliver et al., 1999; Ordás et al., 1999; Villalba et al., 2004; Volety and Chu, 1995; Volety and Chu, 1996). QPX, as other Thraustochytrids, produces a large amount of ECP including proteases (Bongiorni et al., 2005) but their effects on *M. mercenaria* defenses are still unknown.

2.4.4. *Comparison of QPX isolates*

Initial description of QPX by Whyte et al. (1994) in Canada differs from observations made since 1998 in Massachusetts (MA) and New York (NY) (Ragone Calvo et al., 1998; Smolowitz et al., 1998; Whyte et al., 1994). For instance, initial observations by Whyte et al. (1994) described an organism ranging from 5 to 71 µm (CA QPX), whereas VA QPX (Virginia) and MA QPX range from 4 to 25 µm. Some Thraustochytrid characteristics such as sagenosome and biflagellate zoospore stages were identified in CA QPX, but not in other isolates (Ragone Calvo et al., 1998; Smolowitz et al., 1998; Whyte et al., 1994). Moreover, Periodic Acid Schiff (PAS) stains only the wall of CA QPX, whereas both cytoplasm and wall are stained in other isolates. In addition, CA QPX was never found in nodules and produces little mucoid material compared to MA and NY isolates. Observation of pathogenicity of different QPX isolates from two locations (MA and NY) indicates some variations of virulence (Dahl et al., 2008). In the same way, clam populations appear to have different susceptibilities toward QPX. Dahl et al. (2008) suggested the presence of different strains of QPX exhibiting variable virulence despite absence of significant genetic variability among different isolates (Qian et al., 2007). The existence of different strains with specific virulence has been reported for other parasites of marine bivalves and suggested a co-evolution between parasite strains and host populations (Bushek and Allen, 1996b).

3. Host defenses

Invertebrates lack a truly adaptive immune system and rely instead on various innate defenses against invading pathogens. The internal defense system depends on hemocytes and noncellular components in plasma. Few studies have investigated defense factors in *M. mercenaria* (Tripp, 1992a; Tripp, 1992b). This lack of information is particularly obvious at molecular level (Figure 1.6). For instance, genomic information on clam species represented less than 1 % of available information on bivalves in public database in 2006 (Saavedra and Bachère, 2006). More specifically, less than 400 nucleic sequences are available for *M. mercenaria* in

NCBI compared to the 60,000 nucleic acid sequences already deposited for *Crassostrea gigas*. For these reasons, a large part of the introduction on host defenses will be based on studies investigating defense mechanisms in other bivalves and more generally in invertebrates.

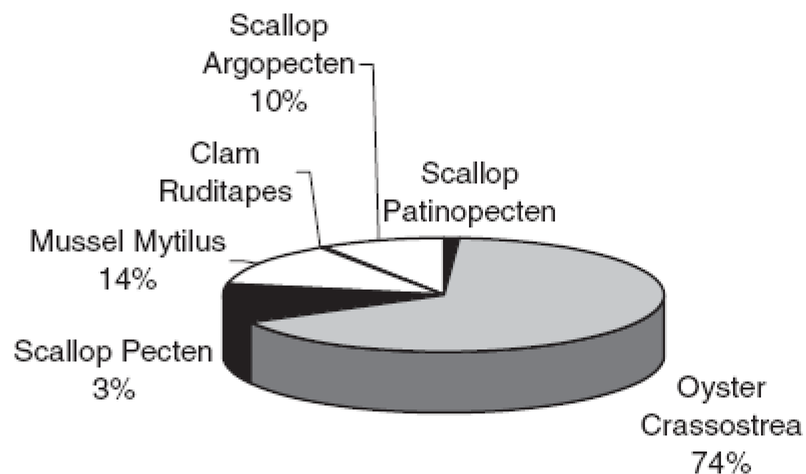


Fig. 1.6. Distribution of bivalve nuclear DNA sequences in GenBank by species or genus in 2006 (From Saavedra and Bachère, 2006).

Based on histological observation of QPX infections, some characteristics of hard clam response can be identified (Figure 1.7). For instance, hard clam is able to recognize the parasite, induce inflammatory processes and encapsulate the parasite. Signaling of pathogen presence induces the recruitment and the migration of hemocytes to the infection site. Additionally, the presence of dead QPX cells suggests effective defense mechanisms in clam (Figure 1.3). However, response mechanisms and specific defense molecules mediating QPX killing remain unknown and general defense mechanisms such as phagocytosis seem to have little impact on QPX. For instance, the size of QPX cells (thallus stage) and the presence of mucus limits hemocyte capacity to phagocytose QPX and only a few observations of phagocytosed QPX cells by hemocytes were previously reported (Smolowitz et al., 1998).

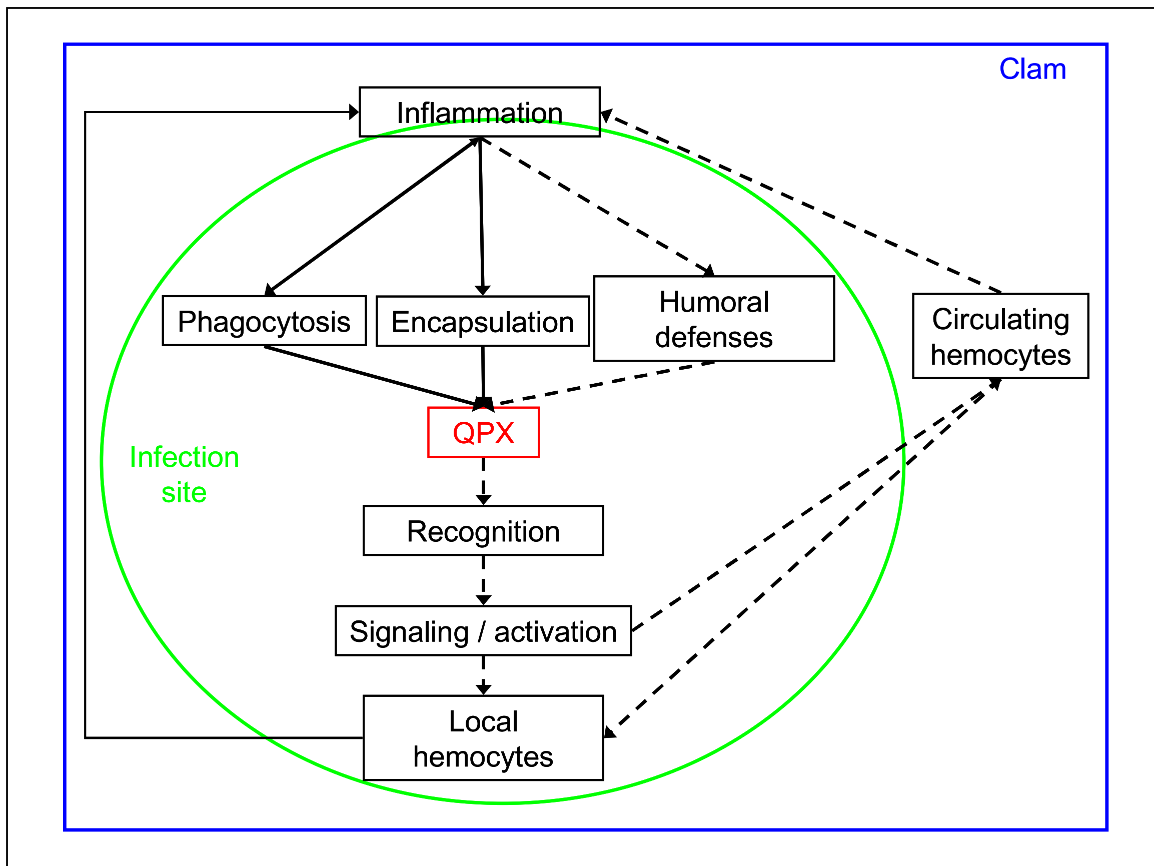


Fig. 1.7. Schematic representation of observed (solid lines) and putative (dashed lines) hard clam responses to QPX infection.

3.1. *Recognition and signaling*

Activation of innate immunity implies its capacity to recognize and initiate a response to the presence of foreign agents. Specific molecular structures of microorganisms, known as pathogen-associated molecular patterns (PAMPs) activate intracellular signaling pathways through pattern recognition receptors (PRRs) which lead to the initiation of the immune response by the host (Iwanaga and Lee, 2005; Medzhitov and Janeway, 1997). Mechanisms of PAMP recognition and signaling pathways are particularly well described in insects (for review Lemaitre and Hoffmann, 2007). For instance, different PRRs with specific PAMP affinities (Gram positive or negative bacteria, fungi, and viruses) were identified in *Drosophila* and were associated to the activation of different signaling pathways. Identified pathways (Toll, Imd, JAK/STAT) were characterized by specific immune responses in *Drosophila* (Lemaitre and Hoffmann, 2007). Several components of the Toll-Rel/ NF-κB (Figure 1.8) and mitogen-activated protein kinases (MAP kinase) pathways

were also identified in mollusks (Gueguen et al., 2003b; Humphries and Yoshino, 2006; Montagnani et al., 2008). For example, some studies associated activation of MAP kinase pathway to phagocytosis activity and reactive oxygen species production in *Biomphalaria glabrata* (Humphries and Yoshino, 2006; Zelck et al., 2007).

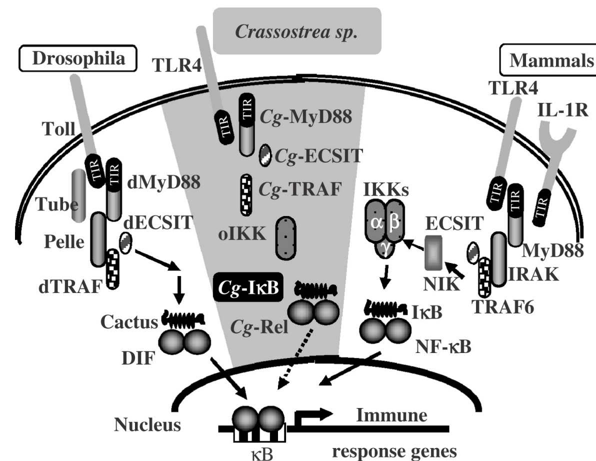


Fig. 1.8. Conservation of Rel/NF-κB pathways in vertebrates *Drosophila* and oyster (From Montagnani et al., 2008).

3.2. *Hemocytes*

Hemocytes are considered to constitute the main line of defense against invaders in invertebrates. Discrepancies exist in the classification of hemocytes in bivalves according to the classification criteria (morphological, cytological or enzymatic) used (Cheng, 1981). In the hard clam, two major types of hemocytes are usually described: hyalinocytes (agranular cells) and granulocytes (Allam et al., 2002a; Tripp, 1992b). Hyalinocyte functions are currently poorly understood. Granulocytes, the most numerous cell type, are associated with phagocytic activity (cellular defenses) (Allam et al., 2002a; Tripp, 1992b). Granules of these cells contain many proteins that could be released in plasma (humoral defenses) by degranulation or involved in intracellular killing / digestion of ingested particles. Both cellular and humoral defenses play important roles against invading microorganisms.

3.3. *Cellular factors*

3.3.1. Phagocytosis

The presence of non-self in tissues stimulates cell-mediated response. Different studies on various pathogens (bacteria and protists) and cellular responses of both resistant and susceptible hosts (clams and oysters) reported some constant characteristics (Allam et al., 2001; Cochennec-Laureau et al., 2003; Ford et al., 1993b; La Peyre et al., 1995a). Successful host response to pathogen challenge is usually characterized by an increase of the total hemocyte count (THC), massive hemocyte infiltration of infected tissues and active phagocytosis of foreign particles. Tripp (1992b) reported capacities of *M. mercenaria* hemocytes to avidly phagocytose a variety of biological particles (red blood cells, yeasts, bacteria). Hard clam hemocytes are also capable of recognizing, phagocytozing and killing some Thraustochytrids related to QPX but not enveloped by secreted material (Anderson et al., 2003b). In addition to changes in THC, prior studies have demonstrated changes in hemocyte composition. Lower granulocyte proportion in THC was observed in susceptible oysters naturally infected with MSX (*Haplosporidium nelsoni*) compared to resistant oysters (Ford et al., 1993b). Similar observations were reported in *C. virginica* and *C. gigas* infected by *P. marinus*. The natural host of *P. marinus*, *C. virginica*, showed lower granulocyte percentage in THC than the resistant oyster *C. gigas*. In addition, phagocytosis and related toxic oxygen metabolites of susceptible oysters were less efficient in eliminating pathogen cells (La Peyre et al., 1995a).

3.3.2. Reactive oxygen species

ROS (reactive oxygen species) activities have been reported as actively involved in defense mechanisms of vertebrates. These compounds have also been identified in invertebrates such as the oysters *C. gigas* and *C. virginica*, but not in the clam *R. decussatus* (López et al., 1994). Anderson (1994) reported the absence of ROS activity in *M. mercenaria* but recent results from Buggé et al. (2007) demonstrated some ROS activity using 2’,74-dichlorofluorescein-diacetate (DCFH-DA). ROS are produced by the membrane-associated NADPH oxidase that is activated by phagocytosis of foreign particles or fixation of an elicitor, such as

Phorbol Myristate Acetate (PMA), to cell membrane receptors. This activation increases NADPH availability, oxygen consumption, and production of superoxide anions ($O_2^{\cdot-}$) (Anderson, 2001). Superoxide anions are enzymatically or naturally converted successively into hydrogen peroxide (H_2O_2) and more harmful compounds such as hydroxyl radicals (OH^-), hypochlorous acid (HOCl) and singlet oxygen ($^1O_2$) (Torreilles et al., 1996). ROS activities in cells / tissues can be harmful for organisms themselves. ROS production and concentration are thus finely controlled by superoxide dismutases (SOD) and other scavenging molecules such as metallothioneins (Anderson et al., 1999). SODs remove superoxide anions and thus prevent generation of hydroxyl radicals (Torreilles et al., 1996). SODs have been characterized in several invertebrate species, including *R. decussatus, Dreissena polymorpha* and *C. gigas* (Geret et al., 2004; Gonzalez et al., 2005).

3.4. *Humoral factors*

In addition to cellular defenses, humoral factors in plasma are involved in inhibition and neutralization of pathogens. Presence and variation of antimicrobial activities against various pathogens have been reported in the plasma of marine bivalves (Anderson and Beaven, 2001a). Plasma of *M. mercenaria* contains constitutive factors (host defenses without the presence of stimulus) that modulate the growth of QPX and other Thraustochytrids (Anderson et al., 2003a; Anderson et al., 2003b). Similar observations were reported for other protistan pathogens; *P. marinus* proliferation is significantly reduced in medium supplemented with plasma from *M. mercenaria* or heavily infected *C. virginica,* but inhibitory effects are less pronounced with plasma from uninfected oysters which indicates the presence of induced humoral factors in response to the pathogen (Gauthier and Vasta, 2002).

3.4.1. Lectins

Lectins play an important role in invertebrate defenses as non-self pattern recognition molecules by promoting agglutination and opsonization of pathogens. La Peyre et al. (1995a) showed a greater haemagglutination titer of *C. gigas* (resistant)

than in *C. virginica* (susceptible) infected with *P. marinus*. Moreover, *C. gigas* increases plasma haemagglutinin titer in response to *P. marinus* challenge (Romestand et al., 2002). Lectins have been characterized in several species, including clams and oysters (Bulgakov et al., 2004; Minamikawa et al., 2004). Kang et al. (2006) demonstrated that several lectins are involved in *Ruditapes philippinarum* defense as microbial challenges (exposure to different bacterial and protistan pathogens) induce expression of different lectins. *M. mercenaria* agglutination abilities over bacteria and fungi were investigated (Tripp, 1992a) and some of these lectins were also isolated and shown to have hemolytic activities (Vasta et al., 1980) as in other marine invertebrates (Kouzuma et al., 2003).

3.4.2. Lysozyme, proteases and protease inhibitors

Specific enzymatic activities have been clearly linked to host defenses. Lysozyme (glycosidase) is well-known and possesses anti-bacterial activities. Lysozyme activity increases significantly in hemolymph of *R. philippinarum* infected by *Vibrio tapetis* (Allam et al., 2000a). However, infection by the protistan parasite *Perkinsus* does not induce similar host response in clams and oysters (La Peyre et al., 1995a; Ordás et al., 2000). Other enzymes from the four major peptidase classes (aspartic-EC 3.4.23, cysteine-EC 3.4.22, serine-EC 3.4.21, metalloendopeptidase-EC 3.4.24) (McKerrow et al., 1993) have also been characterized in different marine invertebrates (Vaseeharan et al., 2006; Ziegler et al., 2002) and were shown to be modulated by protistan challenges (Muñoz et al., 2003). Moreover, specific inhibitors of these protease families have been reported in bivalves (Faisal et al., 1999a; Xue et al., 2006). A comparative study of anti-*P. marinus* activities in sera from different bivalve species showed that the greatest inhibition of pathogen growth and protease activity was observed in naturally resistant bivalves (Anderson and Beaven, 2001b; Faisal et al., 1998). Infection of *C. gigas* with *P. marinus* increases inhibitory activities against *P. marinus* proteases and other peptidases.

3.4.3. Anti microbial peptides (AMP)

Antimicrobial peptides (AMPs) are important components of the innate immune system in plants and animals (Bulet et al., 2004). In insects, 7 families of AMPs were described and characterized by specific regulatory pathways and anti-microbial activities (Lemaitre and Hoffmann, 2007). In bivalves, AMPs have been only reported in a few species, *Mytilus edulis*, *M. galloprovincialis*, *M. trossulus*, *Crassostrea virginica*, *C. gigas*, and *Argopecten irradians*. Among these defense factors, defensins represent an important family of antimicrobial peptides. Defensins are compact cationic peptides that are active against a wide range of bacteria, fungi and viruses but each defensin exhibits specific action for some kind of microbe (Roch et al., 2008; Zhao et al., 2007). Some defensins are associated to hemocytes and expressed during bacterial challenges (Mitta et al., 1999b; Zhao et al., 2007) whereas others are constitutively expressed in tissues (Gonzalez et al., 2007b). Defensins from *C. gigas* exhibit high activities against gram positive bacteria but low activity against fungi (Gueguen et al., 2006b). On the other hand, big defensins from *A. irradians* and the horseshoe crab *Tachypleus tridentatus* exhibit strong fungicidal activities (Saito et al., 1995; Zhao et al., 2007).

3.4.4. Phenoloxidase

In higher invertebrates such as arthropods, phenoloxidase (PO) is known to be involved in melanization, non-self-recognition, opsonization, cellular communication, anti-bacterial activity, wound healing and encapsulation (Söderhäll, 1992; Söderhäll et al., 1994). PO exists in hemolymph as inactive proenzyme and can be activated by both an endogenous activating system (serine proteases such as chymotrypsin and trypsin) and exogenous reagents such as lipids and carbohydrates from bacterial and fungal cell walls (Asokan et al., 1997). PO activity has been detected in several marine bivalves (Carballal et al., 1997a; Jordan and Deaton, 2005; López et al., 1997) including hard clams (Deaton et al., 1999). Muñoz et al. (2006) showed a significant increase of PO activity in plasma of *R. decussatus* infected with *P. atlanticus*.

3.4.5. *Complement components*

The complement system is a major biological reaction system involved in immune responses mediated by the innate and adaptive immune systems in vertebrates (Fujita et al., 2004). The human complement system is composed of more than 30 serum and cell surface components and can be activated through three separate pathways, referred to as the classical, alternative, and lectin (Fujita et al., 2004). Activation of the mammalian classical pathway is initiated by C1q, which is a protein characterized by a globular C1q (gC1q) domain in its C-terminus (Kishore and Reid, 2000). The classical, alternative and lectin pathways merge at the proteolytic activation step of the complement component C3. C3 belongs to the thioester-containing protein (TEP) family which is characterized by relatively large proteins containing a reactive thioester moiety and includes complement proteins C3, C4, C5, the universal protease inhibitor α_2 macroglobulin and the thioester containing proteins (TEPs) (Blandin and Levashina, 2004; Dodds and Law, 1998). The complement system had been initially considered to evolve only after the deuterostomes (Dodds and Law, 1998) but recent advances suggest a more ancient origin and several complement-like components have been described in arthropods and mollusks, including bivalves (Blandin and Levashina, 2004; Lagueux et al., 2000; Prado-Alvarez et al., 2009b; Zhu et al., 2005). Interestingly, TEP in *Anopheles gambiae* was identified to be a major effector of the resistance of the mosquito to *Plasmodium falcipinarum*. (Blandin et al., 2009). Similarly, Zhang et al. (2007; 2008) described a C1q-domain-containing protein and a TEP involved in immune response in the scallop *Chlamys farreri*.

3.5. *Modulation of host defenses*

In addition to the presence of pathogenic organisms, several conditions have been shown to modulate defense factors in metazoans. Metabolic factors such as reproduction status and food quality and quantity were demonstrated to significantly modulate defense parameters in oyster and abalone (Delaporte et al., 2003; Travers et al., 2008). Genetic variations were also associated with differences in susceptibility to pathogens in oysters and clams (Allam et al., 2001; Ford and Haskin, 1987).

Genetic variability was also demonstrated in hard clams from different geographical origins and was suspected to be related to the susceptibility of *M. mercenaria* to QPX (Camara et al., 2006; Ragone Calvo et al., 2007). The effect of environmental factors on defense parameters is particularly important in poikilothermic osmoconformers such as marine invertebrates (Shumway, 1977).

4. Effects of environmental factors

4.1. *Parasites*

Environmental parameters affect both pathogen's virulence and host's ability to resist infections. Several authors highlighted influences of temperature and salinity on *Perkinsus sp.* development, proliferation and environmental distribution (Ahn and Kim, 2001; Auzoux-Bordenave et al., 1995; Chu and Greene, 1989; Villalba et al., 2004). *In vitro* investigations suggested an effect of environmental factors (temperature and salinity) on QPX growth (Brothers et al., 2000). These authors suggested that QPX (a Massachusetts isolate of the parasite) grows optimally at 24 °C and reported a reduction in parasite growth below and above this optimal temperature. These authors also reported that QPX isolates were able to grow under a wide range of salinities, with a regular increase in parasite growth between 20 ppt and 40 ppt.

4.2. *Marine invertebrates*

Environmental factors also affect host resistance against infections by altering both cellular and humoral defenses. Several studies reported influences of temperature, salinity and dissolved oxygen on bivalve defenses and their effects on host susceptibilities to bacterial and protistan pathogens (Cheng et al., 2004b; Chu and La Peyre, 1993a; Chu et al., 1996). Temperature modulates hemocyte phagocytic activity, respiratory burst, anti-bacterial activity, and alters response-time-to-challenge in bivalves (Allam et al., 2002a; Carballal et al., 1997b; Cheng et al., 2004a; Chu and La Peyre, 1993b). Effects of temperature on phagocytosis were also reported in *M. mercenaria* (Tripp, 1992b). Humoral defense factors (phenoloxidase and antibacterial

activities) are affected in abalone and blue mussels exposed to high temperature (Cheng et al., 2004a; Hernroth, 2003). Similarly, environmental salinity affects cellular and humoral defenses such as the concentration of circulating hemocytes, phagocytosis, lysozyme and phenoloxidase activities in clams and oysters (Butt et al., 2006; Reid et al., 2003). Previous works have highlighted the impact of hypoxic conditions on defense mechanisms and susceptibilities to pathogens in marine invertebrates. Several cellular and humoral factors are reduced in bivalves under hypoxic or short term anoxic conditions (Cheng et al., 2004b; Matozzo et al., 2005). Low dissolved oxygen concentration was associated with significant reductions of THC, phagocytosis, lysozyme activity, ROS production and associated SOD (Boyd and Burnett, 1999; Cheng et al., 2004b; Matozzo et al., 2005; Monari et al., 2005).

4.3. *Disease dynamics*

Relationships between defense parameters modulated by environmental factors and susceptibility to pathogens remain unclear and may vary depending on the specific host-pathogen model. For example, high temperature was positively correlated with hemocyte counts and phagocytic activity in *Crassostrea sp*. when the disease progression of *P. marinus* was also high (Chu, 1996; Chu and La Peyre, 1993b). Several studies reported the increase of *P. marinus* prevalence and intensity in *C. virginica* at high salinity (Chu et al., 1993; Chu et al., 1996; Ragone and Burreson, 1993). Deficiency of defense factors in hypoxic environments was associated with elevated mortalities of *C. virginica* infected by *P. marinus* (Anderson et al., 1998).

Field results suggested the important effect of environmental parameters on QPX disease development (Ragone Calvo et al., 2007). For instance, QPX disease has never been identified South of Virginia despite the widespread presence of highly susceptible clam stocks there (Ford et al., 2002b). Similarly, QPX disease was absent in Virginia clamming areas with moderate (15 to 25 ppt) salinities but was present in areas exposed to high (> 25 ppt) salinities (Ragone Calvo et al., 1998). However, relationship of specific environmental factors with the development of QPX disease was not demonstrated.

5. Objectives of this dissertation

In the context of increasing frequency and distribution of diseases in the marine environment, it is essential to enhance our knowledge of mechanisms involved in host – pathogen interactions. QPX disease represents a good model for the investigation of host – pathogen interactions since previous studies suggested the involvement in disease development of both intrinsic (genetic variability) and extrinsic (environmental) factors (Dahl et al., 2008; Dahl et al., 2010; Ragone Calvo et al., 2007).

Therefore, the purpose of this dissertation was three-fold. The first was to characterize the virulence factors of QPX (part II). The cytotoxicity of QPX toward clam hemocytes was investigated trough the development of a biotest to determine and quantify the effect of QPX cells and extracellular compounds on cellular defenses of *M. mercenaria*. QPX cells and extracellular products grown in different media were tested to optimize the biotest and to evaluate the effect of the presence of host compounds on QPX virulence. The biotest was also used to compare the cytotoxicity of different QPX isolates and to assess the sensitivity of hemocytes from different clam populations in an attempt to link the *in vitro* variations of QPX cytotoxicity on hemocytes with previous *in vivo* results on disease development among different clam populations following experimental challenge (Dahl et al., 2008).

The second objective of this dissertation was focused on the identification of host defenses against the parasite. The presence of anti-QPX factors was investigated in different clam tissues using a biotest previously developed to quantify QPX biovolume (Buggé and Allam, 2005, part III - chapter 1). This methodology was used to compare anti-QPX factors within the same clam population and between different clam broodstocks exhibiting various degrees of susceptibility toward QPX *in vivo*. Host response to QPX challenge was also investigated using molecular approaches (part III – chapter 2). Clams were differentially challenged with mucus-coated or washed QPX cells to compare clam response in the presence or absence of virulence factors associated with QPX mucus (see part II). Experiments were performed over 48 days and genomic libraries were generated from tissues using suppression

subtractive hybridization to identify differentially regulated genes. Another set of experiments was designed to compare several hemocyte parameters and the transcription level of two stress-related genes in clams challenged by QPX and by bacteria for 28 days (part III – chapter 3). Bacteria and protists are differentially recognized in insects (Lemaitre and Hoffmann, 2007) and initiate specific responses from the host. The same approach was also applied to compare the response of susceptible and resistant clam populations to QPX. These experiments could allow the identification of specific hemocyte / hemolymph parameters involved in the successful response of hard clams to QPX.

Finally, the last objective aimed at the evaluation of the effect of some environmental parameters on the parasite, the host and host – pathogen interactions. The effects of temperature and salinity on the *in vitro* survival and growth of QPX were tested. Additionally, the effect of environmental factors was tested on QPX isolates from New York and Massachusetts to compare the temperature and salinity optima for these isolates and to determine if they represent distinct strains or ecotypes as suggested in a previous study (Dahl et al., 2008, part II - chapter 1). Long term effects (4 months) of temperature and salinity on constitutive clam defense parameters as well as clam response to QPX and associated disease development were also assessed (part IV – chapter 2 and 3). Susceptible clams from Florida were experimentally infected with QPX and maintained at specific salinities and temperatures. A similar approach was also applied on naturally infected clams from Massachusetts to confirm the results of experimental infections and to evaluate the impact of environmental factors on disease dynamics. Hemolymph parameters were assessed after 2 and 4 months of challenge and were compared with QPX prevalence and intensity (Dahl et al., Accepted, part IV - chapter 3). The final part of the dissertation presents a synthesis of major results associated to a critical discussion of the findings and perspectives on the different axes of the dissertation.

Part II

QPX virulence

Part II

Chapter 1

Cytotoxicity of Quahog Parasite Unknown (QPX) toward hard clam (*Mercenaria mercenaria*) hemocytes and interactions between different pathogen isolates and host strains

Adapted from **Perrigault M.**, Allam B. 2009. Cytotoxicity of Quahog Parasite Unknown (QPX) toward hard clam (*Mercenaria mercenaria*) haemocytes and interactions between different pathogen isolates and host strains. **Parasitology**, 136 (11) : 1281-1289.

Abstract

The ability of pathogens to neutralize host defence mechanisms represents a fundamental requisite in the successful establishment of an infection. Host-pathogen interactions between Quahog Parasite Unknown (QPX) and its hard clam host are poorly understood. Our prior *in vivo* investigations have shown that different QPX isolates display varying levels of pathogenicity toward clams. Similarly, field investigations and laboratory transmission studies revealed some variations in the susceptibility of different hard clam stocks to QPX infection. An *in vitro* approach was developed in this study to evaluate the toxicity of QPX cells and extracellular products toward hemocytes using a neutral red uptake assay. Results demonstrated that QPX produces virulence factors that are cytotoxic to *M. mercenaria* hemocytes. This cytotoxicity appears to be induced by clam factors, suggesting that it may play an important role in supporting QPX infection and proliferation within the host. Moreover, application of this technique to different QPX isolates and clam broodstocks indicates variations of QPX cytotoxicity in agreement with previous *in vivo* experiments, strengthening the existence of different QPX strains.

1. Introduction

Quahog Parasite Unknown (QPX) is a parasite member of the phylum Labyrinthulomycota (Maas et al., 1999; Ragan et al., 2000; Stokes et al., 2002). Despite the ubiquitous nature of this phylum in marine environment, Labyrinthulomycota were poorly studied (Raghukumar, 2002) and only few pathogens were described in this group (Bower, 1987d; Muehlstein et al., 1988). QPX has been associated with mass mortalities of cultured and wild *Mercenaria mercenaria* along the Northeastern coasts of the United States (Dove et al., 2004; Ford et al., 2002b; Ragone Calvo et al., 1998; Smolowitz et al., 1998) and Maritime Canada (Ragan et al., 2000; Whyte et al., 1994).

Despite the economic importance of *M. mercenaria*, little is known about the interactions between hard clam and QPX at cellular and molecular levels, as most information has been gained from histological observations of infected clam tissues. These observations have clearly shown that QPX infections cause a significant inflammatory response in clams, sometimes leading to effective neutralization of parasite cells and healing (Ragone Calvo et al., 1998; Smolowitz et al., 1998, Personal Observations). Histological observations also suggest that QPX displays virulence activities that facilitate the colonization of clam tissues. For instance, QPX produces mucus, forming lucent areas around parasite cells in clam tissues. It has been hypothesized that this mucus plays a role in virulence, acting as a physical barrier to both phagocytosis, as well as diffusion of clam defence molecules (Anderson et al., 2003a; Smolowitz et al., 1998). Lucent areas around parasite cells could also result from tissue degradation by proteases produced and released by QPX (Anderson et al., 2006). As a matter of fact, it is not uncommon to identify necrotic hemocytes near active QPX foci (Allam, unpublished).

The balance between parasite virulence and host resistance factors is probably linked to the fact that QPX is present at extremely low prevalence in some geographic areas (below 0.1 %), while it causes significant mortalities in other locations (Smolowitz et al., 1998, Allam, unpublished). This led Ford (2001) to designate QPX as an opportunistic pathogen that only infects clams under unfavourable conditions. Interestingly, host genetic makeup has been clearly shown to be associated with clam susceptibility, as both field (Ford et al., 2002b; Ragone Calvo et al., 2007) and

laboratory (Dahl et al., 2008) experiments have demonstrated higher susceptibility toward QPX of southern clam strains (originating from South Carolina and Florida) as compared to northern strains (Massachusetts, New York and New Jersey).

Our prior investigations also showed that QPX virulence *in vivo* was specific to the parasite isolate (Dahl et al., 2008). In that study, four clam strains were experimentally challenged with two QPX isolates originating from New York (NY) and one parasite isolate from Massachusetts (MA). Results showed that the NY isolates of the parasite were more virulent than the MA isolate when injected into clams originating from Florida, New York and Massachusetts. In clams originating from Virginia, however, the MA isolate of QPX caused significantly higher disease prevalence and mortality. This was one of a few studies dealing with bivalve diseases that combined investigations on resistances of different host strains toward different parasite isolates. Bushek and Allen (1996a) previously reported variations of both virulence of *Perkinsus marinus* strains and resistance of geographically distinct populations of the host, *Crassostrea virginica*. In *M. mercenaria*, our findings raise questions about the biological bases of QPX virulence and clam resistance.

Because histological observations of infected clam tissues suggest a role for hemocytes during pathogenesis (see above), we initiated research investigating the interactions between hemocytes and QPX on cellular levels. In this study, we developed a technique, using the neutral red (NR) uptake assay, to evaluate the cytotoxicity of QPX cells and extracellular products (ECP) toward hemocytes. This assay is based on the lysosome's ability to retain weakly cationic supravital dye (NR). The dye readily diffuses through the plasma membrane and concentrates in the lysosomes of undamaged cells. Alterations of the cell surface or lysosomal membrane under the effect of cytotoxic compounds (including contaminants and pathogen-derived compounds) lead to gradual leakage of NR from cells to the surrounding medium (Borenfreund and Puerner, 1985b). This is the first study to report the cytotoxicity of QPX products to clam hemocytes. Additionally, we compared the cytotoxicity of ECP obtained from different QPX isolates against hemocytes collected from two clam strains, and discussed results in light of prior information obtained during *in vivo* pathogenicity studies.

2. Materials and methods

2.1. QPX cultures

Three QPX isolates were used in this study. Two isolates, NY0314220AC5 (NY1-QPX) and NY0313808BC7 (NY2-QPX) were established from two infected clams collected from 2 field sites in New York (Qian et al., 2007). QPX isolation and culturing were performed in Minimal Essential Medium Eagle (MEM, Sigma M06440) as described by Kleinschuster et al. (1998). The third isolate (MA-QPX), originally isolated from Massachusetts hard clams in 1997 (Kleinschuster et al., 1998), was purchased from ATCC (Number 50749) as a cryopreserved sample, thawed and maintained in culture the same way as the new isolates. QPX isolate cultures were propagated in MEM, or in clam tissue homogenates (1000 $\mu g.ml^{-1}$ protein of muscle homogenates) according to Perrigault et al. (part III – chapter 1). Sub-culturing of each QPX isolate in both culture media were performed weekly for at least six months before use in the cytotoxicity experiments. Under these conditions, cultures in the exponential phase of growth are typically obtained after one week at 23 °C. QPX growth in cultures was monitored by measuring parasite biomass as described by Buggé and Allam (2005). Briefly, 100 µl of culture aliquots were plated in a black 96-well microplate and 50 µM of fluorescein diacetate (FDA) were added before fluorescence was measured at 485 nm excitation and 535 nm emission (Wallac 1420 plate reader).

2.2. Hemolymph sampling

Adult hard clams (45 to 55 mm in length) from New York (NY) and Massachusetts (MA) were obtained from commercial sources and transferred overnight to our laboratory in refrigerated containers. All clams belonged to the same year class (~2.5 to 3 year old) and were descendants from broodstocks cultured by these companies in each site for several generations. They were maintained in tanks equipped with a closed recirculating seawater system (20 °C, 28 ppt) and fed daily with live algae (DT Plankton, IL, USA). Clams were acclimated for 2 to 4 weeks before hemolymph collection. Hemolymph was withdrawn from the adductor muscle with a 1-ml plastic syringe equipped with 25-gauge needle. According to each assay,

hemolymph samples were used individually or pooled in a 15-ml tube held on ice. The number of hemocytes per ml of hemolymph was microscopically determined using a Neubauer chamber.

2.3. Setup of the neutral red uptake assay

The neutral red uptake assay was adapted from Borenfreund and Puerner (1985a). A neutral red working solution (250 $\mu g.ml^{-1}$) was freshly prepared for each assay by diluting a stock solution of neutral red (Sigma N4638 at 20 $mg.ml^{-1}$ in dimethyl sulfoxide) in filtered sterile seawater (FSSW). Hemolymph from NY hard clams was plated in flat bottom 96-well plates, in four replicates, at concentrations ranging from 3.5×10^3 to 7×10^4 hemocytes per well. Hemolymph samples were serially diluted with FSSW and control wells were made by substituting hemolymph with FSSW. Plates were incubated 1 h at room temperature (RT) to allow hemocyte adhesion to the plates. The supernatant was carefully removed and cells were washed with 200 µl of FSSW. Plates were then incubated at RT with 100 µl of test media (FSSW for controls and setup experiments, QPX cells and ECP for cytotoxicity experiments, see below). After 1 h, liquids were carefully removed and hemocytes were washed with 200 µl of FSSW. Cells were then incubated 1 h at RT with 100 µl of neutral red work solution and washed twice with FSSW. Neutral red in viable cells was eluted into 100 µl of solvent consisting of glacial acetic acid:ethanol:water (1:50:49 by volume). Plates were sealed, agitated for 10 min and absorbance was recorded at 560 nm. Hemocyte viability and morphology were monitored under phase-contrast microscopy before and after cell staining with neutral red.

2.4. Effect of QPX cells and supernatants

Exponentially growing cultures of QPX (isolate NY0313808BC7) in MEM or tissue homogenates were diluted with their respective media at 1×10^5 $cell.ml^{-1}$ and centrifuged at 1000 g for 20 min. Supernatants (henceforth called extracellular products or ECP) were collected and filtered through 0.22 µm syringe filters. Pellets made of QPX cells were washed, centrifuged at 1000 g for 10 min and resuspended in

appropriate culture medium (volume equal to initial culture volume at 1 x 10^5 cell.ml^{-1}). Hemolymph from 6 NY clams was pooled and plated at a final density of 5 x 10^4 hemocytes per well. After 1 h at RT, plasma was discarded and hemocyte preparations were challenged with 100 µl QPX ECP or resuspended parasite cells. Both undiluted or diluted (1/5 and 1/10 dilution by volume in appropriate medium) QPX cells or ECP were added to each well, resulting in 1:5 (undiluted), 1:25 or 1:50 QPX (cells or cell equivalent for ECP):hemocyte ratios. Sterile MEM and tissue homogenate were added to control wells (empty wells and wells containing hemocyte layer). All preparations were made in triplicates. Neutral red uptake assay was performed as described above and absorbance was recorded after 1 and 4 h of interaction between hemocytes and QPX cells or ECP. Data are presented as percentage of optical density of test media (QPX suspension or ECP) to control preparations made with culture media (MEM or tissue homogenates).

2.5. Comparison of QPX isolates and clam populations

Cytotoxicity of ECP from 3 QPX isolates (NY1-QPX, NY2-QPX and MA-QPX) on hemocytes collected from clams originating from NY and MA were investigated. Hemolymph from 5 clams from each population was withdrawn individually and diluted with FSSW at 5 x 10^5 hemocytes.ml^{-1}. One hundred microliters of hemolymph dilution from each clam was plated in 3 replicates and allowed to adhere to the plate for 1 h. QPX cultures (NY1-QPX, NY2-QPX and MA-QPX) grown in tissue homogenates were diluted with sterile media to obtain 1 x 10^5 cells.ml^{-1}, and suspensions were processed as previously to collect ECP. Tissue homogenates were chosen as culture media to produce ECP rather than MEM based on results from preliminary experiments (moderate and consistent cytotoxicity of QPX ECP, see results section). Challenges were initiated by adding 100 µl of ECP to experimental wells, while control wells were added with sterile culture medium. After 1 h of challenge, QPX ECP were removed and NR uptake assay was performed as described above.

2.6. Statistical analysis

Data were analyzed using SigmaStat (Ver3.11. Systat Software, Inc., San Jose, CA) statistical software. Effects of the time-of-incubation and the dilution factor of QPX cells and ECP on NR uptake by hemocytes were analyzed by a two-way ANOVA. A two-way ANOVA was also used to compare differences of cytotoxicity among QPX isolates on hemocytes withdrawn from MA and NY clams. ANOVA treatments that generated probability values below 0.05 were systematically followed by a Holm-Sidak post-hoc test comparing different data points. Differences between data points were considered statistically significant at $p < 0.05$.

3. Results

3.1. Setup of the neutral red uptake assay

Under the tested experimental conditions, the assay indicated a linear relationship between neutral red uptake and the number of hemocytes in the range of 3.5×10^3 to 7×10^4 hemocytes per well (Fig. 2.1, $R^2 = 0.99$).

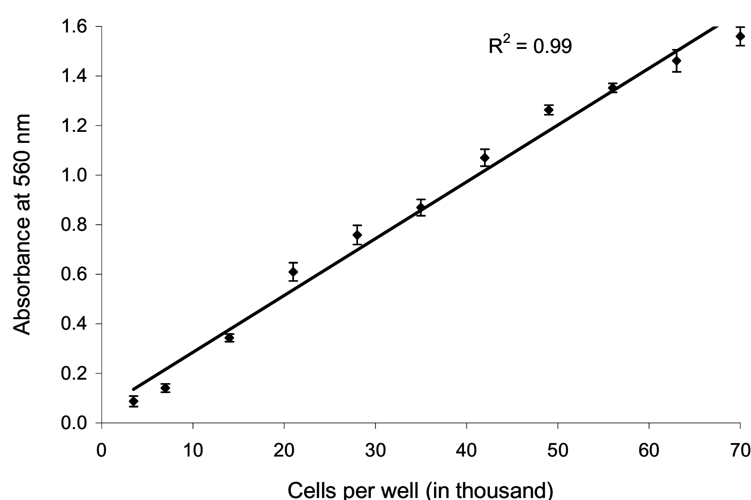


Fig. 2.1. Neutral red uptake (absorbance at 560 nm) as a function of hemocyte counts in experimental wells. Linear relationship ($R^2 = 0.99$) was measured for hemocyte concentrations ranging from 3.5×10^3 to 7×10^4 cells per well. Mean ± SEM (4 replicates / data point).

Higher densities of hemocytes enhanced cell aggregations and hemocyte concentrations were no longer proportional to the absorbance (data not shown).

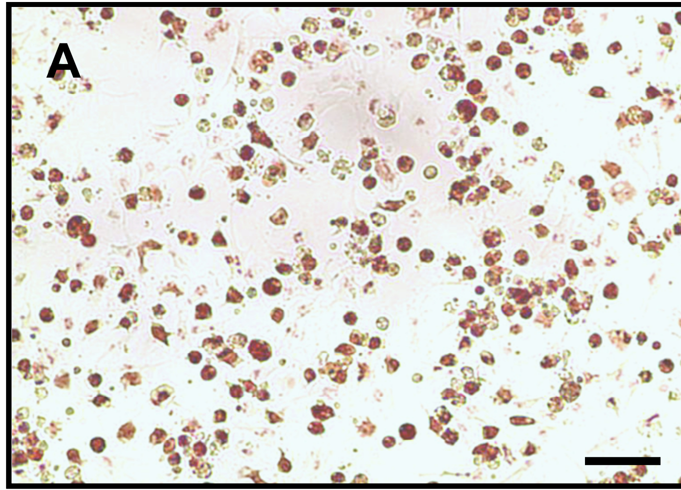
3.2. Cytotoxicity of QPX cells and ECP produced in tissue homogenates

Addition of QPX cells or ECP from parasite cultures caused an increase in cell debris and decrease in the uptake of neutral red in the exposed hemocytes (Fig. 2.2). Challenged hemocyte layers displayed discoloured cytoplasm, sometimes lacking intracellular complexity when compared to control hemocytes exposed to sterile culture media (Fig. 2.2). The cytotoxicity of QPX cells and ECP from parasite cultures grown in clam tissue homogenates was dose-dependent, with NR uptake higher at higher dilutions of QPX cells or ECP (Fig. 2.3A, Table 2.1). When hemocytes were incubated with undiluted (1:5 ratio, see Materials and Methods) QPX ECP (Fig. 2.3A), the decrease in NR uptake was maximal (31.12 ± 3.57 % of the controls) after one-hour exposure to ECP; further incubation (4 h) induced barely significant further decrease of NR uptake by hemocytes (Table 2.1, $p = 0.047$).

Table 2.1. Summary of Multifactor ANOVA testing the effects of QPX cells and ECP (isolate NY0313808BC7) grown in tissue homogenates and MEM on neutral red uptake by hemocytes from NY clams.

Culture Medium	Source of Variation	Degrees of freedom	Sum of squares	Mean squares	*F*	*P*
Clam extracts	**QPX cells**					
	Time	1	1.102	1.102	361.915	<0.001
	Dilution	2	0.727	0.363	119.386	<0.001
	Time x Dilution	2	0.197	0.098	32.306	<0.001
	QPX ECPs					
	Time	1	0.016	0.016	4.907	0.047
	Dilution	2	0.542	0.271	85.922	<0.001
	Time x Dilution	2	0.018	0.009	2.807	0.1
MEM	**QPX cells**					
	Time	1	0.069	0.069	37.012	<0.001
	Dilution	2	0.065	0.032	17.400	<0.001
	Time x Dilution	2	0.004	0.002	1.177	0.341
	QPX ECPs					
	Time	1	0.013	0.013	1.866	0.197
	Dilution	2	0.177	0.089	12.438	0.001
	Time x Dilution	2	0.026	0.013	1.829	0.203

Interestingly, a different trend was observed in hemocytes exposed to QPX cells: NR uptake was significantly lower at longer incubation periods (4 h), as compared to those exposed for 1 h ($p < 0.001$). This was the case for hemocytes challenged with both undiluted and diluted parasite cells (Fig. 2.3A, Table 2.1).



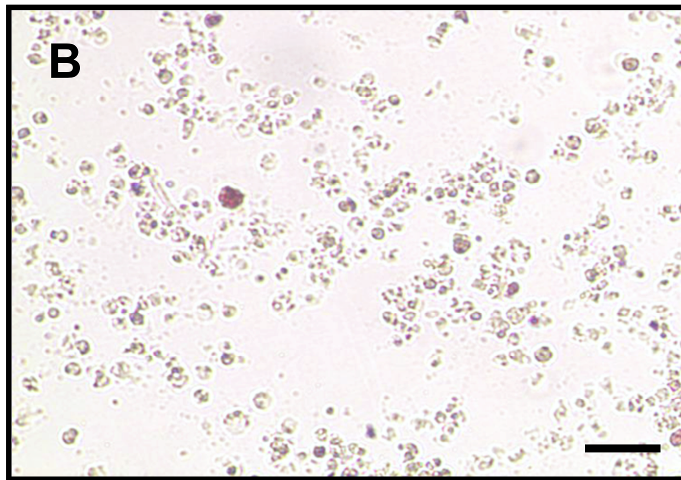


Fig. 2.2. Neutral red uptake by hemocytes exposed for 4 h to (A) sterile culture media or to (B) supernatants of an exponentially growing QPX culture in tissue homogenates (isolate NY0313808BC7). Scale bar = 50 µm.

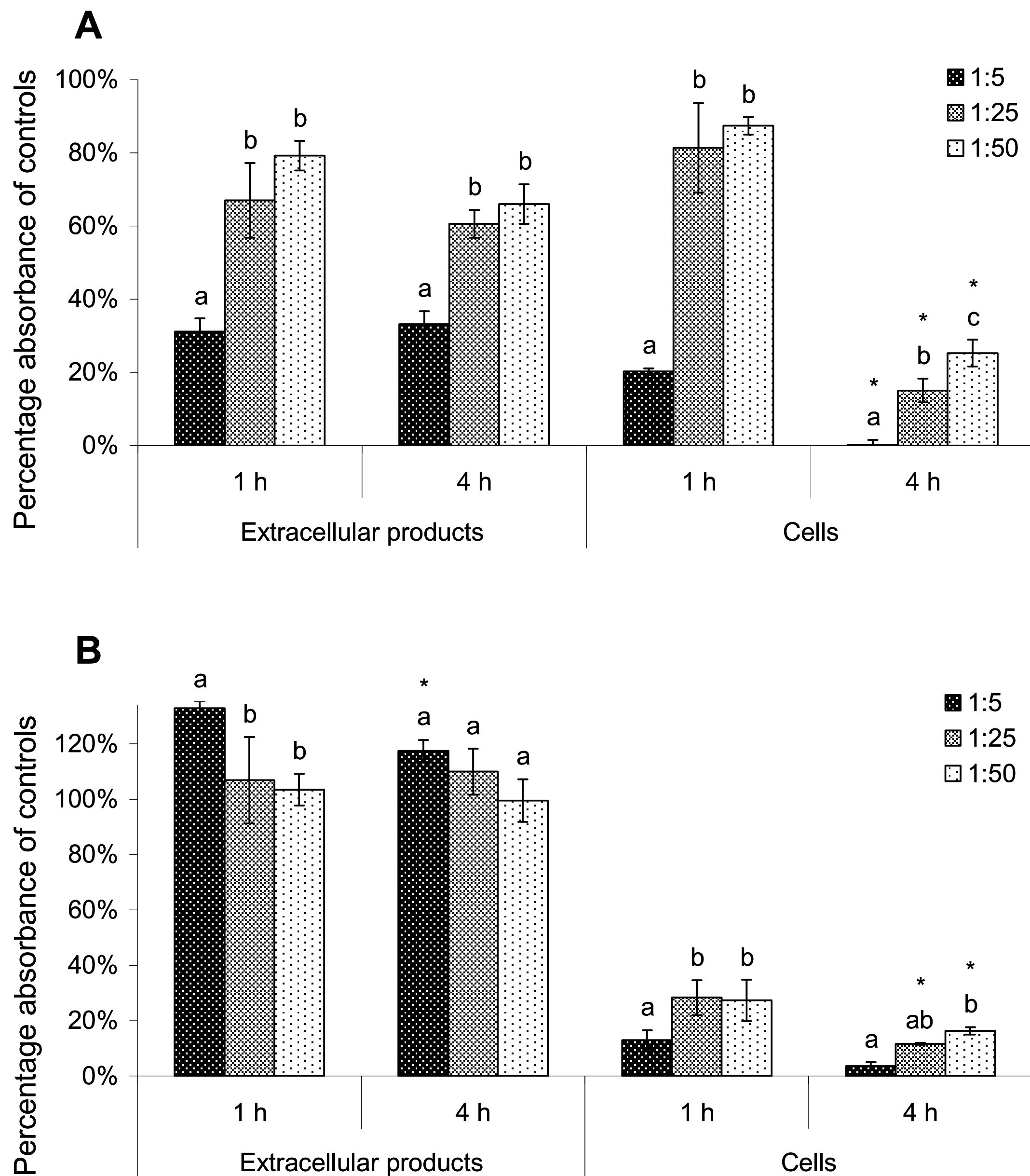


Fig. 2.3. Neutral red uptake by hemocytes exposed to serial dilutions of QPX cells and ECP from isolate NY0313808BC7 grown in tissue homogenate (A) and in MEM (B). Ratios correspond to 1:5, 1:25 and 1:50 QPX (cells or cell equivalent for ECP):hemocyte ratios. Results are expressed as percentage of absorbance of unchallenged controls at 560 nm. Lower NR uptake / absorbance indicates higher QPX cytotoxicity. Letters (a, b and c) represent differences among different dilutions for each time and symbols (*) indicate significant differences between incubation times for each dilution (Two-way ANOVA, $p < 0.05$). Mean ± SEM (3 replicates / data point).

3.3. Cytotoxicity of QPX cells and ECP produced in MEM

The trends of NR uptake were noticeably different in hemocytes exposed to QPX cells and ECP produced in MEM (Fig. 2.3B). Significant differences between

the tested ECP dilutions were detected by ANOVA analysis (Table 2.1). However, these differences were mostly attributed to effects of undiluted ECP. NR uptake in hemocytes exposed to diluted ECP was not statistically different from control hemocytes exposed to sterile MEM. Surprisingly, hemocytes exposed to undiluted ECP displayed higher NR uptake than hemocytes exposed to diluted ECP samples (1 h) or control hemocytes (1 h and 4 h).

On the other hand, QPX cells cultured in MEM exhibited a strong cytotoxicity toward hemocytes after 1 h of challenge and appeared more toxic to hemocytes than parasites grown in tissue homogenates, particularly when diluted parasite cells were used (Fig. 2.3). Both dilution- and time-dependent effects were significant (Table 2.1).

3.4. Comparison of QPX isolates and clam populations

ECP produced by different QPX isolates grown in tissue homogenate displayed different levels of cytotoxicity toward hemocytes (Fig. 2.4). Multifactor ANOVA analysis indicated variations of cytotoxicity between tested QPX isolates ($p < 0.001$) and differences in hemocyte resistance between MA and NY clams ($p = 0.008$). No specific interactions between specific QPX isolates and clam strains were detected ($p = 0.57$, Table 2.2).

Table 2.2. Two-way ANOVA results testing the effects of ECP from three QPX isolates on clam hemocytes from two geographic locations, and interactions between variables.

Source of Variation	Degrees of freedom	Sum of squares	Mean squares	*F*	*P*
Clam	1	0.191	0.191	8.244	0.008
QPX	2	0.569	0.285	12.305	<0.001
Clam x QPX	2	0.0262	0.0131	0.566	0.575

Among all clams, NR uptake by hemocytes was systematically lower when NY isolates of the parasite (particularly isolate NY1-QPX) were used as compared to the MA isolate (MA-QPX). Absorbance readings obtained with ECP from the NY isolates ranged from 39.02 ± 5.6 to 68.49 ± 9.6 % of control values while ECP from the MA isolate resulted in a smaller reduction of NR uptake (ranging from 76.09 ± 4.1

to 92.15 ± 5.76 % of control absorbance). Significant differences of ECP cytotoxicity were observed between NY1-QPX and MA-QPX for both MA and NY clams (p < 0.005), whereas the two NY isolates of the parasite exhibited no significant difference when compared to each other.

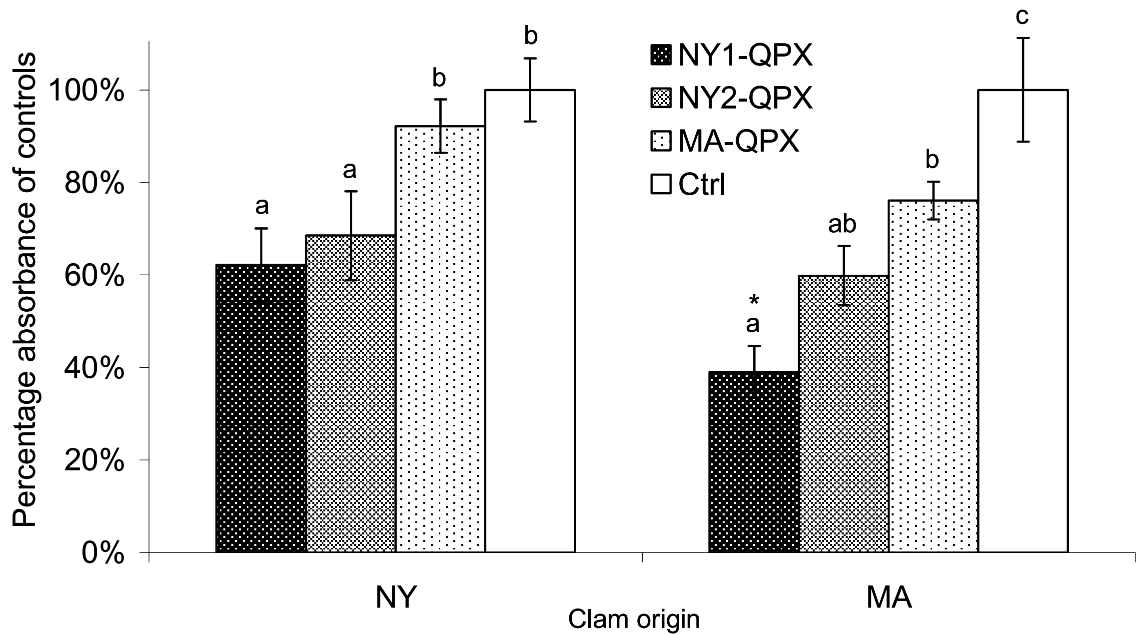


Fig. 2.4. Neutral red uptake by hemocytes from 2 clam stocks exposed to ECP from 3 different QPX isolates. Results are expressed as percentage of absorbance of corresponding controls at 560 nm. Lower NR uptake / absorbance indicates higher QPX cytotoxicity. The letters a, b and c represent differences among treatments for the same clam strain while symbol (*) represents difference among clam strains exposed to the same QPX isolate (Two-way ANOVA, $p < 0.05$). Mean ± SEM (5 replicates).

Hemocytes also presented different levels of resistance to ECP according to the clam stock from which they were withdrawn (Fig. 2.4, Table 2.2). For each specific QPX isolate, hemocytes from NY clams exhibited higher resistance to ECP than hemocytes from MA clams. However, pairwise comparisons demonstrated statistically significant variations of resistance only during challenge with ECP from NY1-QPX (Holm-Sidak post-hoc test, $p = 0.02$, Fig. 2.4).

4. Discussion

The neutral red (NR) uptake assay was initially developed by Borenfreund and Puerner (1985a) to test cytotoxic effects of chemical agents on cells in a monolayer culture. This method was successfully used in bivalves to investigate cytotoxic effects of metals (cadmium, copper, nickel) or chemical compounds (polycyclic aromatic hydrocarbons, tributyltin) (Cajaraville et al., 1996; Chu et al., 2002; Grundy et al., 1996; Matozzo et al., 2002; Pipe et al., 1999). Alteration of NR uptake usually reflects damage to the membrane or, alternatively, changes in the volume of the lysosomal compartment. These alterations are therefore characterized by either a decrease or an increase in NR uptake by hemocytes of exposed bivalves. For instance, an increased NR uptake was reported in hemocytes of the mussel *Mytilus edulis* exposed to cadmium (Coles et al., 1995), whereas the same heavy metal caused a decrease in NR uptake in *M. mercenaria* (Zaroogian et al., 1992). Neutral red assay uptake, however, has been rarely used to investigate cytotoxic effects of parasites or parasite products on cells (Le Sueur et al., 2005; Pichardo et al., 2006). The increase of NR uptake in hemocytes challenged by ECP from QPX grown in MEM (Fig. 2.3B) might be related to the effect of byproducts resulting from MEM degradation as supported by our ongoing studies (data not shown). The observed reduction in NR uptake following exposure to QPX products in this study is, however, a clear indication of the parasite's cytotoxic effects on clam hemocytes. However, the NR uptake technique does not allow for identification of cytotoxicity mechanisms, since reduction in the uptake may result from modification of lysosomal compartment volume, loss of hemocyte adherence, or fragilization of membrane integrity. Microscopic observations supported alterations of cell membranes and cell death in hemocyte monolayers exposed to QPX ECP (Fig. 2.2). Pathogens are known to produce and sometimes release cytotoxic substances that interfere with normal activities of bivalve hemocytes. For instance, the exposure of hemocytes from the clam *Ruditapes philippinarum* to ECP produced by its pathogen *Vibrio tapetis* caused significant reduction in hemocyte viability and phagocytic activity (Allam et al., 2002b). *Vibrio spp.* cells can also induce morphological alterations of bivalve hemocytes, leading to cell rounding and loss of adherence (Choquet et al., 2003; Lane and Birkbeck, 2000). Similarly, ECP of *P. marinus* altered the motility of *C. virginica* hemocytes (Garreis et al., 1996).

Interestingly, variations of cytotoxicity induced by QPX cells and ECP were observed according to culture conditions. ECP from parasite cultures grown in tissue homogenates exhibited significantly higher cytotoxicity compared to ECP from cultures made in MEM. These findings suggest that the presence of clam factors in the culture medium enhanced the production/release of cytotoxic compounds into the surrounding environment. Thraustochytrids are well known to produce and release a large variety of extracellular compounds (Jain et al., 2005) and Anderson et al. (2006) demonstrated the presence of serine protease in QPX ECP. Similar mechanisms have already been described in the oyster parasite *P. marinus*, in which the supplementation of parasite cultures with oyster tissue extracts or plasma significantly induced the secretion of low-molecular weight serine proteases (MacIntyre et al., 2003) and enhanced infectivity of the parasite (Earnhart et al., 2004).

Our results also demonstrated high cytotoxicity of QPX cells toward clam hemocytes for all tested conditions, with parasite cells grown in MEM tending to be more cytotoxic than QPX cells grown in tissue homogenates. The disparity between toxicity profiles of ECP and parasite cells in different culture media is intriguing and cannot be interpreted here without speculations, particularly since the factors responsible for the cytotoxic effects remain unidentified. Factors involved in QPX cell toxicity might be different from those mediating ECP toxicity (cell-cell interactions *versus* ECP proteases for example). Alternatively, factors mediating ECP toxicity might be produced and stored in parasite cells and only released to the extracellular environment in the presence of host tissue extracts or hemocytes. This hypothesis could explain the strong cytotoxicity of ECP obtained in tissue homogenates (Fig. 2.3A) and the rapid (within 1 h) and robust cytotoxicity of QPX cells cultured in MEM (Fig. 2.3B). The delay of cytotoxicity observed between 1 h and 4 h with diluted QPX cells cultured in tissue homogenates may be attributed to the time required to *de novo* produce and release ECP by QPX. This hypothesis is supported by findings of Anderson et al. (2003), who reported that washed QPX cells were able to produce copious amounts of mucus (and probably virulence factors coupled within).

Our results revealed various levels of cytotoxicity among the three tested QPX isolates. New York isolates were significantly more cytotoxic to hemocytes (both

clam strains combined) than the Massachusetts isolate ($p < 0.001$), but no significant difference was detected between the two NY strains. These *in vitro* results closely matched *in vivo* observations we previously reported, where experimental challenges produced higher mortality of naïve MA clams after exposure to QPX isolate NY0314220AC5 (here called NY1-QPX), followed by isolate NY0313808BC7 (NY2-QPX) and finally MA-QPX (Dahl et al., 2008). Obviously, it is difficult to make a link between *in vitro* cytotoxicity and *in vivo* pathogenicity, and potential relationships require further investigations. However, such variations among isolates suggest the existence of different QPX strains between Massachusetts and New York. Qian et al. (2007) observed substantial sequence variability in the rRNA operon intergenic spacers of different QPX isolates without being able to identify specific differences between isolates. The possible absence of sexual life stages (zoospore stages) in QPX could enhance the speciation of the parasite. Investigations of virulence and environmental tolerance among *P. marinus* isolated from different geographical locations also demonstrated the existence of parasite races (Bushek and Allen, 1996b; Reece et al., 2001).

Previous *in vivo* investigations demonstrated that clam resistance toward QPX disease is mediated genetically (Dahl et al., 2008; Ford et al., 2002b; Ragone Calvo et al., 2007). Our *in vitro* results indicated higher susceptibility toward QPX ECP of hemocytes from MA clams as compared to NY clams (Fig. 2.4, Table 2.2). However, the significance of this difference was mostly supported by the effects of ECP from isolate NY1-QPX. Implying that *in vitro* resistance of hemocytes to QPX ECP is linked to higher *in situ* survival of clams exposed to QPX, prior and likely long term exposure of NY clams to NY1-QPX could explain higher resistance of hemocytes from these animals compared to hemocytes from MA clams, which had not been previously exposed to this strain. Additional investigations using a larger number of QPX isolates and clam populations from different geographic locations (particularly from areas where QPX disease is absent) are necessary to discriminate specific interactions between QPX isolates and hemocytes from different clam broodstocks.

This study demonstrated the cytotoxicity of QPX toward clam hemocytes and established the inducible nature of cytotoxic factors. NR uptake assay allowed the comparison of QPX cytotoxicity among different parasite isolates. Results of these *in*

vitro investigations corresponded well with results of prior *in vivo* studies comparing pathogenicity of different QPX isolates. The NR uptake technique described here appears to represent an efficient and sensitive tool for the investigation of QPX virulence factors and alternatively, for evaluation of hemocyte resistance to the parasite.

Acknowledgements

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Part III

Host defenses

Part III

Chapter 1

Modulatory effects of hard clam (*Mercenaria mercenaria)* tissue extracts on the *in vitro* growth of its pathogen QPX

Adapted from **Perrigault M.**, Buggé D.M., Hao C.C., Allam B. 2009. Modulatory effects of hard clam (*Mercenaria mercenaria*) tissue extracts on the *in vitro* growth of its pathogen QPX. **Journal of Invertebrate Pathology**, 100 (1) : 1-8.

Abstract

Quahog Parasite Unknown (QPX) is a fatal protistan parasite affecting cultured and wild hard clams *Mercenaria mercenaria* along the northeastern coasts of the USA and maritime Canada. Field investigations and laboratory transmission studies revealed some variations in the susceptibility of different hard clam stocks to QPX infection. In this study, we used *in vitro* QPX cultures to investigate the effect of plasma and tissue extracts from two different clam stocks on parasite survival and growth. Results demonstrated the presence of factors in clams that significantly modulate QPX growth. Extracts from gills and mantle tissues as well as plasma inhibited *in vitro* QPX growth, whereas foot and adductor muscle extracts enhanced parasite growth. Investigations of anti-QPX activities in plasma from two clam stocks displaying different susceptibility toward QPX disease *in vivo* demonstrated higher inhibition of QPX growth by plasma from New York (resistant) clams compared to Florida (susceptible) clams. Some clams appeared to be deficient in inhibitory factors, suggesting that such animals may become more easily infected by the parasite.

1. Introduction

Quahog Parasite Unknown (QPX) is a protistan parasite of the hard clam *Mercenaria mercenaria*. This organism is a member of the phylum Labyrinthulomycota (Maas et al., 1999; Ragan et al., 2000). It has caused mortalities in hard clams along the Atlantic coast of North America, as far south as Virginia (Ragone Calvo et al., 1998; Smolowitz et al., 1998; Whyte et al., 1994). Field research (Ford et al., 2002; Calvo et al., 2007) and laboratory (Dahl et al., 2008) studies demonstrated variability in susceptibility toward QPX infection according to broodstock origin, with clams originating from states farther south on the East coast being more susceptible to QPX than stocks originating from more northern states. In New York and Massachusetts, QPX infections of clams are usually localized in the mantle and gills, sometimes producing nodules (Dove et al., 2004; Smolowitz et al., 1998). Histological observations demonstrated clam response to the parasite. Areas of QPX infections triggered inflammatory responses by hemocytes and effective elimination of the parasite was observed in some cases by phagocytosis (usually rare) and/or encapsulation and extracellular killing of parasite cells (Ragone Calvo et al., 1998; Smolowitz et al., 1998).

Bivalves are characterized by a non-specific defense system based on both cellular (phagocytosis, respiratory burst, etc.) and humoral (lectins, lysosomal enzymes, antimicrobial factors, etc.) activities. Since bivalves have an open circulatory system, antimicrobial constituents in plasma and hemocytes are present in all tissues. Prior studies investigated the effects of bivalve plasma on pathogens (Anderson and Beaven, 2001a; Anderson and Beaven, 2001b). Gauthier and Vasta (2002) observed an inhibition of *Perkinsus marinus*, a pathogen of the eastern oyster (*Crassostrea virginica*), in the presence of plasma from uninfected oysters. This inhibition increased when plasma from *P. marinus*-infected oysters was used. Similarly, Anderson et al. (2003a) demonstrated the presence of QPX inhibitory agents in *M. mercenaria* plasma at moderate protein concentrations (10-50 $\mu g.ml^{-1}$) when mucoid secretions of QPX were removed by washing. Inhibitory activities of the plasma disappeared when unwashed QPX cells were used (Anderson et al., 2003a), suggesting protective functions of pathogen secretions. Prior studies also investigated the effects of bivalve tissue extracts on pathogen proliferation. For

instance, supplementation of *Perkinsus marinus* cultures with oyster tissue homogenates modulated cell proliferation and protease expressions, suggesting the presence of molecular signals in oysters that regulate *P. marinus* proliferation (Brown et al., 2005; Earnhart et al., 2004). However, few studies focused on the comparison of antimicrobial activities in specific tissues of marine bivalves. Haug et al. (2004) tested antibacterial activities of different tissues (gill, mantle, foot, muscle) including plasma and hemocytes from the horse mussel *Modiolus modiolus* over four different bacterial strains. They demonstrated the presence of specific antimicrobial activities in gill, mantle, foot and muscle as compared to plasma and hemocytes. Given the typical localization of QPX infections in *M. mercenaria*, we hypothesized that various clam tissues modulate QPX growth differently. Siphon tissue is of particular interest for these investigations because it has been hypothesized to represent the portal of entry for QPX as the clam filters seawater from its surrounding environment (Lyons et al., 2005).

In this study, the effects of plasma and extracts of various clam tissues on QPX growth were investigated. Plasma from clams originating from New York and Florida was also compared in an attempt to link differences in susceptibility toward the disease *in vivo* (Ford et al., 2002b; Ragone Calvo et al., 2007) to variations in QPX modulating activities *in vitro*.

2. **Materials and methods**

2.1. Hard clam plasma and tissue homogenates

Naïve *Mercenaria mercenaria* originating from New York (NY) were obtained from Frank M. Flowers Oyster Company (Oyster Bay, NY). Clams originating from Florida (FL) were also obtained from a commercial source (Farm Raised Clams, St James City, FL). Clams from experimental stocks showed no QPX infections. Clams were acclimated at least one week in the laboratory, held in 150-L tanks with re-circulating water (28-30 ppt) and fed daily with commercial algae (DT's Live Phytoplankton, Sycamore, IL). Hemolymph samples were individually withdrawn with a 1 ml-syringe from adductor muscle and held on ice. Plasma was recovered by centrifugation (700 x g, 10 min, 4 °C) and the supernatant was sterilized

by filtration (0.22 µm pore size). Different clam tissues were dissected and used individually or pooled according to each experiment. Tissues were suspended in filter-sterilized artificial seawater (FASW; 1/10, w/v) and homogenized with a tissue homogenizer (Kinematica AG, Switzerland). Homogenates were centrifuged at 5,000 x g for 15 min and the resulting supernatant was sterilized by filtration through 0.22 µm syringe filters. Fifty µl of filtered plasma and homogenates were allocated to protein analysis using the BCA protein assay kit (Pierce, Rockford, IL, USA).

2.2. QPX

QPX strain NY0313808BC7 was isolated from nodules of infected New York clams (Qian et al., 2007) and subcultured in Minimal Essential Medium (MEM) supplemented by 10% fetal bovine serum according to Kleinschuter et al. (1998). QPX cultures were initiated in 25-cm^2 flasks incubated at 23 °C for 1 week in order to reach the exponential phase of growth, then washed according to a protocol adapted from Anderson et al. (2003a). Briefly, a volume of QPX culture was well mixed by repeatedly drawing up and expelling the culture with a 3 ml syringe without a needle. A small volume of well mixed culture was then suspended in at least four times its volume of FASW. This suspension was then vortexed until uniform and centrifuged for 15 min at 600 x g. The supernatant was removed and the QPX pellet was then washed two times. This washing procedure has been thoroughly tested and found not to affect QPX viability (Buggé and Allam, 2005).

In all experiments, biovolume of QPX was measured using a semi-automated fluorometric technique according to Buggé and Allam (2005). This technique uses the dye fluorescein di-acetate (FDA) to measure esterase activity of live cells. The fluorescence signals produced by this assay, expressed as relative fluorescence units (RFU), were used as a measure of cell biovolume to assess viability and growth of a specific QPX culture.

2.3. Effects of M. mercenaria plasma on QPX growth

Two separate experiments were performed to study the effect of clam plasma on QPX growth. Both experiments compared plasma from clams originating from New York (NY) and Florida (FL). In the first experiment, plasma samples (100 µl clam^{-1}) from ten clams belonging to each stock were pooled. A volume of 50 µl of pooled plasma was added to 50 µl of MEM containing 1 x 10^3 QPX cells in black 96-wellplates. Assays were performed in triplicate and an additional replicate without QPX cell was used to quantify the fluorescence signal generated by the medium (MEM and plasma). FASW was substituted for plasma as positive controls. The FDA technique was then used to measure QPX biovolume at time zero (t_0) and after incubation at 23 °C for 4 days. Briefly, plates were added with FDA (12 µM) and incubated in the dark for 30 min. before fluorescence was measured at 485 nm excitation and 535 nm emission (Wallac 1420 plate reader).

In the second experiment, plasma samples from nine NY clams and nine FL clams were processed individually. Protein contents in each plasma sample were normalized to a final concentration of 100 µg.ml^{-1} by dilution with FASW. Assays were then performed individually in black 96-wellplates and QPX biovolume was measured at t_0 and after 4 days as described above.

2.4. Effects of clam extracts on QPX growth

Gill, mantle and adductor muscle tissues from six clams (NY) were dissected, separately pooled, suspended in FASW and homogenized as described above. Protein concentrations of the different tissue homogenates were normalized by dilution with FASW to 2000 µg.ml^{-1}. Controls were composed of MEM diluted with FASW to 2000 µg protein.ml^{-1}. Assays were initiated in 4-ml sterile culture tubes by adding 100 µl of QPX suspension in FASW to 1.5 ml of the different treatments and controls (in triplicate). Tubes were incubated on a rotating shaker at room temperature (21 ± 1 ºC) and QPX growth was measured at t_0 and on days 4, 8 and 15 by the FDA technique. A second assay used these same homogenates as a supplement to MEM to account for potential nutrient limitation that may result when tissue homogenates are used alone as a growth medium. Assay tubes containing 750 µl MEM were mixed

with an equal volume of the appropriate tissue homogenate (in triplicate). One control consisted of 1.5 ml MEM and a second control contained 1.5 ml FASW-diluted MEM (vol:vol). The assay was carried out as above, and biovolume was measured at t_0 and on days 3, 8 and 15.

In a second set of experiments, mantle tissues from twelve NY clams were further dissected into more distinct regions: siphon, edge of mantle, and central part of the mantle. This processing was done in light of available information concerning the microscopic detection of QPX in certain areas of the mantle, particularly siphon and the edge of the mantle, and based on structural differences between siphon (mostly muscular), the edge of the mantle (mix of muscular and connective tissues) and the central part of the mantle (mostly connective). Tissue homogenates were divided into three pools (4 clams.pool^{-1}) and protein concentrations were normalized with FASW at 2000 µg.ml^{-1}. QPX suspension was added to each pool and parasite growth was assessed as described above at t_0 and on days 3, 8 and 15. MEM adjusted with FASW at the same protein concentration (2000 µg.ml^{-1}) was used as control.

In a third set of experiments, clams were processed individually to assess the variability in anti-QPX factors in tissues among different specimens. Ten NY clams were dissected and the following tissues were individually collected and processed: gill, central part of mantle, edge of mantle, foot, adductor muscle and siphon tissues. Protein concentrations of tissue homogenates were normalized with FASW at 2000 µg.ml^{-1} and used with appropriate controls (MEM diluted with FASW to 2000 µg.ml^{-1}). QPX growth was measured at t_0 and on days 2, 8 and 15.

2.5. Dose effect of tissue homogenates

Gill, mantle and adductor muscle tissues from five New York clams were pooled and protein concentrations of tissue homogenates were adjusted with FASW to obtain protein ranges between 100 µg.ml^{-1} and 2500 µg.ml^{-1}. As previously, assays were performed with and without MEM supplementation to ascertain that growth inhibition was not a simple result of trophic limitations of culture media. Briefly, one series was composed of 2 ml of tissue homogenates and another series was performed by adding 1 ml of MEM to 1 ml of each tissue homogenate dilution. Controls were

composed of MEM and MEM diluted in FASW (vol:vol) for unsupplemented and MEM-supplemented assays, respectively. The FDA technique was used to measure QPX growth at t_0 and on days 4, 7 and 14.

2.6. Statistical analysis

Data were analyzed using SigmaStat (Ver. 3.1, Systat Software, Inc., San Jose, California, USA) statistical software. Data were tested for normality and homogeneity of variance before analysis. A Student's *t*-test was used to compare the growth of QPX in plasma from NY and FL clams. A one-way analysis of variance (ANOVA) was used to compare QPX biovolume obtained in different culture media during the last sampling (Day 14 or 15 according to each experiment) and a repeated measure ANOVA was used to assess the evolution of QPX biovolume in each culture over time. When clams were individually processed, a repeated measure ANOVA was also used to compare the growth of QPX in homogenates of different tissues collected from the same specimen. ANOVA tests were followed with a Holm-Sidak post-hoc test when significant. All differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Effects of M. mercenaria plasma on QPX growth

Pooled plasma from both NY and FL clams caused strong inhibition of QPX growth (92 and 87 % inhibition, respectively) as compared to control cultures of QPX grown in MEM (data not shown). The inhibitory effect of the plasma was significantly higher (Student's *t*-test, $p = 0.02$) in clams from NY than those from FL. Protein concentrations were also significantly higher in plasma from NY clams (282 ± 22 $\mu g.ml^{-1}$, mean ± standard error) when compared with FL (118 ± 4 $\mu g.ml^{-1}$) clams (Student's *t*-test, $p < 0.01$).

Results from the second series of experiments, which used plasma from individual clams normalized to 100 μg protein.ml^{-1}, confirmed the findings obtained

above. For instance, averaged biovolume data showed that inhibition was significantly higher in plasma from NY clams when compared to FL clams with a reduction of 65 % and 26 % of parasite growth compared to controls, respectively (p = 0.006, Fig. 3.1). However, results also demonstrated major variations in QPX biovolume using plasma from individual clams within each population (Fig. 3.1). Some plasma samples inhibited QPX growth whereas others enhanced parasite proliferation. Fluorescence signals were stronger than the controls (grown in diluted culture media) using plasma samples from 3 FL clams but none from the NY clams.

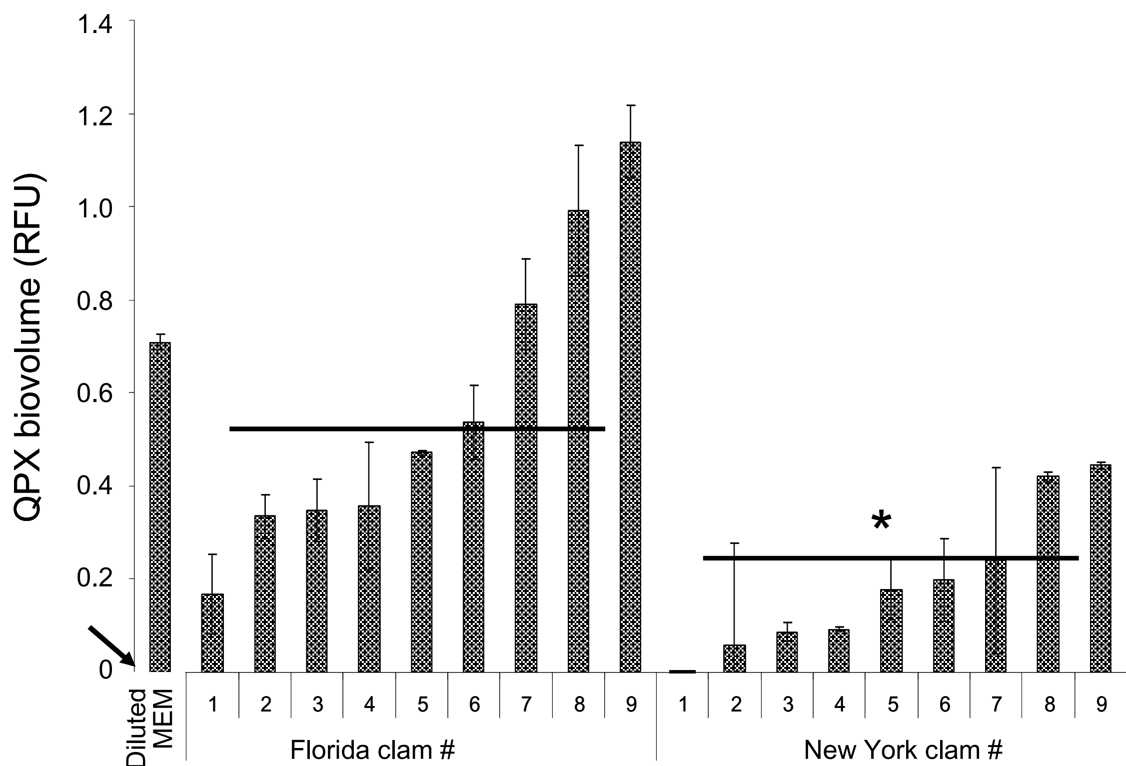

Fig. 3.1. QPX biovolume (relative fluorescent units) after 4 days in cultures made with plasma from 9 clams belonging to two populations of *M. mercenaria*. Protein concentrations were adjusted to 100 $\mu g.ml^{-1}$ in FASW and supplemented to the culture medium (vol:vol). Plasma was substituted with FASW in the MEM control (diluted MEM). The arrow on the Y axis represents QPX biovolume added to each culture at t_0. The lines represent average QPX biovolume in the plasma for each clam population. * denotes significant differences between both populations (Student *t*-test, p = 0.006). All data are presented as Mean ± SEM (n = 2 replicates).

3.2. Effects of clam extracts on QPX growth

Incubation of QPX with *M. mercenaria* tissue homogenates yielded different growth results in various tissues (Fig. 3.2a). QPX biovolume increased in adductor muscle homogenates and surpassed the biovolume of QPX grown in culture media from Day 4 until the end of the experiment. There was no increase in QPX biovolume in gill or mantle tissue homogentates (Fig. 3.2a). The second assay, in which QPX cells were incubated in culture media supplemented with clam tissue homogenates, indicated similar trends with a limited increase in QPX biovolume in cultures supplemented with gill and mantle homogenates (Fig. 3.2b). On the other hand, supplementation of culture media with adductor muscle homogenates resulted in significantly higher QPX biovolumes than in undiluted or diluted MEM (ANOVA, $p = 0.01$).

In the second set of experiments that compared different parts of the mantle, QPX growth was completely inhibited in pools made with homogenates from the central part and the edge of the mantle (data not shown but trends were similar to those reported in Fig. 3.2a). Two pools made with siphon tissue homogenates were also inhibitory, while the third pool significantly enhanced QPX growth, as compared to control preparations grown in culture media (Fig. 3.3, ANOVA, $p = 0.001$).

Trends observed among pools of tissue homogenates were confirmed in the third set of experiments that used tissues from individual clams. QPX growth was maximal in homogenates made with foot and adductor muscle tissues, while significant inhibition of QPX growth was noted when homogenates from gill, and central part and edge of mantle were used (Fig. 3.4a). A repeated measure ANOVA comparing the growth of QPX in homogenates of different tissues collected from the same clam showed the following significant trends: muscle = foot > siphon > edge of mantle > gill = central part of mantle. Interestingly, homogenates made with siphon tissues displayed high inter-individual variability: siphon obtained from one clam displayed significantly higher QPX growth than other siphon homogenate samples (Fig. 3.4b).

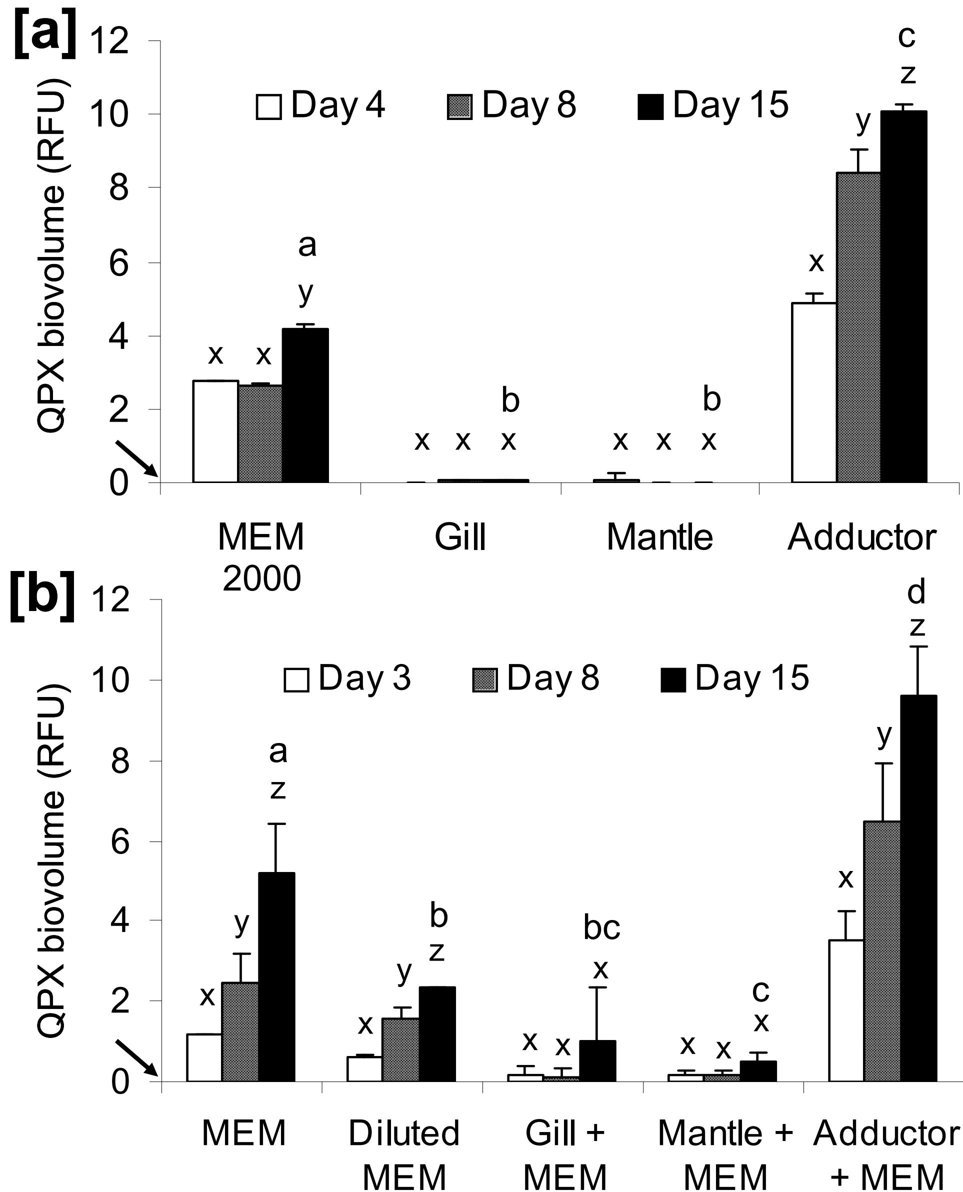

Fig. 3.2. QPX biovolume (relative fluorescent units, Mean ± SEM, n = 3 replicates) in cultures made with different tissue extracts. Extracts were adjusted at 2000 μg protein.ml^{-1} in FASW and used as sole source of nutrient [a] or supplemented (vol/vol) to MEM [b]. Controls were composed of culture medium adjusted to 2000 μg protein.ml^{-1} with FASW (MEM 2000), undiluted culture medium (MEM) and diluted (vol:vol) culture medium in FASW (Diluted MEM). The arrows on the Y axis represent QPX biovolume added to each culture at t_0. Letters indicate which experimental treatments shared statistically equivalent (same letters) or different (different letters) QPX biovolume means among cultures in different experimental media at Day 15 (a, b, c, d), or among measurements taken at different times within each culture (x, y, z).

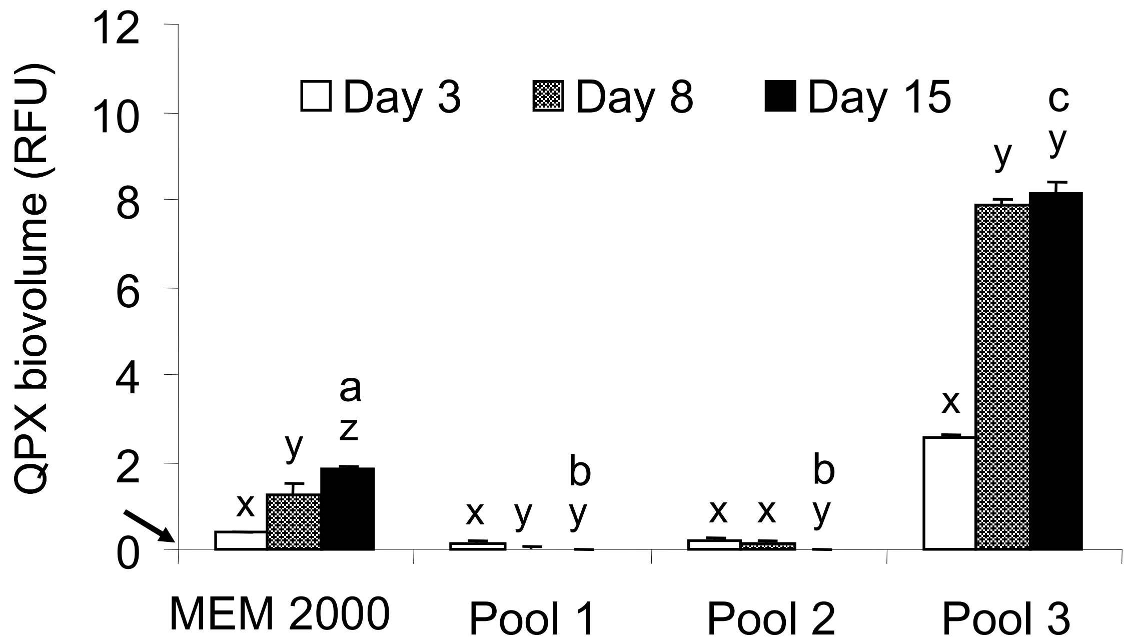

Fig. 3.3. QPX biovolume (relative fluorescent units, Mean ± SEM, n = 3 replicates) in extracts made with three pools of siphon tissues. Extracts and MEM control (MEM 2000) were normalized to 2000µg protein.ml^{-1} with FASW. The arrow on the Y axis represents QPX biovolume added to each culture at t_0. Letters indicate which experimental treatments shared statistically equivalent (same letters) or different (different letters) QPX biovolume means among cultures in different experimental media at Day 15 (a, b, c), or among measurements taken at different times within each culture (x, y, z).

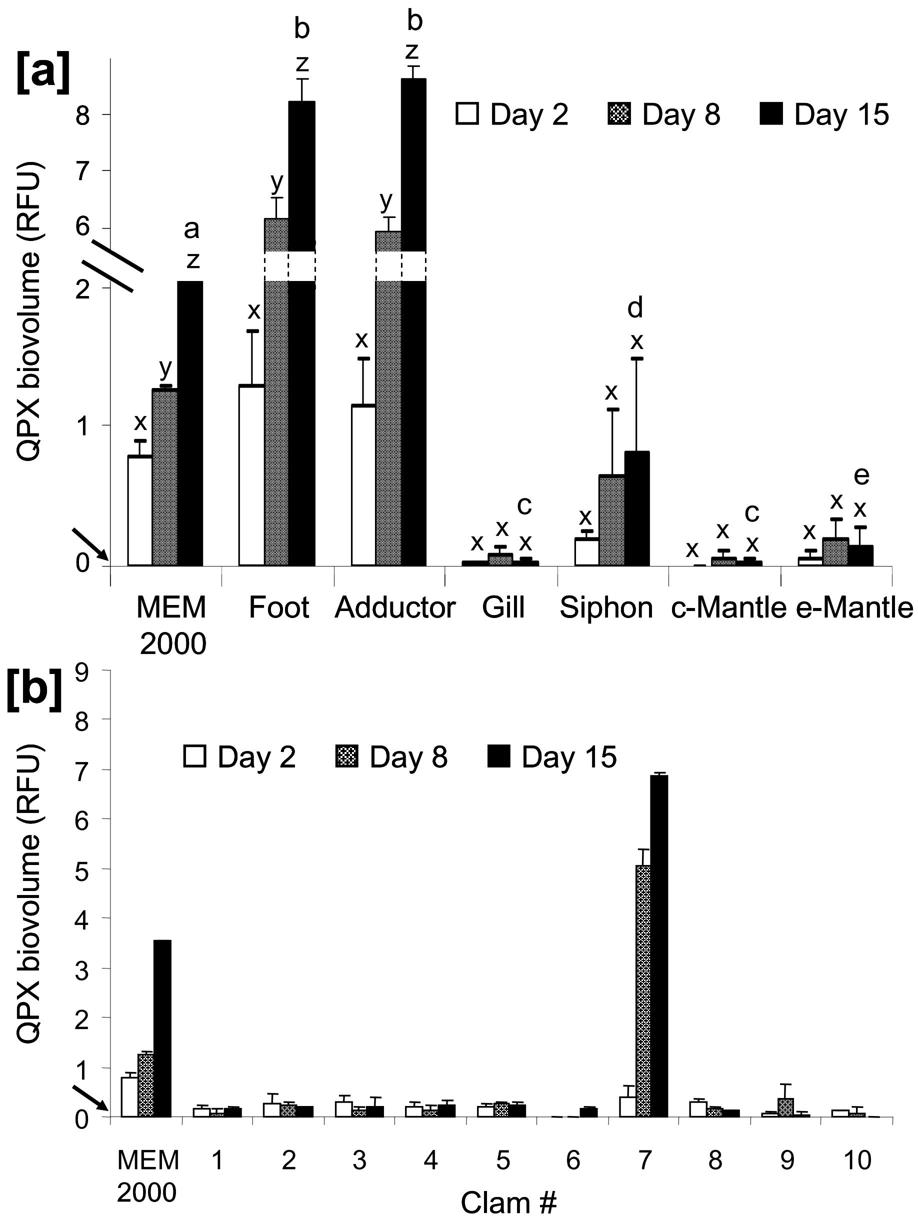

Fig. 3.4. [a] QPX biovolume (relative fluorescent units, Mean ± SEM, n=10 clams) in cultures made with different tissue extracts. c-Mantle and e-Mantle designate the central part and the edge of the mantle, respectively. Tissue extracts and MEM control (MEM 2000) were normalized at 2000 µg protein.ml^{-1} with FASW. [b] QPX biovolume in cultures made with siphon tissue homogenates (same data as in [a] shown individually). The arrows on the Y axis represent QPX biovolume added to each culture at t_0. Letters indicate which experimental treatments shared statistically equivalent (same letters) or different (different letters) QPX biovolume means among cultures in different experimental media at Day 15 (a, b, c, d, e), or among measurements taken at different times within each culture (x, y, z).

3.3. Dose effect of tissue homogenates

Incubation of QPX cells in serially-diluted muscle homogenates showed a clear dose effect of clam extracts at Day 14 (Fig. 3.5a and b). For instance, QPX growth was maximal at 1000 $\mu g.ml^{-1}$ of protein using muscle homogenates for both MEM-supplemented and unsupplemented assays (ANOVA, $p = 0.001$). QPX growth was relatively limited when diluted homogenates (100 and 500 μg $protein.ml^{-1}$) were used as sole source of nutrients since parasite biovolumes in these preparations peaked at Day 7 before decreasing at Day 14 (Fig. 3.5a). The decrease at Day 14 disappeared when muscle homogenates were supplemented with MEM (Fig. 3.5b). Significant decrease in parasite proliferation at Day 14 was observed at high protein concentrations (2000 and 2500 $\mu g.ml^{-1}$) for MEM-supplemented and unsupplemented cultures when compared with values obtained at 1000 μg $protein.ml^{-1}$ of muscle homogenates (ANOVA, $p = 0.016$).

Inhibitory effects of gill homogenates on QPX growth were also dose-dependent (Fig. 3.6). Concentrations of gill homogenates as low as 100 μg $protein.ml^{-1}$ were inhibitory for QPX growth when added to MEM (ANOVA, $p = 0.001$), but this inhibition was only temporary when compared to parasite growth in MEM controls (measured 4 and 7 days after incubation). After 14 days, significant increase in parasite biovolume, compared to the control, was observed at protein concentrations of 100 $\mu g.ml^{-1}$ of gill homogenates (ANOVA, $p = 0.001$). QPX inhibition was total at 1000 $\mu g.ml^{-1}$ and 2500 μg $protein.ml^{-1}$ over the entire length of the experiment. Dose-dependent inhibitory effects of mantle homogenates (data not shown) were similar to those obtained from gill tissue homogenates. No QPX growth was observed when gill and mantle homogenates were used without supplementation with MEM (data not shown), including for QPX cells incubated in diluted gill and mantle homogenates with protein concentrations as low as 100 $\mu g.ml^{-1}$.

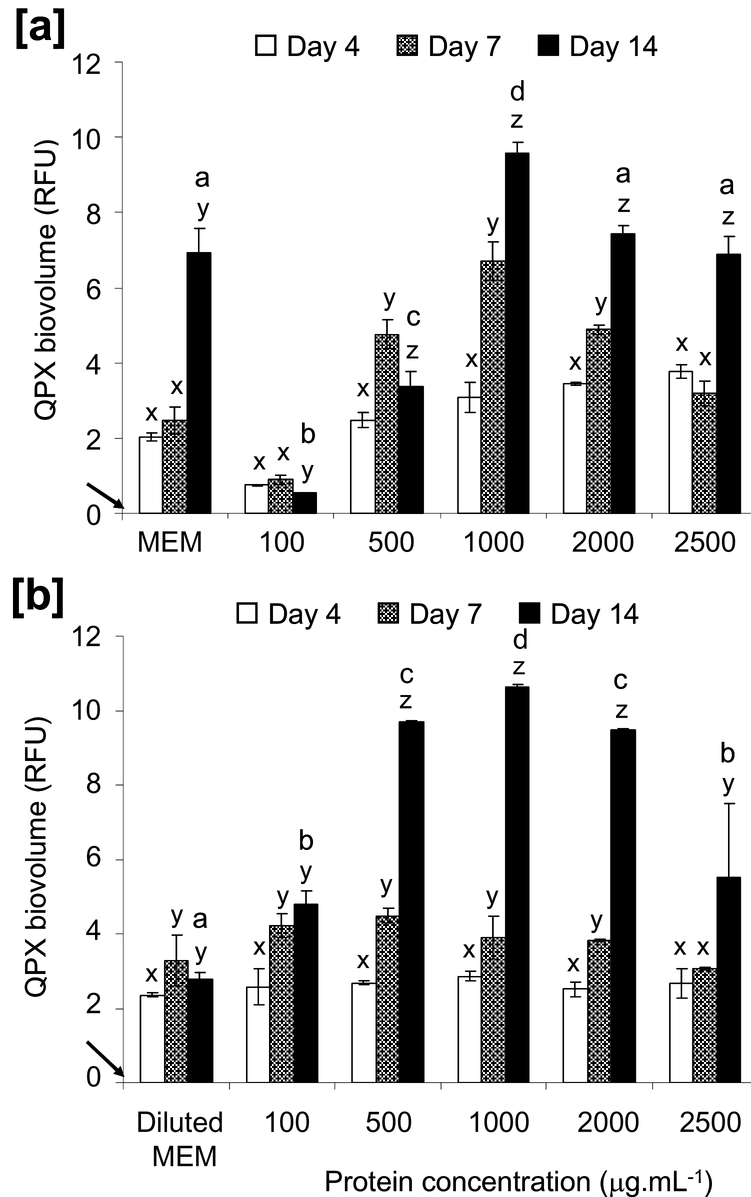

Fig. 3.5. [a] QPX biovolume (relative fluorescent units, Mean ± SEM, n=2 replicates) in cultures made with different dilutions of adductor muscle extracts. Control cultures were grown in undiluted MEM. In [b], muscle extracts were supplemented with MEM (vol/vol) before the initiation of the cultures and FASW was substituted for muscle extracts in control (Diluted MEM). Values on the X axis indicate the final concentration of proteins from muscle extracts in each condition. The arrows on the Y axis represent QPX biovolume added to each culture at t_0. Letters indicate which experimental treatments shared statistically equivalent (same letters) or different (different letters) QPX biovolume means among cultures in different experimental media at Day 14 (a, b, c, d), or among measurements taken at different times within each culture (x, y, z).

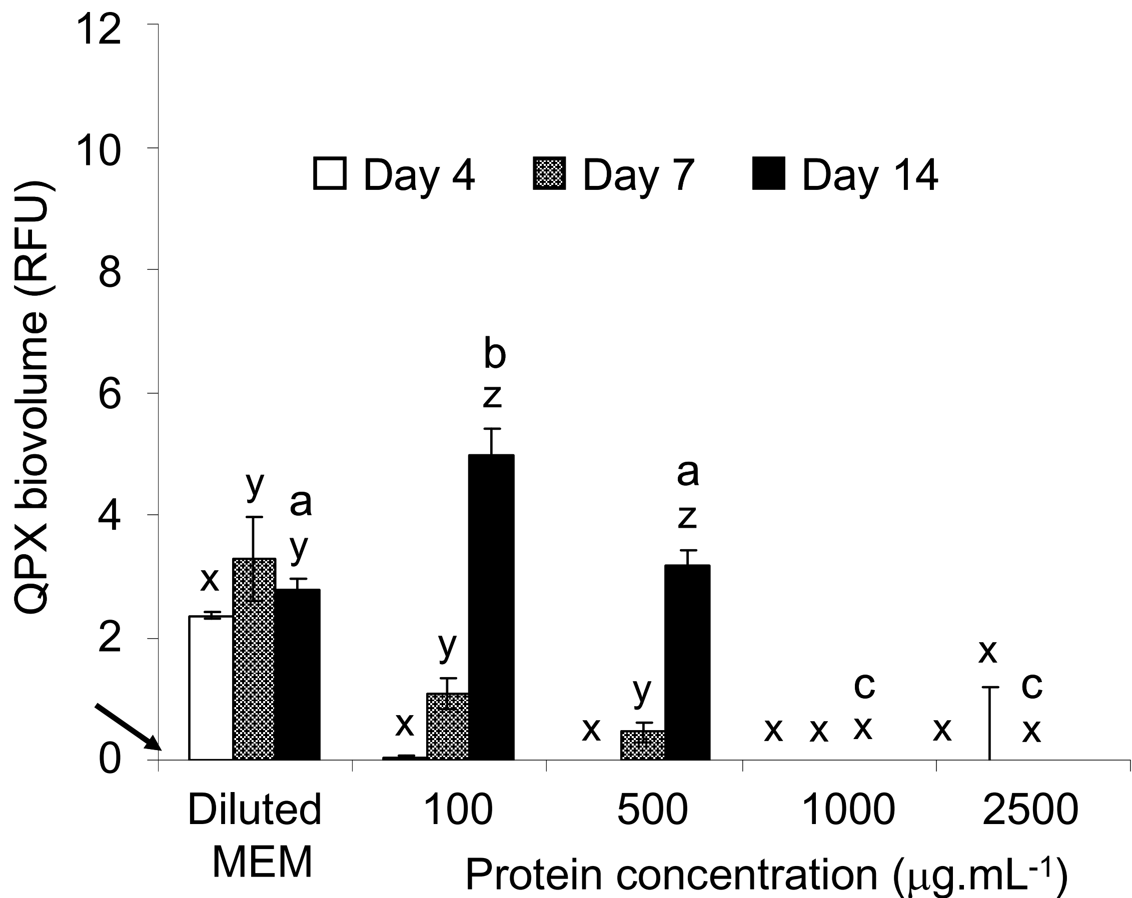

Fig. 3.6. QPX biovolume (relative fluorescent units, Mean ± SEM, n=2 replicates) in cultures made with different dilutions of gill extracts supplemented with MEM (vol:vol). Tissue extracts were substituted with FASW in control (Diluted MEM). Values on the X axis indicate the final concentration of proteins from gill extracts in each condition. The arrow on the Y axis represents QPX biovolume added to each culture at t_0. Letters indicate which experimental treatments shared statistically equivalent (same letters) or different (different letters) QPX biovolume means among cultures in different experimental media at Day 14 (a, b, c), or among measurements taken at different times within each culture (x, y, z).

4. Discussion

In bivalves, plasma is known to contain a wide array of anti-microbial compounds (Anderson and Beaven, 2001a; Bachère et al., 2004; Hubert et al., 1996; Tincu and Taylor, 2004). Anderson et al. (2003a) demonstrated the presence of inhibitory compounds against QPX in the plasma of *M. mercenaria* originating from Virginia. In the same way, this study demonstrated a strong inhibition of QPX

growth by plasma of *M. mercenaria* from New York and Florida. A reduction of up to 92 % of the parasite biovolume, compared to the controls, was observed in cultures made in MEM supplemented with plasma. Moreover, results showed that plasma from NY clams inhibited QPX growth more effectively than plasma from FL clams. These *in vitro* results coincide with results of disease transmission studies provided by Dahl et al. (2008), which showed significantly higher resistance toward QPX in NY clams than in FL clams (same broodstock as those used in this study). Similar results showing higher resistance toward QPX disease in northern strains of clams as compared to southern strains have also been described in previous studies (Ford et al., 2002b; Ragone Calvo et al., 2003; Ragone Calvo et al., 2007), and are thought to be an outcome of the selection process resulting from clam mortalities due to prior exposures to QPX among northern stocks.

Protein concentrations were also significantly higher in plasma from NY clams compared to FL clams. However, there was no correlation between protein levels and inhibitory activity of plasma on QPX growth and higher inhibition levels were also detected when normalized plasma samples were used (Fig. 3.1). Differences in the diversity of plasma proteins, as well as the involvement of non-protein compounds, could explain differences in QPX inhibition of plasma between NY and FL clams. Overall, it is unclear whether or not resistance of clams to QPX disease *in vivo* is linked to the inhibitory activity of plasma on QPX growth *in vitro*, but it is intriguing that both were higher in NY clams. Interestingly, further comparison of QPX inhibitory factors in plasma from individual clams demonstrated also a strong variability within each clam population. Some clam plasma inhibited parasite's growth whereas others had no effect or even stimulated QPX growth compared to the controls. Specific effects of active compounds in the plasma on QPX remain unknown. In some cases, QPX biovolume was below levels inoculated at Day 0, suggesting killing activities of plasma compounds whereas in other cases, only static effects were observed.

The present study also demonstrated the modulatory effects of clam tissue extracts on QPX growth *in vitro*. Our approach based on tissue homogenates could be compared to the microenvironment created by QPX activity within host tissues. For instance, QPX is known to disrupt host tissues and to lyse their cells, releasing cell

contents, as demonstrated by histological observations of infected clam tissues (Dove et al., 2004; Smolowitz et al., 1998). Our *in vitro* results showed that gill and mantle homogenates significantly inhibited QPX growth, while homogenates made with foot and adductor muscle tissues enhanced parasite growth compared to the standard culture medium (MEM).

Prior investigations also demonstrated that extracts of oyster adductor muscle are suitable for the proliferation and the culture of other members of the phylum Labyrinthulomycota (Perkins, 1973b). Except for siphon homogenates, individual variability of QPX modulating compounds at the tested protein concentrations was lower in tissue homogenates as compared to plasma. Despite the relatively low number of clams tested in each assay, results were consistent among assays using different clams for muscle, mantle and gill tissue homogenates.

Our results with adductor muscle extracts showed a clear difference in growth between samples incubated in very low (100 $\mu g.ml^{-1}$ for example) and high (2000 and 2500 $\mu g.ml^{-1}$) protein concentrations (Fig. 3.5a). In both cases, QPX biovolume was below maximal values measured in preparations made with 1000 $\mu g.ml^{-1}$ protein but temporal evolution of QPX biovolume demonstrated trophic exhaustion of the medium after 7 days at very low protein levels. The inhibitory effects of muscle homogenates above 1000 μg protein.ml^{-1} demonstrated the presence of compounds limiting QPX growth, but at much lower levels than gill and mantle tissue extracts.

Overall, these results suggest the existence of a balance between enhancing and inhibitory factors in tissue extracts. In the muscle homogenates, increasing protein concentration could raise the concentration of inhibitory factors, leading to a decrease in QPX growth at protein concentrations exceeding 1000 μg protein.ml^{-1}. Anderson et al. (2003a) noticed that low to moderate concentrations of proteins from *M. mercenaria* plasma also enhanced QPX growth. The existence of both types (enhancing and inhibitory) of factors in clam tissues could also explain the evolution of QPX growth in gill (Fig. 3.6) and mantle (not shown but similar to gills) homogenates. Homogenates from these tissues possess an effective activity against QPX growth at low protein concentration but after 7 days, these factors are no longer active at low protein concentrations. Such loss of activity may result from degradation of inhibitory factors due to parasite's activity and the production of

extracellular proteases (Anderson et al., 2006), but also possibly as a result of spontaneous degradation of inhibitory factors in the homogenates under the effect of proteolytic enzymes released from disrupted clam cells during tissue homogenization.

Clams naturally infected with QPX typically display parasite presence in pallial organs (gills, mantle and siphon), and very rarely in foot or adductor muscle tissues (Dove et al., 2004; Ragone Calvo et al., 1998; Smolowitz et al., 1998). These observations appear to contradict our *in vitro* results showing enhanced parasite growth in muscle and foot extracts. This apparent contradiction can find an explanation in the fact that in the natural environment, the clam tissues most exposed to this waterborne pathogen are gills, mantle and siphon tissues, which are hypothesized to represent the parasite's portal of entry to its host (Lyons et al., 2005). From a host defense standpoint, pallial organs are thus more likely than the adductor muscle to contain defense factors aimed at limiting establishment of the pathogen. It is noteworthy that QPX is considered as an opportunistic pathogen and its prevalence in clams is typically low. It is possible that such a low prevalence is partially due to clam defenses at the parasite's portal of entry, and it is indeed common to identify dead/dying QPX cells in mantle and gill tissues (Smolowitz et al., 1998, Allam, unpublished).

Interestingly, QPX inhibitory activity was not homogeneous in tissue extracts made of different parts of the mantle. The central part of the mantle exhibited the most inhibitory activity, followed by the edge of the mantle and finally siphon tissue homogenates. This is likely the result of differences in tissue composition among these different parts, with the siphon being mostly composed of muscular tissues. These findings are particularly relevant since in naturally-infected clams, QPX lesions are most common in siphon (Smolowitz et al., 1998) and more rarely found in the central part of the mantle or in the gill, leading some scientists to speculate that siphon specifically represents the most important portal of entry for QPX into its host (Lyons et al., 2005). Thus, despite limitations related to the fact that our *in vitro* experiments may not reflect the parasite's behavior *in vivo*, we can speculate that QPX establishment in siphon and the edge of mantle may be the result of lower inhibitory activity in these tissues and not necessarily specific acquisition of the parasite in these areas, as compared to other parts of the pallial organs (central part of the mantle and

gills). The large variability of inhibitory factors in homogenates of siphon tissues (Fig. 3.3 and 3.4) supports our speculation and suggests that some clams appear to be deficient in these factors, allowing parasite's proliferation. These deficiencies might be linked to the sensitivity of some clams to the infection *in vivo*, where a relatively small fraction of clams is typically affected in enzootic areas.

This preliminary research, however, does not provide information about the nature of the inhibitory effects of plasma and tissue homogenates. A variety of antimicrobial factors, including chlorinated acetylenes (Walker and Faulkner, 1981), terpenes (Ireland and Faulkner, 1978), indole derivatives (Benkendorff et al., 2001), glycerol derivatives (Gustafson and Andersen, 1985) and glycoproteins (Yamazaki, 1993) have been isolated from mollusks. Several papers identified specific proteases and protease inhibitors in the oyster *C. virginica* that are active against its pathogen *P. marinus* (Romestand et al., 2002; Xue et al., 2006). Moreover, our actual knowledge about QPX virulence is limited and its response to the presence of different clam extracts is unknown, making extrapolation of *in vitro* results to *in vivo* observations more difficult. Other studies have demonstrated the modulation of *P. marinus* virulence and extracellular products in the presence of oyster tissue extracts (Brown et al., 2005; Earnhart et al., 2004) and similar changes may be expected to occur in the case of QPX.

In conclusion, our *in vitro* experiments demonstrated the presence of tissue-associated factors that modulate QPX growth. QPX growth was significantly enhanced by extracts made with adductor muscle or foot tissues, but was inhibited by extracts made with mantle and gill tissues. Enhancing and inhibitory factors also appeared to be present in plasma, and displayed important inter-individual variability. Differences were also noticed in homogenates from specific parts of the mantle and corroborated well with previously reported *in vivo* observations of QPX lesions in those parts. Large variability in inhibitory activity was also noticed in siphon extracts among different clams. Clams that are deficient in inhibitory factors may become more easily infected by the parasite and thus more readily develop QPX disease. Such susceptible individuals may act to initiate an epizootic in a given area. Further research is necessary to identify and characterize QPX inhibitory factors. Specifically, biological bases of the variability in inhibitory activity in tissues among

different clams should be targeted and potential links between *in vitro* inhibitory activity of plasma and/or tissue extracts and clam susceptibility *in vivo* should be assessed.

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Part III

Chapter 2

Identification and expression of differentially regulated genes in the hard clam, *Mercenaria mercenaria*, in response to Quahog Parasite Unknown (QPX)

Adapted from **Perrigault M.**, Tanguy A., Allam B. 2009. Identification and expression of differentially expressed genes of the hard clam, *Mercenaria mercenaria*, in response to Quahog Parasite Unknown (QPX). **BMC Genomics**, 10 : 377.

Abstract

The hard clam, *Mercenaria mercenaria*, has been affected by severe mortality episodes associated with the protistan parasite QPX (Quahog Parasite Unknown) for several years. Despite the commercial importance of hard clams in the United States, molecular bases of defense mechanisms in *M. mercenaria*, especially during QPX infection, remain unknown.

Our study used suppression subtractive hybridization (SSH), as well as the construction of cDNA libraries from hemocytes to identify genes related to the defense of the hard clam against its parasite. Hard clams were experimentally infected with QPX and SSH was performed on mRNA samples extracted from mantle and gill tissues at different times post-challenge. A total of 298 clones from SSH libraries and 1352 clones from cDNA libraries were sequenced. Among these sequences, homologies with genes involved in different physiological processes related to signal transduction, stress response, immunity and protein synthesis were identified. Quantitative PCR revealed significant changes in the expression of several of these genes in response to QPX challenge and demonstrated significant correlations in terms of levels of gene expression between intermediates of signalling pathways and humoral defense factors, such as big defensin and lysozyme.

Results of this study allowed the detection of modifications caused by QPX at the transcriptional level providing insight into clam immune response to the infection. These investigations permitted the identification of candidate genes and pathways for further analyses of biological bases of clam resistance to QPX allowing for a better understanding of bivalve immunity in general.

1. **Introduction**

The hard clam, *Mercenaria mercenaria,* is exploited along the eastern coast of North America, from Maritime Canada to the Gulf of Mexico. This species is among the most commercially important bivalves in the United States and is well suited for aquaculture as it is characterized by relatively fast growth. The hard clam is a sturdy bivalve and the only infectious agent that causes severe mortality episodes among wild and cultured clams is the protistan parasite QPX (Quahog Parasite Unknown) (Ford, 2001). QPX is a unicellular protist member of the family Thraustochytriidae (Stokes et al., 2002). Despite the ubiquitous nature of this family in marine environments, thraustochytrids were poorly studied and only few pathogens were described in this group (Bower, 1987b). QPX reported in 1989 at Prince Edward Island was linked to almost 100 % of the mortality among cultured clams (Whyte et al., 1994). It was subsequently identified in other locations further south: Massachusetts in 1995 (Smolowitz et al., 1998), Virginia in 1996 (Ragone Calvo et al., 1998), New Jersey (Ford et al., 2002b) and New York (Dove et al., 2004) in 2002, but the parasite has never been detected further south than Virginia. Recent lab-controlled experiments (Dahl et al., 2008) and *in situ* investigations (Ford et al., 2002b; Ragone Calvo et al., 2007) demonstrated variability of susceptibility among hard clam populations, with clams from southern broodstocks being more susceptible to QPX disease than northern broodstocks, suggesting a genetic origin of clam resistance. Field investigations also showed variability in the resistance toward QPX among different local (New York State) clam broodstocks (Dahl et al., 2008). Differences of susceptibility to pathogen infection between different populations were previously observed in other bivalves (Bushek and Allen, 1996a; Culloty et al., 2004). Intra-specific genetic variation in disease susceptibility to *Perkinsus marinus* was indirectly demonstrated by the evolution of resistance in disease-challenged natural populations of oysters (Gaffney and Bushek, 1996).

Like other invertebrates, bivalves lack adaptive immunity and instead rely on various innate defenses against invading pathogens. In hard clams, hemocytes constitute the primary line of defense against materials recognized as non-self (Cheng et al., 1981). The presence of non-self materials in tissues initiates a complex molecular signalling cascade to stimulate cell-mediated immune responses, mainly

involving phagocytosis or encapsulation of foreign materials, and the production of reactive oxygen species (ROS) (Anderson, 1994; Pipe, 1992). Humoral factors, such as defensins, also play an important role because they possess various anti-microbial properties (Cheng, 1992; Chu, 1988). Enzymes, such as peptidases and lysozyme, are particularly crucial because of their ability to hydrolyze protein components of invading microorganisms (Allam et al., 2000a; Allam et al., 2000b; Paillard et al., 2004a). Since bivalves have an open circulatory system, antimicrobial constituents associated with plasma and hemocytes are present in virtually all tissues. Histological observation of naturally- and experimentally- QPX-infected clams demonstrated that some individuals are sometimes able to mount a defense reaction characterized by an intense inflammatory response, phagocytosis (rare) and encapsulation of parasite cells. The presence of dead and necrotic QPX cells was reported in some instances, suggesting that clam's humoral and/or cellular response was sufficient to lead to the healing of infected individuals (Dahl and Allam, 2007; Dove et al., 2004; Ragone Calvo et al., 1998). Histological observations of infected clam tissues and *in vitro* cultures also revealed an abundant production of a mucofilamentous net by QPX. These secretions are suspected to represent virulence factors that protect the parasite from host defense mechanisms (Smolowitz et al., 1998). *In vitro* investigations demonstrated that the mucus layer protects QPX from humoral defense factors in clam plasma and therefore, could be important to the establishment, as well as the development, of the disease (Anderson et al., 2003a). However, clam immune response to QPX cells and/or mucus has never been investigated.

In bivalves, prior studies focusing on the identification of immune-related genes were performed in oysters (Gueguen et al., 2003a; Jenny et al., 2006; Montagnani et al., 2008; Tanguy et al., 2004; Yamaura et al., 2008), mussels (Mitta et al., 1999a) and scallops (Qiu et al., 2007a; Qiu et al., 2007b; Yu et al., 2007; Zhao et al., 2007; Zhu et al., 2007). Molecular bases of defense mechanisms in hard clams, especially during QPX infection, are unknown. The only investigations that have studied molecular aspects of clam immunity were performed in the genus *Tapes* or *Ruditapes*; (Gestal et al., 2007; Kang et al., 2006; Prado-Alvarez et al., 2009a), a relatively distant member of the family Veneridae. Identification of immune-related genes involved in the response of *M. mercenaria* to QPX infection could lead to the development of tools that will contribute to the selection of resistant populations of

clams and develop knowledge about clam immunity by the generation of a nucleic database for the species.

This study aimed for a better characterization of clam's response to QPX infection by investigating differentially expressed genes following parasite challenge. Our study is the first to apply transcriptomic approaches in *M. mercenaria*. Suppression subtractive hybridization (SSH), as well as the construction of cDNA libraries of expressed genes were associated with quantitative PCR to identify genes related to the defense of hard clam against its parasite.

2. Materials and methods

2.1. QPX cultures

QPX strain NY0313808BC7 was isolated from nodules of infected New York clams (Qian et al., 2007) and subcultured in a culture medium based on muscle homogenates from hard clams adjusted to 1000 $\mu g.ml^{-1}$ of protein in filter-sterilized artificial seawater (FASW) (Part III – chapter 1). QPX cultures were initiated in 25-cm^2 flasks incubated at 23 °C for 1 week to reach the exponential phase of growth. Parasite cultures were thereafter subdivided into two aliquots: one aliquot was untreated resulting in QPX cells associated with their typical abundant mucus secretions surrounding parasite cells (cells and mucus – u-QPX) and another aliquot was washed according to a protocol adapted from Anderson et al. (2003a) to remove the mucus from cells (w-QPX). Briefly, a volume of QPX culture was mixed well by repeatedly drawing up and expelling the culture with a 3 ml-syringe without a needle. A small volume of well-mixed culture was then suspended in five times its volume of sterile culture medium. This suspension was then vortexed for 10-15 seconds and centrifuged for 15 min at 600 x g (Anderson et al., 2003a). The supernatant was removed and the QPX pellet was then washed two times and resuspended in sterile culture medium. This washing procedure has been thoroughly tested and found not to affect QPX viability (Buggé and Allam, 2005). QPX biovolume in each aliquot was then measured using a semi-automated fluorometric technique according to Buggé and Allam (2005) and QPX suspensions were adjusted with sterile culture medium to obtain the same parasite biovolume.

2.2. *Hard clams and experimental infections*

QPX-free adult *Mercenaria mercenaria* were obtained from Frank M. Flowers Oyster Company (Oyster Bay, NY). Clams were acclimated one week in the laboratory, held in 150-L tanks with re-circulating water (28-30 ppt) at 21 ± 1 °C and fed daily with commercial algae (DT's Live Phytoplankton, Sycamore, IL). After acclimation, clams were divided into three groups of 30 individuals and challenged with either washed (w-QPX) or unwashed (u-QPX) parasite cells to compare clam response in presence or absence of the mucus layer surrounding QPX cells. Experimental infections were performed according to Dahl and Allam (2007) by injecting 100 µl of culture medium containing 5 x 10^4 QPX cells into clam's pericardial cavity. Control clams were injected with 100 µl of sterile culture medium. Following injection, clams were maintained out of the water for 1.5 h and were thereafter transferred to separate tanks. Mortality was monitored daily. For each experimental condition, 8 clams were sampled at 14, 28 and 48 days after challenge. Hemolymph was withdrawn from the adductor muscle and held individually on ice. Samples were centrifuged at 700 x g for 10 min at 4 °C, plasma was discarded and hemocyte pellets were rapidly frozen in liquid nitrogen before storage at -80 °C. Concomitantly, gill and mantle tissues were dissected and frozen individually until RNA extraction.

2.3. *RNA extraction*

Total RNA was extracted from hemocyte pellets and clam tissues using TRI® Reagent (Invitrogen, Carlsbad, CA, USA). Polyadenylated RNA was isolated using the PolyATtract®mRNA Isolation System (Promega, Madison, WI, USA) according to manufacturer's instructions. Messenger RNAs were resuspended in RNase-free water and both quantity and quality were assessed by spectrophotometry (OD260, OD280).

2.4. *Suppression subtractive hybridization*

The suppression subtractive hybridization technique (SSH) (Diatchenko et al., 1996) was used to identify genes involved in clam's immune response following QPX challenge. Messenger RNAs isolated from gill and mantle tissues at 14 and 48 days were pooled for each treatment and sampling time. Both forward and reverse subtracted libraries were generated on 2 µg of pooled mRNA for each SSH library construction (Fig. 4.1). First and second strand cDNA synthesis, *Rsa*I endonuclease enzyme digestion, adapter ligation, hybridization, and PCR amplification were performed as described in the PCR-select cDNA subtraction manual (Clontech, Palo Alto, CA, USA). Differentially expressed PCR products were purified and cloned into pGEM-T vector (Promega, Madison, WI, USA). Bacteria (DH5α phage resistant) were transformed and cultured in Luria-Bertani medium (with 100 $\mu g.l^{-1}$ ampicillin, final concentration). Vectors from two hundred colonies per library were extracted using an alkaline lysis plasmid minipreparation, and screened by size after digestion. A total of 298 clones from forward and reverse libraries were sequenced using an AB3100 sequencer (Perkins-Elmer) and Big Dye Terminator V3.1 Kit (Perkins-Elmer).

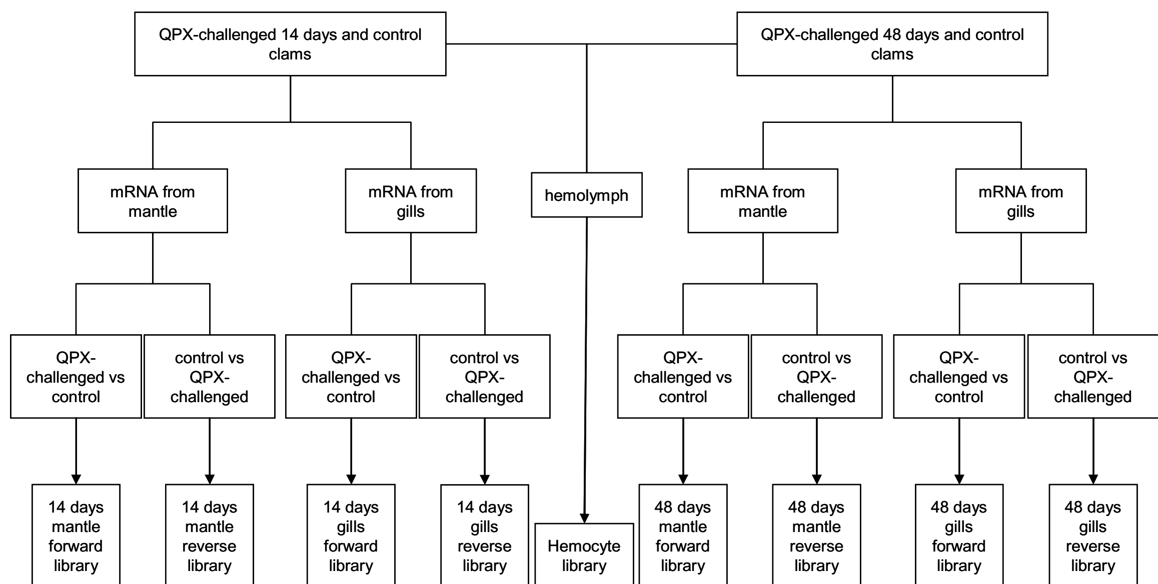


Fig. 4.1. Diagram of the different subtractions and cDNA libraries performed in *M. mercenaria*. Clams challenged with washed and unwashed QPX cells were pooled to perform SSH. The hemocyte library was generated from a pool of all challenged and unchallenged clams collected at 14 and 48 days.

2.5. *Full-length cDNA library construction*

Messenger RNAs isolated from hemocytes were pooled and cDNA library was generated using Creator^{TM} SMART^{TM} cDNA Library Construction Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. The cDNA library was cloned into the pDNR-LIB Vector and transformed in bacteria (DH5α phage resistant) and cultured in Luria-Bertani medium (with 100 $\mu g.l^{-1}$ ampicillin, final concentration). A total of 1352 clones were randomly selected and sequenced using an AB3100 sequencer (Perkins-Elmer) and Big Dye Terminator V3.1 Kit (Perkins-Elmer).

2.6. *Sequence analysis*

The ABI sequence data were basecalled using 'phred' (www.phrap.org) and subsequently clipped for bad-quality and vector using 'lucy' (compbio.dfci.harvard.edu/tgi/software) with standard parameters. cDNA sequences were automatically screened against UniProt (BLASTX) and all ESTs from EMBL (BLASTN).

2.7. *Real-Time PCR Analyses*

Fourteen genes were selected from SSH libraries for further investigations of their expression including 3 stress-related genes (metallothionein, HSP 70 and ferritin), 3 defense-related genes (big defensin, hemocyte defensin and lysozyme) and 2 genes involved in cell signalling (receptor of activated kinase C1, peroxisome proliferator-activated receptor). In addition, several transcripts involved in gene regulation, transcription factors, the cytoskeleton and metabolism were analyzed (elongation factor 1 beta, transcription factor AP-1, actin, NADH4). Two transcripts (senescence-associated protein, cytochrome P450 like TBP) previously identified in other bivalves during parasite challenges (Tanguy et al., 2004) and in our libraries were also selected. Four additional transcripts encoding stress related genes (stress-induced protein – STI1) and components of the NF-kB pathway (tumor necrosis

factor receptor-associated factor – TRAF-6, Toll like receptor - TLR) and complement system (C1q-TNF related protein) were selected from the hemocyte library. Expression of all candidates was compared to controls in gill and mantle tissues at 14, 28 and 48 days post-challenge. For each sample, 10 µg total RNA was individually submitted to reverse transcription using the oligo dT anchor primer (5'-GACCACGCGTATCGATGTCGACT$_{(16)}$V-3') and Moloney murine leukaemia virus (M-MLV) reverse transcriptase (Promega, Madison, WI, USA). The real-time PCR assay was performed with 3µl cDNA (1/20 dilution) in a total volume of 10 µl, using a Chromo 4^{TM} System Q-PCR (Bio-Rad, Hercules, CA USA). Concentrations of the reaction components were as follows: 1X Absolute QPCR SYBR Green ROX Mix (ABgene, UK) and 70 nM of each primer. Primer sequences of the 18 genes selected in the *M. mercenaria* SSH and hemocyte libraries are presented in Table 4.1. Reactions were realized with activation of Thermo-Start® DNA polymerase at 95 °C for 15 min followed by amplification of the target cDNA (50 cycles of denaturation at 95 °C for 30 sec, annealing and extension at 60 °C for 1 min) and a melting curve program from 95 to 70 °C that decreased the temperature by 0.5 °C every 10 sec. Readings were taken at 60 °C. PCR efficiency (E) was determined for each primer pair by determining the slope of standard curves obtained from serial dilution analysis of cDNA from different experimental samples (treatment and control). The comparative CT method (2-ΔΔCT method) was used to determine the expression level of analyzed genes (Livak and Schmittgen, 2001). The expression of the candidate genes was normalized using ribosomal RNA 18S fragment as a housekeeping gene by the specific primers (Table 4.1). Fold units were calculated on normalized expression values by dividing gene expression in tissues from challenged clams by controls. Results are given as the mean and standard deviation of eight replicates per condition.

Table 4.1. Combinations of primers used in quantitative PCR assays.

Gene name	Gene function	Primer sequences
18S	Ribosomal protein	F : CTGGTTAATTCCGATAACGAACGAGACTCTA R : TGCTCAATCTCGTGTGGCTAAACGCCACTTG
Hemocyte defensin	Immune system	F : ACAAATGTAACAGGCATTGTAGGAGCAT R : CATGTGCATCTTCGGTAAAAAGTCCA
Big defensin	Immune system	F : ATGGACACTAGGAAAGTCTACTGTGTGCT R : ACAAGTGCAACCCAGACCCAAGGTGA
Lysozyme	Immune system	F: ATAACGAAAGACCAAGCTCGTGCTCT R: GTTTTGGGTCCTAGATCTCCCCTGTA
C1q - TNF related protein	Immune system	F: ATGCAAGTCAGTGCCGTGATACACCCAGA R: AATAAAGCGCCACTGAAAGTTGTTCCATG
TRAF-6	Immune system	F: GAACTAGCAAACAGGAATTGGGAGGCGCT R:GTCAAGTGATGGCTCATCTTGGATGCTGC
Toll-like receptor	Immune system	F: GTAACAAATTTCACTCTGGCCGCTGACGC R: TAGCTGAAATCCAACGACTGCACCCGTAA
RACK-1	Cell communication	F: CCTAACAGATACTGGCTGTGTGCTGC R: GTCTGTCCATCTGCGGACCATGCAAG
Peroxisome proliferator-activated receptor	Cell communication	F: CATAGCCAATTCCATACCCCTGGCCA R: AGTTGGCATCGCCACTGTCGCTGCTC
HSP70	Stress response	F: AATGACAAAGGCCGTCTCAGCAAGGA R: TCTAACCAACTGATGACCTCGCTACA
Stress-induced protein STI	Stress response	F: GAAGCTGTTGAACAAGCCAAGAGTGGAGC R: GTCTCTTGAATTCGGGGATCTTGAGCTGC
Ferritin	Iron transport	F: ATGTCTGTTTCACGACCTCGACAGAA R: AGTTTCTCGGCATGCTCACGTTCCTC
Metallothionein	Detoxification	F : ACCAGTGATGGTGGCTGCAGGTGTGG R : TTACACGAACAGCCACTATCACACTG
Actin	Cytoskeleton	F : ATTGTGATGGACTCTGGTGATGGTGT R : TCTCTAACAATTTCTCTCTCAGCCGTTGT
Transcription factor AP-1	Transcription	F: AGAAAACTTGAAAGAATTGCGCGACT R : TGTGACATCATTATCTGGCACCCACT
Elongation factor beta	Transcription	F : CCTTGGGATGATGAAACAGATATGGC R : CTAATCTTGGCATCTTCTATAACAGC
NADH sub-unit IV	Mitochondrial respiration	F: CCGTGGGATTTAGGGAGGGATAATATGCT R : ACTCCAGTTAACAACATTGATCCCCTCAA
Senescence associated protein	Unknown	F: AACCTGTCTCACGACGGTCTAAGCCCAGC R: TTACCACAGGGATAACTGGCTTGTGG
Cytochrome P450 like TBP	Unknown	F: GTCTGGAAAACGGCCACAAGGCACCT R: TTATACAAGGTAACCGGCTTGGACGC

2.8. Statistical analysis

Variations in gene expression levels in tissues from clams exposed to different treatments were analyzed with Student's *t*-test using SigmaStat Version 3.10 (Systat

Software, Inc). Effects of sampling times, treatments and tissues on gene expression were analyzed using multifactor analysis of variance (ANOVA) followed by a Holm-Sidak post-hoc test when appropriate. Correlation analyses of the expression of different genes were made using Pearson's method. Finally, Discriminant Analysis (DA) of gene expression was performed using Statgraphics plus Version 2.1. As DA requires a minimum within-treatment sample size of 20, data from the different sampling times within each treatment were combined and DA was separately applied on mantle and gill tissue groups. DA determines linear combinations of variables (genes) that maximize differences among *a priori* defined groups (treatments). The relative contribution of each variable was assessed on the basis of the structure correlations to interpret the discriminating power of the independent variables. In all tests, differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Identification of regulated genes in SSH libraries

The search for homology using the BLASTX program revealed a total of 25 unique sequences in gill libraries and 29 unique sequences in mantle libraries for *M. mercenaria* after 14 days of exposure, 10 unique sequences in gill libraries and 74 unique sequences in mantle libraries for *M. mercenaria* after 48 days of exposure, including sequences corresponding to known genes or unidentified ESTs. Genes regulated by QPX challenge were assigned to 7 major cellular physiological functions using the Gene Ontology (GO) Database: 1) stress response and detoxification; 2) cell communication, immune system and membrane receptors; 3) cell cycle regulation, DNA repair, protein regulation and transcription; 4) cytoskeleton production and maintenance; 5) respiratory chain; 6) metabolism; 7) ribosomal proteins; 8) unknown functions and 9) unidentified sequences. Up-regulated and down-regulated sequences in gill and mantle tissues are listed in tables 4.2 and 4.3, respectively. Sequences were submitted to NCBI with the following accession numbers: [GenBank: GO915165 - GO915272].

Table 4.2: Upregulated genes identified in mantle and gill tissues 14 and 48 days after challenge with washed and unwashed QPX cells.

Homolog proteins	Homolog species	BlastX value	Libraries	GenBank accession no
stress response and detoxification		4 % of sequences identified in libraries from mantle tissue 11 % of sequences identified in libraries from gill tissue		
Metallothionein	*Corbicula fluminea*	1e-11	Mantle (14 days)	GO915213
cytochrome P450 like TBP	*Nicotiana tabacum*	2e-07	Gills (14 days)	GO915227
71kDa heat shock protein	*Haliotis tuberculata*	6e-53	Mantle (48 days)	GO915235
ferritin subunit	*Meretrix meretrix*	3e-50	Mantle (48 days)	GO915233
cell communication, immune system and membrane receptors		7 % of sequences identified in libraries from mantle tissue		
receptor of Activated Kinase C 1	*Mya arenaria*	4e-44	Mantle (48 days)	GO915258
lysozyme (Chain)	*Enterobacteria phage T4*	3e-04	Mantle (48 days)	GO915259
big defensin	*Tachypleus tridentatus*	5e-10	Mantle (48 days)	GO915266
sialic acid binding lectin	*Helix pomatia*	1e-04	Mantle (48 days)	GO915267
C-type lectin A	*Chlamys farreri*	5e-05	Mantle (14 days)	GO915219
nicotinic acetylcholine receptor subunit type H	*Lymnaea stagnalis*	8e-14	Mantle (14 days)	GO915224
cell cycle regulation, DNA repair, protein regulation and transcription		4 % of sequences identified in libraries from mantle tissue 26 % of sequences identified in libraries from gill tissue		
CCAAT/enhancer binding protein	*Aplysia kurodai*	5e-19	Mantle (14 days)	GO915218
translation elongation factor 1-alpha	*Dreissena polymorpha*	3e-108	Mantle (14 and 48 days) and Gills (48 days)	GO915211
elongation factor 1 beta	*Plutella xylostella*	4e-21	Mantle (48 days)	GO915246
eukaryotic translation elongation factor 1 delta	*Bos taurus*	4e-12	Mantle (48 days)	GO915241
similar to H3 histone, family 3B	*Macaca mulatta*	9e-37	Mantle (48 days)	GO915263
cytoskeleton production and maintenance		4 % of sequences identified in libraries from mantle tissue 1 % of sequences identified in libraries from gill tissue		
Actin	*Chlamys farreri*	4e-67	Mantle (48 days)	GO915239
myosin (essential light chain)	*Macrocallista nimbosa*	3e-38	Mantle (48 days)	GO915247
Tropomyosin	*Balanus rostratus*	7e-15	Mantle (48 days)	GO915261
alpha tubulin	*Leishmania braziliensis*	2e-05	Mantle (48 days)	GO915252
beta-tubulin	*Halocynthia roretzi*	2e-59	Gills (14 days)	GO915225
transgelin 3	*Danio rerio*	1e-08	Mantle (48 days)	GO915232
respiratory chain		12 % of sequences identified in libraries from mantle tissue 11 % of sequences identified in libraries from gill tissue		
cytochrome oxidase subunit 1	*Ruditapes philippinarum*	2e-29	Mantle and Gills (14 and 48 days)	GO915255
cytochrome oxidase subunit 3	*Inocellia crassicornis*	3e-19	Mantle (14 and 48 days)	GO915234
NADH dehydrogenase subunit 4	*Ruditapes philippinarum*	3e-30	Mantle and Gills (48 days)	GO915245
ATP synthase subunit 6	*Ruditapes philippinarum*	3e-25	Mantle and Gills (14 days)	GO915223
Metabolism		6 % of sequences identified in libraries from gill tissue		
zinc-dependent alcohol dehydrogenase	*Lysiphlebus testaceipes*	1e-33	Gills (14 days)	GO915228
ribosomal proteins		3 % of sequences identified in libraries from mantle tissue 1 % of sequences identified in libraries from gill tissue		
ribosomal protein L17A	*Argopecten irradians*	8e-11	Mantle (48 days)	GO915262
ribosomal protein L11	*Ictalurus punctatus*	2e-08	Mantle (48 days)	GO915248
ribosomal protein S2e	*Onchocerca volvulus*	8e-25	Gills (48 days)	GO915270
ribosomal protein S3a	*Crassostrea gigas*	5e-99	Mantle (14 days)	GO915220
unknown functions		1 % of sequences identified in libraries from mantle tissue 29 % of sequences identified in libraries from gill tissue		
putative senescence-associated protein	*Pisum sativum*	3e-34	Gills (14 days)	GO915231
hypothetical protein TTHERM_02141640	*Tetrahymena thermophila SB210*	2e-36	Gills (14 days)	GO915230
hypothetical protein TTHERM_00648850	*Tetrahymena thermophila SB210*	8e-09	Mantle (48 days)	GO915260
SJCHGC09076 protein	*Schistosoma japonicum*	4e-03	Mantle (48 days)	GO915249
unknown genes		16 % of sequences identified in libraries from mantle tissue 11 % of sequences identified in libraries from gill tissue		
7 sequences			Mantle (14 days)	GO915212, GO915214-17, GO915221-22
15 sequences			Mantle (48 days)	GO915236-38, 40, 42-44, 50-51, 53-54, 56-57, 64-65
2 sequences			Gills (14 days)	GO915226, GO915229
4 sequences			Gills (48 days)	GO915268-69, GO915271-72

Table 4.3: Downregulated genes identified in mantle and gill tissues 14 and 48 days after challenge with washed and unwashed QPX cells.

Homolog proteins	Homolog species	BlastX value	Libraries	GenBank accession no
stress response and detoxification		8 % of sequences identified in libraries from mantle tissue		
HSP70	*Mytilus galloprovincialis*	1e-52	Mantle (14 days)	GO915169
71kDa heat shock protein	*Haliotis tuberculata*	6e-53	Mantle (14 days)	GO915166
cell communication, immune system and membrane receptors		5 % of sequences identified in libraries from mantle tissue		
hemocyte defensin	*Crassostrea gigas*	1e-05	Mantle (48 days)	GO915199
peroxisome proliferator-activated receptor	*Oncorhynchus keta*	7e-07	Mantle (14 and 48 days)	GO915177
thioester-containing protein	*Euphaedusa tau*	2e-08	Mantle (48 days)	GO915190
cell cycle regulation, DNA repair, protein regulation and transcription		3 % of sequences identified in libraries from mantle tissue		
transcription factor AP-1	*Strongylocentrotus purpuratus*	2e-16	Mantle (48 days)	GO915178
translation elongation factor 1-alpha	*Dreissena polymorpha*	3e-108	Mantle (14 and 48 days)	GO915167
cytoskeleton production and maintenance		2 % of sequences identified in libraries from mantle tissue		
actin	*Cyrenoida floridana*	1e-82	Mantle (48 days)	GO915201
alpha tubulin	*Theromyzon tessulatum*	5e-40	Mantle (48 days)	GO915209
alpha tubulin a1	*Mesenchytraeus solifugus*	1e-09	Mantle (48 days)	GO915208
respiratory chain		1 % of sequences identified in libraries from mantle tissue 1 % of sequences identified in libraries from gill tissue		
cytochrome b	*Ruditapes philippinarum*	8e-91	Gills (14 days)	GO915174
cytochrome c subunit I	*Ruditapes philippinarum*	6e-25	Mantle (14 days)	GO915173
Metabolism		1 % of sequences identified in libraries from mantle tissue		
ADP/ATP carrier	*Leishmania mexicana amazonensis*	2e-05	Mantle (14 days)	GO915170
ribosomal proteins		4 % of sequences identified in libraries from mantle tissue		
ribosomal protein L7	*Argopecten irradians*	8e-03	Mantle (48 days)	GO915202
ribosomal protein L19	*Crassostrea gigas*	9e-21	Mantle (48 days)	GO915205
ribosomal protein L24	*Danio rerio*	3e-12	Mantle (14 days)	GO915172
unknown functions		8 % of sequences identified in libraries from mantle tissue		
SJCHGC02792 protein	*Schistosoma japonicum*	3e-12	Mantle (14 days)	GO915171
hypothetical protein DDBDRAFT_0167791	*Dictyostelium discoideum AX4*	1e-04	Mantle (14 days)	GO915168
hypothetical protein	*Monodelphis domestica*	2e-27	Mantle (48 days)	GO915196
similar to product in *Drosophila melanogaster*	*Schistosoma japonicum*	7e-04	Mantle (48 days)	GO915187
unknown genes		17 % of sequences identified in libraries from mantle tissue 3 % of sequences identified in libraries from gill tissue		
1 sequence			Mantle (14 days)	GO915165
23 sequences			Mantle (48 days)	GO915179-86, 88-89, 91-95, 97-8, GO915200, 03-04, 06-07, 10
2 sequences			Gills (14 days)	GO915175-6

3.2. *Identification of genes from hemocyte cDNA libraries*

The sequencing of 1352 clones from the hemocyte cDNA library resulted in the characterization of a total of 487 ESTs that have been clustered according to their function using the GO Database (Fig. 4.2). Only 29 % of these ESTs present an annotation and 71 % remain unidentified. Several sequences presenting homologies with stress- and defense-related genes have been detected including Stress-Induced Protein STI [GenBank: GR209325] (BlastX value = $8e^{-21}$, *Cryptosporidium parvum* – [GenBank: XP_001388209]), Toll-Like Receptor TLR [GenBank: GR209327] (BlastX value = $2e^{-4}$, *Strongylocentrotus purpuratus* – [GenBank: XP_001201188]),

Tumor Necrosis Factor Receptor-Associated Factor TRAF-6 [GenBank: GR209326] (BlastX value = $4e^{-5}$, *Chlamys farreri* – [GenBank: ABC73694]) and C1q – TNF related protein [GenBank: GR209324] (BlastX value = $3e^{-2}$ *Danio rerio* – [GenBank: NP_001017702]).

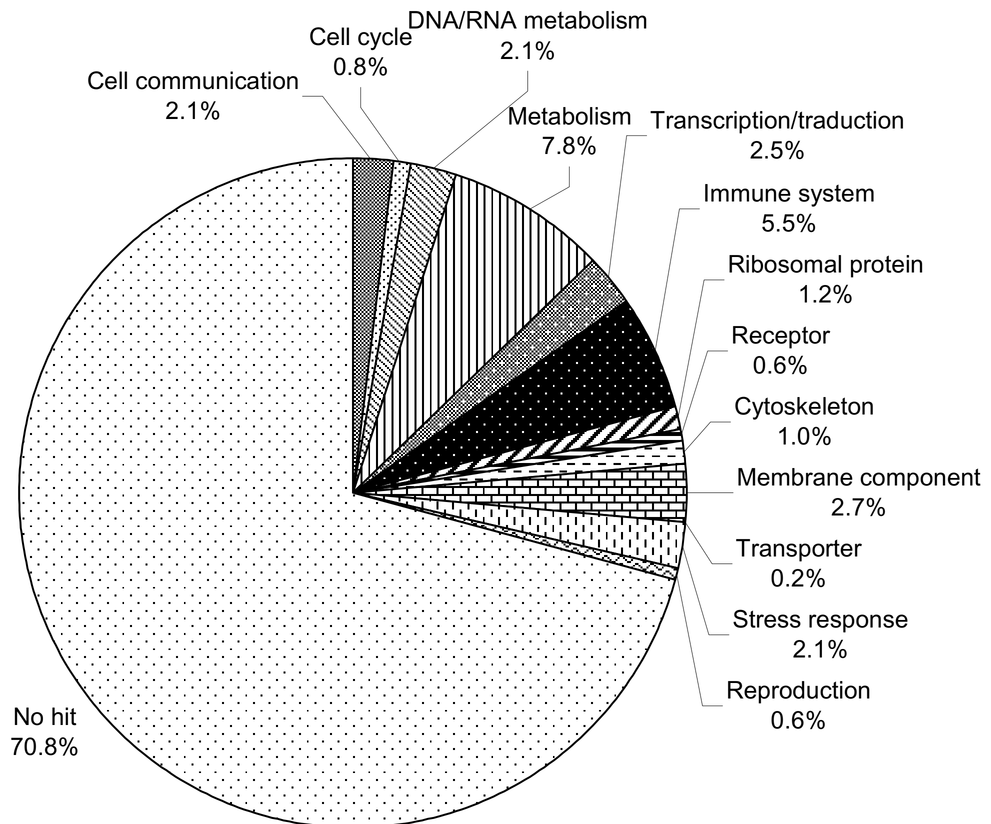


Fig. 4.2. Functional classification of the sequences identified in the hemocyte library (487 ESTs). Genes were clustered into 13 categories according to their putative biological function.

3.3. Expression patterns of differentially-regulated genes

Results showed that QPX challenge induced significant changes in the expression of several of the eighteen investigated genes. This modulation was characterized by a highly variable regulation of these genes 14 days after challenge (Fig. 4.3). At that sampling time, few transcripts were significantly regulated, particularly in w-QPX challenged clams, including hemocyte defensin, C1q, actin, HSP 70 and ferritin (Table 4.4). After 28 and 48 days, significant variations of gene expression were observed in both washed and unwashed-QPX challenged clams with a high variability according to the challenge and the tissue (Table 4.4, Fig. 4.3). Some genes, such as TRAF-6, were more specific to the challenge (washed or unwashed-QPX), whereas other genes tended to be more linked to particular tissues such as the big defensin (Fig. 4.3). In addition, some gene expressions presented continuous trends over time (decrease or increase) as TRAF-6 and ferritin, while other genes displayed strong modulation only at 28 days (TLR expression in mantle tissues, Fig. 4.3). Multifactor analysis indicated significant effects of all individual parameters (time, treatment and tissues) on actin and big defensin expression and a significant effect of combined parameters on AP-1, lysozyme and TLR expression (Table 4.5).

Table 4.4. Summary of the results of Student's t-tests of gene expression data.

	14 days				28 days				48 days			
	Mantle		Gills		Mantle		Gills		Mantle		Gills	
	w-QPX	u-QPX	w-QPX	u-QPX	w-QPX	u-QPX	w-QPX	u-QPX	w-QPX	u-QPX	w-QPX	u-QPX
hemocyte defensin												
big defensin											+++	
lysozyme					+		++				+	---
C1q - TNF related protein			+									
TRAF-6											--	
Toll-like receptor							-					
RACK-1	+++				+++	+++		+		+	++	+++
peroxisome proliferator-activated receptor					+							+
HSP70			-			+						
stress-induced protein STI					+							
ferritin			-								++	+
metallothionein						++					-	+++
actin			+		++							
AP-1					++		+				++	
elongation factor beta	+++								-		---	+++
NADH sub-unit IV								+				
senescente associated protein											--	+
cytochrome P450 like TBP	+++								-			

Symbols + and – respectively indicate significant increase or decrease of gene expression compared to controls and the number of symbols for each condition refers to the p-value: + or -: $p < 0.05$, ++ or --: $p < 0.01$ and +++ or ---: $p < 0.001$.

Table 4.5. Effects of QPX challenge, sampling time and tissue type on gene expression in *M. mercenaria* (Multifactor ANOVA followed by Holm-Sidak post-hoc test).

	Time	condition	tissue	time x condition	time x tissue	condition x tissue	time x condition x tissue
hemocyte defensin					*		
big defensin	***	*	**	**	***		**
Lysozyme				*			*
Toll-like receptor	***		*	*	***		*
Ferritin	***						
Actin	**	*	**	***	**	*	***
AP-1	*			*	*		**
NADH sub-unit IV	*				*		
Senescente associated protein	***		*	*	***		

Only genes showing significant variations are presented. Symbols refer to the p-value: *: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.001$.

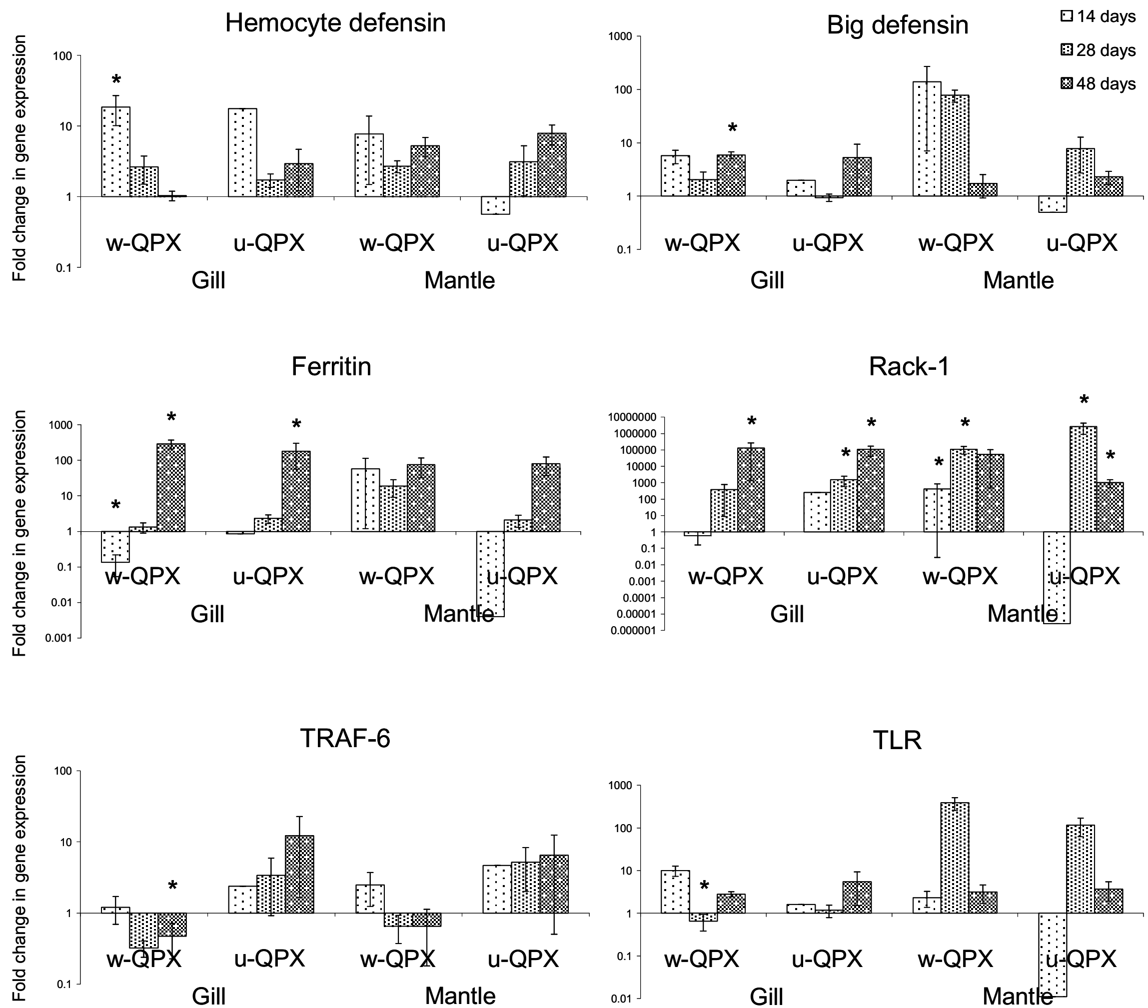


Fig. 4.3. Relative expression by quantitative PCR of selected transcripts from SSH (hemocyte and big defensins, ferritin, RACK-1) and hemocyte (TRAF-6 and TLR) libraries. Expression levels were normalized to 18S RNA and presented as relative expression to controls (mean ± SD, n=8 clams). * indicates significant differences of gene expression compared to controls at $p < 0.05$ (Student's *t*-test).

Discriminant Analysis performed on data from mantle and gill tissues (samples from different time points combined) revealed significant discrimination of the treatments in both tissues by Function 1 ($p = 0.05$ and 0.01, respectively) which explained 68.3 % and 81.5 % of the total variance respectively (Table 4.6). Scatter plots of discriminant functions indicated a small overlapping between treatments with a good discrimination of centroids by Function 1 (Fig. 4.4). Examination of the structure correlation matrix (Table 4.7) revealed that 9 variables were highly loaded in Function 1 for both mantle and gill tissue analyses. Among these variables, the expression of TLR, AP-1, big defensin and lysozyme were highly correlated (Table 4.8, $p < 0.001$).

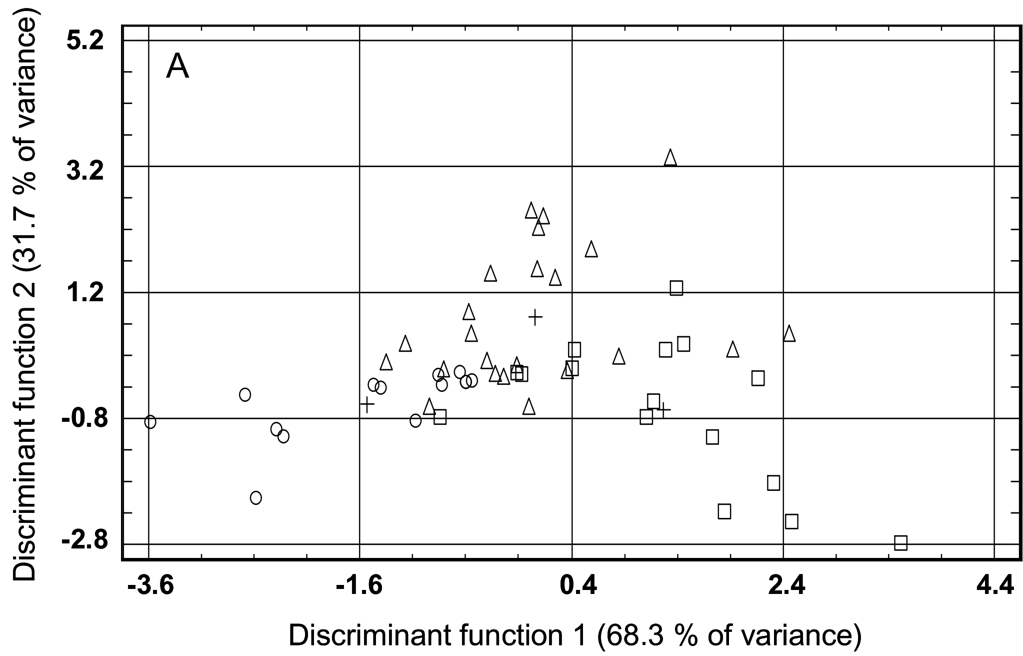
Table 4.6. Gene expression data from different sampling times were combined.

Tissue	Discriminant function	Eigenvalue	relative percentage	Canonical Correlation	Wilks Lambda	Chi-Square	Degrees of freedom	Statistical significance
Mantle	1	1.18	68.3	0.74	0.30	50.4	36	0.05
	2	0.55	31.7	0.59	0.65	18.1	17	0.38
Gills	1	1.54	81.5	0.78	0.29	57.2	36	0.01
	2	0.35	18.5	0.51	0.74	13.9	17	0.67

Table 4.7. Structure matrix of Discriminant Analyses on gene expression data obtained from mantle and gill tissues.

	mantle tissue		gill tissue	
	function 1	function 2	function 1	function 2
Toll-like receptor	1.118*	1.021	2.109*	-1.269
AP-1	4.635*	0.899	1.492*	-0.098
peroxisome proliferator-activated receptor	-0.356*	0.119	-2.771*	1.871
big defensin	-1.164*	-0.197	2.178*	-0.883
lysozyme	-2.662*	0.220	-0.704*	0.163
metallothionein	6.612*	-2.521	0.313*	0.241
actin	-2.452*	-1.006	-2.161*	1.222
NADH sub-unit IV	0.335*	0.073	-1.133*	1.051
senescence associated protein	-0.095*	0.036	0.352*	0.025
hemocyte defensin	1.642*	0.238	0.461	0.775
HSP70	1.413*	-0.295	0.377	-0.613
elongation factor beta	-6.159*	-0.112	-2.472	2.639
cytochrome P450 like TBP	-0.155	-0.270	-0.107*	-0.009
TRAF 6	0.322	-0.364	-1.859*	-0.946
stress-induced protein STI	-1.698	2.122	5.207*	-3.063
RACK-1	0.1242	-0.645	0.033	0.181
C1q - TNF related protein	0.278	0.857	0.142	-0.559
ferritin	0.115	0.139	-0.430	0.992

Largest absolute correlations between variables and discriminant functions are indicated by *.



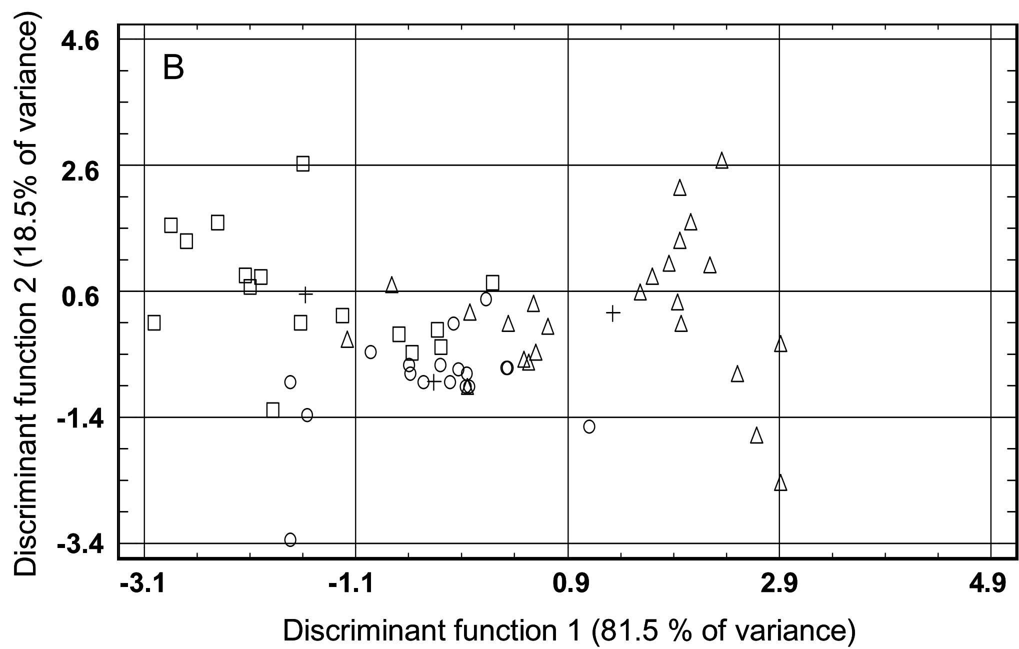


Fig. 4.4. Scatter plots of Discriminant Analysis scores in mantle (A) and gill (B) tissues for un-challenged controls (circles) and clams challenged with washed (triangles) or unwashed (squares) QPX cells. Positions of group centroids for each treatment are indicated by a black cross.

Table 4.8. Pearson's correlation coefficients of genes related to cell signalling (AP-1, TLR, TRAF-6; RACK-1) and humoral defense factors (hemocyte and big defensins, lysozyme).

	lysozyme	big defensin	hemocyte defensin
AP-1	0.934 ($5.3e^{-51}$)	0.736 ($-2.5e^{-20}$)	-0.025 (NS)
Toll-like receptor	0.408 ($7.9e^{-6}$)	0.726 ($1.4e^{-19}$)	-0.030 (NS)
TRAF 6	-0.034 (NS)	0.016 (NS)	0.087 (NS)
RACK-1	-0.015 (NS)	-0.039 (NS)	-0.020 (NS)

p -values are indicated between parentheses and non significant correlations are indicated by NS.

4. Discussion

Our investigations allowed the identification of components involved in different physiological processes related to signal transduction (RACK-1, TLR, TRAF-6), stress response (HSP, metallothionein, ferritin), immunity (lectins, defensins, lysozyme) and protein synthesis (transcription and elongation factors). This new information allowed the detection of modifications caused by QPX at the transcriptional level. Our results depict some integrative aspects of clam responses to QPX infection and more specifically to different forms of QPX challenges (washed versus unwashed cells of the parasite).

Since QPX is an opportunistic parasite usually found in pallial tissues (gills and mantle) of infected clams (Ford et al., 2002b), we focused our investigations on these tissues to identify differentially expressed genes in response to the infection. Interestingly, the comparison of gene distribution from subtracted libraries indicated some variations according to the tissue and the time of sampling. After 14 days of QPX challenge, similar numbers of unique genes were identified in mantle and gill tissues. However, 48 days post-challenge, a clear difference was observed between tissues with a larger proportion of unique genes identified in mantle as compared to gills. In addition, 24 % of identified genes in mantle tissues presented homologies with proteins known to be involved in stress and immune response, whereas only 11 % of identified genes in gills were involved in stress response (Tables 4.2 and 4.3).

These results suggest that molecular changes observed 14 days after challenge represented a systemic acute response of clams resulting from the injection of parasite in the circulation, whereas the differences observed after 48 days could be related to clam response to established QPX infections in mantle tissues. These results fit well with histological observations made following injection of QPX into the pericardial cavity of hard clams that showed an early (2 weeks) systemic distribution of QPX in clam tissues, followed by the development of most intense lesions in mantle tissues (Dahl and Allam, 2007). It is noteworthy that the development of QPX lesions following intrapericardial injection matches well, temporally (> 4 weeks) and spatially (organs infected), with the typical disease development in naturally infected clams (Dahl and Allam, 2007).

Our investigations demonstrated the modulation in QPX-challenged clams of components with strong homologies to stress-related proteins, including heat shock proteins (HSP's) and their co-chaperones STI1 (Stress Induced Protein), metallothionein and ferritin. These molecular chaperones protect the cell and maintain homeostasis under stressful conditions (Flom et al., 2007). HSPs were identified in several bivalve species in response to various chemical, physical and pathogenic stresses and appear to represent a general marker of non-specific stress (David et al., 2005; Dondero et al., 2006; Meistertzheim et al., 2007; Tanguy et al., 2004). Previous studies demonstrated that heat-shock proteins were able to stimulate innate immune system in mammals (Wallin et al., 2002). Our results are consistent with previous studies, which showed that the relationship between HSP and defense response was not established, despite the identification of heat-shock proteins following pathogenic challenge in invertebrates (Gestal et al., 2007; He et al., 2005; Tanguy et al., 2004). HSP 70 was significantly repressed in gill tissues of w-QPX challenged clams after 14 days and up-regulated in mantle tissues of u-QPX challenged clams (unwashed cells) after 28 days (Table 4.4). However, Multifactor ANOVA analysis indicated no significant role of HSP expression in the response of clams to the different treatments (Table 4.5).

MT's are a ubiquitous class of metal-binding proteins that function in the homeostasis of essential metals, as well as serving a detoxification role by sequestering toxic metals. In oysters, Anderson et al. (1999) demonstrated that MT's

were able to scavenge reactive oxygen species. In mammals, immune stimulants have been shown to be effective inducers of MT's (Carrasco et al., 1998). However, the role of MT's in invertebrates, and especially in bivalves, appears more complex. Bacterial challenge induced a repression of MT expression in *Crassostrea virginica* (Jenny et al., 2006) but not in the bay scallop *Agropecten irradians* (Wang et al., 2009) or during trematode parasitism in the cockle *Cerastoderma edule* where MT concentration increased (Desclaux-Marchand et al., 2007). Differences of MT expression could be related to the nature of the pathogen and its capacity to produce toxic compounds against its host. In our study, MT was significantly up-regulated in mantle after 28 days and gill tissues after 48 days in clams challenged by unwashed QPX but not in w-QPX challenged clams (Table 4.4). Discriminant analysis in mantle and gill tissues also revealed the specific importance of MT to separate the treatments in Function 1 (Table 4.7). Ferritin has also been classified as a stress protein due to its similarity with proteins involved in detoxification processes (Gueguen et al., 2003a). However, ferritin was also associated with defense mechanisms because of its role in the regulation of iron availability to infectious agents (Ong et al., 2006). Previous studies demonstrated an increase of ferritin expression in invertebrates following exposures to pathogen-associated molecular patterns (PAMPs) or bacterial challenge (Beck et al., 2002; Gueguen et al., 2003a; Ong et al., 2005). In our study, quantitative PCR revealed an increase of ferritin expression in clams challenged by QPX after 28 and 48 days (Fig. 4.3). However, significant increase of ferritin expression was only observed in gill tissues (Table 4.4). The biological significance of changes in ferritin expression levels is not known in clams since the effect of iron on QPX has never been investigated, but Gauthier and Vasta (1994) demonstrated limited *in vitro* growth of the oyster pathogen *Perkinsus marinus* under low iron concentrations.

Subtracted libraries also allowed the identification of genes coding for several proteins involved in humoral defense including lysozyme, lectins and defensins. Lysozyme is a well-known protein possessing anti-microbial activities; lysozyme activity has been detected in the body fluids and tissues of many bivalve mollusks and is believed to play a role in host defense and digestion (Allam et al., 2000a; Cronin et al., 2001; Takahashi et al., 1986). Different results have been previously reported on the variation of lysozyme concentration in response to parasites in mollusks.

Lysozyme concentrations were unchanged in clams *Tapes decussatus* infected by *Perkinsus atlanticus* (Ordás et al., 2000) and in oysters *Crassostrea virginica* infected by *P. marinus* (Chu and La Peyre, 1993b; Chu et al., 1993). However, a subsequent investigation in oysters showed a slight decrease in lysozyme concentration in *P. marinus*-infected oysters (La Peyre et al., 1995a). Our results indicate a differential response according to the treatment since the injection of washed QPX cells (w-QPX) induced significant expression of lysozyme in mantle and gill tissues, whereas unwashed parasite cells (u-QPX) caused a down-regulation of lysozyme expression in gill tissues after 48 days (Table 4.4).

Two different lectins were also identified in up-regulated libraries (Table 4.2). Lectins play an important role in invertebrate immunity as non-self pattern recognition molecules by promoting agglutination and opsonization of pathogens. In Manila clams *Ruditapes philippinarum,* Kang et al. (2006) demonstrated significant increase in lectin expression following pathogen challenge. Interestingly, lectins isolated from *R. philippinarum* (Bulgakov et al., 2004) and oysters *C. virginica* (Tasumi and Vasta, 2007) bind to the surfaces of purified hypnospores from *Perkinsus sp.* enhancing their phagocytosis by hemocytes. Identified lectins in libraries could as well be associated with the activation of the complement pathway since a thioester-containing protein (TEP) was also found in subtracted libraries (Table 4.3). Complement pathway is activated in reaction to the presence of PAMPs, leading to increased opsonization and phagocytosis activity by defense cells (Fujita et al., 2004). Several TEP with homologies with $_{\alpha}$-macroglobulin were previously characterized in other invertebrates (Bender and Bayne, 1996; Vaseeharan et al., 2007) and in bivalves (Ma et al., 2005; Zhang et al., 2007). A transcript with homologies with C1q was also present in our libraries. C1q is the target recognition of the classical complement pathway that is crucial for the clearance of pathogens in vertebrates (Kishore and Reid, 2000) and invertebrates (Zhang et al., 2008).

Our approach also led to the identification of two different defensins that show different expression patterns in mantle and gill tissues. Defensins are small antimicrobial peptides (AMP) and represent major actors in innate immunity (Mitta et al., 2000). Defensins were isolated from mussels (Charlet et al., 1996), scallops (Zhao et al., 2007) and oysters (Gonzalez et al., 2007a; Gueguen et al., 2006a). These

AMPs can be constitutively expressed, as observed in oysters (Gonzalez et al., 2007a), or induced in response to infection, as in scallop (Zhao et al., 2007). Most previously described defensins were characterized from hemocytes but some were also constitutively expressed in pallial tissues (Gueguen et al., 2006a). In our study, quantitative PCR revealed that the hemocyte defensin was significantly up-regulated in gills only after 14 days, while no significant change was observed in mantle, despite the tendency for an increase of the hemocyte defensin expression after 48 days following challenge with washed and unwashed QPX cells (Fig. 4.3). Initial induction of the hemocyte defensin after 14 days in gills could reflect systemic hemocyte activity as gill tissues are normally rich in hemocytes compared to mantle. Later on, increased PCR signals in mantle tissues could be related to increased expression in hemocytes present near infection sites or might simply reflect the mobilization of hemocytes toward active infection sites as part of the normal inflammatory response (Dove et al., 2004; Smolowitz et al., 1998). This may, in turn, lead to the observed shift of gene expression among tissues. Similar patterns were found in other studies investigating defensin expression in oysters (Gonzalez et al., 2007a). Regarding the big defensin, a significant induction was observed after 48 days in gill tissues of clams challenged with washed QPX whereas a tendency to a decrease of defensin expression was noticeable in mantle tissue (Fig. 4.3). Moreover, the big defensin appeared as an important variable to discriminate treatments in gill and mantle tissues (Table 4.7). Defensins present a great diversity in terms of structural features, biological properties and functions, and also in their tissue distribution and expression. Defensins from *C. gigas* exhibit high activities against gram positive bacteria but low activity against fungi (Gueguen et al., 2006a), whereas big defensins from *A. irradians* and the horseshoe crab *Tachypleus tridentatus* exhibit strong fungicidal activities (Saito et al., 1995; Zhao et al., 2007). Trends of both defensins suggest a certain level of specificity in the response of *M. mercenaria* to washed and unwashed QPX cells in mantle and gill tissues.

Several genes corresponding to membrane receptors and elements of pathways involved in defense responses have also been identified in our libraries. Among them, RACK-1 is involved in the protein kinase C (PKC) pathways and acts as an activator/receptor for this protein (McCahill et al., 2002). RACK-1 plays a key role as the crossroad among several pathways in cell communication by acting as a

scaffold protein on the translocation of the signalling proteins towards the membrane-bound receptors (McCahill et al., 2002). Ron et al. (1999) demonstrated *in situ* association of RACK-1 and PKC during phorbol 12-myristate 13-acetate (PMA) challenge, an activator of reactive oxygen species production (Humphries and Yoshino, 2008; Vidya et al., 2007). Overexpression of RACK-1 also led to enhanced spreading and increased focal adhesion in mammalian cells (Hermanto et al., 2002). These results suggested an involvement of RACK-1 in phagocytosis and ROS production. RACK-1 was previously identified in bivalves exposed to pollutants, physical stress and pathogens (Gestal et al., 2007; Meistertzheim et al., 2007; Siah et al., 2007). In the hard clam, our quantitative PCR results revealed that QPX challenge significantly induced the expression of RACK-1 in both gill and mantle tissues (Fig. 4.3 and Table 4.4). Libraries generated from hemocytes also led to the identification of several elements of the NF-kB pathway. Toll-like receptors (TLRs) are among the most important families of pattern recognition receptors (PRRs) and have already been identified in other bivalves (Qiu et al., 2007a; Tanguy et al., 2004). They are able to selectively recognize and initiate the response against a large number of varied and complex PAMPs (Arancibia et al., 2007). Tumor necrosis factor receptor-associated factor (TRAF), another component of the NF-kB pathway, was also detected in our hemocyte libraries. This intermediary possesses a unique receptor-binding specificity that results in its crucial role as the signalling mediator for both the TNF receptor superfamily and the TLR superfamily (Chung et al., 2002). Activation of this pathway induces expression of immune response genes triggered by transcriptional activator proteins. Among them, the transcription factor AP-1 was identified in our subtracted libraries. Interestingly, our results demonstrated an important involvement of NF-kB components in the differential response to washed and unwashed parasite cells (Tables 4.5 and 4.7), as well as a high correlation ($p < 0.001$) between humoral defenses (lysozyme, big defensins), TLR and the transcriptional factor AP-1 (Table 4.8). These results suggest that activation of the NF-kB pathway occurred following the recognition of QPX by TLR and the activation of AP-1, leading to a specific response characterized by the production of humoral defense factors including lysozyme and the big defensin.

Actin is often used as a house-keeping gene but we clearly observed a modulation of this gene in gills (14 days) and mantle (28 days) tissues following

challenge with washed parasite cells (Tables 4.4, 4.5 and 4.7). Actins are highly conserved proteins that are ubiquitously expressed in all eukaryotic cells. They are involved in the formation of filaments that are major components of the cytoskeleton and participate in many important cellular functions including cell motility, organelle movements and cell signalling (Herman, 1993; Kabsch and Vandekerckhove, 1992). With regard to infections, actin was, up-regulated in *Biomphalaria tenagophila* at a proteomic level in the presence of *Schistosoma mansoni* (Jannotti-Passos et al., 2008). The involvement of actin in QPX disease pathogenesis, if any, is unclear but it may participate in the encapsulation of parasite cells by host hemocytes leading to healing as in other host-parasite models (Turnbull et al., 2004).

Results of quantitative PCR also indicated that some genes were differentially regulated according to analyzed tissue (TLR, big defensin, Table 4.5) or inoculum (big defensin, Tables 4.5 and 4.7). Discriminant Analysis revealed the importance of signalling pathways and humoral defenses to differentiate between QPX-challenged and unchallenged clams or between clams injected with washed or unwashed parasite cells (Fig. 4.4 and Table 4.7). It should be mentioned that, because of sample size requirements of the statistical test, Discriminant Analyses were performed on data obtained throughout the experiment by pooling samples collected at 14, 28 and 48 days post-challenge. Such a holistic approach eliminated the effect of genes that displayed strong temporal modulations within each treatment and might neglect certain specific clam responses. This limitation could explain the barely significant results obtained with Discriminant Analyses in mantle tissues (Table 4.6). Despite this drawback, our analyses discriminated between clams injected with washed or unwashed parasite cells, highlighting the importance of QPX mucus during host-pathogen interactions. QPX mucus was suggested to represent a virulence factor that protects the parasite from host's cellular and humoral defense mechanisms (Smolowitz et al., 1998). Proteases were also detected in QPX mucus (Anderson et al., 2006) and Thrautochytrids are known to produce several proteolytic enzymes as extracellular products (Jain et al., 2005). Injection of unwashed QPX could protect the parasite from constitutive defenses of hard clams and enhance their ability to establish infection within host tissues, as well as prevent the detection of the parasite's PAMP's, thereby limiting the response of hard clams. In contrast, washed cells can be more readily phagocyted or encapsulated by hemocytes and neutralized by

humoral factors (Anderson et al., 2003a); they can also present PAMPs on their surface, enhancing clam's immune response. Thus, injection of washed QPX cells could induce an effective defense response in clams, leading to elimination of parasites and failure of disease establishment.

Conclusion

In conclusion, this study is the first to characterize molecular modulation in clams in response to QPX infection. A large number of new candidate genes was identified including several genes involved in stress and defense response and cell signalling. Quantitative PCR revealed significant changes in the expression of some of these genes in response to QPX challenge, as well as some correlation between gene expression of intermediates of signalling pathways and humoral defenses. Additional experiments are needed to further characterize molecular components involved in *M. mercenaria* response to its parasite. Specifically, further experiments should compare gene expression in susceptible and resistant clam broodstocks. Generated sequence information could also contribute to the construction of the first hard clam micro-array necessary for investigating gene expression on a larger scale.

Authors' contributions

MP carried out SSH and expression libraries, participated in the sequence alignment and drafted the manuscript. AT was in charge of molecular experiments, carried out real time PCR and helped in drafting the methodology section of the manuscript. BA is the lead PI on this work. He conceived the design of the study, coordinated experiments and supervised the statistical analysis of the results. All authors read and approved the final manuscript.

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Part III

Chapter 3

Differential immune response in the hard clam (*Mercenaria mercenaria*) against bacteria and the protistan Quahog Parasite Unknown (QPX)

Abstract

The immune response of the hard clam (quahog) *Mercenaria mercenaria* against live bacteria (*Vibrio alginolyticus*) and the protist QPX (Quahog Parasite Unknown) was investigated. Additionally, we also compared immune responses following QPX challenge in two different hard clam broodstocks exhibiting different degrees of susceptibility toward this parasite. Different immune and stress-related cellular and humoral factors were assessed including general hemocyte parameters (total and differential hemocyte counts, percentage of dead cells, reactive oxygen production, phagocytosis), parameters geared toward QPX (anti-QPX activity in plasma and hemocyte resistance to the cytotoxicity of QPX extracellular products), and the transcription level of ferritin and metallothionein genes. Results indicated that both *V. alginolyticus* and QPX challenge triggered significant immune responses in clams with similar trends in most measured parameters. However, specific defense parameters against QPX, ferritin and metallothionein expression, as well as global analysis of interaction between defense parameters demonstrated differences between challenges with a faster response in clams challenged with QPX and specific responses according to each inoculum. Similarly, immune response toward QPX challenge was significantly different between susceptible and resistant clam stocks. Resistant clams were able to elicit effective response against the parasite leading to the elimination of QPX and the restoration of constitutive defense status whereas QPX-susceptible clams triggered a strong immune response characterized by an acute phase response and associated acute phase protein but appeared to less active to eliminate the parasite. These results suggest that different signaling pathways are triggered during *V. alginolyticus* and QPX challenge. Moreover, differences in the immune response toward QPX might be linked to the susceptibility or resistance of different clam stocks to the infection by this parasite.

1. Introduction

Like other invertebrates, bivalves possess an effective innate immune system that provides protection against infectious agents present in their microbe-rich habitat. The immune system of bivalves relies primarily on both humoral and cellular responses from hemocytes which constitute the main line of defense against invaders (Cheng, 1981). This system is able to recognize molecular structures of microorganisms, known as pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs) (Iwanaga and Lee, 2005; Medzhitov and Janeway, 1997). Studies in vertebrates and insects report the specificity of PRRs to PAMPs and the consequent defense response (Akira et al., 2006; Dong and Dimopoulos, 2009). A number of different PRRs involved in the recognition of invaders have been described in several invertebrate species including bivalves (Itoh and Takahashi, 2008; Marmaras and Lampropoulou, 2009; Ni et al., 2007; Su et al., 2007). Once activated by microorganisms, PRRs lead to the activation of different intracellular signaling pathways that are required for the immune response. Defense responses involve, but are not limited to, phagocytosis or encapsulation of foreign materials and the production of reactive oxygen species (ROS) (Anderson, 1994; Pipe, 1992; Tripp, 1992b). Humoral factors in plasma, including circulating enzymes, lectins and anti-microbial peptides are also components of host innate immune system because of their various anti-microbial properties (Cheng, 1992; Chu, 1988; Roch, 1999). Additionally, proteins involved in metal homeostasis were identified in bivalves following exposure to pathogens or PAMPs (Gestal et al., 2007; Wang et al., 2008b, part III - chapter 2). For example, the iron-binding protein ferritin has been recognized to be involved in the immune response (Neves et al., 2009; Ong et al., 2005). Similarly, the metal-binding protein metallothionein has been considered to be a stress or acute phase protein in vertebrates with significant expression in response to PAMP injection (Coyle et al., 2002).

Members of the gram-negative genus *Vibrio* are among the most frequently isolated bacteria from mollusks and several species belonging to this genus have been associated with mortalities and diseases in bivalves (Allam et al., 2002b; Gomez-Leon et al., 2005; Paillard et al., 2004b; Travers et al., 2008). Some members of this group, such as *Vibrio alginolyticus*, are recognized as “universal” pathogens because of their

ability to infect a wide range of organisms across phyla. Alternatively, some pathogens, including larger microbes, are more specific usually affecting one or a few closely-related species. This is the case for the protistan parasite QPX (Quahog Parasite Unknown) that infects wild and cultured hard clams *Mercenaria mercenaria* causing severe mortality events. This disease has been observed along the eastern coast of North America, from Maritime Canada to Virginia but never south of Virginia (Dove et al., 2004; Ford et al., 2002b; Ragone Calvo et al., 1998; Smolowitz et al., 1998; Whyte et al., 1994). This geographic distribution is likely responsible for variations in clam susceptibility toward QPX among different clam stocks with lower resistance of southern (Florida and South Carolina) clam broodstocks, which are not exposed to selective mortality due to parasite pressure, as compared to northern (New Jersey, New York, Massachusetts) broodstocks growing and reproducing in enzootic areas (Dahl et al., 2008; Ford et al., 2002b; Ragone Calvo et al., 2007). The biological bases for the resistance process to QPX in Northern hard clams are not known although the outcome has been described as a granulomatous inflammatory response and encapsulation of parasite cells ultimately leading to the healing of infected individuals (Dahl and Allam, 2007; Dove et al., 2004; Ragone Calvo et al., 1998). Our previous studies demonstrated the presence of factors inhibiting QPX growth in clam plasma (part III – chapter 1). On the other hand, extracellular products (ECP) secreted by QPX were shown to significantly alter clam hemocytes (part II – chapter 1). Recent molecular investigations in clams also demonstrated significant modulation of the expression of stress- and defense-related genes during QPX disease development (part III – chapter 2).

Only a few studies have investigated comparative defense response in mollusks to different pathogenic or PAMP stimuli (Adema et al., 2010; Costa et al., 2009). In this study, cellular and humoral defense factors as well as the expression of ferritin and metallothionein transcripts were investigated in *M. mercenaria* following challenge with *V. alginolyticus* and QPX. Ferritin and metallothonein were selected because they were identified in suppressive subtractive hybridization libraries following challenge with QPX (part III – chapter 2) and are well known to be involved in defense and stress response (Coyle et al., 2002; Ong et al., 2006). Additionally, we compared defense response to QPX challenge in clams from resistant northern broodstocks (New York) to that of susceptible southern clams

(Florida). Results indicated that some common cellular and humoral defense parameters exhibited similar response to bacteria and QPX challenge but more specific defense factors as well as metallothionein and ferritin expression displayed different trends suggesting the involvement of different defense mechanisms according to the nature of the invader. Additionally, results demonstrated significant differences in immune response against QPX between the two clam stocks.

2. Materials and methods

2.1. Mercenaria mercenaria

Naïve *M. mercenaria* (35-45 mm in length) were obtained from commercial sources in Florida (FL, 100 clams) and New York (NY, 150 clams). We have been successfully using these aquacultured clam lines for comparative studies because of their confirmed differential susceptibility to QPX infections in laboratory settings (Dahl et al., 2008) and in the field (Dahl et al., 2010, part III - chapter 1). No QPX infection was detected in NY and FL clams by standard histopathology techniques (Dahl and Allam, 2007). They were acclimated for one week in the laboratory, held in 150-L tanks with re-circulating water (28-30 ppt) at 21 ± 1 °C and fed daily with commercial algae (DT's Live Phytoplankton, Sycamore, IL).

2.2. QPX and bacterial cultures

QPX strain NY0313808BC7 was isolated from nodules of infected New York clams (Qian et al., 2007) and subcultured in muscle tissue homogenates (MTH) from *M. mercenaria* according to Perrigault et al. (part III – chapter 1). QPX cultures were initiated in 25-cm^2 flasks containing MTH at 1000 $\mu g.ml^{-1}$ protein and incubated at 23 °C for 2 weeks. Cultures were harvested by centrifugation (15 min at 900 g) and QPX cells were resuspended in filtered artificial seawater (FASW). Neubauer chamber and a previously described fluorometric technique based on the uptake of fluorescein di-acetate (FDA) (Buggé and Allam, 2005) were used to determine the

concentration of QPX cells. QPX suspension was adjusted at 1 x 10^6 cells.ml^{-1} in FASW.

Bacterial (*Vibrio alginolyticus*) suspension, obtained from a 48h-old culture grown on marine agar (Difco BD, USA), was adjusted at 1 x 10^8 bacteria.ml^{-1} in FASW (1 OD_{500} = 5 x 10^8 bacteria.ml^{-1}).

2.3. Challenge of M. mercenaria

After 1 week of acclimation, clams were distributed in 40-L recirculating tanks (40 clams per tank, 2 FL treatments and 3 NY treatments, Table 5.1) filled with filtered and ultraviolet treated seawater (21 °C / 30 ppt). Recirculating seawater was filtered using biological and chemical filter cartridges containing activated carbon and was continuously oxygenated to saturation. Water quality, temperature (21 °C) and salinity (30 ppt) were controlled weekly and clams were fed daily with commercial algae (DT's Live Phytoplankton, Sycamore, IL). FL and NY clams were challenged with either bacteria (1 x 10^7 cfu) or QPX (1 x 10^5 cells) injected into the pericardial cavity according to Dahl and Allam (2007). Control clams were injected with 100 µl of FASW. Following injection, clams were maintained out of the water for 1.5 hour before being transferred into their respective tanks.

Table 5.1. Experimental design of the study. Clams were maintained at 21 °C, 30 psu and were fed daily. Each sampled treatment was composed of 10 clams. NS: not sampled.

Time	NY clams			FL clams	
	Controls NY-c	Bacteria NY-b	QPX NY-q	Controls FL-c	QPX FL-q
T1 – 16 h	10	10	10	NS	NS
T2 – 3 days	10	10	10	10	10
T3 – 4 weeks	10	NS	10	10	10

2.4. Hemolymph parameters

After 16 h, 3 days and 4 weeks, 10 clams from each treatment were sampled according to the experimental design presented in Table 5.1. QPX-challenged FL clams (FL-q) and their respective controls (FL-c) were not sampled at 16 h since QPX infection in the hard clam is chronic (Dahl and Allam, 2007). Similarly, NY-b clams were not sampled at the last sampling time (4 weeks) as clam response to bacterial challenge is usually more acute (Allam et al., 2006). All samples were processed individually. Hemolymph samples (generally 1.2 to 1.8 ml) were withdrawn from the adductor muscle with a 1 ml-syringe and held on ice. A volume of 650 µl hemolymph was diluted (vol:vol) in ice-cold FASW and used for assessment of clam parameters according to Perrigault et al. (part IV – chapter 2). Total and differential hemocyte counts as well as percentage of dead cells were assessed on a FACSCalibur flow cytometer (Becton Dickinson Biosciences) equipped with a 488 nm laser by counting 10,000 events. Reactive oxygen species (ROS) production was assessed before (unstimulated or native) and after stimulation (5 min post-stimulation) of hemocytes with zymosan A (part IV – chapter 2). Phagocytic activity of hemocytes was measured using a plate reader technique employing FITC-labeled *Vibrio parahaemolyticus* (part IV – chapter 2). Similarly, our previously described neutral

red (NR) uptake assay was used to assess *in vitro* resistance of clam hemocytes to QPX extracellular products (part II – chapter 1). Plasma from undiluted hemolymph sample was also recovered by centrifugation (700 x g, 10 min, 4 °C). Supernatant was sterilized by filtration (0.22 µm), aliquoted and preserved at -20 °C to determine protein concentration in plasma and to measure anti-QPX activity using a previously described *in vitro* growth inhibition assay (part III – chapter 1). Hemocyte pellets were immediately transferred to -80 °C until RNA extraction.

2.5. Total RNA extraction and cDNA synthesis

Following hemolymph sampling, mantle and gill tissues were individually dissected and frozen at -80 °C until RNA extraction. These tissues were chosen because they are involved in QPX pathology (Dahl and Allam, 2007; Dahl et al., 2010) and because they were shown to consistently express targeted genes (metallothionein and ferritin). For instance, transcription levels of metallothionein and ferritin was assessed in hemocytes, gills, mantle, foot and visceral tissues individually processed from 4 unchallenged NY clams. Total RNA was extracted from individual samples using TRI® Reagent (Invitrogen, Carlsbad, CA, USA). The same extraction procedure was used for hemocyte pellets but samples were pooled by 2 (5 samples of 2 pooled clams each per treatment) because of the small amount of recovered cells per sample. Total RNA quantity and quality were assessed by spectrophotometry (OD260, OD280) using a Nanodrop ND-1000 (Thermo Scientific, Wilmington, USA) and 5 µg total RNA was individually submitted to reverse transcription using oligo dT_{17} and Moloney murine leukaemia virus (M-MLV) reverse transcriptase (Promega, Madison, WI, USA).

2.6. Rapid amplification of metallothionein (Mm-MT) and ferritin (Mm-Fer) cDNA ends

ESTs of 371 bp and 487 bp coding respectively for metallothionein (GO915213) and ferritin (GO915233) were previously identified by suppression substractive hybridization during QPX challenge in NY clams (part III – chapter 2).

Full cDNA of both metallothionein and ferritin were obtained by RACE using specific primers (Table 5.2) and methods described by Scotto Lavino et al. (2006a; 2006b). Briefly, total RNA was extracted from gill tissue of NY clams using methods described above. Reverse transcription for the determination of 5’ cDNA end of the metallothionein (Mm-MT) was performed with the SuperScript II reverse transcriptase (Invitrogen, USA) and MetRT primer (Table 5.2). Similarly, 3’ cDNA end of the ferritin (Mm-Fer) was obtained using reverse transcriptase and Qt primer. Mm-Fer and Mm-MT cDNA ends were amplified by PCR using Qo-MetO for the Mm-MT and Qo-FerO for the Mm-Fer. PCR products were thereafter used as template in nested PCR using Qi-MetI and Qi-FerI (Table 5.2) primer combinations according to Scotto Lavino et al. (2006a; 2006b). PCR products were separated on agarose gel and extracted using Wizard® SV gel and PCR clean up system (Promega, Madison, USA). Purified products were ligated into pGEM-T vector (Promega, Madison, USA) and used to transform DH5α bacteria (Invitrogen, USA). Bacteria were cultured in Luria-Bertani medium (with 100 $\mu g.ml^{-1}$ ampicillin, final concentration) and plasmids containing insert were extracted and sequenced by extension from both ends using T7 and SP6 universal primers. Mm-MT and Mm-Fer sequences were analyzed and compared with other known metallothionein and ferritin sequences available in the NCBI database using BLAST analysis.

Table 5.2. Sequences of the primers used in this study.

	Primer names	Primer sequences
RACE primers		
	Qt	CCAGTGAGCAGAGTGACGAGGACTC GAGCTCAAGCTTTTTTTTTTTTTTTTT
	Qo	CCAGTGAGCAGAGTGACG
	Qi	GAGGACTCGAGCTCAAGC
Metallothionein	MetRT	AGCGGTGCATGGAGTATCT
	MetO	CTCAAACCGCGTGACTTCT
	MetI	CAGCCACTATCACACTGGC
Ferritin	FerO	CCACCTTACGGTCTAAGCC
	FerI	GAGTGTCAACCAGGCTCTC
Quantitative real-time RT-PCR primers		
Metallothionein	Qpcr-MetF	ATCCGTGCAATTGTGCTGAAACGG
	Qpcr-MetR	TTACATCCGGGACACTTGCAGTCA
Ferritin	Qpcr-FerF	AACCAGGCTCTCCTTGACCTTCAT
	Qpcr-FerR	TGATTGCATCGACCTGTTCCTCCA
Elongation factor	Qpcr-EF1F	AGTCGGTCGAGTTGAAACTGGTGT
	Qpcr-EF1R	TCAGGAAGAGACTCGTGGTGCATT

Elongation factor fragment (EF1, GO915211) was used as housekeeping gene.

2.7. *Real-Time PCR analyses*

Transcript levels of metallothionein (Mm-MT) and ferritin (Mm-Fer) in different tissues and during QPX and bacterial challenge were investigated in *M. mercenaria*. Quantitative real-time PCR (QPCR) was carried out in a Mastercycler ep realplex (Eppendorf, Germany). QPCR assays were performed by mixing 3µl of 15-fold diluted cDNA, 5 µl of Brilliant II SYBR Green QPCR master mix (Stratagene, USA), and 100 nM of each primer in a total volume of 10 µl. Primer sequences of the

Mm-MT, Mm-Fer were designed from full cDNA and are presented in Table 5.2. Reactions were realized with activation of SureStart® DNA polymerase at 95 °C for 10 min followed by amplification of the target cDNA (50 cycles of denaturation at 95 °C for 30 sec, annealing and extension at 60 °C for 1 min) and a melting curve program from 95 to 70 °C that decreased the temperature by 0.5 °C every 10 sec. Readings were taken at 60 °C. PCR efficiency (E) was assessed for each primer pair by determining the slope of standard curves obtained from serial dilution analysis of cDNA from different experimental samples (treatment and control). The comparative CT method ($2^{-\Delta\Delta Ct}$ method) was used to determine the transcript level of analyzed genes (Livak and Schmittgen, 2001). The expression of the candidate genes was normalized using elongation factor (EF1, GO915211, Table 5.1) fragment as a housekeeping gene (ΔCt) and relative transcription level of Mm-MT and Mm-Fer in each sample was subtracted from their respective control at each time (ΔΔCt). Results are given as the mean ($2^{-\Delta\Delta Ct}$) and standard deviation of 10 replicates per condition (except hemocytes, n = 5 pools of 2 clams each). Similarly, analysis of Mm-MT and Mm-Fer transcription level in different tissues was performed by subtracting ΔCt value from each sample by the average of ΔCt values from all tissues. Results are presented as the mean ($2^{-\Delta\Delta Ct}$) and standard deviation of 4 replicates per tissue.

2.8. *Statistical analysis*

All variables were analyzed statistically using 2 and 3 way ANOVA to evaluate effects of challenge (NY-c, NY-b and NY-q) and interactive effect of clam broodstock and QPX challenge (NY-c, NY-q, FL-c, FLq). One way ANOVA was used to compare transcription level of Mm-MT and Mm-Fer in different tissues. ANOVA treatments that generated probability values below 0.05 were followed by a Holm-Sidak post-hoc test comparing different conditions. Data were log10 or arcsin transformed whenever data showed a large variance but results are presented as non-transformed data. Multivariate analysis was performed using Principal Component Analysis (PCA) to analyze relationships between variables. PCA analysis was followed by ANOVA on extracted components to evaluate the effect of different conditions (challenge and clam origin) on overall hemolymph profiles. PCA analysis

was performed with Statgraphics plus (Statistical Graphics Corp., Warrenton, Virginia, USA) and SigmaStat (Systat Software, Inc., San Jose, California, USA) was used for ANOVA analyses. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Differential cellular and humoral response of M. mercenaria to bacterial and QPX challenge

Results demonstrated a strong influence of challenge with bacteria and QPX on *M. mercenaria* total hemocyte counts (THC) and percentage of dead cells (PDC) (Tables 5.3 and 5.4). Both bacterial and QPX challenge induced THC increase with significant difference between NY-c (control) and NY-q (QPX-challenged) 16 hours and 3 days post-inoculation ($p < 0.01$, Table 5.4). Similarly, THC in NY-c and NY-b (*V. alginolyticus*-challenged) were significantly different on day 3 ($p < 0.001$). Increase in THC was associated with an increase in the percentage of granulocytes in NY-q and NY-b compared to NY-c after 16 h but trends were reverted 3 days after challenge (Table 5.3). PDC was significantly different between NY-q and controls ($p < 0.001$) at Day 3 whereas no significant variation of PDC was observed 16 h after bacterial or QPX challenge (Table 5.3).

ROS production and phagocytosis exhibited no significant difference between challenged clams and controls (Table 5.3) although a general trend to lower production of ROS after 5 min of zymosan activation was noticed following challenge with bacteria and QPX. Hemocytes from clams challenged with QPX also displayed higher phagocytosis activity compared to NY-c 16 h after challenge whereas phagocytosis activity of hemocytes from NY-b was similar to controls 3-days after challenge (Table 5.3).

Plasma from NY clams was characterized by a slight increase in protein concentration following QPX or *V. alginolyticus* challenge during the first 3 days of the experiment (Table 5.3). Anti-QPX activity was detected in plasma from all clams (Fig. 5.1A) although a decrease in this activity was observed after 3 days in plasma

from QPX challenged clams (differences were not statistically significant, Fig. 5.1A, Table 5.4).

Sensitivity of hemocytes to cytotoxic effects of QPX extracellular products (ECP) was also modulated following challenge. For instance, hemocytes from QPX challenged clams (NY-q) appeared more resistant to QPX ECP after 16 h of challenge compared to control clams and to those injected with bacteria but the pattern was inverted after 3 days ($p < 0.01$, Fig. 5.1B).

Table 5.3. Mean of defense parameters (n = 10 clams per condition) in FL and NY *M. mercenaria* injected with sterile seawater (NY-c and FL-c) or challenged with bacteria (NY-b) or QPX (NY-q and FL-q). THC: total hemocyte counts, PDC: percentage of dead cells.

	T1 – 16 hours			T2 – 3 days					T3 – 4 weeks			
	NY-c	NY-b	NY-q	NY-c	NY-b	NY-q	FL-c	FL-q	NY-c	NY-q	FL-c	FL-q
THC (cells.ml^{-1} x 10^5)	16.8^{a}	20.5^{ab}	23.4^{b}	14.3^{ax}	26.2^{b}	22.2^{bx}	11.7^{y}	44.2^{y}*	13.5	15.5	7.6	20.0*
% granulocytes	62.5	66.2	74.9	78.7	70.8	72.4^{x}	65.8	40.4^{y}*	77.5	78.3^{x}	67.8	42.9^{y}*
PDC (%)	6.4	8.4	6.7	19.7^{ax}	6.9^{ab}	5.4^{b}*	12.3^{y}	8.3*	7.9	7.8	10.1	9.3
basal ROS production (Fluorescence units/10^4 hemocytes)	1.0	0.8	0.5	0.8	0.7	1.0	1.1	3.0	1.0	0.6	1.5	1.0
5 min stimulated ROS production (Fluorescence units/10^4 hemocytes)	2.3	1.8	1.2	1.5	1.0	0.8	2.6	1.4	2.2	1.6	3.6	2.3
Phagocytosis (Fluorescence units/10^4 hemocytes)	3.0	3.1	4.7	5.5	5.2	5.5^{x}	3.5	2.3^{y}*	2.1^{x}	0.9*	0.8^{y}	0.7
Protein concentration (µg.ml^{-1})	652.5	710.1	665.0	620.8^{x}	666.8	646.8	403.7^{y}	675.4*	495.2^{x}	491.2^{x}	322.3^{y}	329.0^{y}

Letters a and b indicate significance at $p < 0.05$ between the different treatments in NY clams. Letters x and y denote significance at $p < 0.05$ between NY and FL clams for the same treatment and sampling time. Symbols (*) denote significance at $p < 0.05$ between control and QPX challenged clams within the same broodstock. Refer to appendix 1 for SEM values.

Table 5.4. Effects of QPX (NY-q) and bacterial (NY-b) challenge on NY *M. mercenaria* cellular and humoral parameters and gene transcription levels at 16 hours and 3 days. THC: total hemocyte counts, PDC: percentage of dead cells.

	Time	Challenge	Time X Challenge
THC	**NS**	**	**NS**
% granulocytes	**NS**	**NS**	**NS**
PDC	**	***	***
Unstimulated ROS	**NS**	**NS**	**NS**
Stimulated ROS production	**NS**	**NS**	**NS**
Phagocytosis	*	**NS**	**NS**
Protein concentration	**NS**	**NS**	**NS**
Anti QPX activities in plasma	**NS**	**NS**	**NS**
Hemocyte resistance to QPX ECP	***	**NS**	*
Mm-MT – gills	***	***	***
Mm-Fer - gills	**	**NS**	**

Non-significant differences are presented as NS and symbols denote significant differences at $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***) (ANOVA).

3.2. Comparison of cellular and humoral defense response to QPX in clams from NY and FL

THC and PDC generally followed the same trends following QPX challenge in both NY and FL clams although some differences were observed during the last sampling (Tables 5.3 and 5.5). For instance, THC in NY clams increased 3 days after challenge but was similar to controls after 4 weeks whereas THC in FL-q clams was higher 4 weeks after challenge compared to FL-c ($p < 0.045$, Table 5.4). Similar percentages of granulocytes were observed in NY-c and NY-q after 3 days and 4 weeks whereas percentages of granulocytes significantly decreased in FL-q after 4 weeks compared to their controls ($p < 0.002$, Tables 5.3 and 5.5). Additionally, significant differences were observed in THC between NY and FL clams with lower THC in FL-c compared to NY-c whereas QPX-challenged clams exhibited the opposite pattern. QPX challenge caused a significant decrease in PDC in both clam

populations at day 3 compared to their respective controls ($p < 0.031$) but this effect disappeared after 4 weeks.

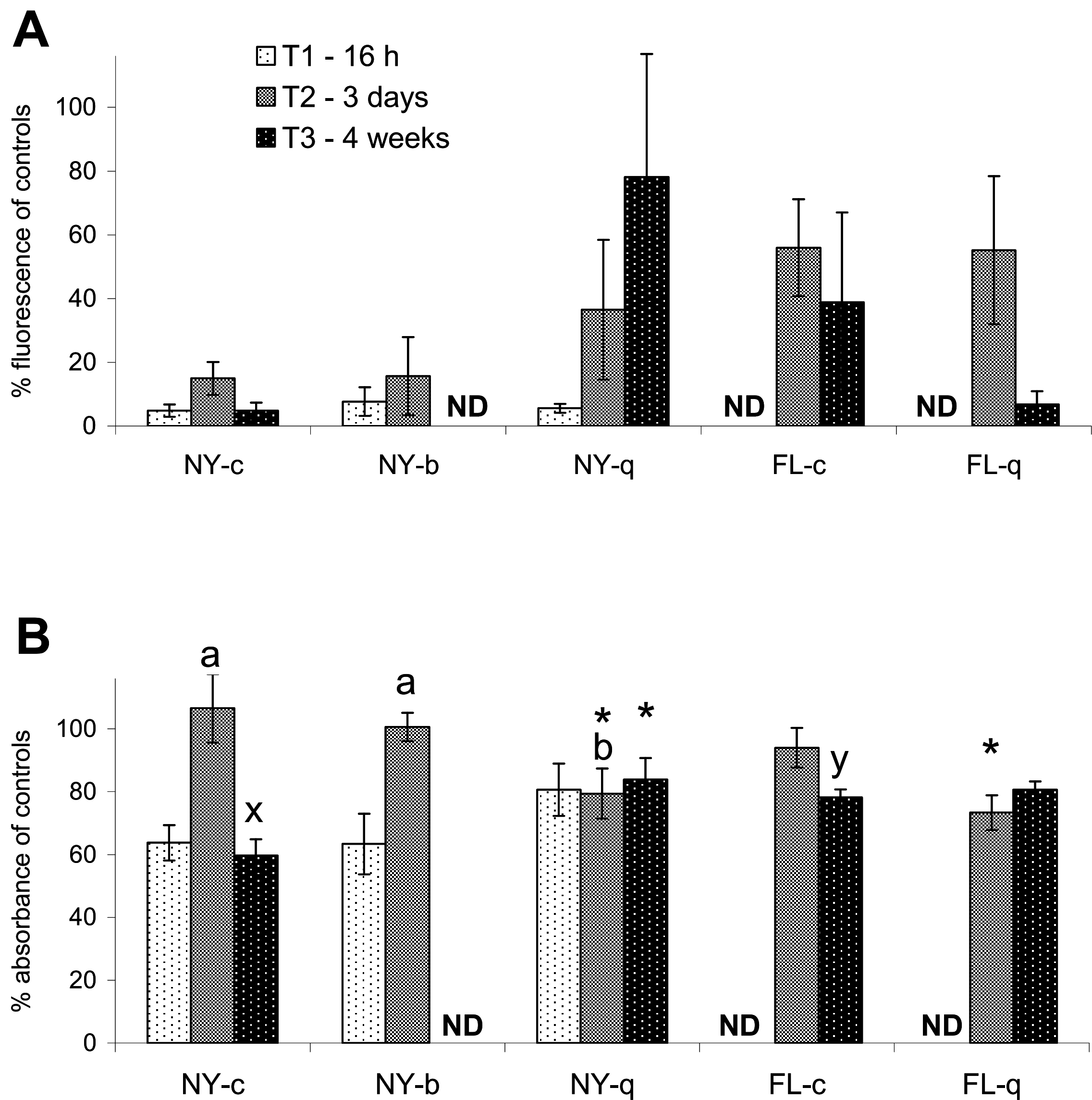

Fig. 5.1. Mean (± S.E., n = 10 clams per data point) of [A] anti-QPX activities in plasma (higher values represent lower activities) and [B] hemocyte resistance to QPX ECP (higher values represent higher hemocyte resistance) in New York and Florida clams injected with seawater (NY-c, FL-c), QPX (NY-q, FL-q) and bacteria (NY-b). ND: not determined. In [B], the letters a and b indicate significant differences (Holm-Sidak post-hoc test, $p < 0.05$) between different treatments in NY clams at day 3, and x and y denote significant differences between control NY and FL clams at 4 weeks. Symbols (*) denote significant differences between QPX challenged clams and their respective controls at each sampling time (Holm-Sidak post-hoc test, $p < 0.05$).

Three-way ANOVA revealed a significant effect of QPX challenge on hemocyte ROS production and phagocytosis activity (Table 5.5). QPX challenge

tended to decrease basal ROS production except in FL-q clams after 3 days (Table 5.3). Similarly, zymosan-stimulated ROS tended to decrease following QPX challenge both in NY and FL clams (Table 5.3). In contrast, different trends in phagocytosis activity were observed between NY and FL clams with similar activity in NY-q at Day 3 and a significant decrease after 4 weeks compared to their controls ($p = 0.008$) whereas phagocytosis significantly decreased in FL-q compared to controls after 3 days ($p = 0.021$) and was similar to FL-c after 4 weeks (Table 5.3).

Differences were also observed in plasma protein concentration between naïve NY and FL clams (Tables 5.3 and 5.5) with NY-c clams ranging from 495 to 652 $\mu g.ml^{-1}$ on average and FL-c clams ranging from 322 to 404 $\mu g.ml^{-1}$ on average ($p < 0.001$). Additionally, QPX challenge caused a temporary increase in protein concentration in plasma from NY and FL clams at Day 3 with significant difference between FL-q and FL-c ($p = 0.001$) before returning to normal after 4 weeks (Table 5.3).

Similarly to protein concentration, plasma from NY-c clams exhibited significant higher anti QPX activity than plasma from FL-c clams ($p = 0.005$, Fig. 5.1A). However, QPX challenge appeared to cause a reduction in anti-QPX activity in NY clams whereas challenged FL (FL-q) clams presented similar activity to their controls at Day 3 and higher anti-QPX activity after 4 weeks compared to FL-c (Fig. 5.1A).

Similar temporal variations were observed in hemocyte resistance to QPX ECP with significantly lower resistance of hemocytes from QPX challenged NY (NY-q) and FL (FL-q) clams after 3 days compared to their respective controls ($p < 0.05$, Fig. 5.1B). Trends were reversed in NY clams at 4 weeks with higher resistance of hemocytes from NY-q compared to NY-c ($p = 0.002$) whereas similar resistance to ECP was noted in hemocytes from FL-q and FL-c at 4 weeks (Fig. 5.1B).

Table 5.5. Effects of clam origin (NY and FL) and QPX challenge on *M. mercenaria* cellular and humoral parameters and gene transcription levels over the 4-week experiment. THC: total hemocyte counts, PDC: percentage of dead cells.

	Time	Clam	Challenge	Time X Clam	Time X Challenge	Clam X Challenge	Time Clam Challenge
THC	*	**NS**	***	**NS**	**NS**	*	**NS**
% granulocytes	**NS**	***	***	**NS**	**NS**	**	**NS**
PDC	**	**NS**	***	*	***	*	**
Unstimulated ROS	**NS**	*	**NS**	**NS**	*	**NS**	**NS**
Stimulated ROS production	*	*	**	**NS**	**NS**	**NS**	**NS**
Phagocytosis	***	**	**	**NS**	**NS**	**NS**	*
Protein concentration	***	***	*	**NS**	*	*	*
Anti QPX activities in plasma	*	**NS**	**NS**	*	**NS**	*	**NS**
Hemocyte resistance to QPX ECP	***	*	**NS**	**NS**	***	**NS**	**NS**
Mm-MT – gills	***	**NS**	**NS**	**NS**	***	*	**NS**
Mm-Fer - gills	**	*	*	**NS**	*	*	**NS**

Non-significant differences are presented as NS and symbols denote significant differences at $p < 0.05$ (*),$p < 0.01$ (**) or $p < 0.001$ (***) (ANOVA).

3.3. Metallothionein (Mm-MT) and ferritin (Mm-Fer) characterization in M. mercenaria and expression during bacterial and QPX challenge

The cDNA sequence coding for metallothionein (Mm-MT) revealed an open reading frame (ORF) of 219 bp encoding a 73 amino acid protein. Mm-MT, which was deposited into GenBank database, exhibited specific characteristics of metallothioneins with high number (21) of cystein residues. Basic Local Alignment Search Tool (BLASTp) yielded the highest homologies with MT from other bivalves: 76 % with the mussel *Unio tumidus* (ABP01350), 75 % with the oyster *Crassostrea gigas* (CAC82788), 74 % with the clams *Venerupis decussatus* (ABS20116) and *V. philippinarum* (ABP57063). The full cDNA coding for ferritin was characterized by an ORF of 513 bp encoding a 171 amino acid protein. Mm-Fer shared high similarity with H-chains of mammalian ferritins and other invertebrate ferritins: 86 % with the clam *Meretrix meretrix* (AAZ20754), 80 % with the razor clam *Sinonovacula constricta* (ACZ65230), 78 % with the worm *Pectinaria gouldii* (ACJ37369) and 77 % with the oysters *Pinctada fucata* (AAQ12076) and *C. gigas* (AAP83794).

Investigation of constitutive transcription level of Mm-MT and Mm-Fer indicated their presence in all tested tissues (Fig. 5.2). However, Mm-MT was expressed to a greater degree in visceral tissues compared to gills and foot ($p < 0.006$, Fig. 5.2A) whereas Mm-Fer was more abundant in mantle tissue and less in visceral tissue, gills or hemocytes ($p < 0.002$, Fig. 5.2B).

Mm-MT and Mm-Fer were significantly up-regulated in gills of NY clams 3 days after challenge with QPX ($p < 0.001$, Fig. 5.3). Similar trends were noted in FL-q clams at the same timeframe whereas bacterial challenge had no effect on Mm-MT and Mm-Fer after 3 days but significant down regulation was noted in Mm-MT 16 h after challenge with *V. alginolyticus* ($p = 0.022$). Identical patterns were observed in Mm-Fer transcription level in hemocytes and gills whereas Mm-MT appeared globally down regulated in hemocytes excepted in FL-q at day 3. Different trends in Mm-MT transcription level were also observed in mantle tissue from NY clams in response to bacterial and QPX challenge with a tendency to an increase in Mm-MT

expression with time in NY-q clams. The transcriptional level of Mm-MT and Mm-Fer in mantle tissue also tended to increase in FL-q clams after 4 weeks (Fig. 5.3).

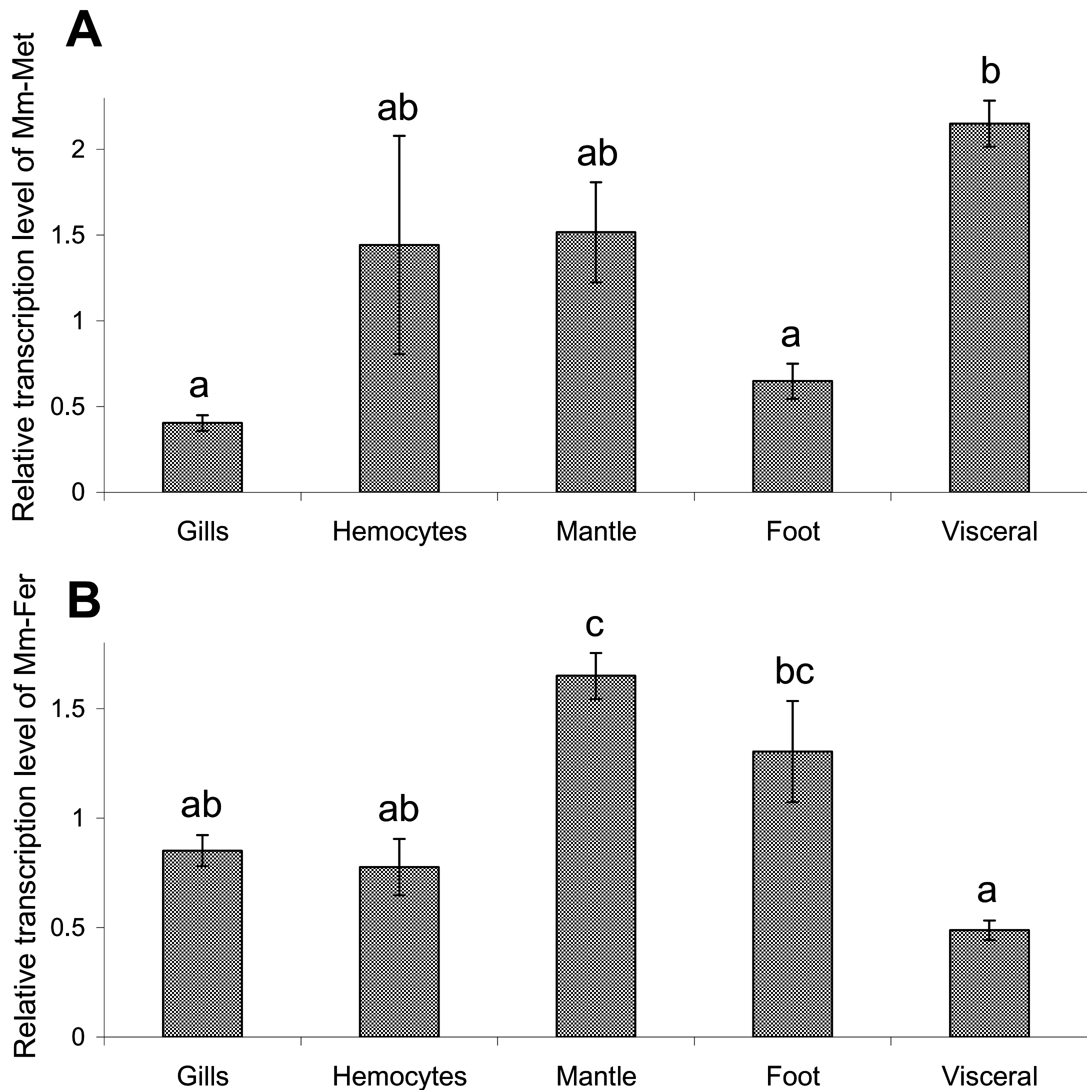

Fig. 5.2. Relative transcription levels (Mean± S.E., n = 4 clams) of [A] metallothionein (Mm-MT) and [B] ferritin (Mm-Fer) mRNA in *M. mercenaria* tissues, analyzed by QPCR. Relative transcription levels of Mm-MT and Mm-Fer were normalized to EF1 transcript level. Letters denote significant differences between tissues (Holm-Sidak post-hoc test, $p < 0.05$).

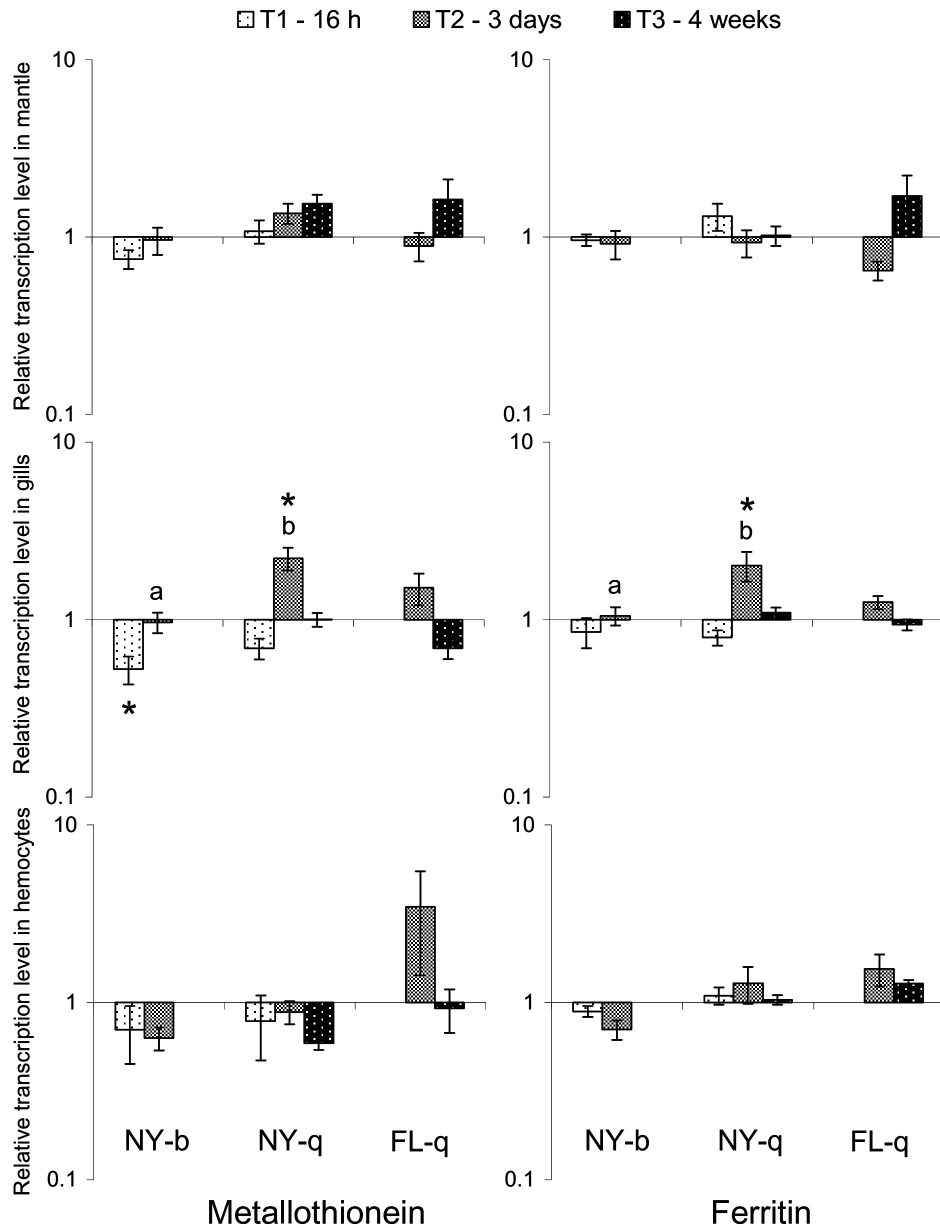

Fig. 5.3. Expression of Mm-MT (left panel) and Mm-Fer (right panel) transcripts in mantle (top, n = 10 per treatment ± S.E.), gill (middle, n = 10 per treatment ± S.E.) and hemocytes (bottom, n = 5 pools of 2 clams per treatment ± S.E.) at different time intervals following QPX (NY-q and FL-q) or *V. alginolyticus* (NY-b) challenge in *M. mercenaria* from New York (NY-b and NY-q) or Florida (FL-q). Relative transcription levels were analyzed by QPCR and were normalized to EF1 transcript level. For each sampling time, letters (a and b) indicate significant differences (Holm-Sidak post-hoc test, $p < 0.05$) between the different challenges (bacteria or QPX) in NY clams and symbols (*) denote significance between challenged clams (FL and NY) and their respective control represented by the x-axis.

3.4. PCA analysis of defense and stress parameters in M. mercenaria in response to bacterial and QPX challenge

Principal Component Analysis was performed on defense and stress parameters combined from all treatments (Fig. 5.4). Expression of Mm-MT and Mm-Fer from gill tissue was incorporated in the analysis instead of MT and ferritin expression in hemocytes because PCA cannot be performed with pooled samples. Components 1 and 2 explained more than 43 % of the total variance (Fig. 5.4). Basal and stimulated ROS production were opposed to phagocytosis, protein concentration in plasma, expression of Mm-MT and Mm-Fer, THC and hemocyte resistance to cytotoxic activity of QPX ECPs on component 1 and to PDC and anti-QPX activity in plasma on component 2.

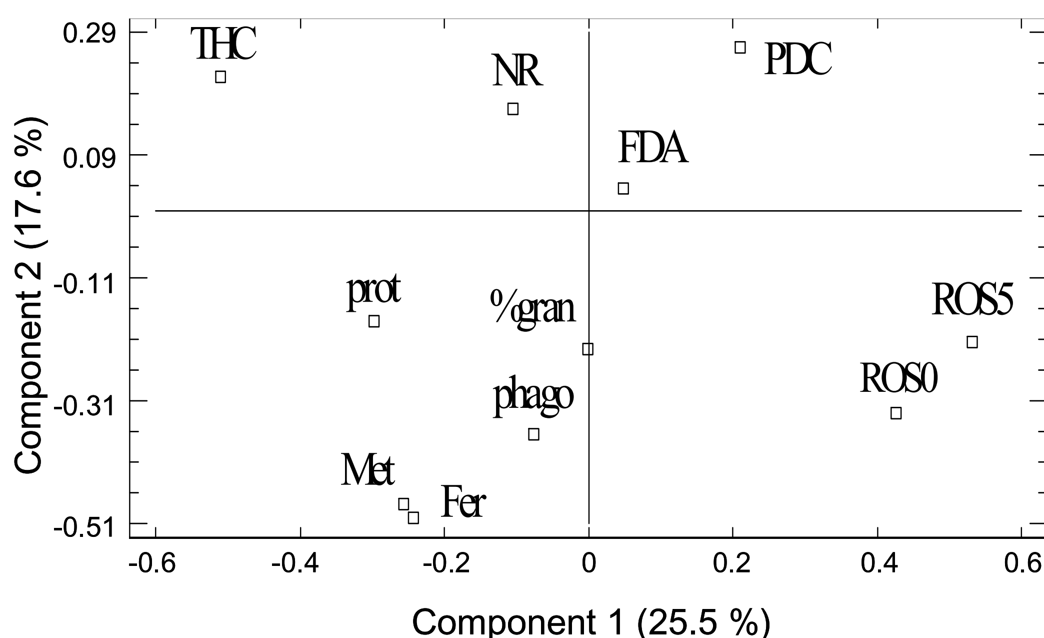

Fig. 5.4. Principal Component Analysis (PCA) plot of all measured parameters (all FL and NY clams combined, n = 120) abbreviated as follows: PDC: percentage of dead cells, ROS0 and 5: basal and zymosan-stimulated ROS production respectively, THC: total hemocyte count, %gran: percentage of granulocytes, phago: phagocytosis, prot: protein concentration in plasma, FDA: anti-QPX activity of clam plasma, NR: hemocyte resistance to cytotoxicity of QPX ECP, FER and MET: Relative transcription level in gill tissue of Mm-Fer and Mm-MT respectively.

Statistical analysis (ANOVA) of extracted component 1 (PC1) revealed interesting trends. For instance, QPX challenge caused a rapid response among NY clams that was visible at the first sampling time (16 hours) as compared to response to bacterial challenge whereas both bacterial and QPX challenges induced significant response in NY clams compared to control after 3 days ($p < 0.018$, Fig. 5.5A). Differences in PC1 between control and challenged NY clams at 3 days were concomitant with increases in THC and protein concentration in plasma and decreases of PDC and ROS production in challenged clams compared to controls (Table 5.3 and Fig. 5.3).

Comparison of extracted PC1 from NY and FL clams challenged by QPX exhibited similar trends but only NY-q was significantly different from its control at day 3 ($p = 0.013$, Fig. 5.5B). However, PC1 from NY-q returned to normal after 4 weeks and was significantly higher than PC1 from the same clam batch at day 3 to ($p < 0.001$) whereas alteration in PC1 among FL clams challenged with QPX (FL-q) remained visible after 4 weeks in comparison to their controls. Additionally, extracted components of NY clams were significantly lower than FL clams ($p = 0.007$, Fig. 5.5B).

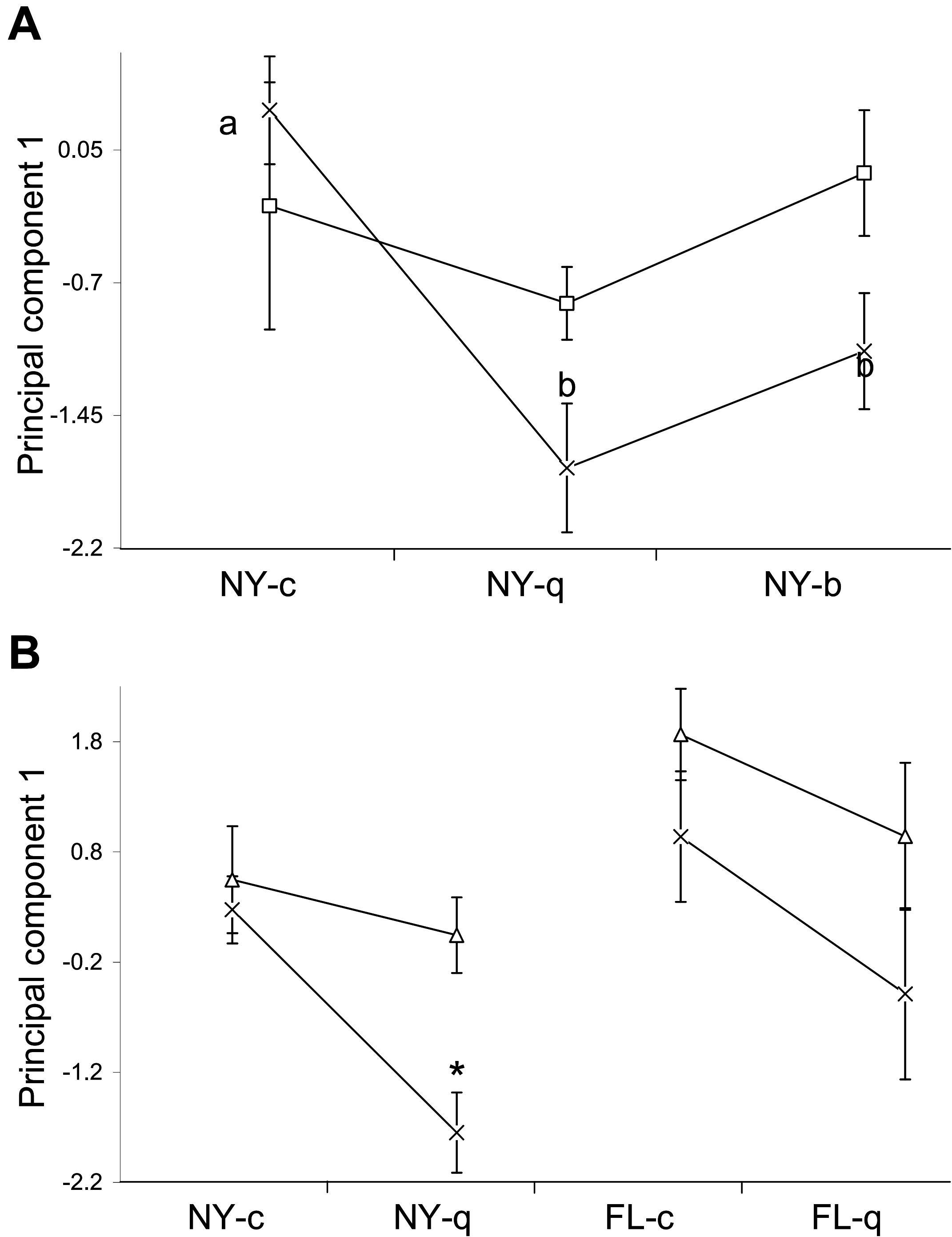

Fig. 5.5. Mean plot (n = 10 per data point ± S.E.) of Component 1 from Principal Component Analysis (Fig. 5.4) for [A] NY clams injected with sterile seawater (NY-c) or challenged with bacteria (NY-b) or QPX (NY-q) after 16 h (□) and 3 days (✕); [B] Controls (NY-c and FL-c) and QPX challenged (NY-q and FL-q) clams from NY and FL after 3 days (✕) and 4 weeks (Δ). Letters (a and b in [A]) denote significant differences between treatments at day 3 and symbols (* in [B]) indicate significant differences between NY-q and control NY-c at day 3.

4. Discussion

By comparing different pathogenic stimuli, this study demonstrated that hard clam can modulate defense-related mechanisms according to the nature of the invader. Additionally, comparison of defense response to QPX challenge in QPX-resistant (NY) and susceptible (FL) clam broodstocks revealed differences that could be related to the susceptibility of clams to this opportunistic parasite.

4.1. Differential defense and stress response of M. mercenaria following bacterial and QPX challenge

Several previous studies investigated bivalve response to pathogenic challenge using cellular and biochemical approaches (Allam et al., 2000a; Cheng et al., 1978; Mohandas and Cheng, 1985; Ordás et al., 2000) as well as molecular approaches (Gueguen et al., 2003b; Tanguy et al., 2004, part III - chapter 2). However, only a few studies focused on the differential response of invertebrate mollusks to different pathogenic stimuli (Adema et al., 2010; Costa et al., 2009).

Our PCA results showed significant effects of both bacterial and QPX challenge on clam hemolymph profile after 3 days (Fig. 5.5A). Differences between control and challenged clams were particularly marked for THC (Table 5.4). Numerous studies have shown changes in the number and types of hemocytes in mollusks experimentally challenged with foreign materials and these changes have been considered as defense response against pathogens (Allam et al., 2000a; Anderson et al., 1995; Ford et al., 1993b; Ordás et al., 2000). Changes in THC are usually difficult to interpret since increase in total number of circulating hemocytes may result from either movement of the cells from tissues into circulation or proliferation of the cells (Pipe and Coles, 1995). Interestingly, QPX caused a rapid change in THC, as well as overall hemolymph profile assessed by PC1 in NY clams as soon as 16 h after challenge as compared to their controls in contrast to individuals injected with bacteria (NY-b) and despite absence of significant difference between extracted components. Difference in response time could be related to the involvement of different mechanisms of pathogen recognition as well as different signaling pathways. Perrigault et al. (part III – chapter 2) showed that the dynamics

of molecular responses in *M. mercenaria* following QPX challenge were different whether the mucoid material surrounding parasite cells was removed (as in this study) or not. For instance, that prior study suggested that the removal of the protective mucus layer may enhance host response by facilitating the presentation and recognition of QPX pathogen-associated molecular patterns (PAMPs) by the host. Absence of mucus surrounding QPX could also explain the rapid increase in phagocytosis activity in NY-q compared to NY-b and NY-c at 16 h (Table 5.3). Stimulated ROS production was generally lower in challenged clams at 16 h and 3 days and could be related to the virulence of living bacteria and QPX. The antioxidant enzymes catalases, which are able to metabolize some ROS, were reported in bacteria and are likely present in *V. alginolyticus* (Bramble and Anderson, 1997; Hauton et al., 2000). Similarly, previous studies demonstrated a reduction in ROS production by hemocytes from *Crassostrea virginica* exposed to extracellular products (ECP) from its alveolate parasite *Perkinsus marinus* (Anderson, 1999a; Volety and Chu, 1995). Cytotoxicity of QPX ECP to *M. mercenaria* hemocytes was previously demonstrated (part II – chapter 1) but the specific effect of QPX on ROS production remains unknown. It should be stated, however, that both ROS production and phagocytosis were normalized to THC. Therefore, the rapid increase in phagocytic activity of hemocytes may be a direct result of the increase in the proportion of granulocytes because this cell type is well known to mediate phagocytosis in different clam species including *M. mercenaria* (Tripp, 1992b). Granulocytes also represent the main contributors to ROS production by clam hemocytes (Hégaret et al., 2003) although our results showed that the increase in percent granulocytes was concomitant to a decrease in ROS production. Production of ROS during immune response is thought to play significant roles in phagocyte-mediated killing of microorganisms (Adema et al., 1991; Torreilles et al., 1996). Our results suggest that the mechanisms controlling phagocytic activity and ROS production in *M. mercenaria* following *V. alginolyticus* and QPX challenge are different. Analysis of phagocytosis and ROS production without normalization to cell numbers (e.g. normalized to blood volume) showed significant increase of basal ROS production in challenged (NY-b and NY-q) clams ($p < 0.021$) as well as increase in hemocyte phagocytic activity at 16 h and 3 days post challenge (Data not shown).

Without taking into consideration the response time following challenge, standard cellular and humoral parameters (THC, PDC, ROS, phagocytosis, and protein concentration in plasma) generally exhibited similar trends during bacterial and QPX challenge. Conversely, anti-QPX activity in plasma and hemocyte resistance to QPX ECP were different whether clams were injected with bacteria or with QPX. These bioassays were specifically developed to investigate the interactions between components of *M. mercenaria* defense system and QPX (part II – chapter 1, part III – chapter 1) in contrast to standard defense parameters measured which are more related to the overall immune status of clams and general response to any microbial challenge. Interestingly, anti-QPX activity in plasma as well as hemocyte resistance to QPX ECP in NY clams challenged with *V. alginolyticus* exhibited a similar profile as controls in opposition to changes in these parameters among NY clams challenged with QPX. These results highly suggest that hard clams differentially respond to challenge with various pathogens. Similarly, differential induction of Mm-Fer and Mm-MT expression in gill tissues was detected in NY clams challenged with QPX as compared to controls and to those exposed to bacteria. Ferritin and metallothionein were previously identified during PAMP or microbial challenge (Gestal et al., 2007, part III - chapter 2; Wang et al., 2008b). Iron is an essential nutrient for virtually all cells and its involvement in innate immunity and pathogenicity has been previously demonstrated (Ong et al., 2006). Host ferritin sequesters iron and makes it unavailable for invading pathogens (Ong et al., 2006). The negative effect of iron chelators has been demonstrated on the *in vitro* growth of the protistan parasite *P. marinus* (Gauthier and Vasta, 1994) although the effect of iron on QPX growth and survival remains unknown. Similarly, metallothioneins are modulated by pathogenic stimuli and inflammatory mediators (Coyle et al., 2002; Jenny et al., 2006) and are thought to provide protection to the host from the deleterious effects of pathologic or environmental stress (Anderson et al., 1999).

4.2. Comparison of defense and stress response following QPX challenge in NY and FL clams

The second objective of this study was to compare defense response of two hard clam broodstocks to QPX challenge. Previous studies demonstrated variation in

M. mercenaria susceptibility toward QPX among different clam stocks with higher resistance of northern clam broodstocks compared to southern broodstocks (Dahl et al., 2008; Ford et al., 2002b; Ragone Calvo et al., 2007). Ragone Calvo (2007) suggested that both host genotype and environmental parameters were important determinants in clam susceptibility to QPX disease and the impact of genetic variation on overall performance of geographically different hard clam populations was previously demonstrated (Camara et al., 2006). Therefore, two different clam broodstocks that were previously shown to display different susceptibility toward QPX disease (Dahl et al., 2008; Dahl et al., 2010) were used in this study with one batch of QPX-susceptible clams from Florida and another of resistant clams from New York.

Assessment of individual defense parameters and results from PCA analysis and extracted component 1 from NY and FL clams demonstrated a significant difference in the hemolymph profile between the clam broodstocks (Fig. 5.5B). Control clams from NY exhibited significantly higher THC and percentage of granulocytes than FL-c clams. Similarly, phagocytosis activity, protein concentration in plasma and anti-QPX activity were lower in FL-c clams compared to NY-c clams. Similar observations of lower immune performances in FL clams, particularly with regard to anti-QPX activity, were also made in a previous study (part III – chapter 1, part IV – chapter 2) and could be related to the susceptibility of this broodstock compared to NY clams (Dahl et al., 2008; Dahl et al., 2010). La Peyre et al. (1995a) compared defense parameters in the closely related oysters *C. virginica* and *C. gigas* and found lower proportions of granulocytes and associated phagocytosis in *C. virginica* . The authors concluded that the difference in granulocyte count could be associated with the higher susceptibility of *C. virginica* to *P. marinus* (La Peyre et al., 1995a). Higher THC was also observed in hemolymph from *C. virginica* populations that are resistant to *Haplosporidium nelsoni* (MSX) as compared to susceptible oysters and lower proportions of granulcoytes were reported in oysters affected by this parasite (Ford et al., 1993b). It should be noted, however, that defense parameters in control NY clams displayed some variability during the experiment with higher phagocytosis activity and hemocyte resistance to QPX ECP at Day 3 compared to the other sampling times. Similarly, significant variations in PDC were observed between controls and challenged NY clams at 3 days but PDC of control

clams was relatively high compared to PDC values at other sampling times. These variations may be related to wounding and stress inflicted during the injection of sterile seawater into the pericardial cavity of control clams. As a matter of fact, prior studies showed significant changes in hemocyte parameters following the injection of sterile seawater into clam tissues including in *M. mercenaria* (Allam et al., 2000a; Allam et al., 2006). Despite this potential limitation, temporal differences between QPX challenged clams and their respective controls were observed.

PCA analysis revealed a significant difference in the effects of QPX challenge on QPX-susceptible (FL) and –resistant (NY) clam broodstocks. For instance, NY clams challenged by QPX exhibited both significant response and dynamics to QPX challenge whereas similar and non-significant relationship was observed between FL-q clams and their respective controls during the 4-week experiment. PC1 from NY-q clams increased significantly between 3 days and 4 weeks ($p = 0.018$) and was similar to that from NY-c at 4 weeks (Fig. 5.5B). Trends in PC1 were concomitant to changes in THC over time. In fact, THC and percentage of granulocytes were similar at 4 weeks in challenged and control NY clams whereas increase of THC as well as reduction in the percentage of granulocytes were observed in FL-q clams at 4 weeks compared to FL-c. The transitory and significant nature of NY clam response to QPX challenge suggests an elimination of QPX in these clams after 4 weeks in agreement with prior findings using the same experimental conditions and procedures (part III – chapter 2). On the other hand, changes in immune parameters in challenged FL clams suggest ongoing infection in this batch after 4 weeks. Of particular interest is the fact that THC in FL-q clams was significantly higher after 4 weeks compared to FL-c clams but was lower between 3 days and 4 weeks. Previous studies on QPX as well as other bivalve diseases reported changes of THC during pathogenic challenge and explained these variations by active infiltration of hemocytes toward infection sites (Ford et al., 1993b), part IV – chapter 2). QPX disease is usually characterized by the presence of focal lesions often located in clam mantle tissues (Dahl and Allam, 2007; Smolowitz et al., 1998). Dynamics of QPX disease could also explain observed trends in Mm-MT and Mm-FER transcription levels with higher expression of metallothionein and ferritin in gills of QPX challenged clams at 3 days (systemic response) whereas gene expression appeared higher in the mantle, especially in FL-q clams, after 4 weeks.

Furthermore, specific responses of FL clams to QPX infection confirmed difference in immune response between northern and southern clam broodstocks to QPX and highlighted the susceptibility of FL clams to the parasite. Basal ROS production in hemocytes from FL-q clams increased 3 days after challenge compared to controls but FL-q capacity to induce production of ROS following zymosan stimulation was reduced. Although ROS have been recognized as important antimicrobial mechanisms in phagocytes, high basal ROS can also have deleterious effects when their level exceeds the capacity of antioxidant mechanisms of the host (Adema et al., 1991; Torreilles et al., 1996). Interestingly, high basal ROS production in FL-q clams was associated with increased transcription levels of Mm-MT and Mm-Fer in hemocytes. Anderson et al. (1999) investigated the relationship between metallothionein and ROS in oyster hemocytes and demonstrated the ROS scavenging properties of metallothionein and its role in protection against oxidative stress. Similar cytoprotectant property from oxidant damage was also suggested for ferritin in cultured endothelial cells (Balla et al., 1992).

Major increase of protein concentrations was noted in plasma from FL-q clams at 3 days compared to their controls. This increase was associated with a massive decrease in the percentage of granulocytes in hemolymph by almost 40 % compared to controls. It is probable that protein increase in plasma was a consequence of protein release by hemocyte degranulation in response to QPX stimulus. For instance, FL-q response at day 3 shares similarities with acute phase response (APR) which is defined as all alterations of homeostasis following inflammatory stimuli causing changes in broad array of metabolic, endocrine and physiologic functions (Kushner, 1988). One characteristic of APR, particularly well studied in vertebrates, is the acute phase protein (APP) that results in an increase of protein concentration in plasma by 25 % or more following stimulus (Bayne and Gerwick, 2001; Kushner, 1988). Acute phase proteins in mammals include complement component proteins, metal binding proteins and are involved in repair of tissue damage, in fighting infection, and in restoring homeostatic state (Gabay and Kushner, 1999; Steel and Whitehead, 1994). In invertebrates, investigation of APR was previously performed in the echinoderm *Asterias forbesi* by Beck et al. (2002) who described ferritin as an APP. APR and associated APP could explain the overexpression of metallothionein and ferritin in

hemocytes from FL-q clams at Day 3, and as a matter of fact both Mm-MT and Mm-Fer covariated with protein concentrations in PCA analysis.

Conclusion

In conclusion, this study characterized metallothionein (Mm-MT) and ferritin (Mm-Fer) genes in the clam *M. mercenaria* and depicted the induction of both cellular and humoral defense parameters in clams in response to bacterial (*V. alginolyticus*) and protistan (QPX) challenge. The response of clams included common processes against both microorganisms but specific assays (anti-QPX activity in plasma and resistance of hemocytes to QPX ECP) as well as Mm-MT and Mm-Fer transcription levels showed specific response of *M. mercenaria* to each challenge, likely as a result of specific molecular interactions between *M. mercenaria* and the pathogens *V. alginolyticus* and QPX. Additionally, comparison of defense response from clams originating from different broodstocks demonstrated that NY clams were able to mount effective response to the presence of QPX that seems to lead to the elimination of the parasite whereas susceptible FL clams were less effective in eliminating the parasite despite the induction of what appears to be an acute phase response.

Acknowledgments

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Part IV

Effect of environmental parameters

Part IV

Chapter 1

Effects of environmental factors on survival and growth of Quahog Parasite Unknown (QPX) *in vitro*

Adapted from **Perrigault M.**, Buggé D.M., Allam B. Effects of environmental factors on survival and growth of Quahog Parasite Unknown (QPX) *in vitro*. **Journal of Invertebrate Pathology**. 104 (2) : 83-89.

Abstract

Quahog Parasite Unknown (QPX) is a protistan microorganism associated with mass mortalities of hard clams (*Mercenaria mercenaria*) along the northeastern coasts of the United States and maritime Canada. Because several studies indicate modulatory effects of prevailing environmental parameters on disease outbreaks, this study tested the effect of major environmental parameters (temperature, salinity and oxygen concentration; individually or combined) on QPX survival in artificial seawater and parasite growth in culture media *in vitro*. Three QPX isolates from two different geographic locations were compared. Results indicated that *in vitro* growth of QPX was optimal in standard culture medium at 34 ppt between 20 and 23 °C. Additionally, significant differences in temperature optima were observed for geographically distinct QPX isolates ($p < 0.001$) confirming previous studies suggesting the existence of different QPX strains (or ecotypes). When tested in seawater, QPX exhibited opposite trends with higher survival at 15 °C and 15 ppt. Results also demonstrated limited survival and growth of QPX under anoxic conditions. Additionally, results showed that the parasite is able to survive extreme temperatures (-12 °C to 32 °C) suggesting that QPX could overcome short periods of extreme conditions in the field. These results contribute to a better understanding of interactions between QPX and its environment, but potential impacts of environmental conditions on QPX disease development need further work as it also involves clam response to these factors.

1. Introduction

Quahog Parasite Unknown (QPX) is a protistan pathogen of the hard clam *Mercenaria mercenaria*, infecting both cultured and wild populations. QPX disease has resulted in hard clam mortalities along the east coast of North America from Virginia to maritime Canada (Dove et al., 2004; Ford et al., 2002b; Ragone Calvo et al., 1998; Smolowitz et al., 1998; Whyte et al., 1994). QPX is a member of the thraustochytrid family within the phylum Labyrinthulomycota (Ragan et al., 2000). Labyrinthulomycota are ubiquitous in marine environments and the ecology of several species belonging to this phylum has been investigated (Raghukumar, 2002). QPX ecology remains relatively understudied. The parasite was successfully detected in seawater and sediment (Gast et al., 2008; Liu et al., 2009) and previous studies also demonstrated its ability to grow on degraded macroalgae material *in vitro* (Buggé and Allam, 2007) but available information on the effects of environmental parameters on the parasite is limited (Brothers et al., 2000).

Environmental parameters strongly affect host-pathogen interactions by modulating host defenses and pathogen survival and virulence. Temperature and salinity have been shown to be key environmental factors controlling infection and disease progression of several pathogens of marine bivalves, including *Perkinsus marinus*, *Haplosporidium nelsoni* and *Bonamia sp.* (Auzoux-Bordenave et al., 1995; Carnegie et al., 2008; Chu and Greene, 1989; Ford and Haskin, 1988). Indications that temperature and salinity affect QPX distribution and/or associated disease development were previously reported. For instance, higher QPX prevalence and associated clam mortalities occur during summer in Canada, Massachusetts and New York (Dove et al., 2004; MacCallum and McGladdery, 2000; Smolowitz et al., 1998). On the other hand, Ragone Calvo et al. (1998) found QPX disease to be absent from areas with moderate salinities (15 to 25 ppt) and surveys of hard clams in New York suggested that low dissolved oxygen could be associated with higher QPX prevalence (Allam, unpublished). Additionally, QPX was identified in different locations along the east coast of the United States but never south of Virginia despite the fact that *in vivo* and *in vitro* studies demonstrated that southern clam stocks were more susceptible to QPX infection than northern stocks (Dahl et al., 2008; Ford et al., 2002b; Ragone Calvo et al., 2007, part II - chapter 1).

Recent studies suggested the presence of different stains of QPX exhibiting variable virulence *in vivo* (Dahl et al., 2008, part II - chapter 1) and *in vitro* (part II – chapter 1). In the case of *P. marinus*, Bushek and Allen (1996a) also suggested that isolates obtained from different locations represented different strains exhibiting various degrees of virulence and different environmental tolerances.

This study was designed to investigate the effect of three major environmental factors (temperature, salinity and oxygen concentration) on QPX survival and growth *in vitro*. The growth of QPX isolates from different geographical locations was compared under ranges of temperatures and salinities to identify specific optima of each QPX isolate. Additionally, some environmental parameters were associated to evaluate their combined effects on QPX survival and growth. Finally, the ability of QPX to survive under extreme temperature conditions that occasionally occur in the field was also investigated.

2. Materials and methods

2.1. QPX cultures

Two New York isolates of QPX, NY0313808BC7 (NY1-QPX) and NY0314220AC6 (NY2-QPX), were obtained from nodules of infected hard clams collected in Raritan Bay, NY in October 2003 (Qian et al., 2007). Massachusetts QPX isolate (MA-QPX) was obtained from the American Type Culture Collection (ATCC 50749). Isolation and subculture were performed in 25-cm^2 culture flasks at 23 ºC using Minimal Essential Medium Eagle (MEM, Sigma M06440) adjusted to 34 ppt according to the methods described by Kleinschuster et al. (1998). In all experiments, survival and growth of QPX *in vitro* were measured using a fluorometric technique based on quantification of QPX biovolume. This technique uses the dye fluorescein di-acetate (FDA) to measure esterase activity of live cells as previously described by Buggé and Allam (2005) and Perrigault et al. (part III – chapter 1).

2.2. Effect of temperature on the growth of different QPX isolates

QPX isolates (NY1-QPX, NY2-QPX and MA-QPX) were subcultured in MEM (34 ppt) at 23 ºC for 7 days to reach exponential growth phase. Aliquots (100 µl) of each culture were transferred in quadruplicate to 24-well plates containing 2.5 ml of MEM culture medium and incubated at 3, 8, 17, 20, 23, 29 and 32 ºC. QPX biovolume was measured by the FDA technique at t_0 and days 1, 3, 7, and 14. Briefly, 100 µl of each culture was transferred into a black 96-well plate (in duplicate) and 12 µM of FDA was added to each well. Plates were incubated in the dark at room temperature (RT) for 30 min and fluorescence was measured at 485 nm excitation and 535 nm emission (Wallac 1420 plate reader). Data are presented as percentage of the maximal fluorescence measured for each isolate.

2.3. Effect of salinity on the growth of different QPX isolates

A similar assay was designed to measure QPX growth under different salinity conditions *in vitro*. Modified culture medium at 19 ppt (MEM-19 ppt) was prepared by removing NaCl from the standard preparation and the different treatments were made by supplementing MEM-19 ppt with specific amount of NaCl to reach 22, 25, 28, 31, 34 and 37 ppt. Aliquots (100 µl) of exponential culture of each QPX isolate were transferred in triplicate into the wells of a 24-well plate and supplemented with 2.5 ml of MEM adjusted to the specific salinities. Plates were incubated at 23 ºC and QPX biovolume was measured at t_0 and days 3, 7, and 14 using the FDA technique as described above. Data are presented as percentage of the maximal fluorescence measured for each isolate.

2.4. Combined effects of temperature and salinity on QPX

Combined effects of temperature and salinity on *in vitro* QPX growth in MEM were investigated in black 96-well plates according to Perrigault et al. (part III – chapter 1). Briefly, an exponential culture of NY1-QPX was harvested by centrifugation (15 min at 600 g) and QPX cells were resuspended in filtered artificial seawater (FASW) (part III – chapter 1). Five hundred QPX cells suspended in FASW were transferred into the wells of a black 96 well-plate containing MEM adjusted to

15, 25 and 35 ppt (final salinities). Replicate plates were incubated at 15 °C or 23 °C. Each condition (temperature/salinity combination) was performed in triplicate. QPX biovolume was measured at t_0 and days 2 and 4 by the FDA technique. Data are presented as relative fluorescence unit (RFU) after removing background fluorescence generated by QPX-free MEM.

A second assay was performed to investigate the combined effects of temperature and salinity on *in vitro* QPX survival in artificial seawater without MEM supplement. As previously, an exponential culture of NY1-QPX was harvested and QPX cells were resuspended in FASW. One hundred µl of QPX suspension (1×10^5 QPX cells) were transferred into 24 well-plates containing 1.9 ml of FASW adjusted to 15, 25 and 35 ppt (final salinities). Plates were incubated at 15 °C and 23 °C. Each condition (temperature/salinity combination) was performed in duplicate. QPX biovolume was measured at t_0 and days 4, 7 and 10 by the FDA technique. Data are presented as relative fluorescence unit (RFU) after removing background fluorescence generated by QPX-free FASW.

2.5. Effect of anoxia on QPX

To evaluate the effect of anoxia on *in vitro* QPX growth, NY1-QPX was subcultured in quadruplicate in 2.5 ml MEM (34 ppt) in 24-well plate and incubated for 25 days in an anoxic glove-box incubator with a 5 % CO_2, 10 % H_2, and 85 % N_2 gas mix at room temperature (22 ± 1 °C). Controls (NY1-QPX in MEM at 34 ppt) were incubated in 24-well plate under ambient air conditions at room temperature. The FDA technique was used to measure QPX biovolume at t_0, day 7 and day 25.

To study the effect of anoxia on *in vitro* QPX survival, 100 µl of QPX cultures exposed to anoxic conditions for 7 and 21 days as well as controls (ambient conditions for 21 days) were transferred to new culture flasks containing 4 ml MEM (34 ppt) and submitted to standard culture condition (ambient air, 23 °C, 3 replicates). QPX biovolume was assessed after 7 days by the FDA technique.

2.6. Tolerance of QPX to extreme temperatures

QPX cultures were also exposed to extreme high and low temperatures that may occasionally occur in the natural environment. Exponential cultures of NY1-QPX were transferred to fresh MEM (34 ppt) and exposed to extreme temperatures for increasing periods of time. Cultures were incubated at -12 ºC (inter-tidal freezing conditions), 3 ºC (sub-tidal winter), or 32 ºC (inter-tidal summer). After exposure to temperatures for the allotted time period (6 h, 24 h, 48 h, 72 h, 1 week and 3 weeks), 100 µl culture aliquots were transferred to new culture flasks containing 4 ml MEM (34 ppt) and incubated at 23 ºC (in triplicate). These transferred cultures were monitored under microscope on a daily basis for 4 weeks to determine whether QPX cells could survive the tested temperatures and propagate when returned to standard conditions.

2.7. Statistical analysis

Data were analyzed using SigmaStat (Ver. 3.1, Systat Software, Inc., San Jose, California, USA) statistical software. Analysis of variance (1- or 2-way ANOVA according to each data set) was used to compare the effects of environmental parameters (temperature, salinity and oxygen concentration) individually or combined on QPX growth and survival within each sampling time. Effect of incubation time within each treatment on QPX growth and survival was analyzed by repeated ANOVA. ANOVA treatments that generated probability values below 0.05 were followed by a Holm-Sidak post-hoc test comparing different conditions. All differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Effect of temperature on the growth of different QPX isolates

Results demonstrated a clear effect of temperature on the *in vitro* growth of QPX with optimal proliferation between 20 and 23 °C (Fig. 6.1). MA-QPX isolate reached maximal biovolume (as measured by fluorescence) at 20 ºC, whereas NY

isolates had optimal biovolume at 23 ºC (Fig. 6.1). Statistical analysis showed that both NY-QPX isolates were significantly different from MA-QPX at 23 °C (p < 0.001, Holm-Sidak post-hoc test). Incubation of all QPX isolates at temperatures below or above these optima reduced the proliferation of QPX to about 6 % and 9 % (of maximal biovolume) at 3 °C and 32 °C respectively.

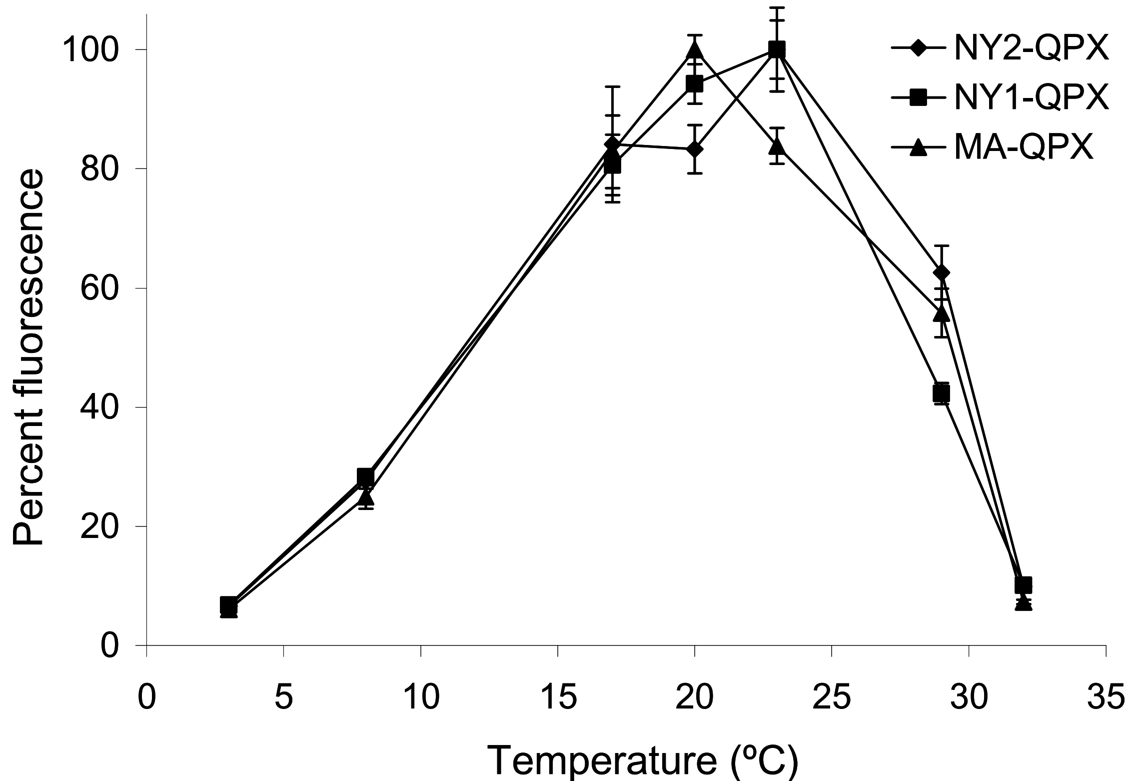


Fig. 6.1. Biovolume of QPX isolates cultured in MEM (34 ppt) and incubated at a range of temperatures from 3 °C to 32 °C. Data are presented as percentage of the maximal fluorescence measured for each isolate at Day 14 (Mean ± SEM, n = 4 replicates).

3.2. Effect of salinity on the growth of different QPX isolates

The growth of QPX at different salinities demonstrated the importance of this parameter on parasite proliferation *in vitro* over the 14 days of culture (Fig. 6.2). However, the range of tested salinities caused limited effect on QPX biovolume compared to temperature effects reported above, with minimal proliferation of MA-QPX at 22 ppt. Overall, no significant differences were observed between NY-QPX and MA-QPX (2-way ANOVA) and all tested QPX isolates exhibited maximal

biovolume at 34 ppt. However, differences of trends were observed among isolates: MA-QPX showed higher susceptibility to low salinity (22 ppt to 28 ppt) with biovolumes ranging from 44 % to 54 % of the maximal biovolume measured at 34 ppt as compared to NY-QPX isolates (55 to 68 %). Interestingly, trends were inverted at higher salinity (37 ppt) with higher biovolume for MA-QPX compared to NY-QPX isolates (Fig. 6.2).

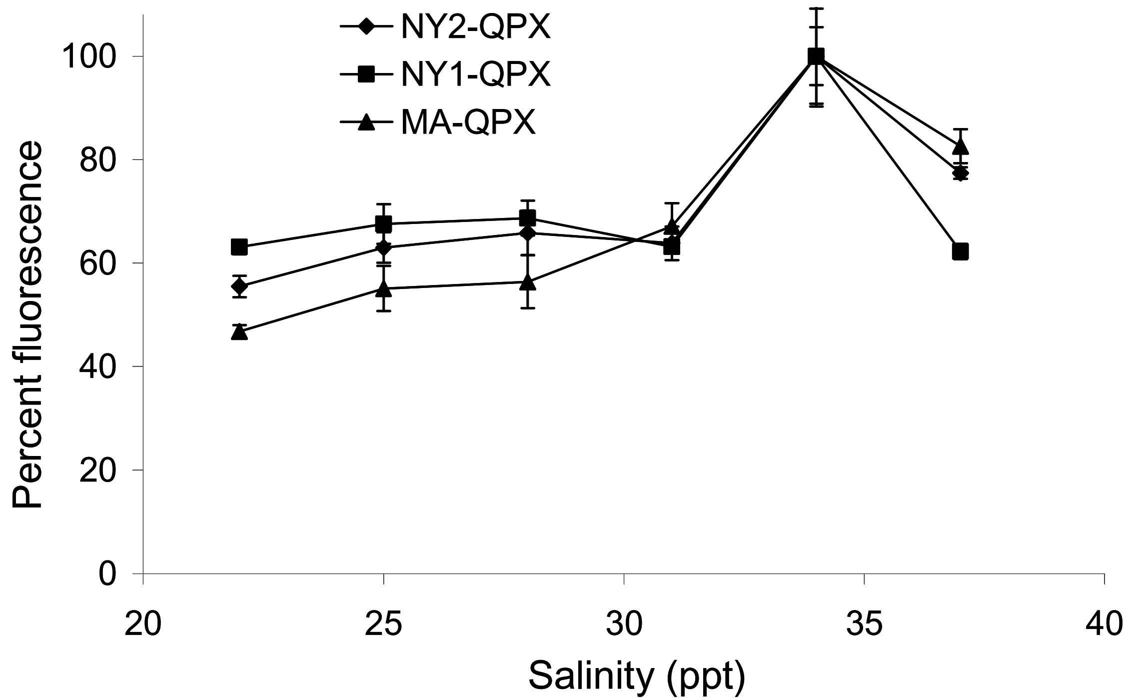


Fig. 6.2. Biovolume of QPX isolates cultured at 23 °C in MEM adjusted to a range of salinities from 22 ppt to 37 ppt. Data are presented as percentage of the maximal fluorescence measured for each isolate at Day 14 (Mean ± SEM, n = 3 replicates).

3.3. Combined effects of temperature and salinity on QPX

The third experiment investigating the combined effects of temperature and salinity on *in vitro* QPX growth confirmed previous assays. For instance, higher QPX biovolumes were observed at 35 ppt and 23 °C and decreasing the salinity to 25 ppt and 15 ppt or the reduction of temperature to 15 °C resulted in a significant decrease of QPX biovolume at Day 4 ($p < 0.02$, 2-way ANOVA and Holm-Sidak post-hoc test, Fig. 6.3, Table 6.1). Additionally, a reduction in QPX biovolume was observed at 15 ppt for both tested temperatures with a significant decrease of QPX biovolume

between t_0 and Day 4 at 23 °C (p = 0.007, repeated ANOVA). Interestingly, incubation of QPX cultures at 25 ppt and 35 ppt under sub-optimal temperature (15 °C) yielded similar biovolumes in contrast to similar cultures maintained at 23 °C (Fig. 6.3).

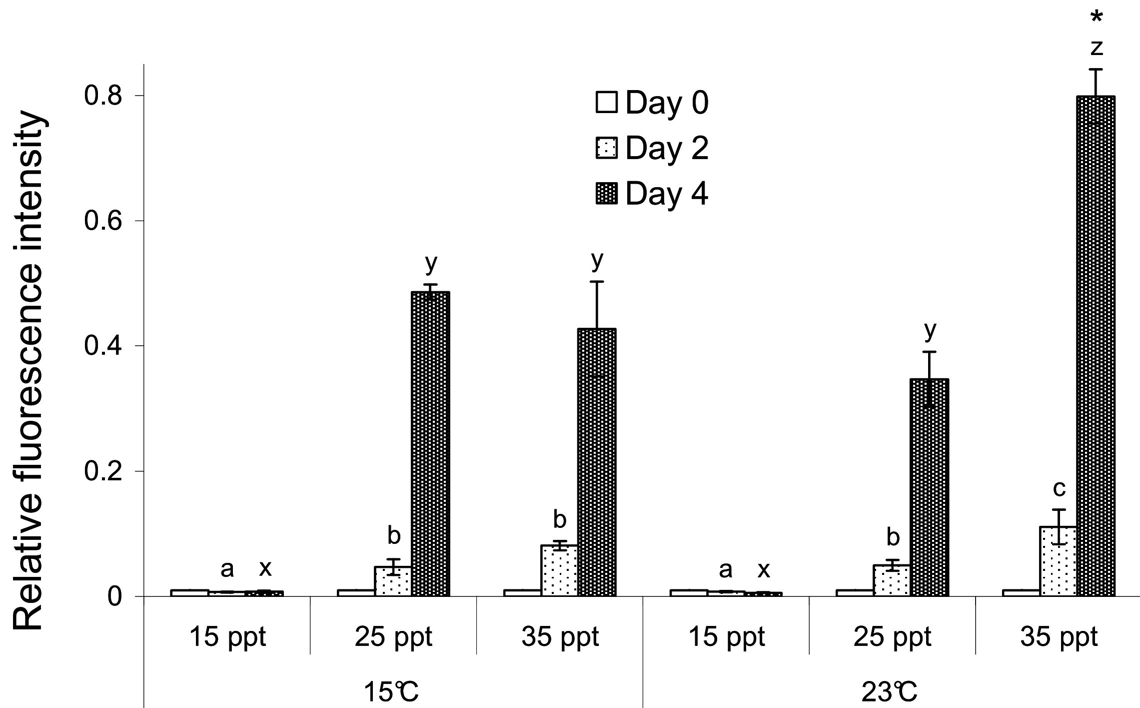


Fig. 6.3. Biovolume (relative fluorescence units, Mean ± SEM, n = 3 replicates) of NY1-QPX (8BC7) in MEM adjusted to different salinities and incubated at 15 °C or 23 °C. Fluorescence was measured at t_0 and after 2 and 4 days of incubation. Letters indicate which experimental treatments shared statistically equivalent (same letters) or different (different letters; Holm-Sidak post-hoc test, $p < 0.05$) QPX biovolume means among cultures at different salinities within each temperature condition at Day 2 (a, b, c), or Day 4 (x, y, z). * indicates significantly higher biovolume in this treatment at Day 4 compared to its counterpart incubated at 15 °C.

Table 6.1. Summary of 2-way ANOVA testing the effect of temperature and salinity on QPX growth in MEM.

Source of variation	Degree of freedom	Sum of squares	Mean squares	F	*p*
Day 2					
Temperature	1	0.019	0.019	0.782	0.394
Salinity	2	3.865	1.933	80.101	<0.001
Temperature x salinity	2	0.004	0.002	0.077	0.926
Day 4					
Temperature	1	0.001	0.001	0.066	0.801
Salinity	2	14.047	7.024	649.398	<0.001
Temperature x salinity	2	0.168	0.084	7.777	0.007

Investigations of *in vitro* QPX survival in artificial seawater under combined environmental conditions showed different trends (Fig. 6.4). Statistical analysis by 2-way ANOVA at each sampling time demonstrated a significant effect of temperature ($p < 0.03$, Table 6.2) on QPX survival as well as significant differences among salinity treatments (15 ppt *versus* 25 ppt and 35 ppt), particularly driven by cultures maintained at 23 °C ($p < 0.01$). QPX biovolume was roughly constant over time at 25 ppt and 35 ppt under 15 °C, and slightly increased at 15 ppt. At 23 °C, parasite biovolume was unchanged over time at 15 ppt whereas a marked decrease of QPX biomass was noted as soon as 4 days at 25 ppt and 35 ppt ($p < 0.008$, repeated ANOVA and Holm-Sidak post-hoc test, Fig. 6.4). Highest QPX biovolumes were observed at low salinity and temperature (Fig. 6.4). Microscopic observation of 35 ppt cultures maintained at 23 °C showed significant cell mortality characterized by the presence of cell debris as opposed to those held at 15 °C (Fig. 6.5), confirming that lower FDA uptake resulted from a reduction of QPX survival.

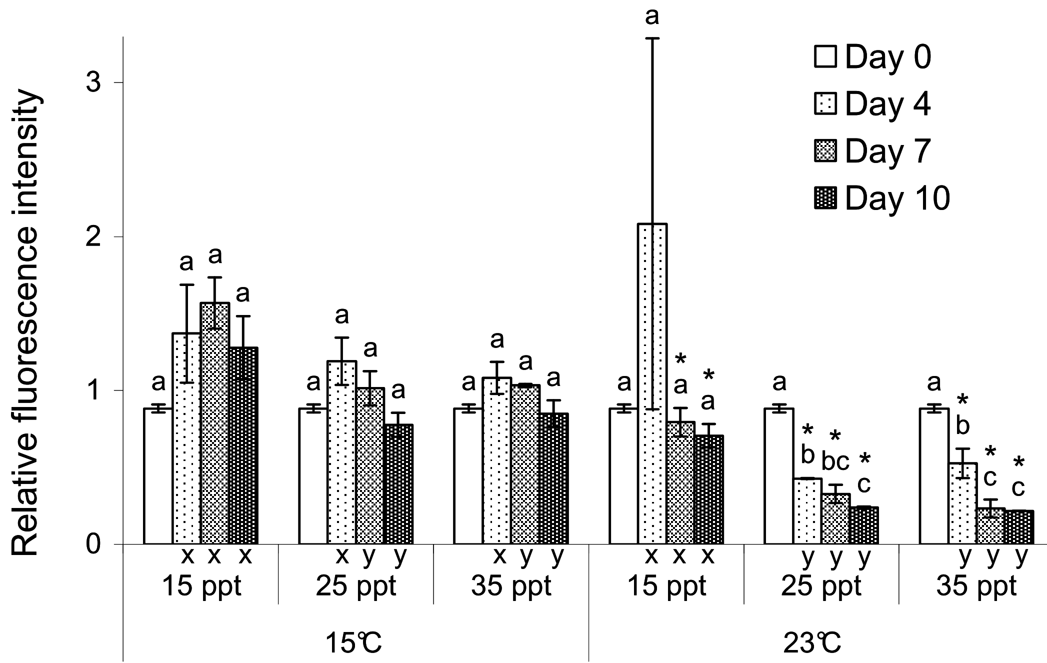
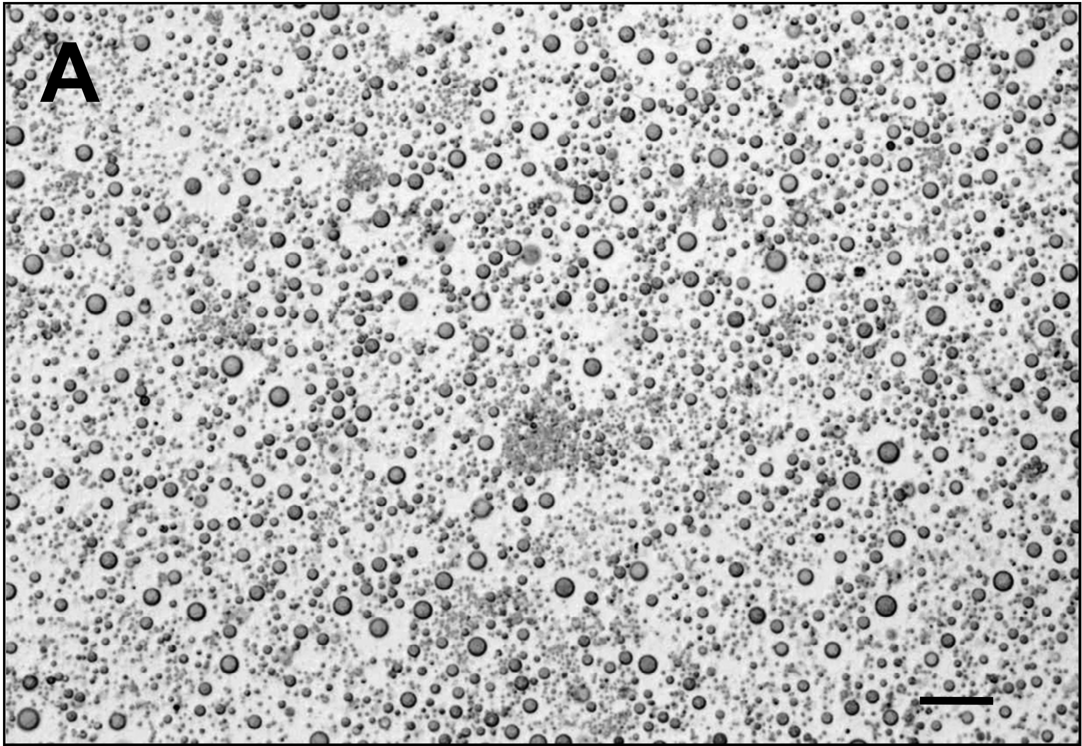


Fig. 6.4. Survival of NY1-QPX (8BC7) in artificial seawater adjusted to different salinities and incubated at 15 °C or 23 °C. Biovolume (relative fluorescence units, Mean ± SEM, n = 2 replicates) was measured for 10 days. Letters (a, b and c) indicate which experimental treatments shared statistically equivalent (same letters) or different (different letters; Holm-Sidak post-hoc test, $p < 0.05$) QPX biovolume means over time within each culture. Similarly, within each temperature, x and y indicate statistical differences in QPX biovolume means among cultures made at different salinities at the same time interval. * indicates significantly lower biovolume in this treatment compared to its counterpart incubated at 15 °C.

Table 6.2. Summary of 2-way ANOVA testing the effect of temperature and salinity on QPX survival in seawater.

Source of variation	Degree of freedom	Sum of squares	Mean squares	F	*P*
Day 4					
Temperature	1	0.124	0.124	8.019	0.03
Salinity	2	0.311	0.155	10.036	0.012
Temperature x salinity	2	0.194	0.097	6.258	0.034
Day 7					
Temperature	1	0.693	0.693	165.455	<0.001
Salinity	2	0.289	0.144	34.475	<0.001
Temperature x salinity	2	0.064	0.032	7.626	0.023
Day 10					
Temperature	1	0.615	0.615	330.77	<0.001
Salinity	2	0.314	0.157	84.452	<0.001
Temperature x salinity	2	0.0612	0.031	16.463	0.004



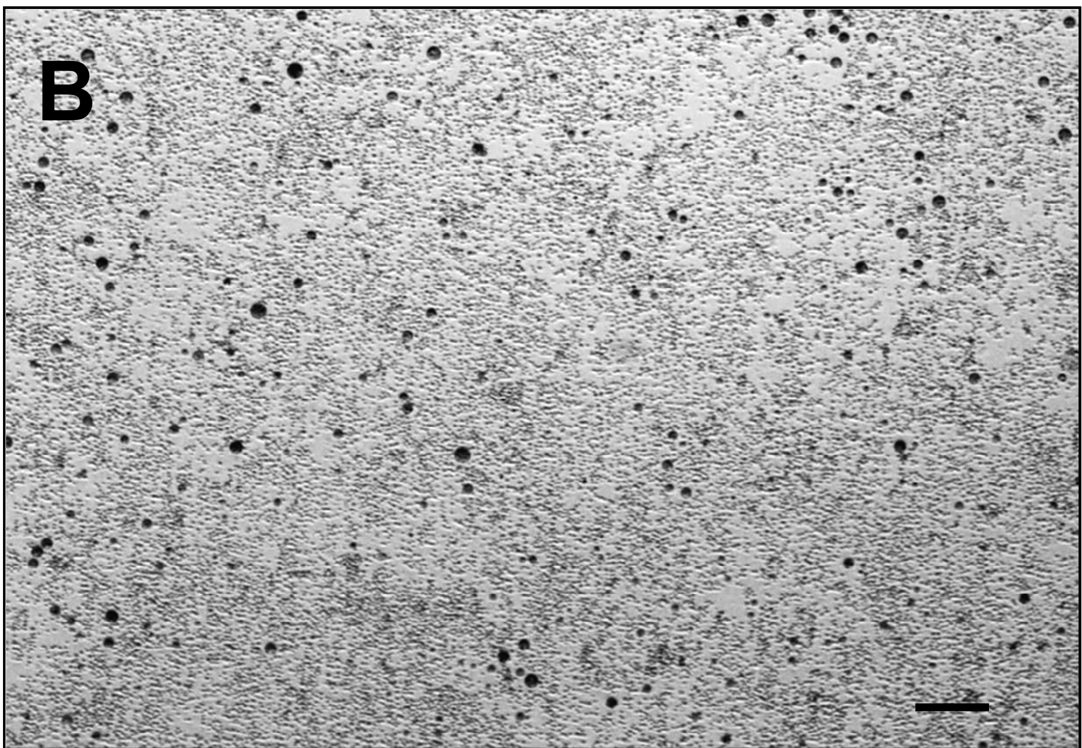


Fig. 6.5. NY1-QPX cells observed after 7 days in seawater at 35 ppt and incubated at 15 °C (A) or 23 °C (B). Scale bar = 50 μm.

3.4. Effect of anoxia on QPX

Anoxic conditions significantly reduced *in vitro* QPX growth ($p < 0.001$, 2-way ANOVA, Fig. 6.6a) without altering the pH of the culture medium as evaluated by change in culture color. Incubation of QPX cultures under anoxic conditions for 7 days slightly increased parasite biovolume compared to t_0 ($p < 0.03$, repeated ANOVA) but QPX growth was significantly reduced compared to controls ($p < 0.001$, 1-way ANOVA). At day 25, QPX biomass under anoxic conditions was no longer different from t_0. Additionally, QPX cultures exposed to anoxic conditions for 7 days displayed limited ability to resume proliferation after transfer to fresh medium under standard conditions (ambient air) compared to normoxic control cultures (Fig. 6.6b, $p < 0.001$, 1-way ANOVA). Longer exposure of QPX to anoxic conditions (21 days) further reduced parasite's survival and subsequent proliferation under standard conditions (Fig. 6.6b).

3.5. Tolerances of QPX to extreme temperatures

Results from experiments exposing QPX cultures to extreme temperatures for varying periods of time showed that parasite cells can survive incubation at -12 ºC for up to 24 h (Table 6.3). Cells from cultures incubated at -12 ºC for longer periods did not propagate when transferred to fresh MEM (34 ppt) and incubated at the standard temperature of 23 ºC. QPX was able to tolerate incubation at 3 ºC for up to one week without any observed change in the microscopic appearance of cultures. After 3 weeks of incubation at 3 ºC, QPX cells displayed morphological alterations characterized by a loss of membrane integrity and subcellular structures. These cells were unable to resume proliferation after transfer to standard culture conditions. Similarly, tolerance of QPX to 32 °C was limited to 1 week and no parasite proliferation was noted after subsequent transfer of cultures to standard conditions.

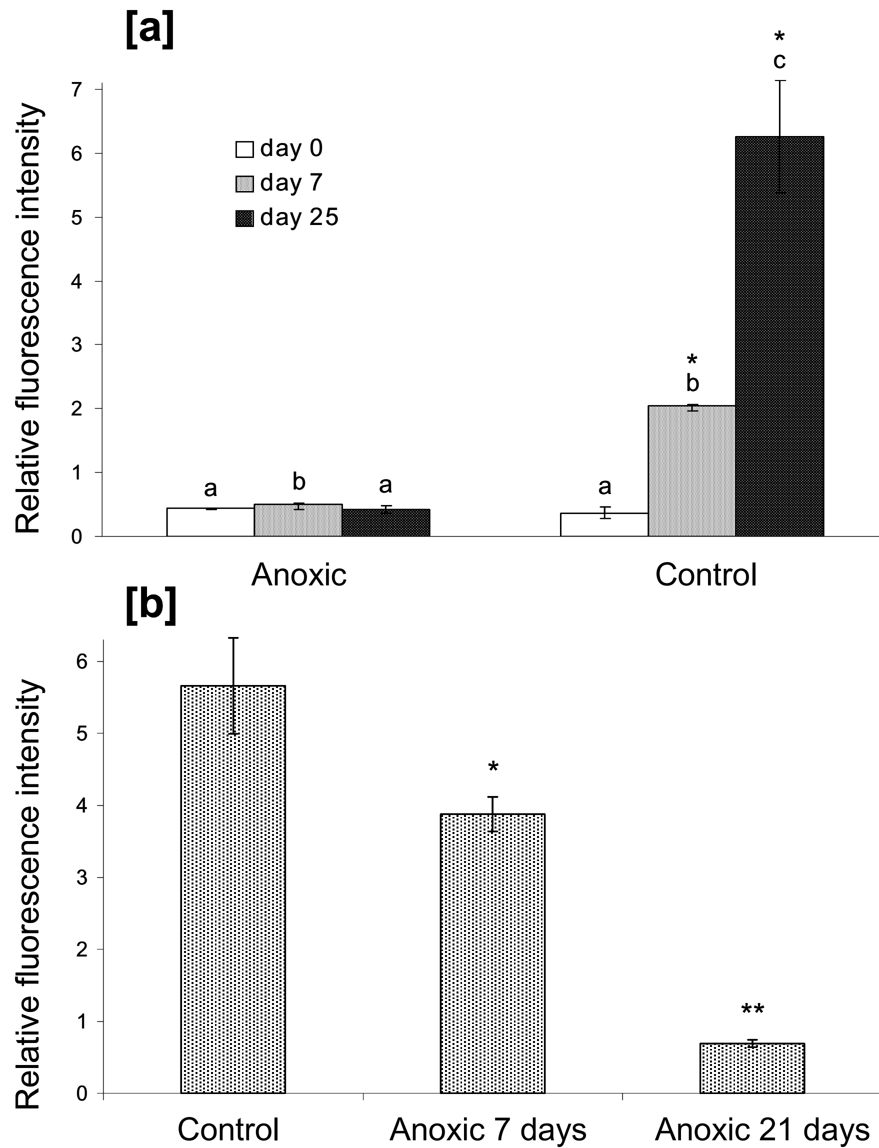


Fig. 6.6. Effects of anoxic conditions on QPX survival and growth. A: Biovolume (relative fluorescence units, Mean ± SEM, n = 3 replicates) of NY1-QPX (8BC7) in MEM (34 ppt) incubated under anoxic or standard conditions for 25 days at room temperature. Letters indicate which experimental treatments shared statistically equivalent (same letters) or different (different letters, Holm-Sidak post-hoc test, $P < 0.05$) QPX biovolume means within each culture over time. * indicates significant differences between both treatments for the same time interval. B: QPX abilities to grow under standard conditions after incubation in anoxic conditions for 7 and 21 days. Biovolume (relative fluorescence units, Mean ± SEM, n = 3 replicates) was measured after 7 days of incubation in normoxic conditions. Letters (a, b, c) indicate that all treatments are significantly different from each other (Holm-Sidak post-hoc test, $p < 0.05$).

Table 6.3. Effect of extreme temperatures on QPX survival. NY1-QPX (8BC7) cultures were maintained at -12 °C, 3 °C and 32 °C for different time intervals before transfer to standard culture conditions (MEM, 34 ppt, 23 °C) and monitoring of QPX growth over 4 weeks.

Treatment	Duration					
	6 hours	24 hours	48 hours	72 hours	1 week	3 weeks
-12 °C	+	+	-	-	-	-
3 °C	+	+	+	+	+	-
32 °C	+	+	+	+	+	-

(+) and (-) indicate presence and absence of QPX growth, respectively.

4. Discussion

Our experiments testing the effects of temperature, salinity and oxygen concentration demonstrated that all tested parameters influence the *in vitro* growth of QPX. All QPX isolates were able to grow under the tested range of salinities (22 to 37 ppt at 23 °C) with an optimal growth at 34 ppt (Fig. 6.2). Salinities below and above this optimum decreased QPX biovolume up to about 40 % of controls and only salinity as low as 15 ppt appeared to inhibit parasite growth in culture medium (Fig. 6.3). Temperature optima were also relatively narrow (20 °C – 23 °C) and temperatures below 15 °C and above 29 °C significantly reduced QPX growth to less than 50 % of the optimal growth measured for all tested isolates (Figs. 6.1).

MA-QPX isolate had optimal growth at a lower temperature (20 ºC) than NY-QPX isolates (23 ºC) (Fig. 6.1, $p < 0.001$). Similarly, differences between the NY and MA isolates were noted in their performance at salinities below and above 34 ppt (Fig. 6.2). These differences may be related to genetic or adaptive differences among the isolates to their environment. The existence of different QPX strains is also suggested by our previous studies that identified differences in *in vivo* (Dahl et al., 2008) and *in vitro* (part II – chapter 1) virulence of the same isolates. The existence of different strains has been proposed for other parasites of marine bivalves. For instance, Bushek and Allen (1996a) and Reece et al. (2001) demonstrated the

existence of different *P. marinus* strains based on virulence and genetic variability they observed among different isolates. Attempts to discriminate *P. marinus* isolates based on their temperature optima were not conclusive, but important regional components to the proliferation rates of the pathogen were noted (Ford and Chintala, 2006).

Results from our *in vitro* investigations of temperature effects on QPX growth matched well with the timing of major QPX epizootic events in the field. MacCallum and McGladdery (2000) observed highest QPX prevalence during summer time with temperatures at about 20 – 25 °C which cover the range of optimal QPX growth *in vitro*. Similarly, major previous QPX epizootics also occurred during summer in Massachusetts (Smolowitz et al., 1998) and New York (Dove et al., 2004). On the other hand, the absence of QPX disease south of Virginia despite intensive clam aquaculture could be explained by higher temperatures in these locations limiting the presence of QPX. As a matter of fact, Gast et al. (2008) reported that QPX was more abundant in the environment in Massachusetts compared to more southern locations (Virginia). Similarly, QPX disease was not detected in moderate salinity waters (15 to 25 ppt) of the Chesapeake Bay while it was present in the more saline ocean-side bays of Virginia (Ragone Calvo et al., 1998). The distribution of other bivalve parasites has been also reported to be dictated by salinity. For example, *in vitro* studies by Chu and Greene (1989) clearly demonstrated the impact of salinity on the survival and sporulation of *P. marinus*. However, the extrapolation of *in vitro* studies to the distribution of QPX disease in the field (MacCallum and McGladdery, 2000; Ragone Calvo et al., 1998; Smolowitz et al., 1998) is not simple since QPX disease investigations do not take into account the impact of environmental factors on host fitness and immune performance.

Our study showed about 20 % growth reduction for MA-QPX at 23 ºC compared to 20 ºC while previous work by Brothers et al. (2000) concluded that cultured QPX originating from Massachusetts clams had optimal growth at 24 ºC. Discrepancy between that study and our results could be related to the microscopic approach used by these authors to assess QPX growth which is problematic because of the production of viscous mucus by the parasite. Additionally, differences in temperature optima for MA-QPX may not be as contradictory as they appear, since

Brothers et al. (2000) did not investigate the specific range of temperature used in our study. It is highly possible that they also would have found optimal growth at 20 ºC if that temperature (± 1 ºC) was among their tested range of temperature. Similarly, these authors observed a regular increase of Massachusetts QPX growth between 20 ppt and 40 ppt and reported a maximal mucus production by the parasite at 34 ppt. It is unclear from our study whether the optimal proliferation of all isolates at 34 ppt truly reflects QPX requirements in the field or is more related to the *in vitro* maintenance of these cultures. For instance, all QPX isolates were routinely subcultured in MEM at 34 ppt for several months before their use in our study. Observed optima could be the result of QPX adaptation to the salinity of the standard culture medium. Long term subcultures at different salinities before testing might help determine if the optimal growth observed in our study at 34 ppt was related to intrinsic properties of QPX isolates or to specific adaptation to its culture environment.

Interestingly, our *in vitro* experiments demonstrated modulatory effects of combined environmental parameters. For instance, salinity had a major effect on QPX growth at 23 °C but parasite biovolume was similar at 25 ppt and 35 ppt when cultures were incubated at 15 °C (Fig. 6.3). As a matter of fact, QPX growth at 25 ppt was similar at 15 °C and 23 °C whereas parasite growth at 35 ppt increased only when cultures were maintained at 23 °C. This suggests that some environmental parameters could limit the impact of sub optimal conditions and facilitate QPX presence in its environment.

Our results on the combined effects of salinity and temperature on QPX survival in seawater exhibited opposite trends compared to assays performed in standard culture media. Reduced survival of QPX in seawater (25 to 35 ppt) at 23 °C described here (Fig. 6.4) is in agreement with a prior report (Buggé and Allam, 2007). Interestingly, our results suggest that QPX survives well in seawater at low temperature, and even better when both salinity and temperature are low. Cold temperatures could decrease the metabolic rates of QPX and therefore delay mortality of the parasite in nutrient-poor seawater whereas in nutrient-rich standard culture medium, higher temperature (23 °C) might stimulate parasite's metabolism and growth rates. Similar contradictions were observed between the *in vivo* and the *in*

vitro performances of the oyster pathogen *Bonamia ostreae* (Arzul et al., 2009). In that study, the survival of the parasite was higher at low temperature whereas *in vivo* studies highlighted the importance of warm temperature on seasonal parasite cycling (Carnegie et al., 2008). Alternatively, our fluorescein diacetate-based biotest is based on the activity of esterase in QPX cells which is directly linked to parasite's biovolume (Buggé and Allam, 2005). Despite the fact that the technique was validated in several studies and that microscopic observations clearly supported differences observed here (Fig. 6.5), the impact of QPX starvation on FDA uptake has never been investigated. Similar remarks about limitations of metabolic assays to assess cell proliferation (variation in cell size and metabolic activity) were made by Ford and Chintala (2006) on another metabolic biotest developed by Dungan and Hamilton (1995) for *P. marinus*.

Our results showed that QPX is sensitive to anoxia and a prolonged anoxic episode significantly reduced survival of the parasite *in vitro*. These results might not be surprising as anoxia induces severe alterations in many organisms. Similar studies of various degrees of hypoxia could provide additional information on QPX sensitivity to oxygen concentration in the environment. It is possible that very low levels of dissolved oxygen could limit the distribution of QPX in the marine environment. However, low levels of dissolved oxygen are known to depress immune systems of marine mollusks (Chen et al., 2007a; Matozzo et al., 2005). Therefore, hypoxic conditions in the field could affect both QPX and hard clams and resulting disease development might be difficult to forecast from laboratory experiments alone.

Our results also showed that QPX is able to survive extreme warm and cold temperatures. QPX cells tolerated 32 ºC and 3 ºC for up to 7 days, and -12 ºC for up to 24 hours. The ability of QPX cells to survive 3 and 32 ºC for up to 1 week suggests that QPX can tolerate very warm summer and cold winter temperatures that occur in the marine environment within the natural range of the parasite. These *in vitro* results could explain the persistence of QPX in enzootic areas (Gast et al., 2008).

In conclusion, this study provides new information about the effect of environmental parameters on QPX survival and growth *in vitro*. Most available information on QPX distribution in the field is based on disease prevalence in clams and may provide a partial picture of the real distribution of QPX itself in the

environment. Despite this limitation, conditions for optimal growth of the parasite *in vitro* observed in this study matched well with temperature and salinity ranges observed during highest QPX prevalence *in situ*. Additionally, tested QPX isolates reacted differently to tested temperatures suggesting specific adaptation to their geographical location and confirming previous work proposing the existence of different QPX strains. Our results also demonstrated that the effects of temperature and salinity as well as other parameters (environmental or nutritional) on QPX were different whether these factors are applied individually or combined with each other. Differences in QPX performance in assays using standard culture media and seawater highlight the need for extreme care in the extrapolation to the field of *in vitro* studies made under "standard" conditions for marine pathogens available in culture. Overall, further knowledge on QPX ecology as well as on the effects of environmental factors on its host, *M. mercenaria*, are needed to better determine QPX distribution in marine environments and factors controlling QPX disease development in hard clams.

Acknowledgments

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Part IV

Chapter 2

Long term effects of temperature on hard clam (*Mercenaria mercenaria*) immunity and Quahog Parasite Unknown (QPX) disease development: Defense parameters

Adapted from **Perrigault M.**, Dahl S.F., Gambino L., Pales-Espinosa E., Allam B. Long term effects of temperature on hard clams (*Mercenaria mercenaria*) immunity and QPX (Quahog Parasite Unknown) disease development: I. defense parameters. **Journal of Invertebrate Pathology**.

Abstract

QPX (Quahog Parasite Unknown) is a protistan parasite affecting hard clams *Mercenaria mercenaria* along the Northeastern coast of the United States. The geographic distribution and occurrence of disease epizootics suggests a primary role of temperature in disease development. This study was designed to investigate the effect of temperature on constitutive and QPX-induced defense factors in *M. mercenaria*. Naive and QPX-challenged (both experimentally and naturally) clams were maintained at 13, 21 and 27 °C for 4 months. Naïve and experimentally-infected clams originated from a southern broodstock (Florida, no prior reports of disease outbreak) while naturally-infected clams originated from a northern broodstock (Massachusetts, enzootic area). Standard and QPX-specific cellular and humoral defense parameters were assessed after 2 and 4 months. Measured parameters included total and differential hemocyte counts, reactive oxygen species production, phagocytic activity of hemocytes, lysozyme concentration in plasma, anti-QPX activity in plasma and resistance of hemocytes to cytotoxic QPX extracellular products. Results demonstrated a strong influence of temperature on constitutive clam defense factors with significant modulation of cellular and humoral parameters of naive clams maintained at 13 °C compared to 21 and 27 °C. Similarly, clam response to QPX challenge was also affected by temperature. Challenged clams exhibited no difference from controls at 27°C whereas different responses were observed at 21 °C and 13 °C compared to controls. Despite differences in infection mode (experimentally or naturally infected) and clam origin (northern and southern broodstocks), similarities were observed at 13 °C and 21 °C between QPX infected clams from Florida and Massachusetts. Clam response to temperature and to QPX exhibited interesting relationship with QPX disease development highlighting major influence of temperature on disease development.

1. Introduction

The success or failure of a pathogen in establishing disease in its host depends upon the efficiency of host's constitutive defense parameters and factors induced in response to pathogen invasion as well as ability of the pathogen to avoid or overwhelm host defenses. It is generally accepted that both host resistance and pathogen virulence are governed by genetic determinants and environmental parameters. The effect of environmental factors is particularly important in poikilothermic osmoconformers such as marine invertebrates (Shumway, 1977). Importance of temperature in disease development was previously demonstrated in several invertebrate taxa, including bivalve mollusks (Chu and La Peyre, 1993b; Paillard et al., 2004a). In these organisms, the immune system primarily relies on the performance of hemocytes which constitute the main line of defense against invaders (Cheng, 1981). The presence and recognition of non-self stimulates cell-mediated immune responses, mainly involving phagocytosis or encapsulation of foreign materials, and the production of reactive oxygen species (ROS) (Anderson, 1994; Pipe, 1992). Humoral factors, especially circulating enzymes and anti-microbial peptides are also involved in host responses because of their various anti-microbial properties (Cheng, 1992; Chu, 1988; Roch, 1999). The importance of enzymes such as peptidases and lysozyme was demonstrated during bacterial challenge in bivalves (Allam et al., 2000a; Allam et al., 2000b).

The protistan parasite QPX (Quahog Parasite Unknown) infects and has been associated with severe mortalities in wild and cultured hard clams *Mercenaria mercenaria*. QPX disease was observed along the eastern coast of North America, from Maritime Canada to Virginia but never south of Virginia (Dove et al., 2004; Ford et al., 2002b; Ragone Calvo et al., 1998; Smolowitz et al., 1998; Whyte et al., 1994) despite significant clam aquaculture activities in more southern states. Previous studies showed variation in clam susceptibility toward QPX among different clam stocks with higher resistance of northern clam broodstocks compared to southern broodstocks (Dahl et al., 2008; Ford et al., 2002b; Ragone Calvo et al., 2007). Histological observation of QPX-infected tissues demonstrated that some clams are able to mount an effective defense reaction characterized by an intense inflammatory response ultimately leading to the healing of infected individuals (Dahl and Allam,

2007; Dove et al., 2004; Ragone Calvo et al., 1998). Our previous studies demonstrated the presence in clam plasma of factors inhibiting QPX growth (part III – chapter 1). On the other hand, extracellular products (ECP) secreted by QPX were shown to significantly alter clam hemocytes (part II – chapter 1). Interestingly, both anti-QPX activity in clam plasma and resistance of hemocyte to cytotoxic effects of ECP were correlated with clam resistance to QPX disease. Recent molecular investigations in clams also demonstrated significant modulation in the expression of stress- and defense-related genes during QPX challenge (part III – chapter 2). All together, these findings suggest that QPX disease development (or clam resistance) largely depends upon interrelated extrinsic (environmental) and intrinsic (immune performances) factors that result in unbalanced host-pathogen interactions that favor the parasite (or the host) leading to disease and subsequent mortality (or to healing).

Further indication for the involvement of temperature in QPX disease development from field studies highlighted seasonality in prevalence and associated clam mortality (Smolowitz et al., 1998 and Allam, unpublished). The *in vitro* growth of QPX was also strongly modulated by temperature (Brothers et al., 2000, part IV - chapter 1). Despite this evidence, no prior studies have been conducted to evaluate the effect of temperature on clam immune defenses and response to QPX challenge in *M. mercenaria*. In this study, the effect of long term exposure (4 months) to different environmentally-relevant temperatures on constitutive and QPX-induced immune factors was investigated in hard clams. Experiments compared cellular and humoral defense parameters of clams naturally (Massachusetts broodstock) and experimentally (Florida broodstock) infected by QPX. Results demonstrate the modulatory effect of temperature on constitutive and QPX-induced defense factors. Attempts were also made to correlate defense parameters in clams from different broodstocks with the progression of QPX disease (Dahl et al., Accepted).

2. Materials and methods

2.1. Mercenaria mercenaria

This study used two aquacultured clam broodstocks. Eight hundred naïve *Mercenaria mercenaria* (30-35 mm in length) were obtained from a commercial source in Florida (FL). Four hundred clams (40-50 mm in length) presumed to be naturally infected by QPX were obtained from an enzootic clamming area in Massachusetts (MA). This sampling strategy was chosen to cover clams originating from broodstocks cultured in a wide geographical range, and therefore temperature range, along the east coast of the US. Clams were acclimated for 1 week in 150-L tanks with re-circulating water (28-30 ppt) at 21 ± 1 °C and fed daily with commercial algae (DT's Live Phytoplankton, Sycamore, IL, Espinosa and Allam, 2006). Thirty clams from each batch were sampled before the beginning of the experiment to assess their disease status using standard histological and quantitative PCR techniques (Liu et al., 2009). No detectable QPX infections were observed in FL clams by histology or quantitative PCR. Histopathological analysis of MA clams indicated active QPX lesions and quantitative PCR revealed a 63.3 % prevalence of the parasite in this batch at the beginning of the experiment(Dahl et al., Accepted).

2.2. QPX

QPX isolate NY0313808BC7 was cultured from nodules of infected New York clams (Qian et al., 2007) and subcultured in muscle tissue homogenates (MTH) from *M. mercenaria* according to Perrigault et al. (part III – chapter 1). QPX cultures were initiated in 25-cm^2 flasks containing MTH at 1000 µg.ml^{-1} protein and incubated at 23 °C for 2 weeks (part III – chapter 1). Neubauer chamber counts and a previously described fluorometric technique based on the uptake of fluorescein di-acetate (FDA) (Buggé and Allam, 2005) were used to monitor the growth and determine the concentration of QPX cells.

2.3. Temperature treatment

Following a 1-week acclimation (21 °C / 30 ppt), clams were distributed in 40 L recirculating tanks (20 clams per tank; 36 tanks in total for the FL clams and 18 tanks for the MA clams) filled with filtered and ultraviolet treated seawater (21 °C / 30 ppt). Seawater was filtered using biological and chemical filter cartridges containing activated carbon and was continuously oxygenated to saturation. Water quality and ammonia level were controlled weekly. Three groups composed of 18 tanks each (6 controls FL [FL-c], 6 QPX-challenged FL [FL-q] and 6 MA [MA]) were exposed to 13, 21 or 27 °C. Temperature adjustments (13 and 27 °C) were performed within 8 days in water baths equipped with electronically-controlled heaters and chillers by increasing or decreasing the temperature by 1 °C per day according to each treatment. Temperature and salinity in each tank was monitored over the 4-month experiment. Clams were fed daily with commercial algae (DT's Live Phytoplankton, Sycamore, IL, Espinosa and Allam, 2006) and monitored twice a day for mortality.

2.4. QPX challenge of Florida clams

After 1 week of acclimation at each experimental temperature, FL clams were challenged with QPX by injecting 5 x 10^4 parasite cells into the pericardial cavity according to Dahl and Allam (2007). Control clams were injected with sterile medium (MTH at 1000 $\mu g.ml^{-1}$ protein) that was maintained under the same conditions as QPX cultures (2 weeks at 23 °C). Following injection, clams were maintained out of the water for 1.5 hours before being returned to their respective tanks.

2.5. Hemolymph parameters

After 2 and 4 months of temperature challenge, 30 FL-c, 30 FL-q and 30 MA clams (5 clams per tank) were sampled for each condition with a total of 360 FL clams and 180 MA clams processed during the experiment. All samples were processed individually. Hemolymph samples (generally 1.2 to 1.8 ml) were withdrawn from the adductor muscle with a 1 ml-syringe and held on ice. A volume

of 650 µl hemolymph was diluted (vol:vol) with ice-cold filtered artificial seawater (FASW) and used for assessment of hemocyte concentration and differential counts (section 2.5.1), reactive oxygen species production (section 2.5.2), phagocytosis activity (section 2.5.3) and cytotoxicity of QPX ECP on hemocytes by the neutral red uptake assay (section 2.5.6). Plasma from undiluted hemolymph was recovered by centrifugation (700 x g, 10 min., 4 °C). Supernatant was sterilized by filtration (0.22 µm), aliquoted and preserved at -20 °C for the determination of protein concentration and lysozyme activity (section 2.5.4) and anti-QPX activity (section 2.5.5).

2.5.1. Hemocyte counts

Hemocyte counts were assessed microscopically (FL clams) or using flow cytometry methods (MA clams) which were preferentially employed but were not applied on FL clams due to the availability of the equipment. For microscopic counts, a volume of diluted hemolymph (vol:vol in FASW) was added to a volume of ice-cold anti-aggregant solution (AASH - 30 mM EDTA, 430 mM NaCl in 100 mM phosphate buffer at pH 7.4) containing 0.2 % trypan blue. Total hemocyte count (THC) and percentage of dead cells (PDC) were microscopically determined using a Neubauer chamber. For flow cytometry counts, one volume of diluted hemolymph was added to a volume of AASH containing Calcein AM (1 mM, Sigma) and ethidium homodimer-1 (1 mM, Sigma) to identify viable and dead cells, respectively. Samples were incubated with both dyes for 30 min. on ice in the dark. Data was acquired on a FACSCalibur flow cytometer (Becton Dickinson Biosciences) equipped with a 488 nm laser by counting 10,000 events. Differential counts of granulocytes and hyalinocytes were assessed by their specific size and cell complexity according to Allam et al. (2002a). Hemocyte counts were expressed as cells.ml^{-1} and hemocyte mortality was presented as percentage of dead cells (PDC).

2.5.2. Reactive oxygen species (ROS) production

Quantification of ROS production by *M. mercenaria* hemocytes was adapted from Buggé et al. (2007). Briefly, 100 µl of diluted hemolymph (vol:vol in FASW)

was transferred into black 96-well plates in triplicate and 10 mM of 2',7'-dichlorofluorescein-diacetate (DCFH-DA, Calbiochem) was added to each well. Fluorescence was immediately recorded at 485 nm excitation and 535 nm emission (Wallac 1420 plate reader, Perkin Elmer) to assess basal (native) ROS production. Production of ROS was thereafter stimulated by adding 10 µl of a zymosan A suspension (20 $mg.ml^{-1}$ in FASW, Sigma) in two wells and fluorescence was measured after 5 min. and 30 min. of incubation in the dark at room temperature (RT). Signals measured in wells with added zymosan A were corrected with values obtained from the third replicate (unstimulated). ROS activity was expressed as mean fluorescence in arbitrary units (A.U.) per 10^4 hemocytes.

2.5.3. Phagocytosis activity

Phagocytosis assay was adapted from Allam et al. (2001) and Blaise et al. (2002). Diluted hemolymph samples were plated in three wells (100 $µl.well^{-1}$) of a black 96-well plate and incubated for 1 hour at RT. Supernatant was then discarded and FITC-labeled *Vibrio parahaemolyticus* (1 x 10^8 CFU in FASW) was added to adherent hemocytes in two replicates. The third well (control) received FITC-labeled bacteria in 2 % formalin. Plates were incubated for 2 hours in the dark at RT, supernatants were discarded and 100 µl of a freshly made trypan blue solution (250 $µg.ml^{-1}$ in 50 mM citrate buffer, pH 4.4) were added for 1 min. to quench fluorescence of non engulfed bacteria. Fluorescence was measured at 485 nm excitation and 535 nm emission (Wallac 1420 plate reader, Perkin Elmer). Phagocytosis activity was expressed as relative fluorescence (A.U.) per 10^4 hemocytes. Because of logistical difficulties, the phagocytosis assay was not performed on hemocytes from MA clams sampled after 4 months.

2.5.4. Lysozyme activity and protein concentration in plasma

Lysozyme concentration was determined spectrophotometrically according to Allam and Paillard (1998). Forty µl of undiluted plasma was added to 180 µl of a bacterial suspension (lyophilized *Micrococcus lysodeikticus*, 20 $mg.ml^{-1}$ in 66 mM

phosphate buffer, pH 5.5) and turbidimetric changes were recorded for 100 s at 450 nm (Wallac 1420 plate reader, Perkin Elmer). Chicken egg white (CEW) lysozyme was used as standard. Lysozyme activity was calculated using the change in absorbance between 10 and 100 s. Lysozyme activity was reported as the concentration of CEW lysozyme equivalent expressed in $\mu g.ml^{-1}$ of plasma. Fifty µl of undiluted plasma was allocated to protein analysis using the BCA protein assay kit (Pierce). Protein concentrations were expressed as µg of protein per ml of plasma.

2.5.5. Anti QPX activity in clam plasma

The measurement of anti-QPX activity in plasma was performed according to Perrigault et al. (part III – chapter 1). Briefly, an exponentially-growing culture (1 week) of QPX in MTH (1000 $\mu g.ml^{-1}$ proteins) was centrifuged for 15 min. at 600 x g. QPX cell pellet was then washed two times and resuspended in Minimal Essential Medium (MEM). Fifty µl of undiluted plasma was added to 50 µl of MEM containing 1 x 10^3 QPX cells in black 96-well plate. Assays were performed in duplicate and an additional replicate without QPX cells was used to quantify the fluorescence signal generated by the mixture of MEM and each plasma sample. FASW was substituted for plasma in another set of controls to monitor QPX growth (no inhibition control). Microplates were incubated at 23 °C for 4 days before measuring QPX biovolume by the FDA technique (Buggé and Allam, 2005). Results were expressed as the percentage of fluorescence related to QPX growth in presence of plasma compared to the FASW controls ([fluo QPX in MEM_plasma – fluo MEM_plasma] / [fluo QPX in MEM_FASW – fluo MEM_FASW] x 100).

2.5.6. Cytotoxicity of QPX ECP on hemocytes

Assessment of cytotoxicity of QPX ECP on hemocytes was performed according to Perrigault and Allam (part II – chapter 1). An exponentially growing culture of QPX in MTH (1000 $\mu g.ml^{-1}$ proteins) containing about 1 x 10^5 $cell.ml^{-1}$ was transferred to 2-ml tubes and centrifuged at 1000 x g for 20 min. Supernatants (henceforth called extracellular products or ECP) were collected and filtered through

0.22 μm syringe filters (part II – chapter 1). Filtered ECP and sterile MTH medium were frozen at -20 °C until assays. One hundred μl of diluted hemolymph (vol:vol in FASW) were plated in 4 replicates in flat bottomed 96-well plates and incubated for 1 hour at RT to allow hemocyte adhesion to the plate. Supernatant was then carefully removed and hemocytes were washed with 200 μl of FASW. One hundred μl of QPX ECP (2 test wells) or sterile MTH (2 control wells) were then added. After 1 hour of incubation at RT, liquids were carefully removed and hemocytes were washed with 200 μl of FASW. One hundred μl of freshly prepared neutral red solution (250 $\mu g.ml^{-1}$ in FASW, Sigma) was then added. Following incubation (1 hour at RT), hemocytes were washed twice with FASW and neutral red incorporated by viable cells was eluted into 100 μl of solvent composed of glacial acetic acid:ethanol:water (1:50:49 by volume). Plates were sealed, agitated for 10 min. and absorbance (OD) was recorded at 560 nm (Wallac 1420 plate reader, Perkin Elmer). Results were expressed as cytotoxic index ($[(OD\ controls - OD\ tests)/THC]*10^8$). Because of logistical difficulties, the neutral red assay was not performed on hemocytes from FL clams sampled after 4 months.

2.6. Statistical analyses

All variables were analyzed statistically using Multifactor ANOVA to evaluate interactive effects of temperature, sampling time and QPX challenge on hard clam hemolymph parameters. ANOVA treatments that generated probability values below 0.05 were followed by a Holm-Sidak post-hoc test comparing different conditions. Data were log10 or arcsin transformed before statistical testing whenever data showed a large variance but results shown in tables and Figs. are presented as non-transformed values. Multivariate analyses were performed using Principal Component Analysis (PCA) and Discriminant Analysis (DA) to analyze relationships between variables and identify variable contribution to the discrimination among treatments, respectively. PCA analyses were followed by ANOVA on extracted components to test the effect of treatments (temperature and QPX challenge) on overall hemolymph profiles. DA and PCA were performed with Statgraphics plus software (Statistical Graphics Corp., Warrenton, Virginia, USA) and SigmaStat

(Systat Software, Inc., San Jose, California, USA) was used for ANOVA analyses. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Clam mortality and QPX prevalence

During the 4 month experiment, mortality was not significant (below 4 %) in any of the FL clam batches without regard to challenge or temperature treatment. Mortality levels were different, however, among MA clams exposed to different temperatures with significantly higher mortality ($p = 0.01$) in clams maintained at 13 °C (19 %) as compared to those held at 21 or 27 °C (6 and 8 %, respectively). None of the FL-c clams displayed QPX disease while significantly higher QPX prevalence was detected 4 months after the beginning of the experiment in MA (13 %) and FL-q clams (70 %) maintained at 13 °C as compared to 21 °C (0 and 10 % for MA and FL, respectively) or 27 °C (0 and 7 % for MA and FL, respectively). A complete presentation of mortality and QPX disease data can be found in Dahl et al. (Dahl et al., Accepted).

3.2. Effect of temperature on individual defense parameters of M. mercenaria

3.2.1. Effects of temperature on defense parameters in naïve clams (FL-c)

Results demonstrated a strong influence of temperature on both cellular and humoral defense factors of unchallenged *M. mercenaria* (Table 7.1). Total hemocyte count (THC) exhibited variations according to temperature treatment and sampling time with significant differences between 21 and 27 °C at 2 months and between 13 and 27 °C at 4 months (Holm-Sidak $p = 0.005$ and $p = 0.004$ respectively, Fig. 7.1A). Temperature did not significantly affect the percentage of dead cells (PDC) but a general increase of PDC was noted between 2 and 4 months (Fig. 7.1B). Unstimulated ROS production was also significantly affected by temperature with higher levels at 13 °C at both sampling times ($p < 0.001$, Fig. 7.2A). However, no significant difference between treatments was observed after 5 min- and 30 min-

stimulation of ROS production by zymosan A (5 min stimulation not shown, Fig. 7.2B) except between FL-c clams maintained at 21 °C and 27 °C for 4 months (p = 0.01, Fig. 7.2B). Higher phagocytosis activity was observed at 21 °C at 2 and 4 months as compared to levels measured at 13 °C or 27 °C ($p < 0.005$, Fig. 7.3). Humoral components such as protein concentration and lysozyme activity in plasma exhibited relatively less variation. For instance, significantly higher lysozyme activity ($p = 0.001$) was only observed after 4 months in clams maintained at 13 °C as compared to those held at 21 °C or 27 °C (Fig. 7.4A). Similarly, higher protein concentration ($p < 0.001$) was measured after 2 months in clams reared at 13 °C as compared to those incubated at 27 °C (Fig. 7.4B). Anti-QPX activity in plasma was not significantly modulated by temperature despite a slight tendency of lower inhibitory activity at 21 °C as compared to both extreme temperatures (Fig. 7.5A). QPX ECP cytotoxicity on hemocytes from FL-c exhibited no significant difference between temperature treatments after 2 months (Fig. 7.5B).

Table 7.1. Summary of 2-way ANOVA results assessing the effect of temperature (13, 21 or 27 °C) and sampling date (2 or 4 months) on cellular and humoral defense parameters in control (FL-c) and QPX-challenged (FL-q) Florida clams.

	FL-c			FL-q		
	Time	T°C	Time/T°C	Time	T°C	Time/T°C
THC	**NS**	*	**	**NS**	***	**NS**
PDC	***	**NS**	**NS**	**NS**	***	**NS**
Unstimulated ROS	***	***	***	**NS**	***	**
Stimulated ROS (5 min)	**NS**	*	**NS**	**NS**	***	*
Stimulated ROS (30 min)	**NS**	**NS**	*	**NS**	***	**
Phagocytosis	***	***	***	***	***	***
Lysozyme	**NS**	***	**NS**	**NS**	**NS**	**NS**
Protein concentration	**NS**	*	*	**NS**	***	*
Anti-QPX activities in	**	**NS**	**NS**	***	**NS**	**NS**
Cytotoxicity of QPX ECP	**nd**	*	**nd**	**nd**	**NS**	**nd**

Non-significant differences are presented as NS and symbols denote significant differences at $p < 0.05$ (*),$p < 0.01$ (**) or $p < 0.001$ (***). Cytotoxicity of QPX ECP on hemocytes was not assessed at 4 months (nd).

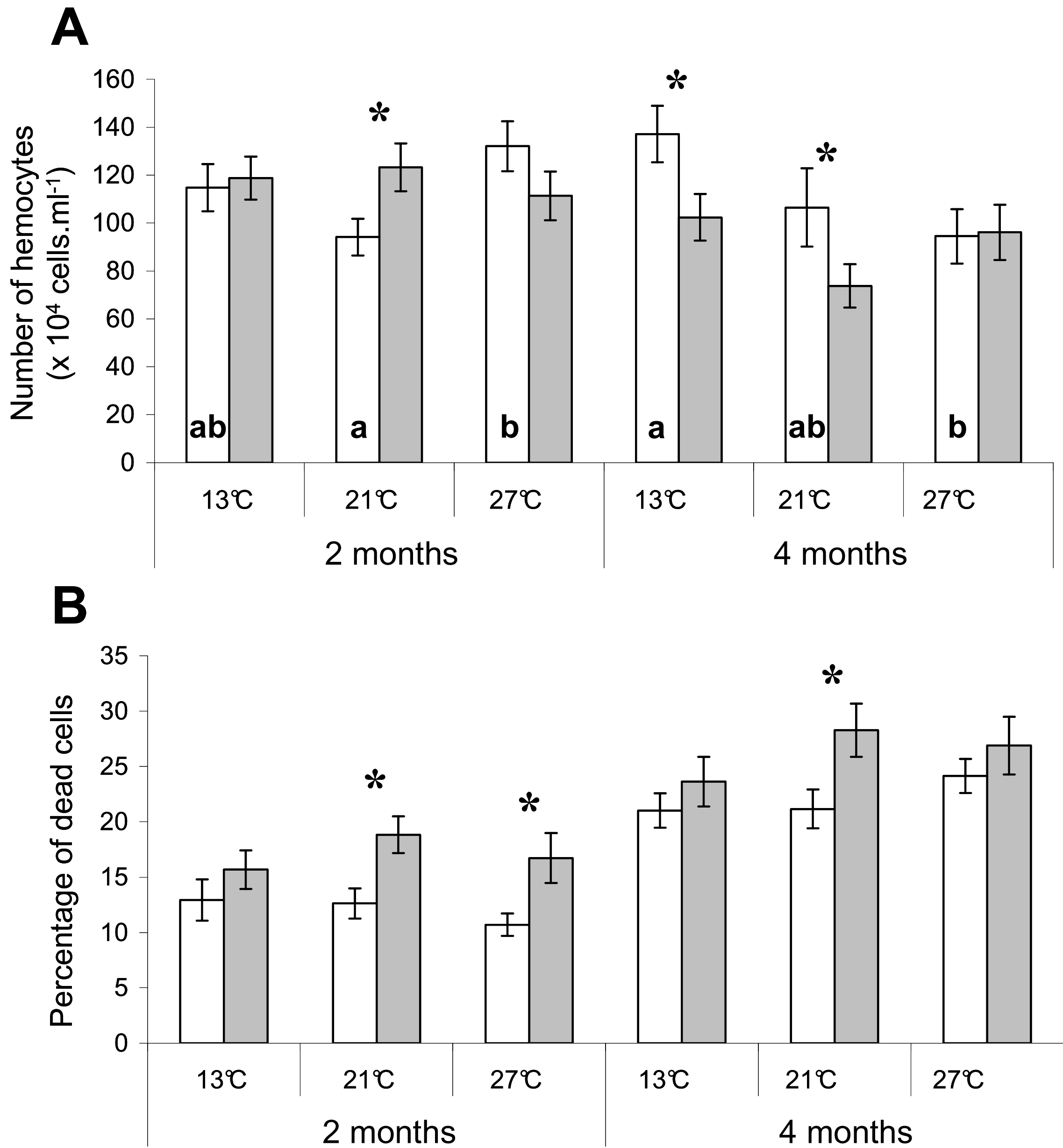

Fig. 7.1. Mean (± SEM, n = 30 clams per condition) of [A] total hemocyte counts (THC) and [B] percentage of dead cells (PDC) from (FL-c) controls (□) and (FL-q) QPX challenged (▣) Florida clams after 2 and 4 months at 13, 21 and 27 °C. For each sampling time, letters (a and b) denote significant differences (Holm-Sidak post-hoc test) in THC among control clams maintained at different temperatures (differences among FL-q clams and for PDC were not significant). * denotes significant differences between FL-c and FL-q maintained at the same temperature and sampled at the same time.

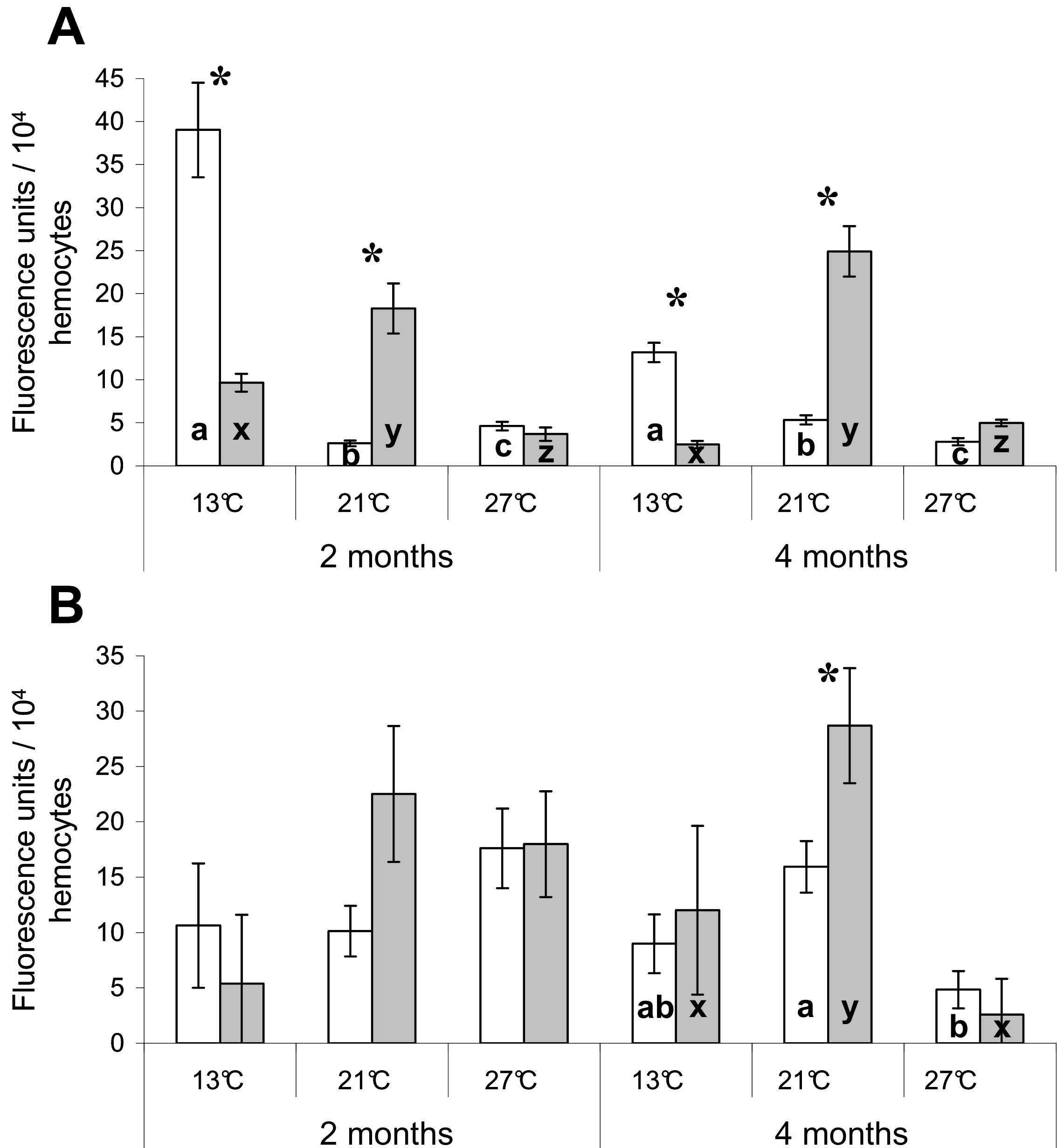

Fig. 7.2. Mean (± SEM, n = 30 clams per condition) of [A] unstimulated (basal) and [B] zymosan A stimulated ROS production (after 30 min) in hemocytes from (FL-c) controls (□) and (FL-q) QPX challenged (▩) Florida clams after 2 and 4 months at 13, 21 and 27 °C. For each sampling time, letters (a, b and c for FL-c and x, y and z for FL-q) denote significant differences (Holm-Sidak post-hoc test) between clams maintained at different temperatures within each challenge treatment. * denotes significant differences between FL-c and FL-q maintained at the same temperature and sampled at the same time.

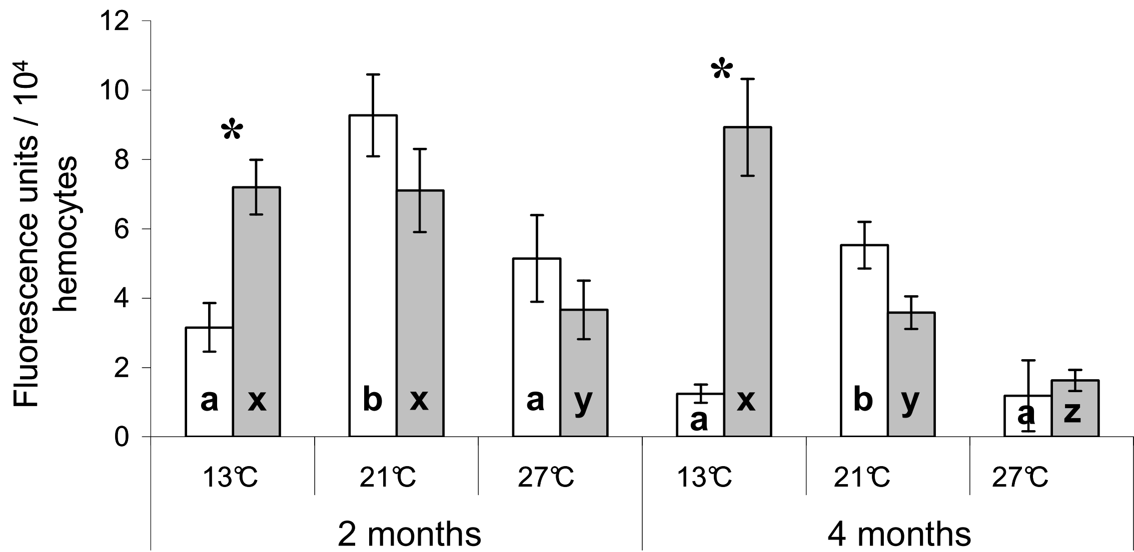

Fig. 7.3. Mean (± SEM, n = 30 clams per condition) of phagocytic activity in hemocytes from (FL-c) controls (□) and (FL-q) QPX challenged (■) clams after 2 and 4 months at 13, 21 and 27 °C. For each sampling time, letters (a and b for FL-c and x, y and z for FL-q) denote significant differences (Holm-Sidak post-hoc test) between clams maintained at different temperatures within each challenge treatment. * denotes significant differences between FL-c and FL-q maintained at the same temperature and sampled at the same time.

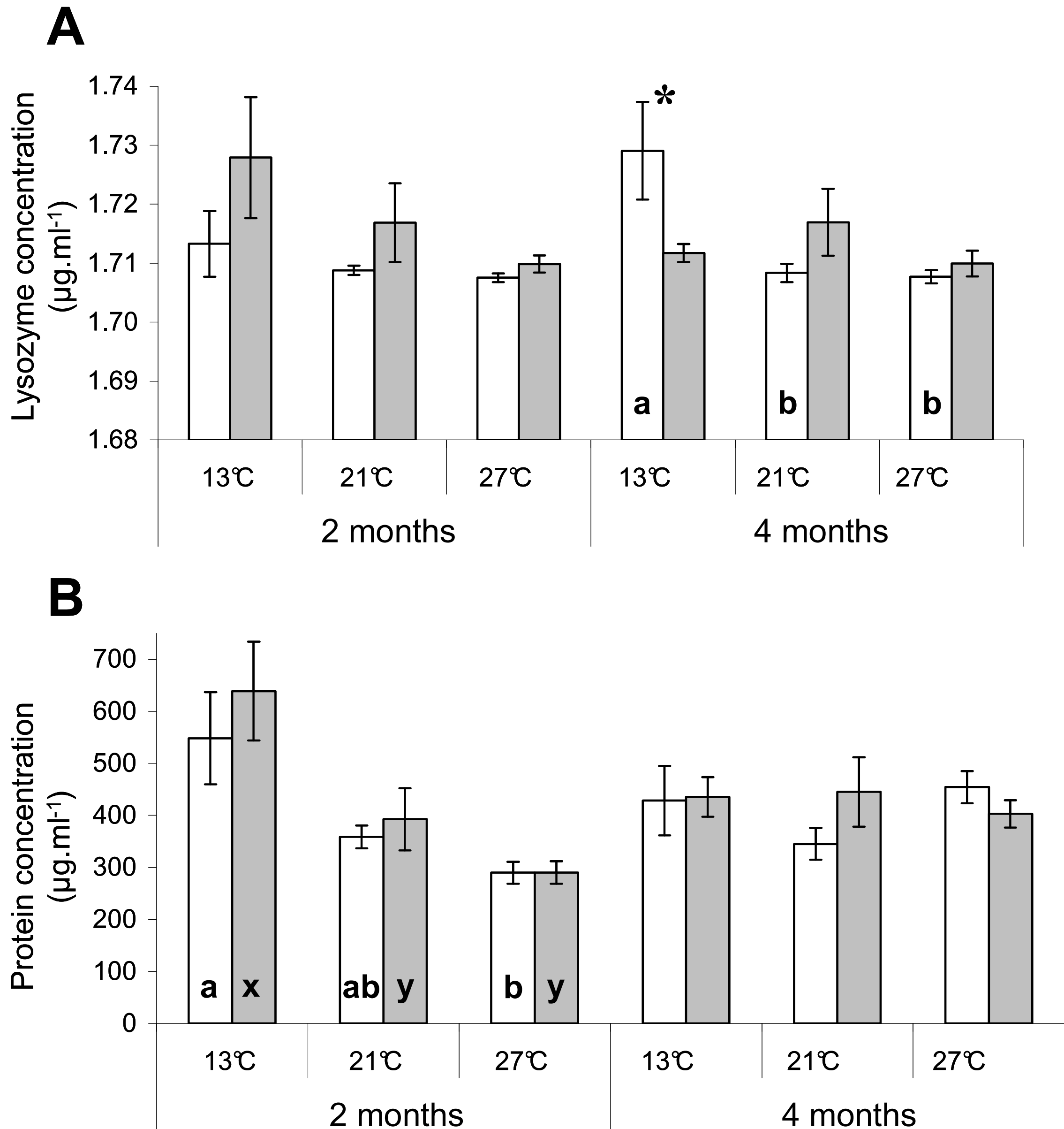

Fig. 7.4. Mean (± SEM, n = 30 clams per treatment) of [A] lysozyme and [B] protein concentrations in plasma from (FL-c) controls (□) and (FL-q) QPX challenged (▩) clams after 2 and 4 months at 13, 21 and 27 °C. For each sampling time, letters (a and b for FL-c and x and y for FL-q) denote significant differences (Holm-Sidak post-hoc test) between clams maintained at different temperatures within each challenge treatment. * denotes significant differences between FL-c and FL-q maintained at the same temperature and sampled at the same time.

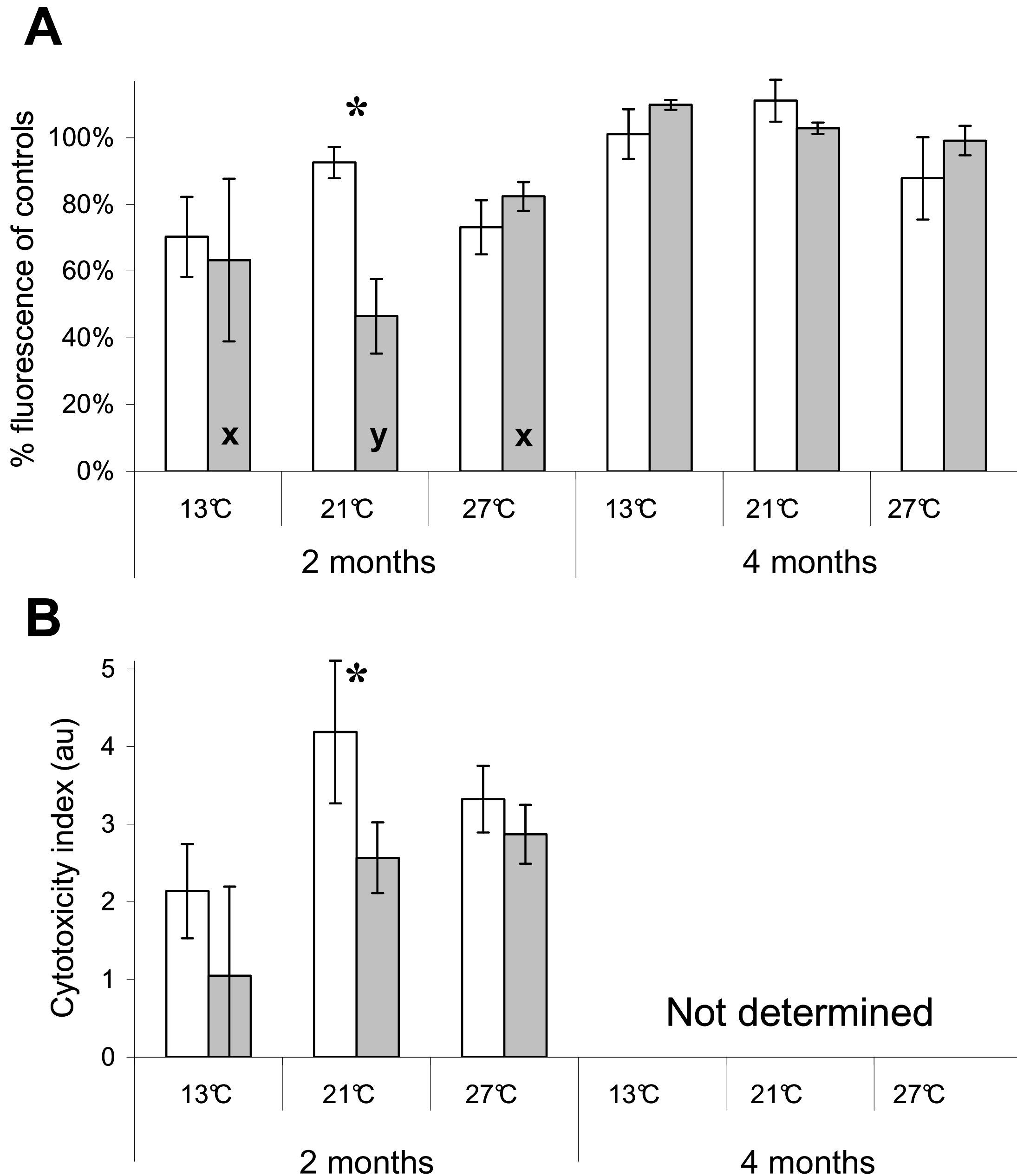

Fig. 7.5. Mean (± SEM, n = 30 clams per condition) of [A] anti-QPX activity of plasma (higher readings represent lower activities) and [B] cytotoxicity of QPX ECPs on hemocytes (higher readings represent lower resistance of hemocytes) from control (□) and QPX challenged (■) FL clams maintained at 13, 21 and 27 °C. Significant differences between temperature treatments were only detected for anti-QPX activity in challenged clams sampled after 2 months and are designated by the letters x and y (Holm-Sidak post-hoc test). * denotes significant differences between FL-c and FL-q maintained at the same temperature and sampled at the same time.

3.2.2. *Effects of temperature on defense parameters of infected clams (FL-q and MA)*

Combined effects of temperature and QPX disease demonstrated significant modulation of defense parameters in clams experimentally (FL-q) or naturally (MA) infected by QPX (Tables 7.1, 7.2 and 7.3). THC did not exhibit large variations in FL-q clams exposed to different temperatures but temperature clearly affected clam response to QPX challenge in comparison to unchallenged controls. For instance, significant increase of THC was observed after 2 months in challenged clams maintained at 21 °C ($p = 0.032$) but not in clams held at 13 °C or 27 °C. Four months after challenge, THC was significantly lower ($p < 0.022$) in clams maintained at 13 °C and 21 °C as compared to their respective controls, but not in those held at 27 °C (Fig. 7.1A). In MA clams, higher THC was observed in clams maintained at 13 °C as compared to those maintained at 27 °C and the difference was highly significant 4 months after the beginning of the experiment ($p < 0.001$, Table 7.2). The differences in THC in MA clams were associated to variations of granulocyte percentage as significantly lower values were measured at 13 °C as compared to 21 and 27 °C at 2 and 4 months ($p < 0.001$, Table 7.2).

Similarly to FL-c, PDC in FL-q exhibited no significant variation among different temperature regimes but global increase of hemocyte mortality was observed between 2 and 4 months after challenge (Fig. 7.1B). Significant increase in PDC was, however, measured after 2 and 4 months in QPX-challenged FL clams (FL-q) maintained at 21 °C as compared to their controls ($p < 0.015$, Fig. 7.1B). Overall PDC in MA clams presented no significant variation between treatments (Table 7.2).

ROS production by hemocytes from FL clams was highly modulated by QPX and temperature challenge. For instance, unstimulated ROS was significantly lower in FL-q clams maintained at 13 °C as compared to their unchallenged controls ($p < 0.001$, Fig. 7.2A). Interestingly, trends were inverted at 21 °C with significantly higher basal ROS production in FL-q compared to their controls ($p < 0.001$). At 27 °C, no difference between FL-c and FL-q was noted. MA clams had significantly lower basal ROS production at 13 °C compared to the other temperature treatments ($p < 0.009$, Table 7.2). When stimulated by zymosan A for 30 min., ROS production by FL-q was higher at 21 °C as compared to challenged clams maintained at 13 °C and

27 °C but differences were significant only at 4 months ($p = 0.037$, Fig. 7.2B). No significant variation in stimulated ROS production (after 30 min.) was observed in MA clams maintained at different temperatures (Table 7.2).

Table 7.2. Cellular and humoral defense parameters in Massachusetts (MA) clams naturally infected by QPX and exposed to 13, 21 or 27 °C for 2 and 4 months.

	2 months			4 months		
	13°C	21°C	27°C	13°C	21°C	27°C
THC (cells.ml^{-1} x 10^5)	11.7	11.0	9.5	13.9^{x}	10.7^{xy}	7.4^{y}*
% granulocytes	50.2^{a}	59.4^{b}	69.6^{c}	49.5^{x}	65.8^{y}	67.9^{y}
PDC (%)	5.6	6.9	5.5	5.1	5.0	5.6
Basal ROS production (Fluorescence / 10^4 hemocytes)	1.7^{a}	1.9^{b}	2.4^{b}	1.6^{x}	2.9^{y}	2.3^{y}
30 min stimulated ROS production (Fluorescence / 10^4 hemocytes)	7.9	7.5	6.6	11.2	7.0	7.3
Phagocytosis (Fluorescence / 10^4 hemocytes)	6.0^{a}	2.2^{ab}	1.4^{b}	nd	nd	nd
Lysozyme (µg.ml^{-1})	7.5	7.3	6.7	5.8	5.5	7.5
Protein concentration (µg.ml^{-1})	400.9^{a}	532.8^{b}	450.3^{a}	436.9	392.4*	431.9
Anti QPX activities in plasma (% controls)	112.7^{a}	76.8^{b}	105.4^{a}	118.7^{x}	53.2^{y}	75.0^{y}*
Cytotoxicity of QPX ECP (cytotoxicity index - A.U.)	6.0^{a}	9.7^{a}	0.6^{b}	9.0^{x}	5.7^{xy}	2.9^{y}

Letters (a, b and c for 2 months and x and y for 4 months) denote significant differences (Holm-Sidak post-hoc test) between clams maintained at different temperatures. * denotes significant differences between 2 and 4 months for clams maintained at the same temperature. Phagocytosis was not assessed at 4 months (nd). Refer to appendix 2 for SEM values.

Phagocytosis activity in FL and MA clams was also strongly affected by temperature and QPX challenge (Fig. 7.3, Tables 7.2). Low temperature caused an increase in phagocytosis in hemocytes from FL-q ($p < 0.005$) compared to their unchallenged controls whereas clams maintained at 21 °C tended to exhibit lower phagocytosis activity as compared to their controls. When clams were maintained at

27 °C, QPX challenge did not induce any change in phagocytic activity of hemocytes (Fig. 7.3). Similarly, higher phagocytosis activity was also observed in hemocytes from MA clams maintained at 13 °C as compared to clams held at the 2 other experimental temperatures (differences were significant between 13 and 27 °C $p < 0.001$, Table 7.3).

Effects of temperature and QPX challenge on lysozyme activity and protein concentration in plasma displayed no large variations in FL-q clams but significant decrease of lysozyme activity in FL-q compared to controls was observed at 13 °C after 4 months of challenge ($p = 0.005$, Fig. 7.4A). Additionally, protein concentration in plasma of FL-q clams was significant higher after 2 months at 13 °C compared to 27 °C and 21 °C treatments ($p < 0.001$, Fig. 7.4B). In naturally infected clams (MA), protein concentration was significantly higher at 21 °C compared to other treatments after 2 months ($p < 0.016$, Table 7.2).

Anti-QPX activity in plasma from FL clams increased following QPX challenge (FL-q) only when clams were incubated at 21 °C and sampled 2 months following challenge ($p = 0.003$, Fig. 7.5A). At 27 °C, a slight and non significant decrease in anti-QPX activity was noted whereas small variations were observed at 13 °C (Fig. 7.5A). Similarly, higher anti-QPX activity was observed in plasma from MA clams maintained at 21 °C as compared to the other tested temperatures (differences were significant between 13 and 21 °C at both sampling times, $p < 0.004$, Tables 7.2).

Results from the neutral red uptake assay (cytotoxicity of QPX ECP on hemocytes) revealed an enhanced resistance (reduced cytotoxicity) of hemocytes from FL-q at all temperature treatments, but differences between QPX-challenged and unchallenged clams were significant only at 21 °C ($p = 0.001$, Fig. 7.5B). Overall, higher hemocyte resistance to ECPs was observed at 13 °C for FL-q whereas hemocytes from MA clams were more resistant at 27 °C (Fig. 7.5B, Table 7.2).

Table 7.3. Summary of 2-way ANOVA results assessing the effect of temperature (13, 21 or 27 °C) and sampling date (2 or 4 months) on cellular and humoral defense parameters in MA clams.

	Time	T°C	Time/T°C
THC	NS	**	NS
% Granulocytes	NS	***	NS
PDC	NS	NS	NS
Unstimulated ROS	NS	***	NS
Stimulated ROS (5 min)	NS	**	NS
Stimulated ROS (30 min)	NS	NS	NS
Phagocytosis	nd	***	nd
Lysozyme	NS	*	NS
Protein concentration	*	NS	***
Anti-QPX activities in plasma	*	***	NS
Cytotoxicity of QPX ECP	NS	***	*

Non-significant differences are presented as NS and symbols denote significant differences at $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***). Phagocytosis was not assessed at 4 months (nd).

3.3. Global effects of temperature and QPX challenge on immune status of M. mercenaria

Integrative effect of temperature and QPX challenge on clam immune status was assessed using discriminant (DA) and principal component (PCA) analyses. Results of DA revealed significant impact of temperature treatments for all tested conditions (Fig. 7.6, DA of defense parameters in MA clams not shown but followed similar trends as FL-q clams). Function 1 of DA on control (FL-c) and challenged (FL-q) clams explained 96.4 % (Eigenvalue = 2.595, Wilks Lambda = 0.25, $p < 0.001$) and 92.7 % (Eigenvalue = 1.989, Wilks Lambda = 0.28, $p < 0.001$) of the total variance, respectively. Scatter plots of discriminant functions of defense parameters of FL control (FL-c) clams indicated a good discrimination of centroids by Function 1 between clams maintained at 13°C as compared to clams held at 21 °C and 27 °C (Fig. 7.6A). In the case of FL-q and MA clams, centroids appeared to be equally discriminated between temperature treatments with marked separation of clams maintained at 21 °C as opposed to the other 2 treatments (Fig. 7.6B, MA data not shown). To enhance the power of the statistical testing, PCA was performed on

defense parameters combined from all clams (FL-c, FL-q and MA) after 2 months (Fig. 7.7) and 4 months (data not shown but clustering of selected parameters were similar to Fig. 7.7 although NRU and phagocytosis were excluded from the analyses because data were missing at 4 months for FL and MA clams, respectively). In clams sampled at 2 months, Components 1 and 2 explained more than 52 % of the total variance of the data (Fig. 7.7). Several defense parameters appeared highly clustered, including phagocytosis, lysozyme activity, anti-QPX activity in plasma and hemocyte susceptibility to cytotoxic activity of QPX ECPs. This first cluster was opposed to ROS production (unstimulated and stimulated) on component 1 and to THC and PDC on component 2. Extraction of component 1 (PC1) and statistical analysis (ANOVA) of the effect of temperature and QPX challenge on clam defense factors indicated significant differences within unchallenged (FL-c) clams between animals maintained at 13 °C as compared to the other 2 temperatures ($p < 0.01$, Fig. 7.8A). Extracted component 1 was significantly higher in challenged (FL-q) clams maintained at 13 °C when compared with FL-c clams maintained at the same temperature ($p = 0.008$) likely as a result of higher phagocytosis activity and lower unstimulated and stimulated ROS production (Fig. 7.7 and 7.8A). The effect of QPX challenge was inverted at 21 °C ($P = 0.05$) and no difference was observed at 27 °C (Fig. 7.8A). Extracted component 1 for MA clams decreased with increasing temperature and differences were significant between 13 °C and 27 °C treatments. ($p < 0.001$, Fig. 7.8B). Extraction of component 1 (PC1) and statistical analysis of defense parameters (without NRU and phagocytosis activity) at 4 months and globally (2 and 4 months combined) exhibited similar results (data not shown) with the exception that differences between FL-q and FL-c significantly increased at 21 °C ($p < 0.001$) whereas differences between these 2 groups were no longer significant at 13 °C.

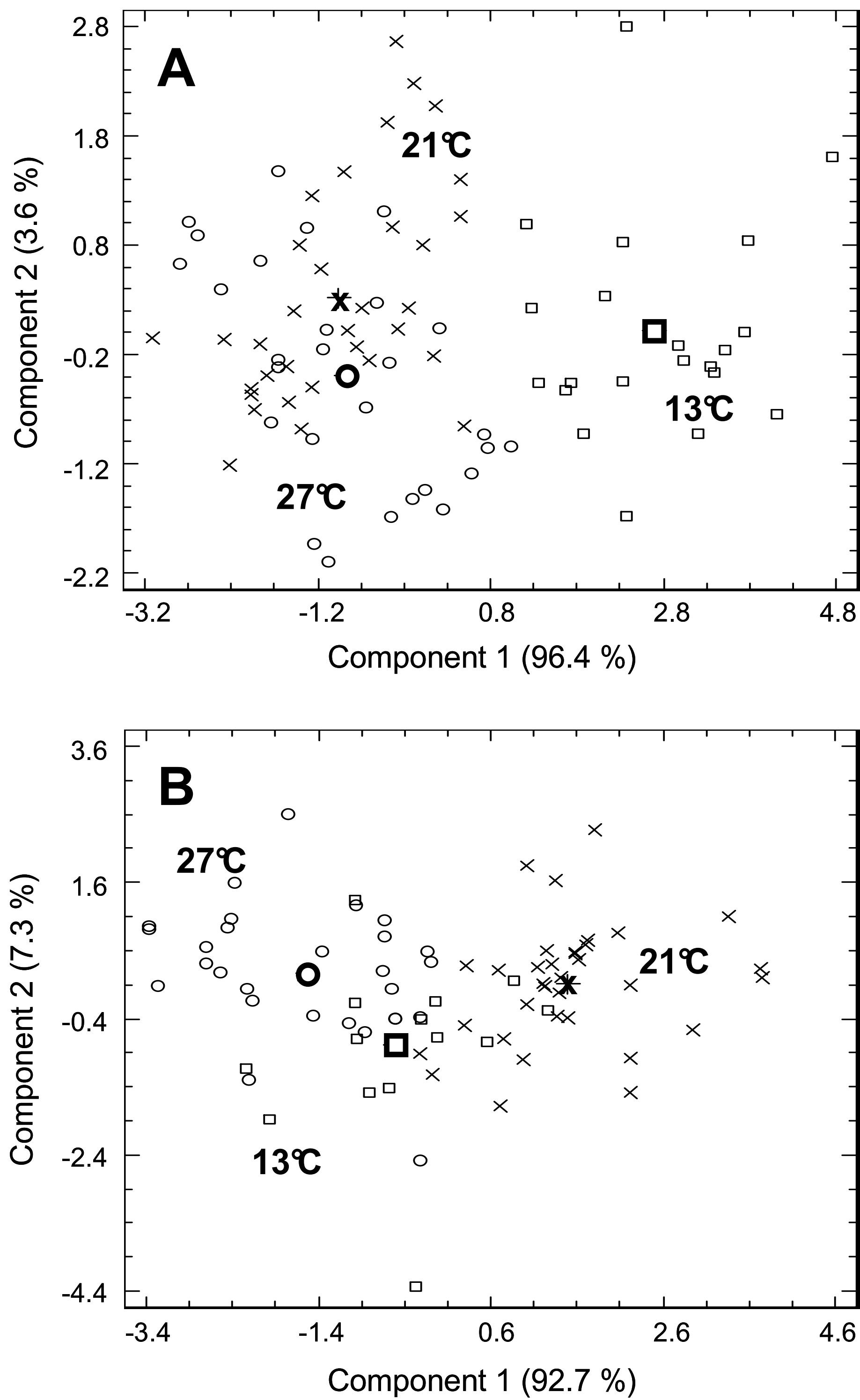

Fig. 7.6. Discriminant Analysis of immune parameters from (FL-c) unchallenged [A] and (FL-q) QPX-challenged [B] clams maintained at 13 °C (□), 21 °C (✕) and 27 °C (○). Positions of group centroids for each treatment are indicated by bold large symbols.

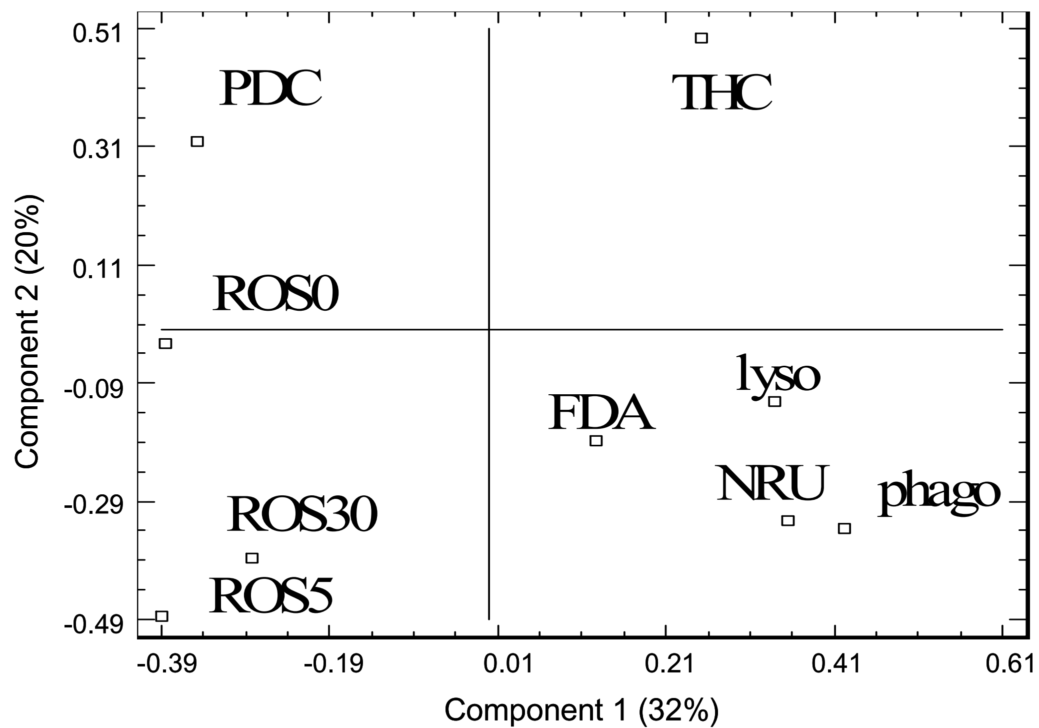

Fig. 7.7. Principal Component Analysis (PCA) plot of immune parameters from clams (FL-c, FL-q and MA combined) sampled at 2 months. PDC: percentage of dead cells, ROS0: basal ROS production, ROS5 and 30: zymosan-stimulated ROS production after 5 min and 30 min respectively, THC: total hemocyte count, phago: phagocytosis, lyso: lysozyme activity, FDA: anti-QPX activity of plasma (increasing values represent lower activities), NRU: cytotoxicity of QPX ECP on hemocytes.

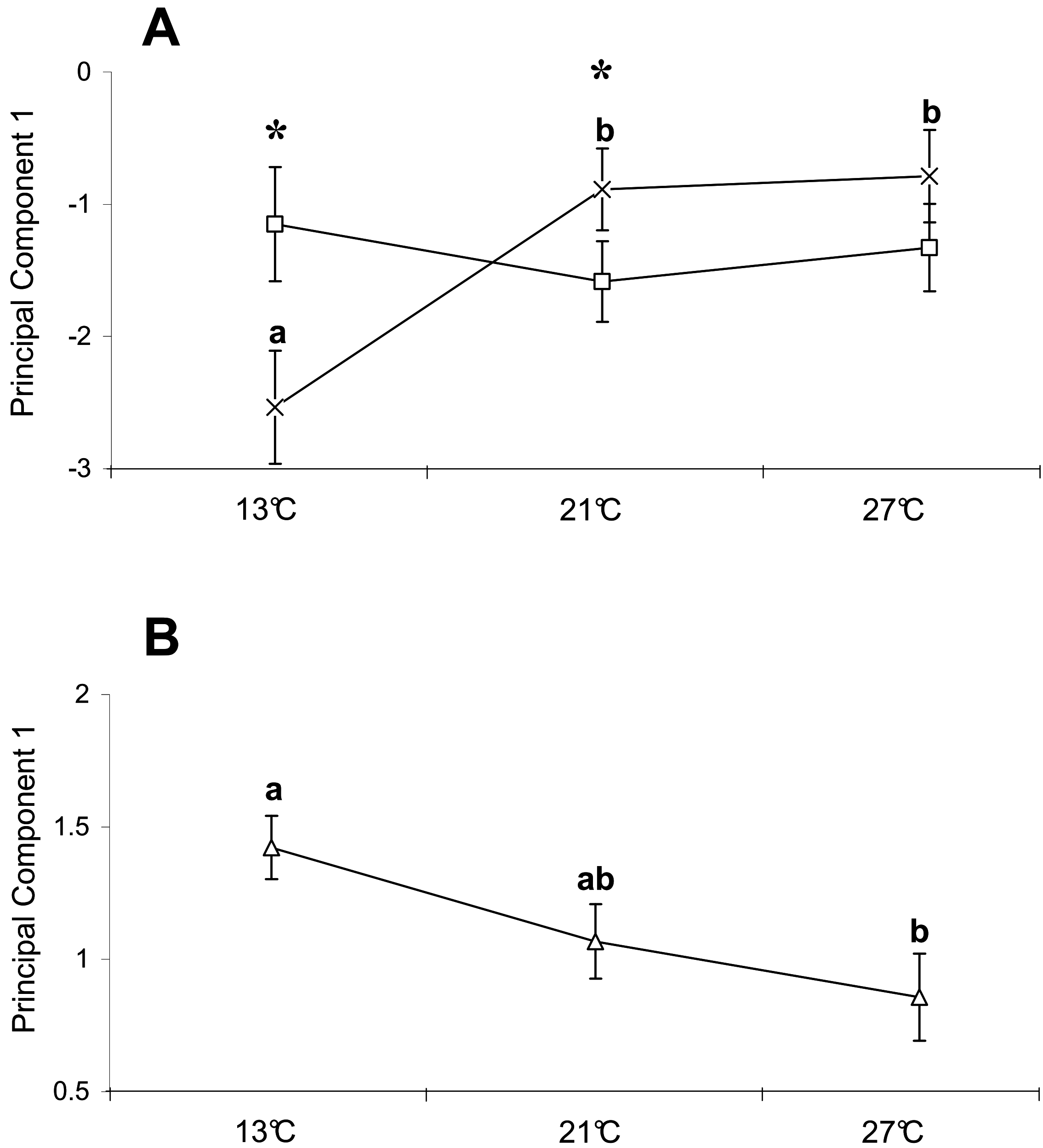

Fig. 7.8. Mean plots of Component 1 from Principal Component Analysis shown in Fig. 7.7 for (FL-c) unchallenged (✕) and (FL-q) QPX challenged (□) clams [A] and MA clams [B] exposed to three different temperatures. Letters (a and b) denote significant differences between clams maintained at different temperatures and symbols (*) indicate significant differences between FL-c and FL-q within each temperature treatment ($p < 0.05$, Holm-Sidak post-hoc test).

4. Discussion

The objectives of this study were to investigate the effect of temperature on cellular and humoral defense parameters of the hard clam *Mercenaria mercenaria*, and to evaluate the combined effects of temperature and QPX challenge on immune factors to identify conditions enhancing disease development or host resistance. QPX is a relatively slow and chronic disease (Dahl and Allam, 2007). Extended time experiments (4 months) were used to provide sufficient time for QPX disease development to assess specific clam response to established infections (Dahl and Allam, 2007, part III - chapter 2). Two groups of infected clams, including a naturally infected batch from a Massachusetts (MA) broodstock and an experimentally infected group from Florida (FL) were used to investigate the effect of temperature on clam response to the presence of QPX. Results demonstrated significant impact of temperature on defense factors in naïve clams and on clam response to QPX.

4.1. Effects of temperature on defense parameters of naïve clams (FL-c)

Changes in temperature were previously shown to modulate bivalve defense parameters both *in vivo* and *in vitro* (Carballal et al., 1998; Travers et al., 2008; Yu et al., 2009). Similarly, our study demonstrated a strong influence of temperature on most investigated defense parameters (cellular and humoral) in naïve FL clams (Table 7.1). PCA analysis and ANOVA on extracted PC1 demonstrated significant differences in defense factors between clams held at 13 °C and those maintained at 21 °C and 27 °C (Fig. 7.8). Clams exposed to lower temperature were characterized by higher production of unstimulated reactive oxygen species (ROS) by hemocytes and higher percentage of dead cells among hemocytes (PDC), as well as lower phagocytic activity and total hemocyte counts (THC) (Fig. 7.7). Temperature effects on individual defense parameters for FL-c exhibited some classic patterns observed in previous studies. For example, previous studies in oysters (Chu and La Peyre, 1993b; Paillard et al., 2004a) and clams (Chu and La Peyre, 1993b; Paillard et al., 2004a) also showed higher lysozyme activity at lower temperature. Phagocytosis activity

measured here was characterized by an optimal activity at 21 °C matching well with previous studies in *M. mercenaria* and other bivalve species (Allam et al., 2002a; Cheng et al., 2004a; Chu and La Peyre, 1993b; Tripp, 1992b). However, other defense parameters displayed unusual patterns. For instance, modulation of the THC by environmental parameters was described in several studies and is usually characterized by higher THC at higher temperatures (Chu and La Peyre, 1993b; Monari et al., 2007; Paillard et al., 2004a). In the Manila clam *Ruditapes philippinarum*, THC displayed a seasonal pattern with higher levels during summer (Soudant et al., 2004). Our results at 4 months revealed opposite trends with higher THC at lower temperature (Fig. 7.1A). Similarly, most studies investigating the effect of temperature on ROS production reported an increase of ROS production (unstimulated and stimulated) with increasing temperature (Chen et al., 2007b; Cheng et al., 2004a; Hégaret et al., 2003). Unstimulated ROS production of FL-c was highest at 13 °C after 2 and 4 months. However, stimulated ROS production at 13 °C was not different from those measured in the other temperature treatments (Fig. 7.2). The involvement of reactive oxygen species in bivalve defense mechanisms was previously demonstrated (Anderson, 1994; Pipe, 1992). However, uncontrolled ROS activities in cells / tissues are also harmful for hosts themselves (Torreilles et al., 1996). Pattern of ROS production by FL-c at 13 °C suggested high oxidative stress and reduced capacity of response to pathogen challenge (high unstimulated but low stimulated ROS levels). It is noteworthy that, in opposition to our study, previous investigations were mostly based on acute or short term changes of temperature. Stress related to rapid increase of temperature was associated with ROS release (Abele and Puntarulo, 2004) and could explain trends obtained in other studies, at least with regard to high unstimulated ROS levels. Additionally, temperature significantly affects physiological parameters. For instance, studies on several bivalves demonstrated correlation between increase of respiration, clearance and heart rate with increasing temperature according to specific optimum and threshold of each species (Han et al., 2008; Haure et al., 1998; Pandolfo et al., 2009). Consequences of physiological variations on defense parameters were also demonstrated such as the correlation of increasing temperature and respiration with increasing ROS production (Heise et al., 2003). It is likely that temperature also affected physiological status of hard clams and contributed to the variations of measured parameters.

Cytotoxicity of QPX ECP on hemocytes from FL-c clams exposed to different temperatures exhibited variability without significant difference between treatments (Fig. 7.5B). Constitutive anti-QPX activity in plasma also exhibited also variability among treatments with a maximal parasite growth inhibition of 29.7 % (compared to control cultures with FASW added instead of plasma) at 13 °C after 2 months (Fig. 7.5A) which could be related to high protein concentration observed at low temperature (Fig. 7.4B). However, anti-QPX activity measured here in FL-c plasma was lower than reported in a previous study using similar Florida clams (part III – chapter 1). This may result from the freezing of plasma samples before the assessment of anti-QPX activity since Anderson et al. (2003a) reported alterations in clam plasma modulatory activity after freezing which could be a limitation in our study.

Overall, our results suggest a global alteration of defense parameters of unchallenged *M. mercenaria* (FL-c) at lower temperature. This was clear in the clustering of data points on the PCA biplots (Fig. 7.6A). Tested temperatures were selected according to cyclic patterns measured in NY coastal environment between spring and fall (Allam, unpublished). However, water temperature in Florida (clam source) ranges on average from 17 °C to 28°C (NODC, NOAA, http://www.nodc.noaa.gov/dsdt). Alterations of FL-c defenses at 13 °C could be exacerbated by poor adaptation of that clam population to colder temperature. In fact, the impact of genetic variation on overall performance of geographically different hard clam populations has been previously demonstrated (Camara et al., 2006). Similarly, Calvo et al. (2007) noted reduced growth and higher QPX prevalence of FL clams planted in colder locations (New Jersey) compared to local clam populations.

4.2. Effects of temperature and QPX on clam defense parameters

Despite potential differences in the effect of temperature on defense parameters in southern clam broodstocks as compared to northern broodstocks, results demonstrated a clear effect of temperature on QPX disease development with higher QPX prevalence in both FL-q (southern, susceptible) and MA (northern, resistant) clams at 13 °C (Dahl et al., Accepted). Obviously, the development of QPX disease

is not a simple result of "reduced" performance of baseline immune defense factors in certain clam populations maintained under a given environment but also includes the effects of environmental parameters on host response to pathological challenge and on parasite virulence during host pathogen interactions. More important, host response (or lack of) to pathological challenge largely depends upon the virulence of the pathogen and its ability to colonize host tissues and cause harm, or alternatively, to mute sentinel detection mechanisms leading to immune avoidance.

At 27 °C, comparison of individual constitutive defense factors as well as extracted Principal Component 1 representing combined immune parameters exhibited no significant difference between challenged (FL-q) and control (FL-c) Florida clams. Interestingly, histological observations and quantitative PCR assessing disease development revealed that QPX challenge failed to induce infection in FL clams held at 27 °C (Dahl et al., Accepted). Similarly, naturally infected clams (MA) maintained at 27 °C also showed significant improvement in their disease status indicated by reduced disease prevalence and intensity as compared to clams held at 13 or 21 °C. These findings matched well with our *in vitro* investigations that showed significant decrease of parasite growth in cultures incubated at temperatures exceeding 25 °C as compared to maximal growth measured between 20 and 23 °C (part IV – chapter 1). Failure of QPX disease development at higher temperature could be related to the inability of the parasite to establish infection as a result of limited survival/virulence under high temperature. This could explain the absence QPX disease outbreaks in the field south of Virginia.

Most dramatic changes in immune parameters in response to QPX challenge were observed at 13 and 21 °C (Fig. 7.8A) while parasite challenge did not elicit a significant response in FL clams maintained at 27 °C. Interestingly, the extracted PCA component (PC1) displayed similar trends in FL-q and MA clams in response to temperature between 13 and 21 °C (Fig. 7.8A and B). The interpretation of findings from MA clams is more difficult as this batch was composed of presumably infected clams and the initial screening revealed a 63 % prevalence of diseased MA clams at the beginning of the experiment. This means that MA clam samples at different time intervals likely contained both QPX infected and non infected clams. An important finding was that QPX disease developed at 13 °C while clams maintained at 21 °C

displayed the typical healing processes characterized by the presence of dead and degrading parasite cells in clam tissues (Dahl et al., Accepted). This is in agreement with previous studies that demonstrated healing in clams maintained in lab controlled environment at 21 °C (Dahl and Allam, 2007). The healing process observed in FL-q clams maintained at 21 °C was associated with effective defense response in these animals as compared to those held at 13 °C as demonstrated by a significant increase of hemocyte resistance to the cytotoxicity of QPX ECP and induction of anti-QPX activity in plasma (Fig. 5A and B). A similar increase in anti-QPX activities in plasma was also visible in MA clams maintained at 21 °C as compared to 13 °C (Table 7.2) and was higher in MA compared to FL clams supporting prior findings of higher performance of humoral defenses in northern clam stocks against QPX (part III – chapter 1). It should be noted that our previous molecular investigations identified significant increases in the expression of genes involved in humoral immunity such as defensins and lysozyme in clams exposed to QPX and maintained at 21 °C (part III – chapter 2). These findings suggest that clams maintained at 21 °C not only have strong native activity against the parasite but are also capable of mounting an effective response following QPX challenge.

QPX disease is usually characterized by the presence in clam tissues of lesions often located in vascular and sinusoidal spaces and connective tissues of infected organs (Dahl and Allam, 2007; Smolowitz et al., 1998). The infection elicits a strong granulomatous inflammatory response characterized by the migration of hemocytes toward the lesions. Changes in number and type of circulating hemocytes observed in the present study following QPX/temperature treatment appear to reflect the involvement of these primary defense cells in the response against the parasite. THC increased in FL-q after 2 months at 21 °C but decreased after 4 months at 13 and 21 °C as compared to their respective unchallenged controls (FL-c). A similar phenomenon was previously described during bacterial challenge of the Manila clam *Ruditapes philippinarum* and was explained by an initial response of clams to challenge by increasing hemocyte counts followed by the migration of defense cells to infection sites (Paillard et al., 2004a). Hemocytes differential counts in MA clams maintained at 27 °C revealed proportions of hyalinocytes (~35 %) and granulocytes (~65 %) similar to those reported in a previous study (Tripp, 1992b). This ratio was altered at 21 °C after 2 months but was restored after 4 months whereas similar

proportions of both hemocyte populations (~50 %) were observed during the entire experiment at 13 °C (Table 7.2). Restoration of hemocyte ratio at 21 °C after 4 months may indicate a "back to normal" situation following elimination of QPX and clam healing. Similar changes of ratio between hemocyte subpopulations were previously documented in other bivalves affected by infectious diseases (Ford et al., 1993a; La Peyre et al., 1995a). It is interesting to note that increasing hyalinocyte proportions in MA clams was associated with an increase in hemocyte phagocytic activity and stimulated ROS production. One interpretation of these findings would be a possible involvement of hyalinocytes in these defense processes although such role is not supported by previous studies showing limited capacities of hyalinocytes in these mechanisms (Hégaret et al., 2003). Therefore, an alternative scenario would be that enhanced phagocytosis activity in granulocytes (but also potentially in hyalinocytes) from infected clams may result from pro-inflammatory processes that take place during infection. Such scenario is supported by our prior molecular investigations that showed significant increase in the expression of pro-inflammatory genes following QPX challenge (part III – chapter 2). A combined impact of QPX challenge and temperature on ROS production was also detected. ROS production (basal and stimulated) increased following QPX challenge in FL-q maintained at 21 °C. In contrast, Fl-q clams incubated at 13 °C displayed a reduction in both basal and stimulated ROS production further highlighting the modulatory effect of temperature on host-pathogen interactions. ROS mediated responses are an important component of invertebrates defense systems (Anderson, 2001). Alteration of ROS production by bivalve pathogens was previously reported (Anderson, 1999a) but the ability of QPX to modulate ROS production by clam hemocyte remains unknown.

Overall, clams maintained at 13 °C appeared to be unable to control QPX disease development (Dahl et al., Accepted). The slight increase of lysozyme at 13 °C appeared to have no effect on the *in vitro* growth of QPX (Fig. 7.4A and 7.5A) or on disease outcome. The FDA assay demonstrated failure of plasma from clams held at 13 °C to alter QPX growth and parasite proliferation was even stimulated with plasma from MA clams maintained at this temperature (Fig. 7.5A, Table 7.2). Similarly, greater prevalence and intensity of QPX disease at low temperature (Dahl et al., Accepted) suggested that higher phagocytosis activities observed at 13 °C in FL-q and MA clams (Fig. 7.3, Table 7.2) provided limited or no protective effect

against QPX. Phagocytosis of QPX by clam hemocytes is restricted by parasite's large cell size and the mucus layer surrounding QPX cells in clam tissues. As a matter of fact, phagocytosis of parasite cells *in vivo* is anecdotal and only a few observations of QPX engulfment by multinucleate giant cells (inflammatory cells) have been reported (Smolowitz et al., 1998).

Conclusion

In summary, this is the first study to provide direct evidence for the impact of temperature on constitutive defenses of hard clam and their response to QPX infection. The tested experimental temperatures provided a range of conditions that favored disease development (13 °C) or healing (21 and 27 °C). While the failure of disease development and healing observed at 27 °C may simply result from direct deleterious effect of this temperature on QPX itself, our findings showed more subtle host-pathogen interactions at 21 and 13 °C. For instance, our results demonstrated a significant response of hard clams to QPX at 21 °C resulting in a better resistance toward the infection and healing. Higher disease development at 13 °C may be related to the alteration of constitutive clam defense and inability to mount effective response against QPX. This is interesting because *in vitro* growth of the parasite is significantly higher at 21 °C when compared to 13 °C (Brothers et al., 2000, part IV - chapter 1). This suggests that despite the suboptimal condition for QPX, 13 °C appears to be even more adverse to the host. Similar conclusions were previously made for another "cold water disease" affecting a different clam species (Paillard et al., 2004a). It should be noted that mortality outbreaks linked to QPX in the field usually occur in summer (Dove et al., 2004; Smolowitz et al., 1998, Allam, unpublished), which appears to contradict our results showing no response and no disease development at high temperature (21 and 27 °C). QPX is however a chronic disease and mortality seen during summer is the end point of an infectious process that developed several weeks if not several months before. Although this study demonstrated a strong effect of temperature on QPX and *M. mercenaria* interactions, additional environmental parameters as well as clam physiological conditions related to other natural factors including seasonal cycles might also have an impact on disease development and outcome.

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Part IV

Chapter 3

Long term effects of salinity on hard clam (*Mercenaria mercenaria*)

defense parameters and QPX disease dynamics

Abstract

QPX (Quahog Parasite Unknown) is a protistan parasite affecting hard clams (*Mercenaria mercenaria*) along the Northeast coast of the United States. The fact that QPX disease epizootics are usually observed in field sites with high salinities led to the general assumption that salinity represents an important factor for disease distribution. This study was designed to investigate the effect of salinity on QPX disease development as well as constitutive and QPX-induced defense factors in *M. mercenaria*. Naive and QPX-infected (both experimentally and naturally) clams were maintained at 17 and 30 psu for 4 months. Standard and QPX-specific cellular and humoral defense parameters were assessed after 2 and 4 months. These included total and differential hemocyte counts, reactive oxygen species production, phagocytic activity of hemocytes, lysozyme concentration in plasma, anti-QPX activity in plasma and resistance of hemocytes to cytotoxic QPX extracellular products. Results demonstrated higher QPX-associated mortality in naturally infected and experimentally challenged clams exposed to high salinity although microscopic observations failed to detect any active lesions in clam tissues following experimental injection of the parasite. Additionally, healing processes were noted in naturally infected clams exposed to both salinities. Constitutive clam defense factors and the response to QPX challenge were also affected by salinity. QPX challenge caused significant but transitory changes in immune parameters that were obvious at 2 months but disappeared at 4 months. Overall, our results suggest that salinity was not the main environmental factor involved in QPX disease development but association of salinity with other parameters (such as temperature) might modulate the development of disease outbreaks in *M. mercenaria*.

1. Introduction

During host-pathogen interactions, success or failure to establish infection is largely determined by the virulence of the pathogen and the immunological response of the host. The effect of environmental factors on these interactions is particularly important in poikilothermic osmoconformers such as marine invertebrates (Shumway, 1977). Impact of salinity on host-pathogen interactions in bivalve mollusks has been well-documented (Chu et al., 1993; Ford and Haskin, 1988; Reid et al., 2003), often via modulation of bivalve immunity and defense response to invaders. Like in other invertebrates, bivalve immune system primarily relies on the performance of hemocytes which constitute the main line of defense against invaders (Cheng, 1981). Among other functions, hemocytes mediate phagocytosis of foreign particles (Pipe and Coles, 1995), generate reactive oxygen and nitrogen species (Adema et al., 1991; Anderson, 1994; Arumugan et al., 2000) and produce a wide array of humoral factors, including hydrolytic enzymes (such as lysozyme, peptidases, etc.) and anti-microbial peptides (such as defensins) which are also involved in host responses because of their various anti-microbial properties (Allam et al., 2000a; Cheng, 1992; Chu, 1988; Roch, 1999).

The protistan parasite QPX (Quahog Parasite Unknown) infects and has been associated with severe mortalities in wild and cultured hard clams (*Mercenaria mercenaria*). In recent years, several studies have focused on the pathobiology of QPX disease and on factors affecting disease development. For example, previous studies showed variation in host susceptibility toward QPX among different clam stocks with higher resistance of broodstocks from northern enzootic locations (Massachusetts, New York, New Jersey) as compared to broodstocks from southern locations (North Carolina and Florida) where the disease has never been described (Dahl et al., 2010). Histological observation of QPX-infected tissues demonstrated that some clams are able to mount an effective defense reaction against the parasite characterized by an intense inflammatory response ultimately leading to the healing of infected individuals (Dahl and Allam, 2007; Dove et al., 2004; Ragone Calvo et al., 1998). Our prior investigations demonstrated the presence in clam plasma of factors inhibiting QPX growth (part III – chapter 1). On the other hand, extracellular products (ECP) secreted by QPX were shown to significantly alter clam hemocytes

(part II – chapter 1). Interestingly, both anti-QPX activity in clam plasma and resistance of hemocytes to cytotoxic effects of ECP were correlated with clam resistance to QPX disease. Molecular investigations in hard clams also demonstrated significant modulation in the expression of stress-and defense-related genes after exposure to QPX (part III – chapter 2). In a recent study, we demonstrated significant effects of temperature on clam defense factors and QPX disease development and showed significant development of QPX disease in clams maintained at low temperature (13 °C) whereas higher temperatures (21 and 27 °C) were associated with a reduction of QPX disease development and enhanced healing processes of previously infected clams (Dahl et al., Accepted, part IV - chapter 2). All together, these findings suggest that QPX disease development (or clam resistance) largely depends upon interrelated extrinsic (environmental) and intrinsic (immune performances) factors that result in unbalanced host-pathogen interactions that favor the parasite (or the host) leading to disease and subsequent mortality (or to healing).

Further progress in understanding factors affecting QPX disease development is limited by the lack of information regarding the effect of environmental parameters on clam immune responses toward the infection. For instance, field observations of QPX disease distribution in Virginia noted that QPX disease was absent from areas with moderate salinities (Ragone Calvo et al., 1998). Additionally, *in vitro* growth of QPX was strongly modulated by salinity with a reduction in growth at 15 psu compared to standard culture condition (30 psu) (part IV – chapter 1). These results suggest a role of salinity in QPX disease development in clams although no prior study has been conducted to confirm or reject this suggestion or to evaluate the effect of salinity on clam immune defenses in *M. mercenaria*. In this study, the effect of long term exposure (4 months) to low (17 psu) and high (30 psu) salinities on disease development as well as constitutive and QPX-induced immune factors were investigated in hard clams. Experiments compared QPX disease development and defense parameters of clams naturally (Massachusetts broodstock) and experimentally (Florida broodstock) infected by QPX. Results demonstrated that salinity significantly modulates constitutive and QPX-induced defense factors and impacts QPX disease dynamics in hard clams.

2. Materials and methods

2.1. Mercenaria mercenaria

Five hundred naïve *M. mercenaria* (30-35 mm in length) were obtained from a commercial source in Florida (FL) and 300 clams (40-50 mm in length) were collected from an enzootic clamming area in Massachusetts (MA). Clams were acclimated for 1 week in 150-L tanks with re-circulating water (28-30 ppt) at 21 ± 1 °C and fed daily with commercial algae (DT's Live Phytoplankton, Sycamore, IL, Espinosa and Allam, 2006). Thirty clams from each batch were sampled to assess their disease status using standard histological and quantitative PCR techniques (Dahl and Allam, 2007; Liu et al., 2009). No detectable QPX infections were observed in FL clams whereas histopathological analysis of MA clams indicated active QPX lesions (37 % prevalence) and quantitative PCR revealed a 72 % prevalence of the parasite in this batch at the beginning of the experiment.

2.2. QPX

QPX strain NY0313808BC7 was isolated from nodules of infected New York clams (Qian et al., 2007) and subcultured in muscle tissue homogenates (MTH) from *M. mercenaria* according to Perrigault et al. (part III – chapter 1). QPX cultures were initiated in 25-cm^2 flasks containing MTH at 1000 µg.ml^{-1} protein and incubated at 23 °C for 2 weeks. Neubauer chamber and a previously described fluorometric technique based on the uptake of fluorescein di-acetate (FDA) (Buggé and Allam, 2005) were used to monitor the growth and determine the concentration of QPX cells.

2.3. Salinity treatment

After one week of acclimation to laboratory conditions (21 °C / 30 psu), clams were distributed in 40 L recirculating tanks (20 clams per tank; 24 tanks in total for FL clams and 12 tanks for MA clams) filled with filtered and ultraviolet treated seawater (21 °C / 30 psu). Seawater was filtered using biological and chemical filter cartridges containing activated carbon and was continuously oxygenated to saturation.

Water quality and ammonia level were controlled weekly. Two groups composed of 18 tanks each (6 controls FL [FL-c], 6 QPX-challenged FL [FL-q] and 6 MA [MA]) were maintained at 17 and 30 psu. Salinity adjustment (17 psu) was performed by decreasing the salinity (2 units per day) by adding freshwater. All tanks were maintained at 18 °C to favor disease development and enhance the differential effect of the salinity treatments (Dahl et al., Accepted). Temperature was adjusted in water baths equipped with electronically-controlled heaters and chillers by decreasing the temperature by 1 °C per day. Temperature and salinity of each tank was monitored over the 4-month experiment. Clams were fed daily with commercial algae (DT's Live Phytoplankton, Sycamore, IL, Espinosa and Allam, 2006) and monitored twice a day for mortality.

2.4. QPX challenge of Florida clams

After 2 weeks of acclimation at each salinity, FL clams (FL-q) were challenged with QPX by injecting 5 x 10^4 parasite cells into the pericardial cavity according to Dahl and Allam (2007). Control (FL-c) clams were injected with sterile medium (MTH at 1000 µg.ml^{-1} protein) maintained under the same conditions as QPX cultures (2 weeks at 23 °C). Once injected, clams were maintained out of the water for 1.5 hours and then transferred to their respective tanks.

2.5. Defense parameters

Before QPX challenge (t_o, after 2 weeks at the 2 experimental salinities), 60 FL clams were sampled (5 clams per tank, 30 clams per treatment). Similarly, after 2 and 4 months, 30 FL-c, 30 FL-q and 30 MA clams (5 clams per tank) were sampled from each salinity condition with a total of 240 FL clams and 120 MA clams processed during the experiment. All samples were processed individually. Hemolymph samples (generally 1.2 to 1.8 ml) were withdrawn from the adductor muscle with a 1 ml-syringe and held on ice. A volume of 650 µl hemolymph was diluted (vol:vol) in ice-cold FASW and used for assessment of clam parameters according to Perrigault et al. (part IV – chapter 2). Total (THC) and differential

(DHC) hemocyte counts as well as percentage of dead cells (PDC) were assessed on a FACSCalibur flow cytometer (Becton Dickinson Biosciences) equipped with a 488 nm laser by counting 10,000 events. Reactive oxygen species (ROS) production was assessed before (unstimulated or native) and after stimulation (5 min post-stimulation) of hemocytes with zymosan A (part IV – chapter 2). Phagocytic activity of hemocytes from FL clams (because of logistical difficulties, this assay was not performed on hemocytes from MA clams) was measured using a plate reader technique employing FITC-labeled *Vibrio parahaemolyticus* (part IV – chapter 2). Similarly, our previously described neutral red (NR) uptake assay was applied to assess *in vitro* resistance of clam hemocytes (not performed at t_o) to QPX extracellular products (part II – chapter 1). Plasma from undiluted hemolymph was also recovered by centrifugation (700 x g, 10 min, 4 °C). Supernatant was sterilized by filtration (0.22 µm), aliquoted and preserved at -20 °C to determine lysozyme activity and protein concentration in plasma (part IV – chapter 2). Anti-QPX activity in plasma of clams (not performed at t_o) was measured using a previously described *in vitro* growth inhibition assay (part III – chapter 1).

2.6. Disease diagnosis

Following hemolymph sampling, clams were individually processed for histological analysis. A transverse section about 5 mm in thickness was made through the central region of the clam to include the visceral organs, gills, mantle and the base of the siphon (Dahl and Allam, 2007). After fixation in formalin (10 % buffered), tissue pieces were embedded in paraffin, sectioned (5 to 6 µm in thickness), mounted on histology slides and stained (Harris's hematoxylin and Eosin Y). QPX intensity was assessed and scored based on a classification system described in Ragone Calvo et al. (1998) resulting in the following rankings: rare (< 10 QPX cells on the section), light (11–100), moderate (101–1000) and heavy (> 1000).

2.7. *Statistical analyses*

All variables were analyzed statistically using Multifactor ANOVA to evaluate interactive effects of salinity, sampling time and QPX challenge on hard clam hemolymph parameters. ANOVA treatments that generated probability values below 0.05 were followed by a Holm-Sidak post-hoc test comparing different conditions. Data were log10 or arcsin transformed whenever the variance was large but results are presented as non-transformed values. Two-sample comparisons (mortality data) were made using Student's *t*-test. Multivariate analyses were performed using Principal Component Analysis (PCA) to analyze relationships between variables. PCA analysis was followed by ANOVA on extracted components to assess the effect of treatments (salinity and QPX challenge) on overall hemolymph profiles. PCA analysis was performed with Statgraphics plus (Statistical Graphics Corp., Warrenton, Virginia, USA) and SigmaStat (Systat Software, Inc., San Jose, California, USA) was used for ANOVA analyses. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. *Clam mortality and QPX disease*

Mortality levels were overall low (≤ 9 %) in challenged (FL-q) and control (FL-c) Florida clams maintained at different salinities although interesting trends were noticed among different treatments (data not shown). For instance, exposure of naive *M. mercenaria* (FL-c) to low salinity induced significant mortalities (8 % after 4 months) when compared to clams maintained at 30 psu (0.7 %, $p = 0.001$, Student *t*-test). Mortality in clams exposed to 17 psu was not significantly greater, however, following QPX challenge (9 % after 4 months). Conversely, mortality levels of FL-q clams maintained at 30 psu (5 %) was significantly higher as compared to mortality level in the controls (FL-c) maintained at the same salinity (0.7 %; $p = 0.036$, Student *t*-test). Microscopic observations did not detect any active QPX infection during the 4-month experiment as only dead QPX cells were occasionally seen in vascular tissues of challenged (FL-q) clams.

Mortality differed also between salinity treatments in naturally QPX-infected clams (MA), with significantly higher mortality at 30 psu compared to the low salinity treatment after 4 months (Fig. 8.1A, $p < 0.001$, Student *t*-test). QPX disease prevalence was higher in MA clams maintained at 30 psu for 2 months (17 %) as compared to those reared at 17 psu (10 %) but the difference between treatments disappeared at 4 months (Fig. 8.1B). QPX prevalence among moribund clams collected throughout the experiment was higher in clams maintained at 30 psu (91 %) as compared to those maintained at 17 psu (67 %). Disease intensity among positive clams exhibited no significant difference between treatments (Fig. 8.1B). Healing signs ranging from 10 % to 30 % were noted in naturally infected clams during the 4-month experiment with higher healing signs observed at 2 months in clams maintained at 30 psu compared to those maintained at 17 psu (Fig. 8.1B).

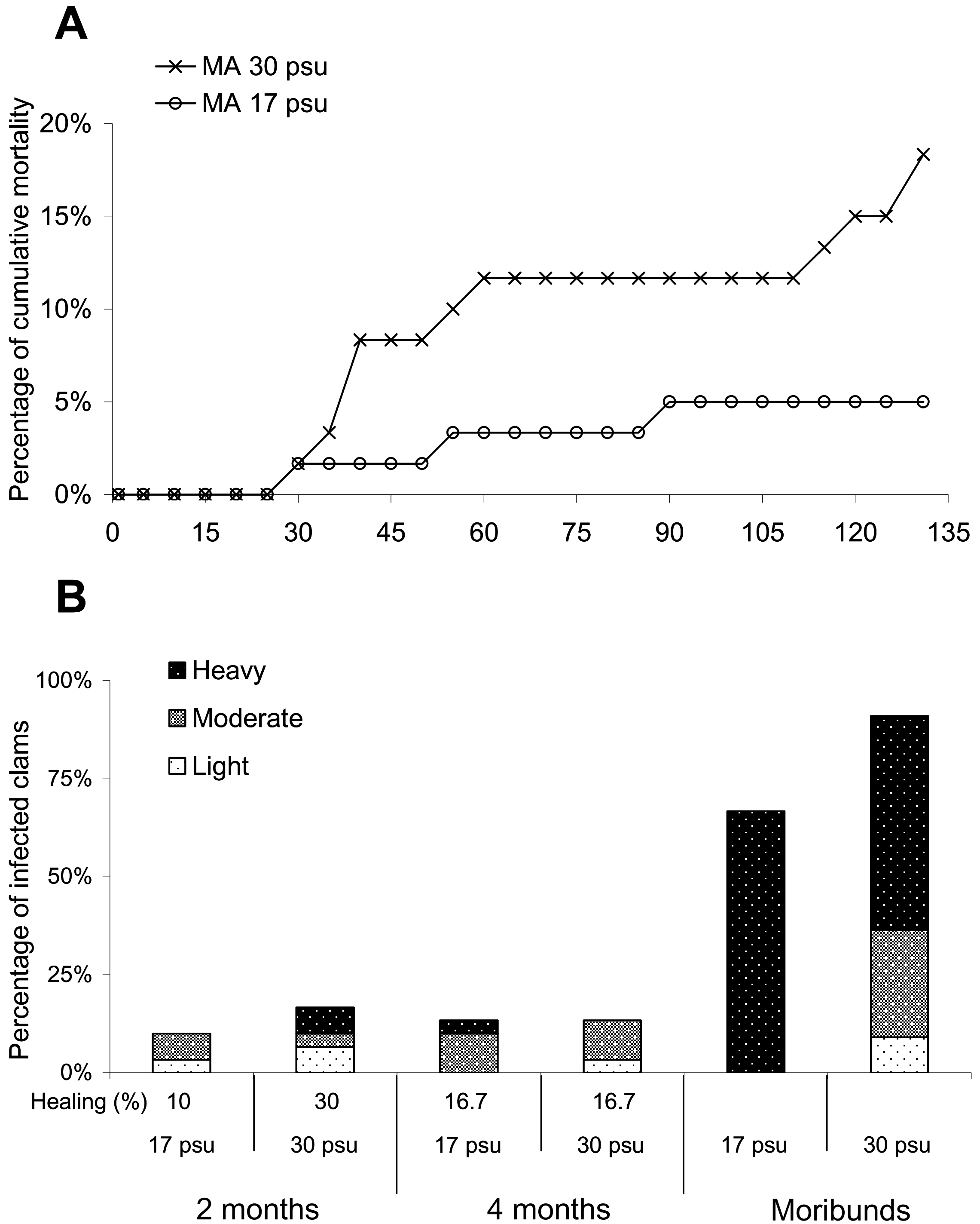

Fig. 8.1. Cumulative mortality [A] and QPX disease prevalence and intensity [B] in naturally-infected clams (MA) maintained at 17 and 30 psu for 4 months. QPX disease prevalence and intensity were determined in 30 clams per treatment excluding moribund clams where n=4 and 10 clams for 17 psu and 30 psu, respectively. * in [A] designates significant difference between treatments (Student *t*-test, $p < 0.001$). Values along the x-axis in [B] indicate percentage of clams presenting healing signs.

3.2. Effect of salinity on individual defense parameters of M. mercenaria

3.2.1. Effect of salinity on defense parameters in naïve clams (FL-c)

Variation of salinity induced significant changes in cellular and humoral defense parameters of naïve *M. mercenaria* from Florida (Tables 8.1 and 8.2, Figs. 8.2A and 8.3A). Total hemocyte counts (THC) tended to be higher in clams exposed to the lower salinity although no significant differences were observed between clams maintained at 30 psu and 17 psu (Table 8.1). Differential hemocyte counts of clams maintained at to 17 psu also tended to decrease in the proportion of granulocytes over time from 48.6 % at 2 weeks to 44.3 % at 4 months compared to the high salinity treatment where hyalinocyte and granulocyte ratios were roughly equivalent and constant over the 4 month experiment. Percentage of dead cells (PDC) was significantly higher ($p < 0.001$, Table 8.1) in naïve clams maintained at 17 psu as compared to those held at 30 psu for 2 weeks and 2 months and a general tendency of higher PDC was noticed at 4 months. Reactive oxygen species (ROS) production in naïve and FL-c clams was also modulated by salinity. Significantly lower basal ROS production was observed in clams after 2 weeks and 2 months of exposure to 17 psu ($p < 0.032$, Table 8.1). Similarly, the production of ROS following stimulation with zymosan A was also less in clams exposed to the lower salinity as compared to those held at 30 psu with a significant differences observed at 2 months ($p < 0.001$), although the difference between treatments was not significant at 4 months (Table 8.1). Phagocytosis activity was also strongly modulated by salinity after 2 months with significantly higher activity in FL-c clams maintained at 30 psu as compared to clams held at 17 psu ($p < 0.001$) although no difference between treatments was observed at 2 weeks or 4 months (Table 8.1). Protein concentration of plasma was roughly similar in both treatments after 2 weeks but higher at 30 psu after 2 and 4 months as compared to the lower salinity group (significant difference at 2 months, $p < 0.001$, Table 8.1). Conversely, lysozyme activity was higher in FL-c clams maintained at 17 psu for 2 weeks as compared to the higher salinity treatment ($p = 0.005$, Table 8.1) but differences between treatments disappeared during the following 2 samplings.

Constitutive anti-QPX activity of plasma from unchallenged (FL-c) clams was higher at 17 psu compared to 30 psu (Fig. 8.2A). Results from the neutral red uptake assay

showed that hemocyte resistance to QPX ECPs was not significantly modulated by salinity treatment although it tended to be slightly higher at 30 psu after 2 months and at 17 psu after 4 months (Fig. 8.3A).

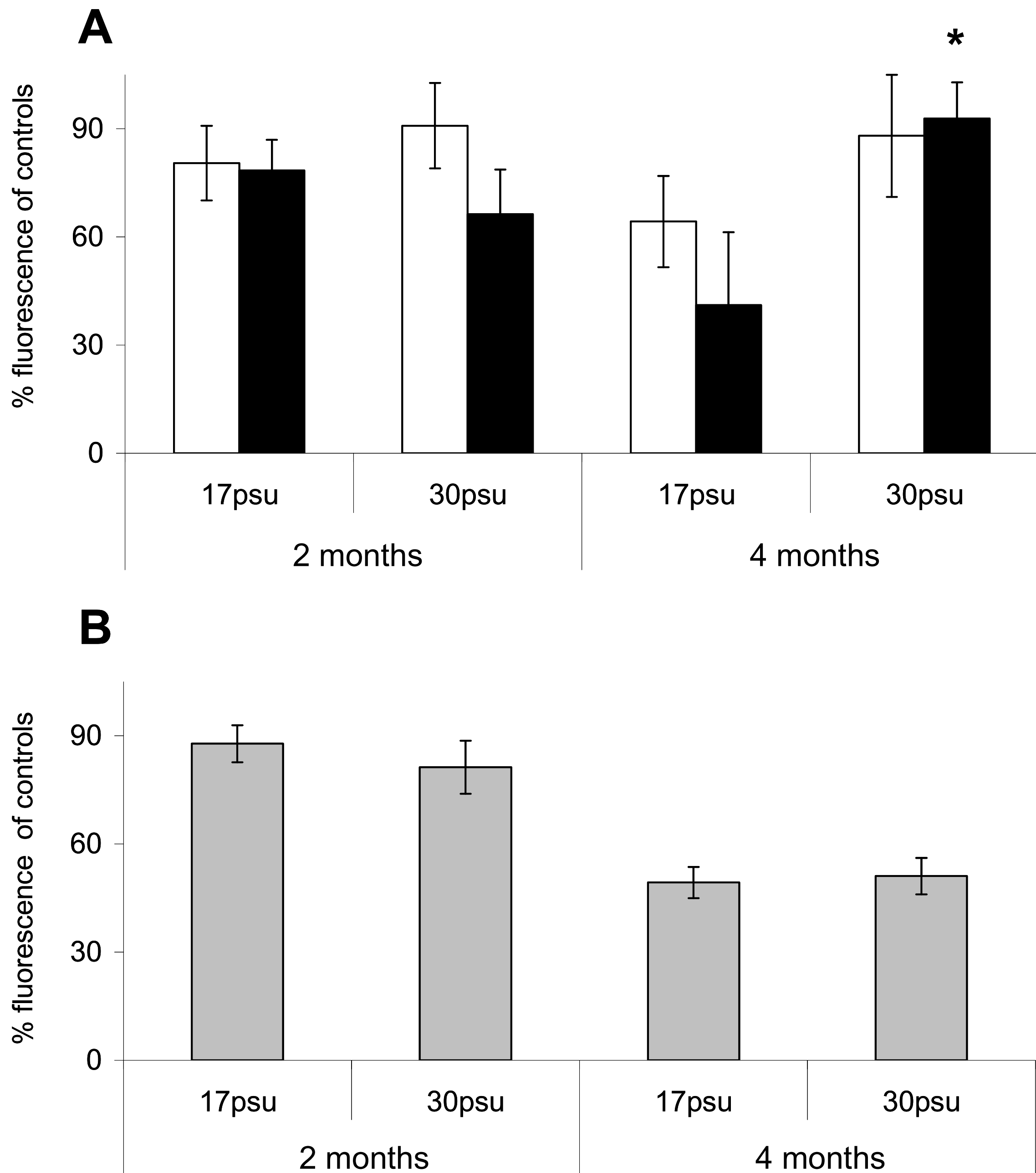

Fig. 8.2. Anti-QPX activity in plasma from [A] control (□) and QPX challenged (■) FL clams and [B] naturally-infected MA clams after 2 and 4 months at 17 and 30 psu (mean ± S.E., n = 30 clams / data point). Higher readings represent lower activities. Symbols (*) denote significant differences (Holm-Sidak post-hoc test, $p < 0.05$) between salinity treatments within each clam group (FL-c, FL-q or MA clams) and sampling time (2 or 4 months).

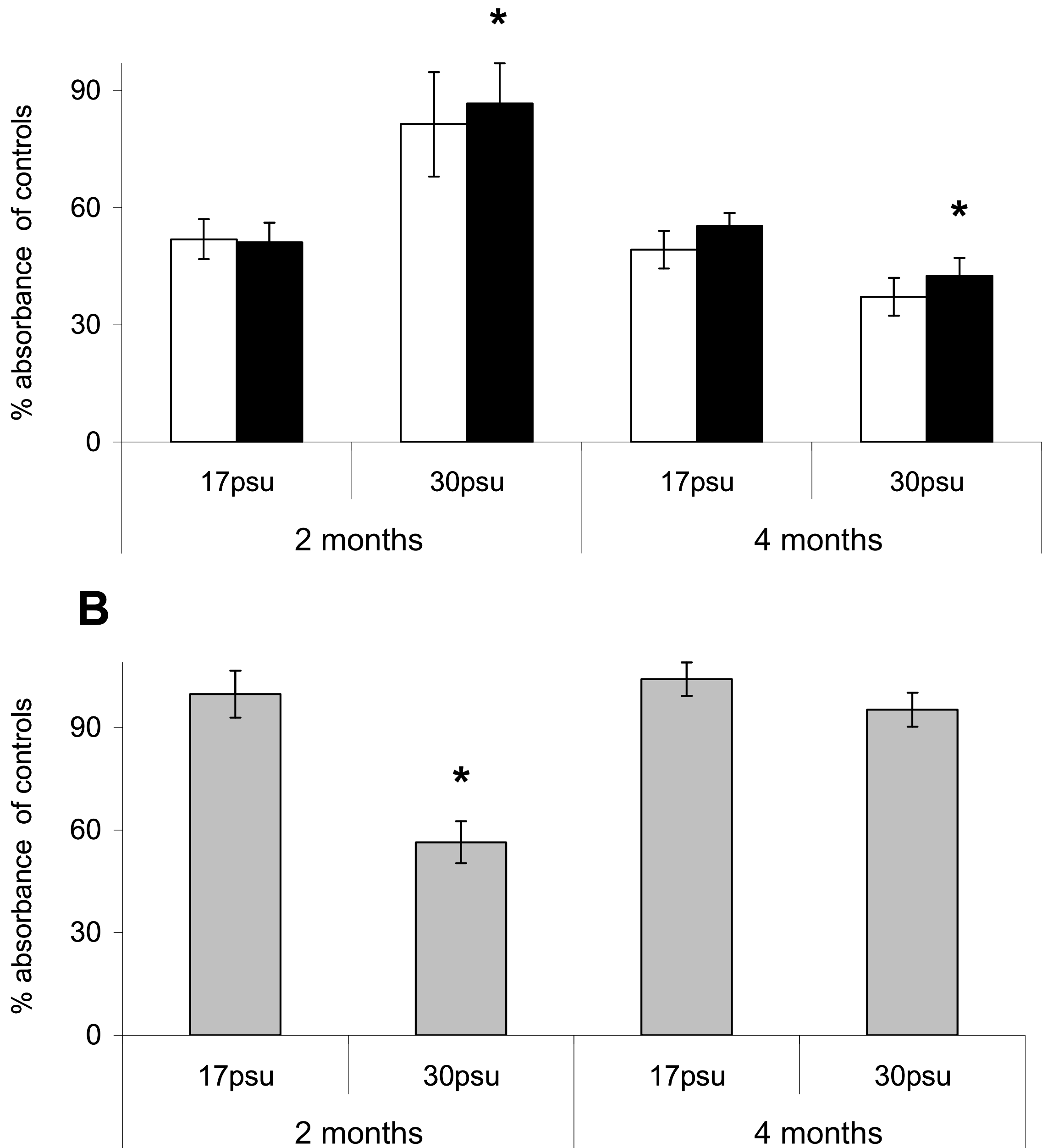

Fig. 8.3. Hemocyte resistance to QPX ECPs in [A] control (□) and QPX challenged (■) FL clams and [B] naturally-infected MA clams after 2 and 4 months at 17 and 30 psu (mean ± S.E., n = 30 clams/data point). Higher readings represent higher resistance of hemocytes to QPX ECPs. Symbols (*) denote significant differences (Holm-Sidak post-hoc test, $p < 0.05$) between salinity treatments within each clam group (FL-c, FL-q or MA clams) and sampling time (2 or 4 months).

Table 8.1. Cellular and humoral parameters in *M. mercenaria* (FL) 2 weeks after acclimation to 17 and 30 psu (T0) and in control (FL-c) and QPX-challenged (FL-q) clams maintained at 17 psu and 30 psu for 2 or 4 months.

	T_o – 2 weeks		2 months				4 months			
	17 psu	30 psu	17 psu		30 psu		17 psu		30 psu	
	FL	FL	FL-c	FL-q	FL-c	FL-q	FL-c	FL-q	FL-c	FL-q
THC (cells.ml^{-1} x 10^5)	12.2	9.5	5.2	5.3	4.0	6.5*	8.5	6.5	7.3	7.1
% granulocytes	48.6	50.7	42.9	51.2	46.5	57.5§*	44.3	51.1	52.8§	58.14
PDC (%)	13.9	7.8§	12.3	13.1	6.2§	11.3*	10.4	10.8	9.4	8.5§
basal ROS production (Fluorescence units / 10^4 hemocytes)	1.8	2.3§	3.4	3.4	5.7§	2.3§*	3.4	3.6	3.0	2.4
5 min stimulated ROS production (Fluorescence units / 10^4 hemocytes)	2.6	3.0	3.4	4.9	8.5§	3.4*	3.9	2.2	3.0	3.1
Phagocytosis (Fluorescence units / 10^4 hemocytes)	2.0	1.6	1.5	2.6	7.1§	4.2*	2.0	1.9	1.9	2.5
Protein concentration (µg.ml^{-1})	411.5	405.5	313.1	310.7	433§	446.6§	300.4	330.9	346.9	394.1§
Lysozyme (µg.ml^{-1})	7.6	7.1§	8.9	8.9	8.8	9.1	8.9	9.0	8.9	8.8§

§ denotes significance at $p < 0.05$ between salinity treatments within the same treatment (FL, FL-c or Fl-q) and sampling time (2 weeks, 2 or 4 months). * denotes significant differences at $p < 0.05$ between FL-c and FL-q within same salinity treatment and sampling time. (Mean, n = 30 per condition). Refer to appendix 3 for SEM values.

Table 8.2. Effects of salinity and QPX challenge on Florida *M. mercenaria* cellular and humoral parameters during 4 months (2 way ANOVA).

	FL-c			FL-q		
	Time	PSU	Time/PSU	Time	PSU	Time/PSU
THC	***	*	*	ns	**	ns
% Granulocytes	ns	*	Ns	***	ns	***
PDC	ns	***	***	***	**	ns
Unstimulated ROS	**	*	**	ns	***	ns
Stimulated ROS (5 min)	**	*	***	*	ns	*
Phagocytosis	**	***	***	***	***	ns
Protein concentration	*	***	Ns	ns	**	ns
Lysozyme	ns	ns	Ns	ns	ns	ns
Anti QPX activities in plasma	*	**	Ns	ns	ns	ns
Hemocyte resistance to QPX ECP	**	ns	*	**	*	***

Non-significant effects are designates by ns and symbols denote significant differences at $p < 0.05$ (*),$p < 0.01$ (**) or $p < 0.001$ (***) (ANOVA).

3.2.2. Effects of salinity on clam responses to QPX (FL-q and MA)

Salinity treatment induced significant modifications of defense parameters in both experimentally challenged (FL-q) and naturally infected (MA) clams (Tables 8.1-8.4, Figs. 8.2 and 8.3). QPX challenge caused a significant increase of THC only in FL-q clams maintained at 30 psu for 2 months as compared to FL-c controls maintained at the same salinity (Table 8.1, $p < 0.001$). Additionally, THC in FL-q and MA clams was generally higher after 4 months in clams maintained at 30 psu as compared to those held at 17 psu (Tables 8.1 and 8.3). QPX challenge also caused an increase in the percentage of granulocytes in FL-q clams maintained at 30 psu as compared to FL-c clams reared at the same salinity or to FL-q clams held at 17 psu (Table 8.1). Similar differences were observed in MA clams with significantly higher percentage of granulocytes in clams maintained at 30 psu compared to those held at 17 psu for 2 and 4 months ($p < 0.005$, Table 8.3). QPX challenge caused a significant increase in PDC among FL-q clams maintained at 30 psu for 2 months (11.3 %, Table 8.1) as compared to their respective controls (FL-c) held at the same salinity (6.2 %, *p*

< 0.001). Challenge with the parasite did not affect, however, the already elevated PDC among clams maintained at 17 psu or among clams collected at 4 months. Overall, FL-q and MA clams presented similar PDC profiles characterized by lower PDC at 30 psu compared to 17 psu with significant differences in MA clams sampled at 2 and 4 months ($p < 0.019$, Table 8.3) and FL-q clams after 4 months ($p < 0.001$, Table 8.1). ROS production by hemocytes from FL clams was highly modulated by both QPX challenge and salinity treatment (Table 8.2). Unstimulated (basal) ROS production was not modulated by QPX challenge in clams maintained at 17 psu but was significantly lower in FL-q clams maintained at 30 psu as compared to their unchallenged controls sampled after 2 months (Table 8.1, $p < 0.001$). Additionally, decreasing salinity caused an increase in basal ROS production with significant effect at 2 months in FL-q clams (Table 8.1, $p = 0.001$) and after 4 months in MA clams ($p < 0.016$, Table 8.3). Zymosan-activated ROS production generally followed similar trends as unstimulated ROS and was lower in FL-q clams maintained at 30 psu as compared to their unchallenged controls sampled after 2 months (Table 8.1, $p < 0.001$), although differences among MA clams were not statistically significant (Table 8.3). Phagocytosis activity of Fl-q clams exhibited inverted trends with higher activity at 30 psu compared to similar treatments at 17 psu (Table 8.1). Interestingly, significant reduction of phagocytosis activity was also observed in FL-q clams maintained at 30 psu for 2 months compared to their unchallenged (FL-c) controls maintained under the same conditions ($p = 0.01$) whereas the effect of QPX challenge was not detectable after 4 months. Protein concentration in plasma displayed identical trends in naturally and experimentally infected clams and was significantly higher in both MA and FL-q clams maintained at 30 psu compared to those held at 17 psu ($p < 0.023$, Tables 8.1 and 8.3). Lysozyme activity also displayed similar trends among MA and FL-q with slightly higher lysozyme activity at 30 psu after 2 months and lower activity after 4 months although differences were only significant among FL-q clams sampled at 4 months ($p = 0.007$). Experimental challenge did not cause any significant alteration in protein concentration in plasma or lysozyme activity over the entire duration of the study despite a tendency of higher protein concentration in plasma of FL-q clams compared to their respective controls (Table 8.1).

Table 8.3. Hemocyte and plasma parameters of MA *M. mercenaria* naturally infected by QPX and maintained at 17 psu and 30 psu during 2 and 4 months.

	2 months		4 months	
	17 psu	30 psu	17 psu	30 psu
THC ($cells.ml^{-1} \times 10^5$)	12.0	18.6	10.2	10.5
% granulocytes	49.0	60.1§	44.5	66.0§
PDC (%)	6.3	4.9§	7.6	4.4§
basal ROS production (Fluorescence units/ 10^4 hemocytes)	2.4	1.1§	1.9	1.5§
5 min stimulated ROS production (Fluorescence units / 10^4 hemocytes)	2.1	1.3	2.7	2.6
Protein concentration ($\mu g.ml^{-1}$)	348.8	442.2§	349.8	451.4§
Lysozyme ($\mu g.ml^{-1}$)	6.1	6.4	6.7	4.6

Symbols (§) denote significant differences ($p < 0.05$, ANOVA) between salinity treatments for the same sampling time (2 or 4 months). (Mean, n = 30 per condition). Refer to appendix 4 for SEM values.

Anti-QPX activity in plasma from MA and FL-q clams was lower at 17 psu after 2 months (Fig. 8.2) but higher at 4 months with significantly higher anti-QPX activity among FL-q clams maintained at 17 psu compared to the 30 psu treatment ($p = 0.019$, Fig. 8.2A). Hemocyte resistance to QPX ECP was less consistent between FL-q and MA clams with significant and opposite effects between salinity treatments at 2 months (Fig. 8.3, Tables 8.2 and 8.4). For instance, hemocyte resistance was higher in FL-q clams maintained at 30 psu for 2 months as compared to those held at 17 psu ($p < 0.009$) while it was lower in MA clams maintained at the higher salinity as compared their 17 psu counterparts ($p < 0.001$). The opposite was observed in FL-q clams at 4 months with higher hemocyte resistance in clams maintained at 17 psu compared to 30 psu ($p = 0.047$). Hemocyte resistance to QPX ECPs decreased over time in FL-q clams maintained at 30 psu while it increased in MA clams held at the same salinity. In contrast, hemocyte resistance to QPX ECPs in FL-q and MA clams maintained at 17 psu remained constant during the 4-month experiment. Overall, experimental challenge did not cause any significant alteration in anti-QPX activity or

hemocyte resistance to QPX ECP despite general trends for higher anti-QPX activity and hemocyte resistance in FL-q clams compared to their controls (Figs. 8.2 and 8.3).

Table 8.4. Effects of salinity on cellular and humoral parameters of QPX infected clams from MA during 4 months.

	Time	PSU	Time/PSU
THC	**	Ns	ns
% Granulocytes	ns	***	ns
PDC	ns	***	ns
Unstimulated ROS	ns	***	ns
Stimulated ROS (5 min)	**	Ns	ns
Protein concentration	ns	***	ns
Lysozyme	ns	Ns	ns
Anti-QPX activity in plasma	***	Ns	ns
Hemocyte resistance to QPX ECP	***	***	*

Symbols denote significant differences: $p < 0.05$ (*),$p < 0.01$ (**),$p < 0.001$ (***) (ANOVA).

3.3. Global effects of salinity and QPX challenge on immune status of M. mercenaria

Principal Component Analysis was performed on defense parameters combined from all FL (Fig. 8.4) and MA clams (Data not shown) sampled at 2 and 4 months. Components 1 and 2 explained more than 45 % of the total variance in FL clams. Phagocytosis, percentage of granulocytes and hemocyte resistance to cytotoxic activity of QPX ECPs clustered together and were opposed to THC, PDC, protein concentration and anti-QPX activity in plasma on component 1 and to ROS and PDC on component 2. Similar clustering was also observed in PCA analysis of MA clams except PDC and lysozyme activity were opposed to THC and protein concentration on component 1 (Data not shown). Extraction of component 1 (PC1) and statistical analysis (ANOVA) from clams sampled at 2 and 4 months confirmed the significant impact of salinity treatment and QPX challenge on the hemolymph profiles of both Florida and Massachusetts clams, mostly driven by major differences among treatments at 2 months. For instance, PC1 values were significantly different

between FL-c clams maintained to 17 and those maintained at 30 psu for the 2-month sampling and for both sampling times combined (*p* = 0.0012, Fig. 8.5A). QPX challenge also caused a significant change in PC1 (*p* = 0.002) at 2 months and for combined sampling times in FL-q clams maintained at 30 psu as compared to their unchallenged controls (FL-c, Fig. 8.5A) likely as a result of higher THC and lower unstimulated and stimulated ROS production (Table 8.1). Interestingly, the effect of QPX challenge was inverted at 17 psu where PC1 from FL-q clams was slightly higher than that from FL-c at 2 months and for both sampling times (Fig. 8.5A). After 4 months, PC1 was slightly higher in FL-q clams as compared to FL-c at both salinities but differences between groups were no longer significant (Fig. 8.5C). Similarly to trends observed in FL-q clams at 2 months, MA clams exhibited a significant decrease in PC1 with increasing salinity during the 4-month experiment ($p < 0.001$, Fig. 8.5B and 8.5D).

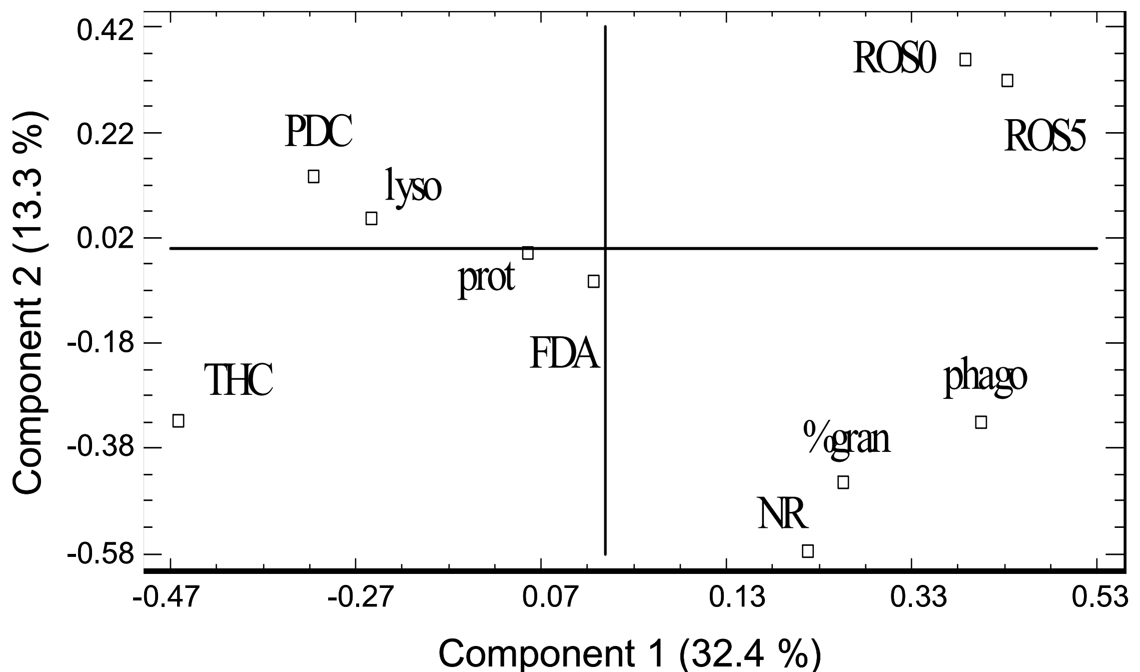

Fig. 8.4. Principal Component Analysis (PCA) plot of immune parameters (2 and 4 months) of FL clams abbreviated as follows. PDC: percentage of dead cells, ROS0: basal ROS production, ROS5: zymosan-stimulated ROS production after 5 min, THC: total hemocyte count, %gran: percentage of granulocytes, phago: phagocytosis, prot: protein concentration in plasma, lyso: lysozyme activity, FDA: anti-QPX activity of clam plasma, NR: hemocyte resistance to cytotoxicity of QPX ECP.

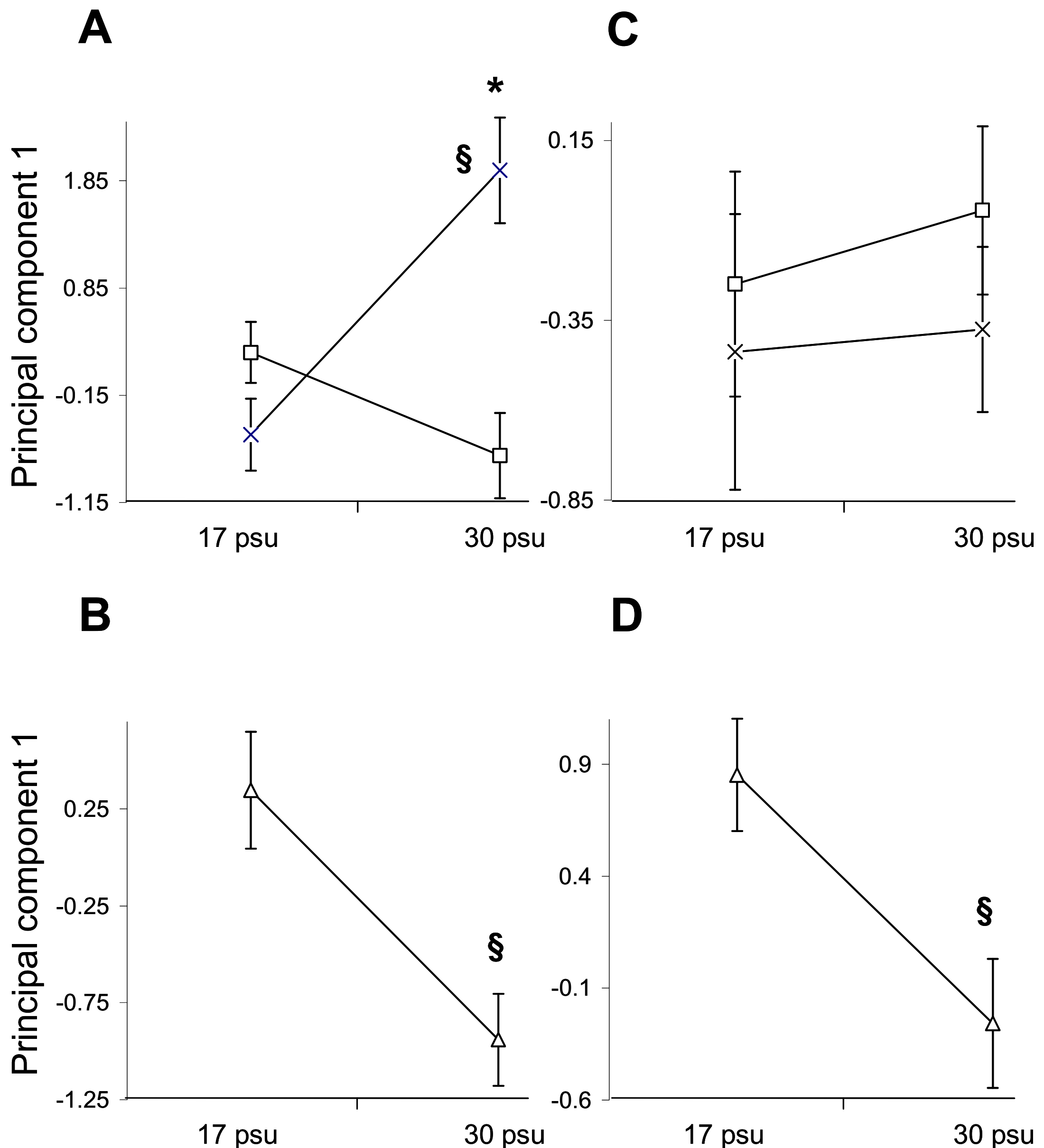

Fig. 8.5. Mean plots of Component 1 from Principal Component Analysis of immune parameters in [A and C] unchallenged (✕) and QPX challenged (□) FL clams and [B and D] MA clams maintained at 17 and 30 psu. Components were extracted at [A and B] 2 and [C and D] 4 months of temperature challenge. Symbols (§) denote significant differences between salinity treatments and (*) indicate significant differences between FL-c and FL-q at 30 psu ($p < 0.05$, ANOVA).

4. Discussion

The aim of this study was to investigate the effect of salinity on defense parameters (both cellular and humoral) of naive hard clams *M. mercenaria*, and to evaluate the combined effects of salinity and QPX challenge on host defenses and

disease development. Naturally- infected and experimentally-challenged clams were used to investigate the effect of salinity on QPX disease progress and clam response to the parasite. Results demonstrated significant impacts of salinity on defense factors in naïve clams, QPX disease development and clam response to the parasite.

QPX is a relatively slow and chronic disease (Dahl and Allam, 2007; Dahl et al., Accepted). The duration of the experiment (4 months) and temperature conditions (18 °C) were selected based on data from previous experiments to enhance the differential effect of salinity treatments on clam defense parameters and the response to QPX challenge (Dahl and Allam, 2007, part III - chapter 2, part IV - chapter 2). Additionally, intrapericardial injection of the parasite into naïve Florida clams was used to produce the disease since this is the only reliable method for disease induction in the laboratory (Dahl and Allam, 2007).

4.1. Effect of salinity on naïve clams(FL-c)

Our study demonstrated a strong influence of salinity on clam survival as well as on cellular and humoral defenses of naïve FL clams (Tables 8.1 and 8.2). Although mortality levels among FL clams were overall low, they were significantly higher in naive clams maintained at 17 psu compared to those maintained at 30 psu. Hard clams grow optimally between 24 and 28 psu but they are occasionally found in waters with salinities as low as 4 psu (Chanley, 1958). Castagna and Chanley (1973) reported negative effects of salinities below 15 psu on *M. mercenaria* growth and survival. Interestingly, mortality in FL-c clams maintained at 17 psu occurred mostly during the first month of the experiment (data not shown) suggesting a failure of clams to adapt to this low salinity treatment. It is possible that stress induced by the wound inflicted during intrapericardial injection of MTH represented an aggravating factor that further enhanced clam susceptibility to the salinity treatment although the injection alone caused very limited mortality in FL-c clams maintained at 30 psu. Additionally, decreasing salinities were associated to reduction of physiological parameters such as clearance and heart rate which could be related to observed mortalities as well as the modulation of some measured parameters (Bakhmet et al., 2005; Sara et al., 2008).

PCA analysis and ANOVA on extracted PC1 demonstrated significant differences in measured defense factors between clams held at 17 psu and those maintained at higher salinity after 2 months (Fig. 8.5A). Clams exposed to lower salinity generally had higher THC, PDC, and lysozyme activity as well as low phagocytosis activity and ROS production (Table 8.1). Our findings of high hemocyte counts at low salinity are in opposition to previous studies using other marine mollusks species. This may reflect the fact that most prior investigations were performed over short and/or acute exposure to low salinity (Bussell et al., 2008; Cheng et al., 2004c; Hauton et al., 2000). In fact, except for the study by Reid et al. (2003), changes in THC in response to exposure of marine bivalves to low salinity are usually not significant. For example, previous studies in the oyster *Crassostrea virginica* showed statistically insignificant changes in THC following exposure to salinities ranging from 10 to 36 psu (Chu et al., 1993; Fisher and Newell, 1986), although the study by Fisher and Newell (1986) showed gradually decreasing THC with increasing salinity after 1.5 months of exposure. Overall, long term exposure to 17 psu is probably stressful to *M. mercenaria* as this salinity falls below the optimal range for hard clam growth. Stress, caused either by environmental change or pathogen challenge, has been associated with THC increase in bivalves (Ford et al., 1993b; Pipe and Coles, 1995). Additionally, THC increase at low salinity was associated with a significant increase of PDC (Tables 8.1 and 8.2) highlighting stressful conditions for clams at 17 psu. Similar increase of PDC with decreasing salinity was previously observed in *C. gigas* (Gagnaire et al., 2006).

ROS production (both unstimulated and zymosan-stimulated) was higher at 30 psu than 17 psu (Tables 8.1 and 8.2), in agreement with prior findings of higher ROS production with higher salinity in abalone (Cheng et al., 2004c) and oysters (Hauton et al., 2000). The involvement of reactive oxygen species in defense mechanisms was previously demonstrated (Anderson, 1994; Pipe, 1992), although increasing unstimulated (baseline) ROS activities in cells and tissues are also harmful for hosts themselves (Torreilles et al., 1996). It should be noted however that, in our study, hemocytes may have been "activated" during hemolymph sampling and processing (which involved plating in a microplate) before the addition of zymosan. Nevertheless, higher stimulated ROS production at 30 psu likely reflects higher immunocompetency among clams maintained at this salinity. This is supported by

the fact that trends of ROS production at 2 and 4 months matched well with phagocytosis activity of hemocytes (Table 8.1). This is not surprising given known links between phagocytosis and respiratory burst that leads to ROS production (Torreilles et al., 1996). Our results of modulatory effects of salinity on phagocytosis activity in hemocytes are also supported by findings from previous studies (Carballal et al., 1997b; Cheng et al., 2004c; Gagnaire et al., 2006; Matozzo et al., 2007; Reid et al., 2003; Wang et al., 2008a) which demonstrated variation of phagocytic activity after accute and long term exposure to different salinities. However, mechanisms involved in this modulation are still unknown.

Protein concentrations in plasma were generally higher at high salinity as compared to 17 psu (Table 8.1). The effect of salinity on protein concentration in plasma was previously suggested (Fisher and Newell, 1986) and demonstrated in *C. virginica* (Gullian and Aguirre-Macedo, 2009). Bivalves are osmoconformers and are able to modulate their osmotic pressure in response to environmental changes (Shumway, 1977). This response involves quantitative and qualitative changes in proteins as demonstrated in oyster hemocytes in response to salinity changes *in vitro* (Tirard et al., 1997). It is therefore likely that *M. mercenaria* is able to modulate plasma protein concentration and composition in response to salinity / osmotic change. Interestingly, slightly higher lysozyme activity was measured in plasma from clams maintained at 17 psu as compared to those held at 30 psu despite generally lower protein contents in this batch (lysozyme activity was normalized to plasma volume and not to protein contents). This finding is in agreement with those of Chu et al. (1993) who observed continuous decrease of lysozyme activity in plasma of *C. virginica* maintained at increasing salinities ranging from 3 to 20 psu. As for lysozyme, higher anti-QPX activity was measured in plasma from FL-c clams maintained at 17-psu compared to 30 psu suggesting a relationship between lysozyme and anti-QPX activity.

4.2. Effects of salinity and QPX on clam defense parameters

Change in salinity has been correlated with disease incidence in several bivalve species. Infection intensities of oyster parasites *Haplosoridium nelsoni*

(MSX) and *Perkinsus marinus* in *C. virginica* increase with increasing salinity (Chu et al., 1993; Ford and Haskin, 1988) whereas development of brown ring disease by *Vibrio tapetis* in the Manila clam *Ruditapes philippinarum* is higher at 20 psu than 40 psu (Reid et al., 2003). Similarly, our study demonstrated an influence of salinity on QPX disease development, resulting mortality and immune responses to the parasite in naturally infected and experimentally challenged *M. mercenaria* (Tables 8.1-8.4, Fig. 8.1). Mortality was significantly higher among experimentally infected clams maintained at 30 psu (5 %) as compared to their controls (0.7 %). Similarly, naturally infected clams exhibited significantly higher mortality at high salinity compared to 17 psu treatment with 18.3 % and 5 % respectively. Differences between salinity treatments were also noted for disease prevalence among moribund clams with 91 % and 67 % for MA clams maintained at 30 and 17 psu, respectively. These findings contrast with relatively low and similar disease prevalence detected in MA clams sampled at 2 and 4 months from both salinity treatments (Fig. 8.1B). It should be noted that our MA clams displayed 37 % QPX disease prevalence at the beginning of the experiment. Therefore, the decrease in disease prevalence at 2 and 4 months is likely the result of 2 different processes: (1) mortality of diseased clams; this is particularly true among clams held at 30 psu that displayed high mortality (18.3 %) and disease prevalence among moribund clams (91 %), and (2) healing of infected clams, as observed in clams maintained at both salinities.

Salinity significantly affected defense factors in clams naturally and experimentally infected with QPX (Tables 8.2 and 8.4). To help assess the global effect of salinity on all measured factors combined, we used PCA analysis and tested changes in extracted principal component 1 (PC1). PC1 changes in response to salinity treatment displayed similar trends among naturally and experimentally infected clams at 2 months (Fig. 8.5A and B), but differences between 17 psu and 30 psu were significant only in MA clams. Interestingly, PC1 trends in both challenged (FL-q) and MA clams were opposed to those observed in FL-c and significant difference was observed in PC1 between FL-q and FL-c clams at 30 psu after 2 months (Fig. 8.5A) and at both sampling times combined (but not at 4 months alone, Fig. 5C).

Significantly lower ROS production and phagocytosis activity were observed in FL-q clams as compared to unchallenged controls after 2 months at 30 psu (Table 8.1). These results are in agreement with our prior report changes in phagocytosis and ROS production in naturally-infected clams or following experimental challenge (part IV – chapter 2). QPX challenge induced a significant increase in THC among FLq clams maintained at 30 psu for 2 months as compared to their controls. This was associated with a significant increase in the percentage of granulocytes (Table 8.1). Hemocytes constitute the main line of defense for bivalves against invaders (Cheng, 1981). Increase of THC in bivalves following bacterial or protistan challenge was previously reported to be an important part of host response to pathogens (Cheng et al., 2004c; Ford et al., 1993b). QPX disease is usually characterized by the presence in clam tissues of lesions often located in vascular and sinusoidal spaces and connective tissues of infected organs and is associated with a strong granulomatous inflammatory response characterized by the migration of hemocytes toward the lesions (Dahl and Allam, 2007; Smolowitz et al., 1998). Therefore, changes in THC and percentage of granulocytes observed here may be related to the involvement of hemocytes in the inflammatory response against QPX. Interestingly, there were not significant changes in hemocyte counts or composition in response to QPX challenge at 17 psu or after 4 months of challenge for both salinity treatments. Differences between challenged (FL-q) clams exposed to 17 and 30 psu show significant modulation of hemocyte counts and composition only in animals exposed to the high salinity suggesting significant response of clams to parasite challenge only under these conditions. The fact that this response was transitory (disappeared at 4 months) and that QPX challenge under these conditions failed to induce the development of histologically detectable QPX disease highlights the ability of clams to neutralize this opportunistic parasite. In that regard, "specific" clam activities against QPX can be more informative than general defense responses such as hemocyte counts and phagocytosis. At 2 months, higher anti-QPX activity was measured in plasma from challenged (FL-q) and naturally infected (MA) clams maintained at 30 psu in comparison to those held at 17 psu. On the other hand, hemocyte resistance to QPX ECPs was significantly higher in FL-q clams maintained at 30 psu as compared to their 17 psu counterparts. Trends were generally inverted at 4 months further supporting the significant, transitory, response of clams to QPX observed at 2 months.

Microscopic observations failed to detect any active lesions in clam tissues following experimental injection with QPX under our current experimental conditions and only old granuloumatous lesions containing dead parasite cells were detected in challenged (FL-q) clams after 2 and 4 months. These findings contrast with our prior reports of successful experimental transmission under similar timeframes using the same technique (Dahl and Allam, 2007; Dahl et al., Accepted). These "negative" results should be carefully interpreted, however, since QPX lesions in clam tissues are usually focal and histophatological methods have already shown their limitations for the detection of light QPX infections (Liu et al., 2009). Despite the lack of microscopic evidence of active infection, a significant immune response was identified in FL-q clams compared to unchallenged (FL-c) clams maintained at 30 psu for 2 months (Fig. 8.5A). Differences between treatments were no longer significant at 4 months and no significant differences were observed between FL-q and FL-c clams held at 17 psu for either 2 or 4 months. Both absence of histological evidence of active infections and the transitory immunomodulation following experimental challenge suggest an effective response of clams that succeeded in eliminating the parasite. On the other hand, a significant effect of salinity on disease development and resulting mortality and defense response was identified in MA clams. Together, these results suggest that at 18 °C (temperature used in the current study), a salinity of 17 psu appears unsuitable for QPX since, not only QPX-related mortalities were reduced in naturally infected clams, but also disease did not develop and clams did not immunologically respond to experimental QPX challenge clams. This interpretation agrees well with findings by Perrigault et al. (part IV – chapter 1) that demonstrated significant alteration of the *in vitro* growth of QPX at low salinity (15 psu) compared to high salinity (30 psu). Additionally, defense factors of clams could be more effective in eliminating the parasite at 17 psu particularly since QPX cells are likely already stressed by the low salinity treatment itself. Such improvement of immune response against weakened parasites has been proposed by Andrews (1983) who suggested active elimination of *H. nelsoni* by *C. virginca* host defenses, which in turn were enhanced by low salinity. Alternatively, the failure of the parasite to establish itself and cause infection at 30 psu in experimentally challenged clams (and improvement in naturally infected clams) may be related to clams ability to mount a significant immune response leading to elimination of the parasite. The impact of

salinity on clam response against QPX was obvious in naturally infected clams (Figs. 8.5B and D).

In conclusion, this study demonstrates a significant impact of salinity on clam immunity and QPX disease progress in naturally infected clams. Our prior investigations demonstrated that QPX establishment in clams is strongly affected by temperature as experimental challenge of Florida clams (same broodstock as that used in the current study) with QPX caused significant disease development at 13 °C and 30 psu whereas similar challenge at 21 °C induced an effective clam immune response aborting disease development (part IV – chapter 2). The fact that our current experiments were performed at 18 °C limited our abilities to effectively assess the effect of salinity on disease development following experimental challenge and future experiments investigating the impact of salinity should be performed at lower temperatures. Overall, our results suggest a weaker impact of salinity than temperature on disease development.

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Part V

Conclusions and perspectives

The objectives of this study were to investigate QPX-*Mercenaria mercenaria* interactions by using several approaches including cellular and molecular biology, biochemistry and proteomics. These approaches allowed the identification of QPX virulence factors and *M. mercenaria* defense mechanisms involved during QPX disease development and provided new insights into the modulatory effect of temperature and salinity on parasite growth and survival, host defenses and QPX disease development.

1. Factors associated with the variability of *Mercenaria mercenaria*–QPX interactions and QPX disease development

The comprehension of host–pathogen interaction is essential to understand a disease as the modulation of any parameter involved in the system can result in the development of the disease or the effective healing of hosts. Our study demonstrated that each element (host, parasite and environment) was actively involved in the modulation of *M. mercenaria*–QPX interactions (Figure 9.1).

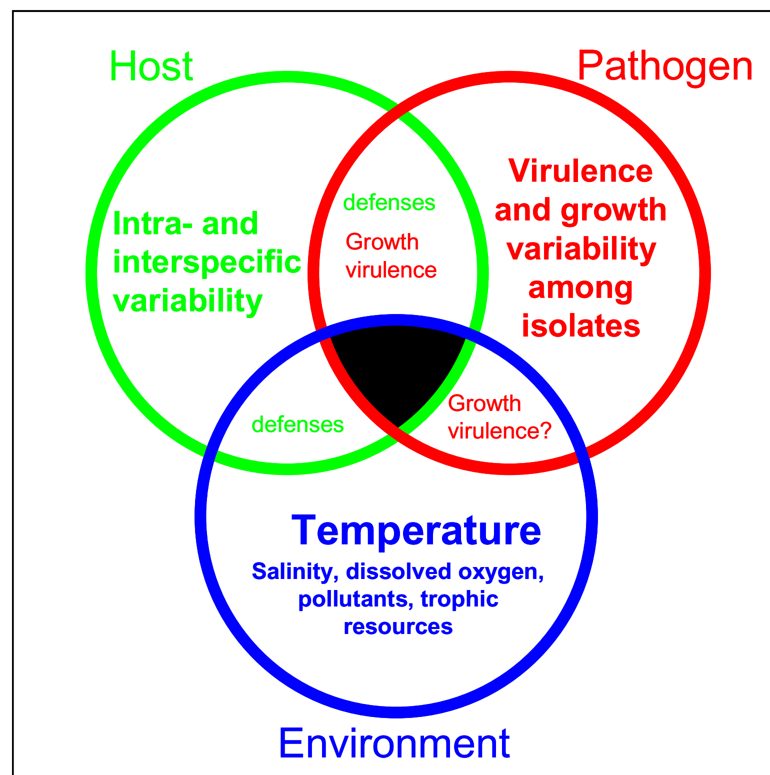

Fig. 9.1. Schematic representation of interactions between *M. mercenaria*, QPX and environmental parameters. Host defenses include cellular and humoral defenses.

Evidence for the existence of **different QPX strains** with specific characteristics was observed. For instance, QPX isolated from different locations (New York and Massachusetts) exhibited significantly different degrees of virulence (part II – chapter 1) and temperature optima (part IV – chapter 1). These *in vitro* results are consistent with *in vivo* observations made by Dahl et al. (2008) on the variation of QPX pathogenicity in hard clams. Similar observations were reported for the oyster parasite *Perkinsus marinus* (Bushek and Allen, 1996b). Despite the absence of molecular information on the difference between QPX isolates, it is likely that QPX is locally adapted to its environment (local physico-chemical conditions and hard clam populations).

Results also demonstrated **differences in immune parameters between clam populations**. Camara et al. (2006) previously reported variation in growth among clam broodstocks. In our study, striking differences between southern and northern clam broodstocks included lower total hemocyte count (THC) and protein concentration (part III – chapter 1, chapter 3) as well as the low constitutive transcriptional level of defensins (data not shown) in naïve Florida (FL) clams compared to northern (NY) clam populations. Since hemocytes are the main line of defense in invertebrates, these observations could support a higher susceptibility to diseases among FL clams compared to NY and MA clams. However, FL clams are not particularly more prone to diseases (other than QPX) or mass mortalities, suggesting the presence of compensatory mechanisms. Differences in defense parameters observed between naïve clam populations were not related to the alteration of overall resistance to pathogens and could even be unrelated to the specific susceptibility of FL clams to QPX. For example, no correlation was noted between protein concentration in plasma and anti-QPX activity (part III – chapter 1). Additionally, large variability of anti-QPX factors as well as immune parameters was observed within each clam population.

Finally, investigation of environmental parameters highlighted the importance of **temperature** in determining QPX growth and survival, clam defense parameters and host – pathogen interactions (part IV). The impact of environmental parameters and especially temperature on pathogens and bivalves has been well described. However, few studies have assessed long term effects of different environmental

conditions on bivalve defense parameters. Our results demonstrated that temperature significantly affected clam immunity over the 4-month experiment (part IV – chapter 2) whereas variation of salinity induced significant effects after 2 months but not after 4 months suggesting clam's capacity to adapt to salinity changes (part IV – chapter 3). Similarly, QPX was more sensitive to temperature variation than salinity changes. Additionally, comparison of QPX disease to *in vitro* QPX growth and extracted component 1 from PCA analysis in naïve clams exposed to different temperatures demonstrated difference of conditions providing optimal QPX growth and disease development. For instance, temperatures favorable to QPX disease development were below the optimum for *in vitro* growth of QPX whereas temperature favoring *in vitro* parasite growth was associated with effective immune response and healing processes in infected clam (Figure 9.2).

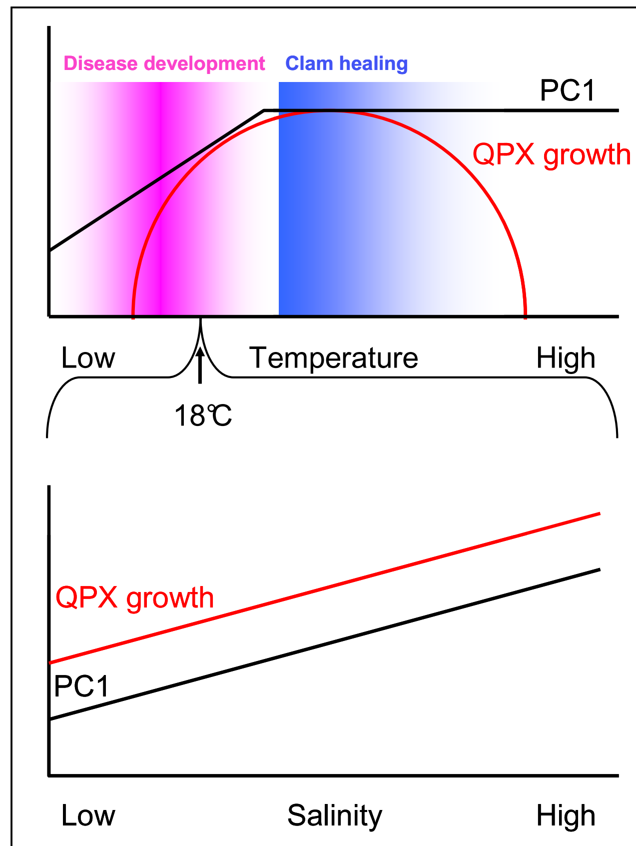

Fig. 9.2. Schematic representation of the effect of environmental parameters on *in vitro* QPX growth (Part IV – Chapter 1) and principal component 1 extracted from PCA analysis in naïve Florida clams (proxy for immune performances; Part IV – Chapters 2 and 3).

These results illustrate well the complexity of host-pathogen interactions and resulting diseases. Similar complex interactions were previously suggested in the *Vibrio tapetis*-*Ruditapes philippinarum* (Manila clam) association in the framework of another "cold water disease" called brown ring disease (Paillard, 2004). Development of QPX disease at low temperature in our experiments may contrast with field observations of higher disease prevalence during summer (Liu, 2010) but should be interpreted in light of the chronic nature of QPX disease. For instance, acquisition of QPX by clams and initial disease development may occur during early spring but overt disease and associated clam mortality may not appear until summer months when clam metabolic requirements are high. Additionally, other environmental parameters are also involved in the interaction between QPX and *M. mercenaria*, including salinity and dissolved oxygen (part IV – chapter 2 and 3, data not shown) but their effects are less important than temperature. In the field, a combination of these parameters as well as the physiological status of clams (reproduction, food quality and quantity) is likely to affect host defenses and QPX disease dynamics. Effects of salinity and temperature on physiological parameters such as respiration, clearance and heart rates were previously demonstrated in bivalves (Bakhmet et al., 2005; Han et al., 2008; Haure et al., 1998; Pandolfo et al., 2009; Sara et al., 2008). Consequences of physiological modifications on defense parameters were also demonstrated. For example, Heise et al. (2003) demonstrated that increasing temperature was associated to increasing respiration rate in the bivalve *Laternula elliptica*. Such increase of respiration also induced an increase of ROS production in *L. elliptica*. However, this pattern was not observed in our study (part IV – chapter 2) where basal ROS production of FL-c clams was not correlated to increasing temperature. Nevertheless, it is likely that physiological response of *M. mercenaria* exposed to different environmental conditions as well as the "tank effect" (maintenance of clams in tanks for an extended time period) modulated cellular and humoral parameters and therefore the response to QPX assessed in our experiments.

2. Virulence factors and QPX pathogenicity

Previous studies suggested the involvement of the mucus produced by the parasite in QPX virulence. Similarly, we demonstrated the presence of factors toxic

to clam hemocytes in QPX extracellular products (part II – chapter 1). Interestingly, the presence of host factors (tissue extracts, hemocytes) appears to enhance the production and release of cytotoxic factors by parasite cells. QPX mucus plays an important role in the cytoxicity of the parasite and is thought to provide protection from phagocytosis by clam hemocytes (Anderson et al., 2003a). In addition, QPX mucus could be involved in the avoidance of parasite recognition by the clam defense system and may serve as barrier for the diffusion of clam humoral defense factors (part III – chapter 2, Figure 9.3). Interestingly, significant reduction of ROS production by clam hemocytes was observed during QPX challenge and could be related to specific degradation of these compounds by QPX (by mean of catalases, SODs) or alteration of signaling pathways leading to ROS production.

Following QPX injection into the pericardial cavity, a large number of QPX infections and most important clam molecular responses were localized in pallial tissues (mantle, siphon) (part III - chapter 2) despite the fact that mantle (and gill) homogenates significantly reduced QPX growth *in vitro* compared to foot and muscle tissue homogenates (part III – chapter 1). This conflicting result could be related to the presence of specific compounds in tissues enhancing QPX virulence factors and resistance to host defenses since we demonstrated the presence of both pro- and anti-QPX compounds in tissue homogenates (part III – chapter 1). Additionally, the role of culture medium in QPX virulence could also explain some discrepancies among experiments. For instance, in chapter 2 of part III, clams were challenged with QPX cells with or without mucus grown and suspended in culture medium made with clam extracts whereas in chapter 3 of part III, clams were challenged with washed QPX cells resuspended in seawater (to allow for comparison with clams challenged with bacteria). Despite the fact that molecular response may not necessarily reflect variation of measured cellular and humoral parameters, results showed a molecular response of clams throughout the 48 days of the experiment (part III – chapter 2) whereas in the next experiment clams from the same population (NY) restored hemolymph parameters after 28 days. Interestingly, our results indicated that medium composition causes morphological changes of QPX cells (part IV – chapter 1, data not shown). Similarly, transfer of QPX to seawater induced a reduction of cell size and limited production of mucus. Injection of these typical forms of QPX could enhance

host response and parasite elimination especially through phagocytosis activity over a short time period.

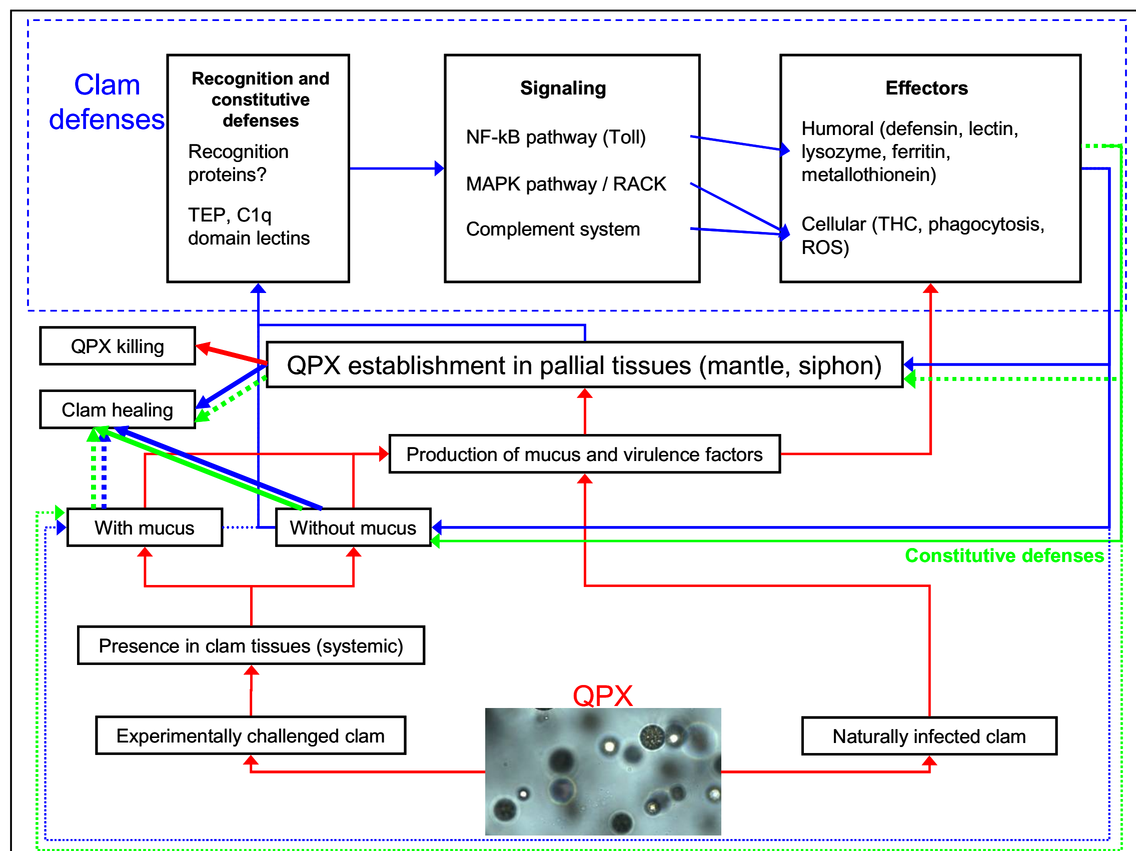

Fig. 9.3. Representation of observed (solid lines) and putative (dashed lines) interactions between QPX (virulence factors, red lines) and its host, *M. mercenaria*. Constitutive (green lines) and induced (blue lines) defense factors of *M. mercenaria* were represented.

Effects of temperature and salinity on *in vitro* growth and survival of QPX could explain some results observed in part IV of the dissertation. Low temperature (13 °C) was associated to significant disease development in clams whereas higher temperature contributed to clam response (healing). Difference of clam response between 21 °C and 27 °C suggested that QPX was rapidly eliminated at 27 °C compared to 21 °C where clam response was significantly different from control (part IV – paper 2). Despite the narrow range of temperature optimum for *in vitro* QPX growth, lower temperature (15 °C) appeared more favorable to parasite survival under stressful conditions (starvation in seawater, part IV – chapter 1) as compared to 23 °C. It is likely that clam represents also a stressful environment for QPX. In addition to alteration of clam defenses at low temperature, observation of QPX disease

development in clams maintained at 13 °C could be related to the higher resistance of QPX at low temperature compared to 27 °C. Similarly, only salinity as low as 15 psu affected QPX growth *in vitro,* partially explaining the results of the experiment testing the effect of salinity on disease development (part IV – chapter 1 and 3). For instance, defense responses and healing processes of naturally infected clams maintained at low salinity (17 psu) were more effective than QPX-infected clams exposed to high salinity (30 psu).

Experiments also provided interesting observations on potential QPX transmission between clams. For instance, we were able to detect, for the first time, the release into seawater of secretions containing QPX cells from heavily infected clams (Figure 9.4). Despite the inability of QPX to survive for an extended period in seawater without a source of nutrient (part IV – paper 1), QPX cells, in this case, may have survived longer using clam secretions as source of nutrient. It should be noted that the release of parasite cells in this clam lasted for several weeks (before clam death). Such process could provide mode of transmission for QPX in the field particularly in areas with high clam density as previously suggested by Ford et al. (2002b).

Fig. 9.4. Observation of naturally infected clam (Massachusetts) [A] releasing mucoid secretions and [B] microscopic detail of a QPX-like cell present in released secretions.

3. Host response to QPX infection

Hard clams exhibited both constitutive and induced defenses against QPX. Experiments assessed cellular and humoral parameters as well as molecular responses

of clams under various conditions and time frames. Clam response to experimental challenge with QPX can be divided into two steps: An initial and systemic response of the clams to the presence of the parasite was followed by a more specific response in particular tissues such as mantle as a likely result of the establishment of QPX in these tissues (Figure 9.5).

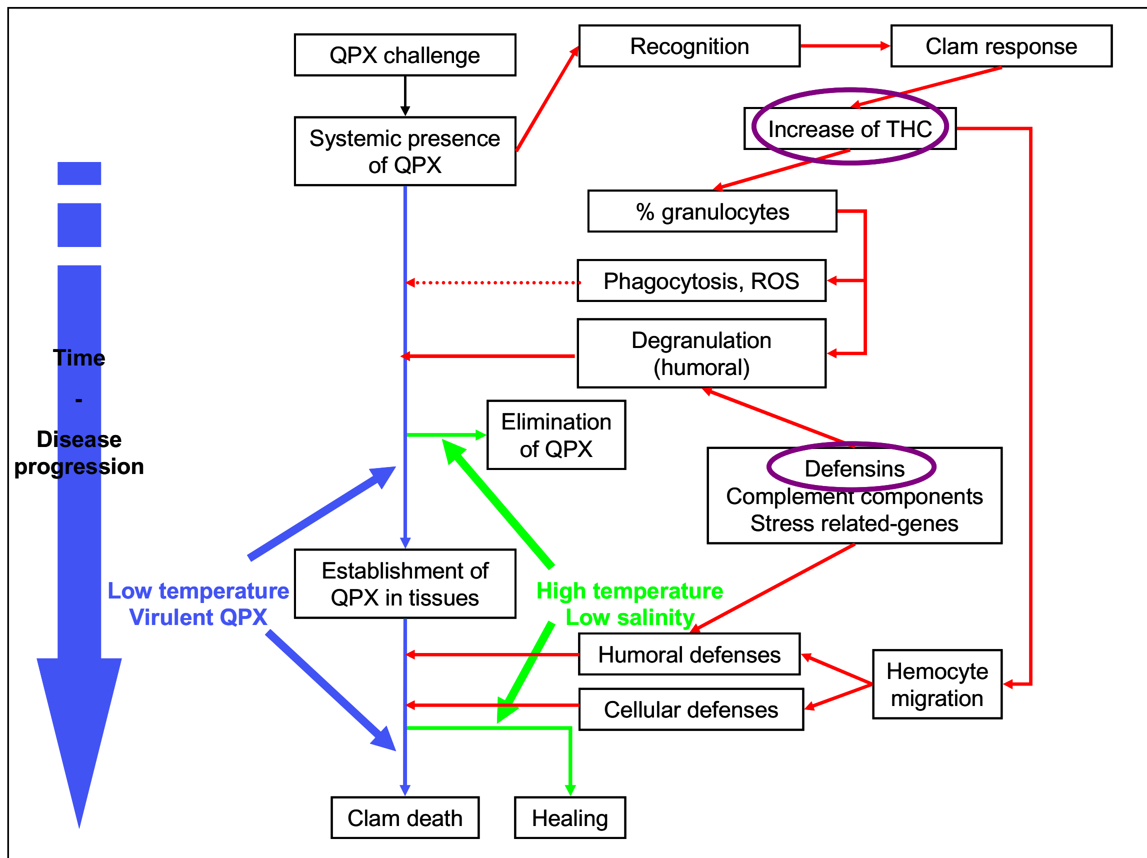

Fig. 9.5. Schematic representation interactions between clam response, QPX disease development, and effect of environmental parameters. Purple circles indicate variation of constitutive parameters between NY and FL clams. THC: Total Hemocyte Count, ROS: Reactive Oxygen Species.

During initial response, (few days, part III – chapter 3) significant variations of THC were observed in clams but this response appeared to be non-specific to QPX as similar changes were observed in clams challenged with bacteria. On the other hand, phagocytosis and ROS production did not appear to significantly contribute to the initial response although both were presented as fluorescence unit per hemocyte, so overall phagocytosis and ROS production per ml of hemolymph did increase with challenge as a result of THC changes. Conversely, stress- (ferritin, metallothionein, part III – chapter 3) and defense-related genes (data not shown) as well as anti-QPX

activity of hard clam plasma and hemocyte resistance to QPX extracellular products (bioassays developed during this study) were differentially modulated following bacterial or QPX challenge. These results suggest a control of these components by specific pathways differentially activated according to the nature of the invader (Lemaitre and Hoffmann, 2007). Comparison of QPX-resistant (NY) and –susceptible (FL) broodstocks also exhibited differences in defense response and dynamics. These differences of response could have a genetic and metabolic origin as significant differences between clam populations were also observed for constitutive THC and transcriptional level of defensins (Figure 9.5, data not shown). Similarly, different mechanisms of recognition and signaling may be involved in the two broodstocks (part III – chapter 3). For instance, recognition proteins and several components of signaling pathways were differentially expressed during QPX challenge in NY clams (part III – chapter 2, data not shown). Although both FL and NY clams displayed a response to QPX challenge, significant differences between them could be related to the activation of different signaling pathways. Our study identified a TEP (part III – chapter 2 and data not shown) which is involved in the recognition of pathogens and activation of complement components. A similar TEP was identified in *Anopheles gambiae* and was specifically associated with the resistance of the mosquito to *Plasmodium falcipinarum* (Blandin et al., 2009). Susceptibility of some mosquito populations was related to a mutation of the TEP which was unable to recognize the parasite and trigger a response in susceptible mosquitoes. Similarly, Zhang et al. (2009) demonstrated differential splicing of TEP gene in the scallop *Chlamys farreri* according to different factors such as the nature of the pathogen and the sex of the host. In addition to the TEP and complement components, other signaling pathways were identified in our study and could be linked to the difference of defense response between FL and NY clams. For example, NF-kB pathway has been extensively described in insects and its activation leads to the induction of specific anti-microbial peptides (Lemaitre and Hoffmann, 2007). Surprisingly, most variations in transcription levels of studied stress- and defense-related genes (part III – chapter 3 and data not shown) were observed in gills and mantle but not in hemocytes. It is likely that QPX cells quickly colonized tissues and were not present in hemolymph circulation for a long time. This hypothesis would imply that transcriptional regulation of studied genes was controlled by local

signaling such as cell interactions rather than systemic signaling using for example interleukins.

Following the initial response of clams, a second phase can be observed in response to the establishment of QPX in tissue (Figure 9.5). This statement is motivated by both *in vivo* and *in vitro* results. For instance, natural QPX infections were typically observed in pallial tissues and experimental challenges also resulted in the presence of QPX in mantle tissues (Dahl et al., 2008; Dahl et al., Accepted). Additionally, a large number of differentially regulated genes was identified in mantle tissues after 48 days (part III – chapter 2) and shifts in transcription levels among several stress- and defense-related genes were observed in gill and mantle tissues during the first month (Part III – chapter 2 and 3 and data not shown). Changes in THC (chapter 2 and 3 of part IV) also support a focal response of clams to QPX. For instance, in all temperature and salinity treatments (except at 27 °C), THC was higher in experimentally infected clams as compared to controls whereas opposite trends were observed at 4 months suggesting hemocyte migration from hemolymph to infected tissues. Conversely to the initial phase response (chapter 2 of part IV), at 21 °C phagocytosis was reduced in experimentally challenged clams compared to controls whereas ROS activity was stimulated compared to controls. Inverted trends were observed at 13 °C where clams were not able to control QPX disease development. As previously discussed, phagocytosis is inefficient against QPX cells (because of their large size and their mucous protective layer) whereas ROS are highly toxic products that could effectively kill encapsulated QPX cells. The importance of ROS against parasites encapsulated by hemocytes was previously documented (Torreilles et al., 1996). However, our assessment of hemolymph parameters may be altered by the nature of the response. For example, hemocyte and plasma parameters measured in hemolymph may not necessarily reflect response to local infection and could be more related to the global physiological status of clams.

4. On going and future research

This study notably increased our comprehension of QPX disease by documenting the virulence of QPX, the genetic basis of differences in clam

susceptibility to QPX and effects of environmental parameters on host-pathogen interactions. Our investigations also generated valuable information on cellular, humoral and molecular immunity in bivalves, and their modulation by pathogens and environmental parameters.

Further characterization of QPX virulence factors has been initiated through large scale sequencing of QPX expressed genes allowing the identification of candidate genes involved in virulence, such as proteases and proteins involved in mucus production. Investigation of these candidates in different QPX isolates and culture media should help the characterization of factors involved in QPX pathogenicity against *M. mercenaria* defenses as well as the origin of variation of parasite virulence between different QPX isolates. Additionally, as our results demonstrated existence of different QPX strains with specific temperature optima and cytotoxicity on clam hemocytes, experimental challenge of clams with different QPX isolates could be associated to differences of disease development at specific temperature according to each isolate.

Similarly, large scale sequencing of *M. mercenaria* expressed genes will identify genes involved in clam response to QPX. Especially, characterization of other components of signaling pathways and comparison of their expression between resistant and susceptible clam broodstocks appears promising. Additionally, our investigations of defense-related genes identified in this study (defensins, complement components) already provided interesting differences between clam broodstocks and may be related to variations in the susceptibility of clams to QPX. However, large scale analysis by oligoarray is underway and is expected to provide a wider view of the biological bases of clam resistance since it is unlikely that clam susceptibility to QPX only relies on a few components. Especially, metabolism- and defense-related factors were closely related and might be involved in the dynamics of the disease.

Finally, future laboratory and field investigations should take into consideration environmental parameters, especially temperature, in association with the physiological status of clams for a better characterization of conditions associated with the development of QPX disease and the development of potential remediation strategies.

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Appendices

Appendix 1. SEM of defense parameters (n = 10 clams per condition) in FL and NY *M. mercenaria* injected with sterile seawater (NY-c and FL-c) or challenged with bacteria (NY-b) or QPX (NY-q and FL-q). THC: total hemocyte counts, PDC: percentage of dead cells.

	T1 – 16 hours			T2 – 3 days					T3 – 4 weeks			
	NY-c	NY-b	NY-q	NY-c	NY-b	NY-q	FL-c	FL-q	NY-c	NY-q	FL-c	FL-q
THC (cells.ml^{-1} x 10^5)	4.5	3.9	2.1	1.6	4.0	2.5	2.9	15.0	2.2	1.8	1.1	7.4
% granulocytes	7.2	3.8	3.0	2.2	5.5	4.7	4.3	7.7	2.3	2.4	5.0	7.4
PDC (%)	1.3	0.8	0.5	2.5	0.9	0.8	0.7	1.4	0.5	0.8	1.1	1.5
basal ROS production (Fluorescence units/10^4 hemocytes)	0.3	0.2	0.0	0.1	0.1	0.2	0.2	1.9	0.3	0.1	0.3	0.2
5 min stimulated ROS production (Fluorescence units/10^4 hemocytes)	0.8	0.3	0.1	0.2	0.1	0.2	0.5	0.9	0.4	0.3	0.8	0.5
Phagocytosis (Fluorescence units/10^4 hemocytes)	10	0.8	0.9	1.6	0.7	0.6	0.7	0.9	0.5	0.3	0.3	0.3
Protein concentration (µg.ml^{-1})	39.4	48.0	33.0	20.5	41.2	25.7	31.9	116.8	22.5	19.0	15.8	27.2

Appendix 2. SEM of cellular and humoral defense parameters in Massachusetts (MA) clams naturally infected by QPX and exposed to 13, 21 or 27 °C for 2 and 4 months.

	2 months			4 months		
	13°C	21°C	27°C	13°C	21°C	27°C
THC (cells.ml^{-1} x 10^5)	1.5	0.8	0.7	0.2	0.1	0.7
% granulocytes	3.1	3.4	2.6	2.3	3.5	3.8
PDC (%)	0.7	0.7	0.4	0.5	0.4	0.4
Basal ROS production (Fluorescence / 10^4 hemocytes)	0.2	0.3	0.4	0.5	0.5	0.3
30 min stimulated ROS production (Fluorescence / 10^4 hemocytes)	1	1.1	1.3	1.8	2.1	1.5
Phagocytosis (Fluorescence / 10^4 hemocytes)	1.2	0.9	1.0	nd	nd	nd
Lysozyme (µg.ml^{-1})	0.5	0.6	0.7	0.8	0.8	0.5
Protein concentration (µg.ml^{-1})	20.8	31.7	22.6	17.0	28.6	20.3
Anti QPX activities in plasma (% controls)	9.8	7.3	9.5	12.4	4.3	5.0
Cytotoxicity of QPX ECP (cytotoxicity index - A.U.)	1.6	2.3	1.2	1.8	1.0	1.0

Appendix 3. SEM of cellular and humoral parameters in *M. mercenaria* (FL) 2 weeks after acclimation to 17 and 30 psu (T0) and in control (FL-c) and QPX-challenged (FL-q) clams maintained at 17 psu and 30 psu for 2 or 4 months.

	T_o – 2 weeks		2 months				4 months			
	17 psu	30 psu	17 psu		30 psu		17 psu		30 psu	
	FL	FL	FL-c	FL-q	FL-c	FL-q	FL-c	FL-q	FL-c	FL-q
THC (cells.ml^{-1} x 10^5)	1.2	0.8	0.3	0.5	0.5	0.5	1.4	0.8	0.6	0.5
% granulocytes	3.0	3.2	2.4	2.6	2.6	2.9	3.2	2.4	2.6	2.8
PDC (%)	1.3	0.4	0.5	0.8	0.8	1.0	0.6	0.6	0.5	0.6
basal ROS production (Fluorescence units / 10^4 hemocytes)	0.3	0.2	0.3	0.4	0.7	0.2	0.4	0.5	0.2	0.1
5 min stimulated ROS production (Fluorescence units / 10^4 hemocytes)	0.5	0.4	0.4	0.5	1.5	0.3	0.5	1.1	0.3	0.2
Phagocytosis (Fluorescence units / 10^4 hemocytes)	0.3	0.2	0.2	0.3	1.4	0.5	0.2	0.2	0.2	0.2
Protein concentration (µg.ml^{-1})	31.5	16.7	22.2	17.1	20.0	39.7	15.7	32.8	16.8	23.8
Lysozyme (µg.ml^{-1})	0.6	0.7	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0

Appendix 4. SEM of hemocyte and plasma parameters of MA *M. mercenaria* naturally infected by QPX and maintained at 17 psu and 30 psu during 2 and 4 months.

	2 months		4 months	
	17 psu	30 psu	17 psu	30 psu
THC (cells.ml^{-1} x 10^5)	1.0	4.5	1.0	0.8
% granulocytes	3.0	3.3	3.3	3.2
PDC (%)	0.5	0.8	0.5	0.4
basal ROS production (Fluorescence units/ 10^4 hemocytes)	0.7	0.1	0.2	0.4
5 min stimulated ROS production (Fluorescence units / 10^4 hemocytes)	1.0	0.1	0.3	0.5
Protein concentration (µg.ml^{-1})	24.0	23.5	17.3	19.3
Lysozyme (µg.ml^{-1})	0.7	0.7	0.7	0.8