Journal of Invertebrate Pathology 106 (2011) 322–332

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

Effects of temperature on hard clam (*Mercenaria mercenaria*) immunity and QPX (Quahog Parasite Unknown) disease development: II. Defense parameters

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ARTICLE INFO

Article history:
Available online 27 November 2010

Keywords:
Hard clam
Mercenaria mercenaria
QPX
Thraustochytrid
Parasite
Immune response
Temperature
Environment

ABSTRACT

Quahog Parasite Unknown (QPX) 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*. Control and QPX-challenged (both experimentally and naturally) clams were maintained at 13, 21 and 27 °C for 4 months. Control 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 control 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, 1993; Paillard et al., 2004). 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,b).

The protistan parasite Quahog Parasite Unknown (QPX) 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., 2002; 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., 2002; Ragone Calvo et al., 2007). Histological observation of QPX-infected tissues demonstrated that some clams are able to mount an effective

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doi:10.1016/j.jip.2010.11.004

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 (Perrigault et al., 2009a). On the other hand, extracellular products (ECP) secreted by QPX were cytotoxic to clam hemocytes (Perrigault and Allam, 2009). 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 (Perrigault et al., 2009b). 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; Allam, unpublished). The *in vitro* growth of QPX was also strongly modulated by temperature (Brothers et al., 2000; Perrigault et al., 2010). 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 exposure 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., 2011).

2. Materials and methods

2.1. M. mercenaria

This study used two aquacultured clam broodstocks. Eight hundred naïve *M. mercenaria* (30–35 mm in length) were obtained in the fall (30 ppt, 24 °C) 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) during the fall (30 ppt, 16 °C). 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 (>55% heavy infections) of the parasite in this batch at the beginning of the experiment (Dahl et al., 2011).

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. (2009a). QPX cultures were initiated in 25-cm^2 flasks containing MTH at 1 mg ml^{-1} protein and incubated at 23 °C for 2 weeks (Perrigault et al., 2009a). 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 re-circulating 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 (six controls FL [FL-c], six QPX-challenged FL [FL-q] and 6 MA [MA]) were exposed to 13, 21 or 27 °C. These temperatures represent the range observed in New York clamming waters between spring and fall (Dahl et al., 2010) and have been shown to differentially affect the *in vitro* performance of QPX (Perrigault et al., 2010). 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 5×10^4 parasite cells into the pericardial cavity according to Dahl and Allam (2007). Control clams were injected with sterile medium (MTH at 1000 μg 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 h 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 1.2–1.8 ml) were withdrawn from the adductor muscle sinus 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, 30 ppt) 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*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. (2002). 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 30 min of incubation in the dark at room temperature (RT, about 21 °C). 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 (AU) 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 h at RT. Supernatant was then discarded and FITC-labeled *Vibrio parahaemolyticus* (1×10^8 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 h 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 nonengulfed 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 (AU) 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 microliters 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 µg ml^{-1} of plasma. Fifty microliters of undiluted plasma was allocated to protein analysis using the BCA protein assay kit (Pierce). Protein concentrations were expressed as micrograms 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. (2009a). Briefly, an exponentially-growing culture (1 week) of QPX in MTH (1000 µg ml^{-1} proteins) was centrifuged for 15 min at 600*g*. QPX cell pellet was then washed two times and resuspended in Minimal Essential Medium (MEM). Fifty microliters of undiluted plasma was added to 50 µl of MEM containing 1×10^3 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] × 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 (2009). An exponentially-growing culture of QPX in MTH (1000 µg ml^{-1} proteins) containing about 1×10^5 cell ml^{-1} was transferred to 2-ml tubes 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 (Perrigault and Allam, 2009). Filtered ECP and sterile MTH medium were frozen at −20 °C until assays. One hundred microliters of diluted hemolymph (vol:vol in FASW) were plated in four replicates in flat bottomed 96-well plates and incubated for 1 h at RT to allow hemocyte adhesion to the plate. Supernatant was then carefully removed and hemocytes were washed with 200 µl of FASW. Cells received 100 µl of QPX ECP (two test wells) or sterile MTH (two control wells). After 1 h of incubation at RT, liquids were carefully removed and hemocytes were washed with 200 µl of FASW. One hundred microliters of freshly prepared neutral red solution (250 µg ml^{-1} in FASW, Sigma) was then added. Following incubation (1 h 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 log 10 or arcsin transformed before statistical testing whenever data showed a large variance but results shown in tables and figures are presented as non-transformed values. Multivariate analyses were performed using Discriminant Analysis (DA) to determine linear combinations of variables (hemolymph parameters) that maximize differences among *a priori* defined groups (treatments) and Principal Component Analysis (PCA) to analyze relationships between variables and simplify correlated variables into principal components. 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 treatments 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. (2011).

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

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

Results demonstrated a strong influence of temperature on both cellular and humoral defense factors of unchallenged *M. mercenaria* (Table 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. 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. 1B). Unstimulated ROS production was also significantly affected by temperature with higher levels at 13 °C at both sampling times ($p < 0.001$, Fig. 2A). However, no significant difference between treatments was observed after stimulation of ROS production by zymosan A (Fig. 2B) except between FL-c clams maintained at 21 °C and 27 °C for 4 months ($p = 0.01$, Fig. 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. 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. 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. 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 lowest and highest temperatures (Fig. 5A). QPX ECP cytotoxicity on hemocytes from FL-c exhibited no significant difference between temperature treatments after 2 months (Fig. 5B).

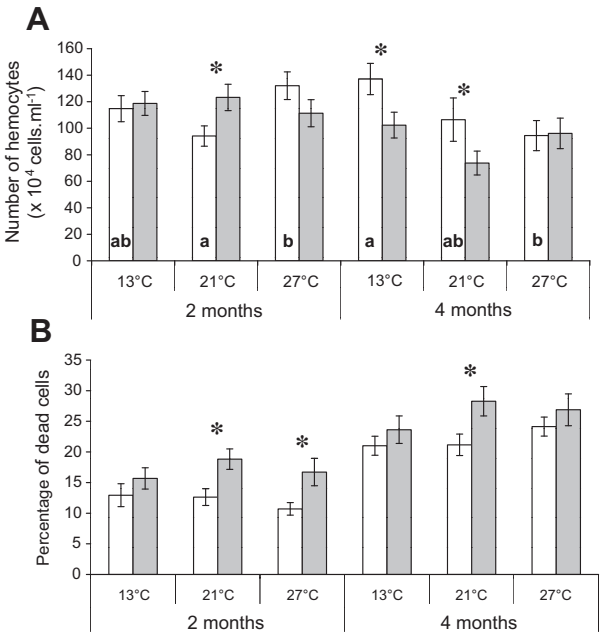

Fig. 1. Mean (±SEM, $n = 30$ clams per condition) of [A] total hemocyte counts (THC) and [B] percentage of dead cells (PDC) from (FL-c) controls (□) and (FL-q) QPX-challenged (▩) Florida clams after 2 and 4 months at 13, 21 and 27 °C. For each sampling time, letters (a and b) denote significant differences (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.

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

Table 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 (30 min)	NS	NS	*	NS	***	**
Phagocytosis	***	***	***	***	***	***
Lysozyme	NS	***	NS	NS	NS	NS
Protein concentration	NS	*	*	NS	***	*
Anti-QPX activities in plasma	**	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).

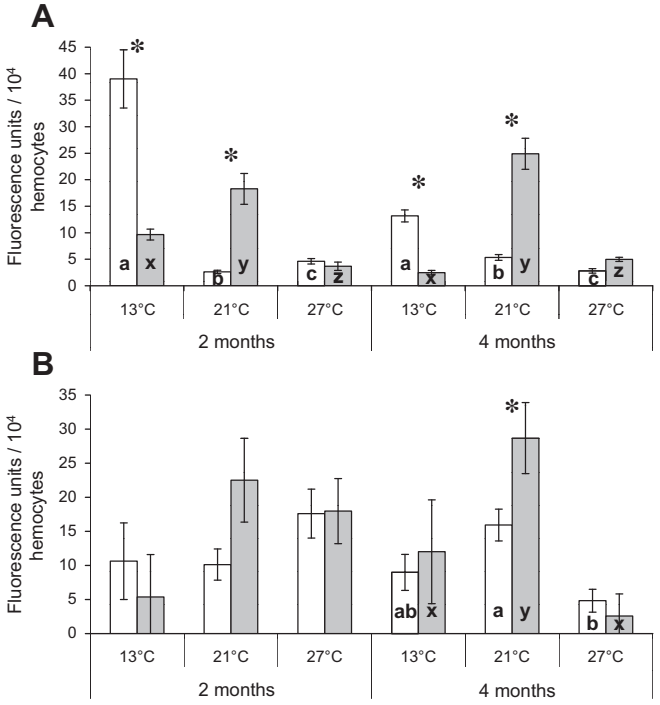

Fig. 2. Mean (±SEM, $n = 30$ clams per condition) of [A] unstimulated (basal) and [B] zymosan A stimulated ROS production in hemocytes from (FL-c) controls (□) and (FL-q) QPX-challenged (▩) Florida clams after 2 and 4 months at 13, 21 and 27 °C. For each sampling time, letters (a, b and c for FL-c and 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.

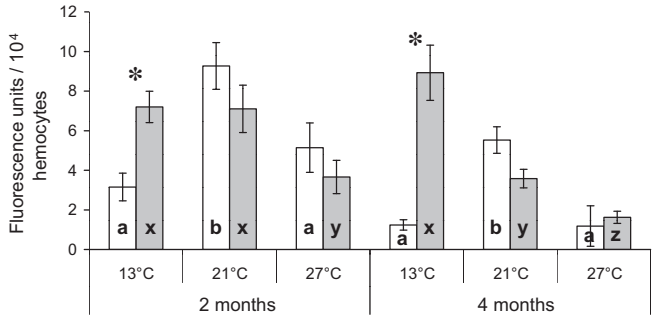

Fig. 3. Mean (±SEM, $n = 30$ clams per condition) of phagocytic activity in hemocytes from (FL-c) controls (□) and (FL-q) QPX-challenged (▩) clams after 2 and 4 months at 13, 21 and 27 °C. For each sampling time, letters (a and b for FL-c and x, y and z for FL-q) denote significant differences (Holm-Sidak 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.

experimentally (FL-q) or naturally (MA) infected by QPX (Tables 1–3). 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. 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

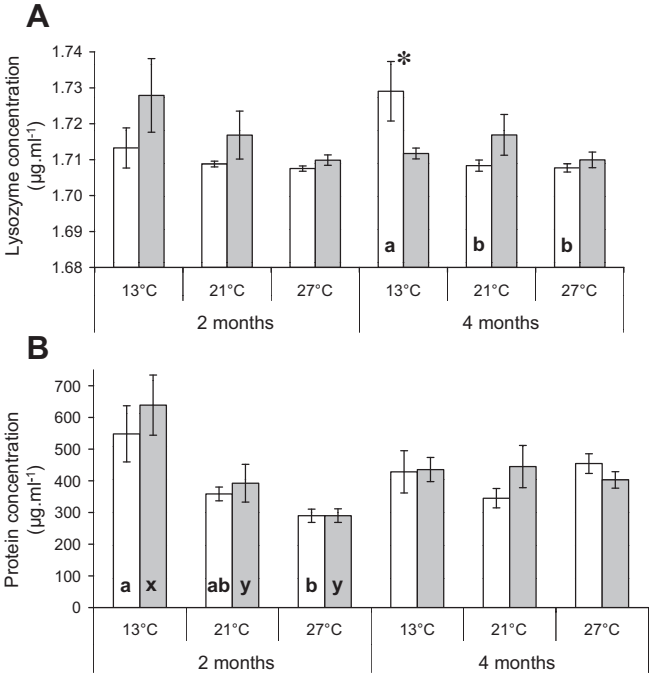

Fig. 4. Mean (±SEM, $n = 30$ clams per treatment) of [A] lysozyme and [B] protein concentrations in plasma from (FL-c) controls (□) and (FL-q) QPX-challenged (▩) clams after 2 and 4 months at 13, 21 and 27 °C. For each sampling time, letters (a and b for FL-c and x and y for FL-q) denote significant 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.

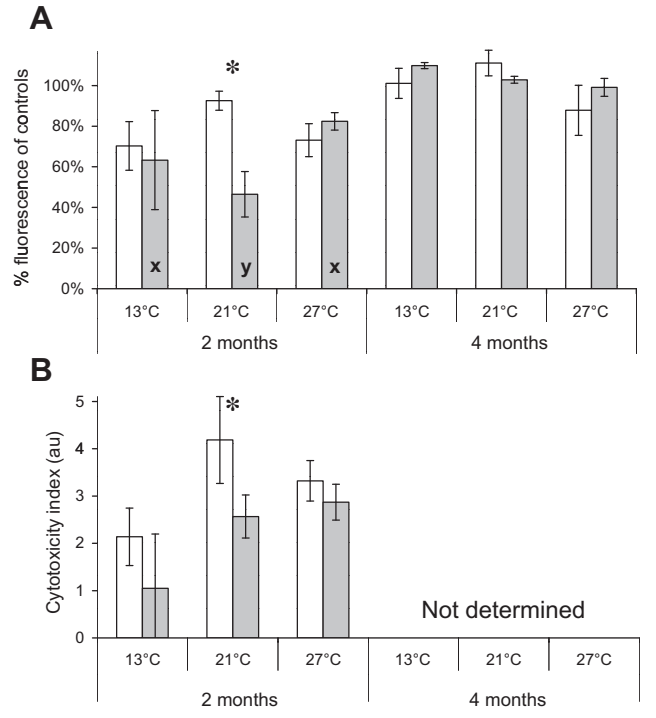

Fig. 5. Mean (±SEM, $n = 30$ clams per condition) of [A] anti-QPX activity of plasma (higher readings represent lower activities) and [B] cytotoxicity of QPX ECPs on hemocytes (higher readings represent lower resistance of hemocytes) from control (□) and QPX-challenged (▩) FL clams maintained at 13, 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.

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

	2 Months			4 Months		
	13 °C	21 °C	27 °C	13 °C	21 °C	27 °C
THC (cells $ml^{-1} \times 10^5$)	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}
Thirty minutes 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 ($\mu g\ ml^{-1}$)	7.5	7.3	6.7	5.8	5.5	7.5
Protein concentration ($\mu g\ ml^{-1}$)	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 – AU)	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).

Table 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 (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).

experiment ($p < 0.001$, Table 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 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. 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. 1B). Overall PDC in MA clams presented no significant variation between treatments (Table 2).

ROS production by hemocytes from FL clams was highly modulated by QPX challenge and temperature. 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. 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 2). When stimulated by zymosan A, 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. 2B). No significant variation in stimulated ROS production was observed in MA clams maintained at different temperatures (Table 2).

Phagocytosis activity in FL and MA clams was also strongly affected by temperature and QPX challenge (Fig. 3 and Table 2). Low temperature caused an increase in phagocytosis in hemocytes from FL-q ($p < 0.005$) compared to their unchallenged controls (FL-c) whereas FL-q 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. 3). Similarly, higher phagocytosis activity was also observed in hemocytes from MA clams maintained at 13 °C as compared to clams held at the two other experimental temperatures (differences were significant between 13 and 27 °C, $p < 0.001$, Table 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. 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. 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 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. 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. 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 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. 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. 5B, Table 2).

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. DA was performed on all parameters (at 2 and 4 months) measured in FL-c (Fig. 6A), FL-q (Fig. 6B) and MA clams (data not shown but hemolymph parameters followed similar trends as FL-q clams). Results of DA revealed a significant impact of temperature treatments for all tested conditions (Fig. 6). 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 hemolymph 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. 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 two treatments (Fig. 6B, MA data not shown). To enhance the power of the statistical testing, PCA was performed on hemolymph parameters combined from all clams (FL-c, FL-q and MA) after 2 months (Fig. 7) and 4 months (data not shown but clustering of selected parameters was similar to Fig. 7 although the neutral red uptake and phagocytosis assays 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 54% of the total variance of the data (Fig. 7). Several hemolymph 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 ($p < 0.001$, Fig. 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 ($p = 0.043$) likely as a result of higher phagocytosis activity and lower unstimulated and stimulated ROS production (Fig. 7 and 8A). The effect of QPX challenge was inverted at 21 °C ($p = 0.046$) and no difference was observed at 27 °C (Fig. 8A). Extracted component 1 for MA clams decreased with increasing temperature and differences were significant between 13 °C and 27 °C treatments. ($p < 0.012$, Fig. 8B). Extraction of component 1 (PC1) and statistical analysis of defense parameters (without neutral red uptake assay 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 two groups were no longer significant at 13 °C.

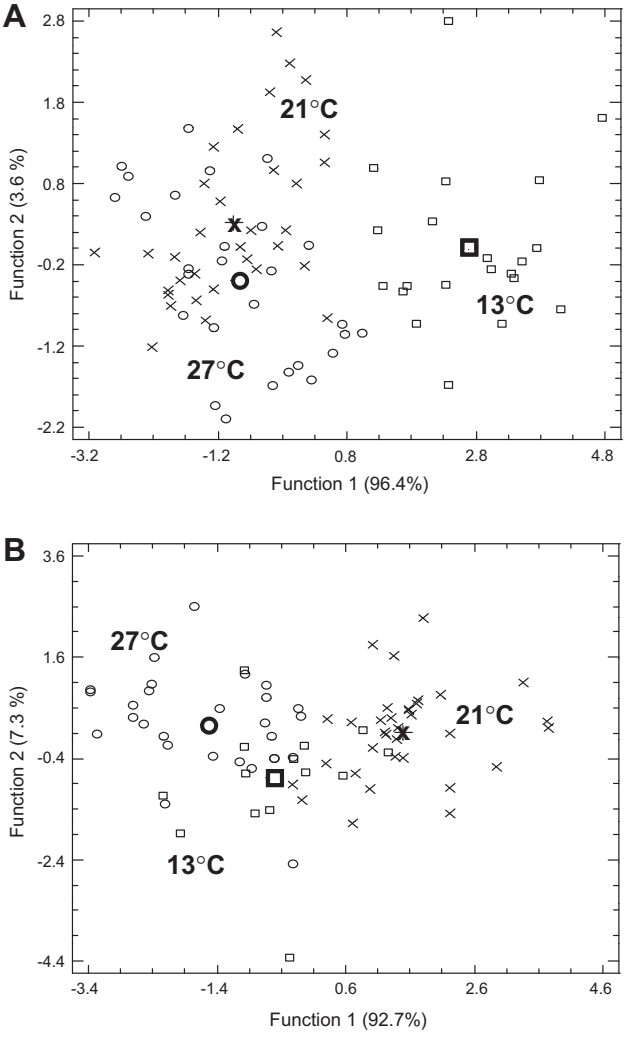

Fig. 6. Discriminant Analysis of all hemolymph parameters (2 and 4 months combined) from (FL-c) unchallenged [A] and (FL-q) QPX-challenged [B] clams submitted to 13 °C (□), 21 °C (×) and 27 °C (○). Positions of group centroids for each treatment are indicated by bold large symbols.

4. Discussion

The objectives of this study were to investigate the effect of temperature on cellular and humoral defense parameters of the hard clam *M. 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). Experiments were performed over 4 months because this timeframe is sufficient to assess specific clam response to established QPX infections (Dahl and Allam, 2007; Perrigault et al., 2009b). Results demonstrated a major impact of temperature on defense factors in control clams with significant modifications of hemolymph parameters in clams maintained at 13 °C compared to those held at 21 °C or 27 °C. More interestingly, temperature also modulated clam response to QPX and disease dynamics resulting in maximal development of the infection in clams maintained at low temperature (Dahl et al., 2011).

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

Temperature is among the most important factors affecting the physiology of marine organisms and prior studies have

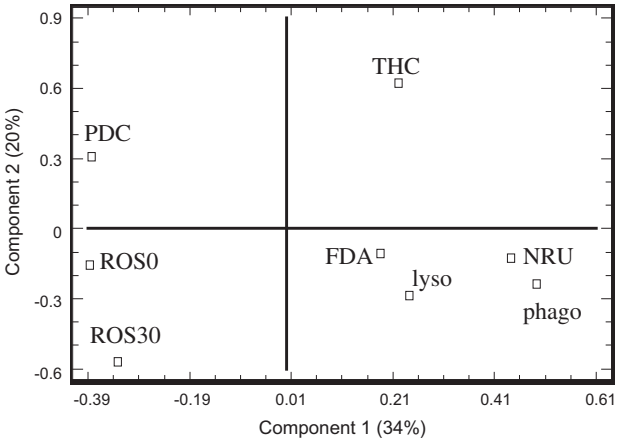

Fig. 7. Principal Component Analysis (PCA) plot of immune parameters from clams (FL-c, FL-q and MA combined) sampled at 2 months. PDC: percentage of dead cells, ROS0: basal ROS production, ROS30: zymosan-stimulated ROS production, THC: total hemocyte count, phago: phagocytosis, lyso: lysozyme activity, FDA: anti-QPX activity of plasma (increasing values represent lower activities), NRU: cytotoxicity of QPX ECP on hemocytes.

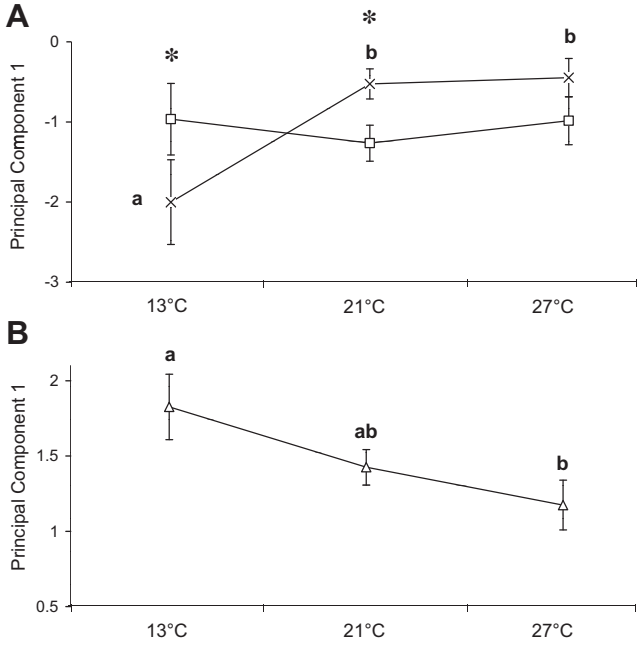

Fig. 8. Mean plots of Component 1 from Principal Component Analysis shown in Fig. 7 for (FL-c) unchallenged (×) and (FL-q) QPX-challenged (□) clams [A] and MA clams [B] submitted to three different temperatures. Letters (a and b) denote significant differences between clams maintained at different temperatures and symbols (*) indicate significant differences between FL-c and FL-q within each temperature treatment ($p < 0.05$, Holm-Sidak post hoc test).

demonstrated the impact of temperature on basic physiological functions in bivalves such as clearance rate, respiration and heart rate (Feng, 1965; Han et al., 2008; Haure et al., 1998; Pandolfo et al., 2009). Changes in temperature have also been previously shown to modulate bivalve defense parameters both *in vivo* and *in vitro* (Carballal et al., 1998; Yu et al., 2009). Similarly, our study demonstrated a strong influence of temperature on most investigated defense parameters (cellular and humoral) in control clams (Table 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. 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). 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, 1993) and clams (Paillard et al., 2004) also showed higher lysozyme activity at lower temperature. Phagocytosis activity measured in hemocytes from control clams was characterized by an optimal activity at 21 °C matching well with previous studies in *M. mercenaria* and other bivalve species (Allam et al., 2002; Cheng et al., 2004; Chu and La Peyre, 1993; Tripp, 1992). 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, 1993; Monari et al., 2007; Paillard et al., 2004). In the Manila clam *Ruditapes philippinarum*, THC displayed a seasonal pattern with higher levels during summer (Soudant et al., 2004). Higher THC with increasing temperature described in these studies may result from the increase in heart rate as shown in oysters by Feng (1965). Interestingly, our results at 4 months revealed opposite trends with higher THC at lower temperature (Fig. 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., 2007; Cheng et al., 2004; Hégaret et al., 2003). Increase in ROS production is thought to be related to increase in respiration at high temperature (Heise et al., 2003). In contrast, results from our study showed that 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. 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). The 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 contrast 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. It should be noted however that, in our study, all cellular parameters were assessed at room temperature (21 to 22 °C), which may introduce a bias in our measurements (as a result of rapid temperature change) particularly for clams maintained at 13 °C and 27 °C.

Cytotoxicity of QPX ECP on hemocytes from FL-c clams exposed to different temperatures exhibited variability without significant difference between treatments (Fig. 5B). Constitutive anti-QPX activity in plasma also exhibited 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. 5A) which could be related to high protein concentration observed at low temperature (Fig. 4B). However, anti-QPX activity measured here in FL-c plasma was lower than reported in a previous study using similar Florida clams (Perrigault et al., 2009a). This may result from the freezing of plasma samples before the assessment of anti-QPX activity since Anderson et al. (2003) 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. 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, Ragone Calvo et al. (2007) noted differences in growth rate and 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 constitutive 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., 2011). 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 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 hemolymph parameters 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., 2011). 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 (Perrigault et al., 2010). 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 of 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. 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. 8A and B). The interpretation of findings from MA clams is more difficult as the initial screening of this batch revealed a 63% prevalence of QPX infection. This means that MA clam samples at different time intervals likely contained both QPX infected and noninfected 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 and Allam, 2007). This is in agreement with our 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 and 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 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 (Perrigault et al., 2009a). 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 (Perrigault et al., 2009b). 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 *R. 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., 2004). Hemocyte 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, 1992). 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 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., 1993; La Peyre et al., 1995). 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 of granulocytes (but also potentially of hyalinocytes) from infected clams may result from stimulation of defense processes that take place during infection. Such scenario is supported by our prior molecular investigations that showed significant increase in the expression of defense and signaling genes following QPX challenge (Perrigault et al., 2009b). 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, 1999) 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., 2011). The slight increase of lysozyme at 13 °C appeared to have no effect on the *in vitro* growth of QPX (Figs. 4A and 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. 5A, Table 2). Similarly, greater prevalence and intensity of QPX disease at low temperature (Dahl et al., 2011) suggested that higher phagocytosis activities observed at 13 °C in FL-q and MA clams (Fig. 3, Table 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).

5. 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 the promotion of 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 enhanced 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; Perrigault et al., 2010). 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., 2004). 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.

Acknowledgments

Authors thank M. Homerding, S.M. Winnicki, W.E. Carden, S. Pawagi, J. Hornstein for their help with processing biological samples. We also thank colleagues and aquaculturists who provided experimental clams from Massachusetts and Florida. This research was partially supported by the NSF (Project EF0429051 to BA) and is a resulting product from Projects R/FBM-33 and R/XG-19, funded under award NA07OAR4170010 from the National Sea Grant College Program of the US Department of Commerce's National Oceanic and Atmospheric Administration to the Research Foundation of State University of New York on behalf of New York Sea Grant. It was also partially supported by the New York State Department of Environmental Conservation. The statements, findings, conclusions, views and recommendations are those of the authors and do not necessarily reflect the views of any of those organizations.

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