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# Effects of salinity on hard clam (*Mercenaria mercenaria*) defense parameters and QPX disease dynamics

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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*. Naïve and QPX-infected (both experimentally and naturally) clams were submitted to 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 clams maintained at high salinity compared to those held at 17 psu. Our findings also showed an increase in mortality following experimental challenge with QPX in clams submitted to 30 psu but not in those held at 17 psu. Constitutive clam defense factors and the response to QPX challenge were also affected by salinity. QPX challenge caused significant but transitory changes in hemolymph parameters that were obvious at 2 months but disappeared at 4 months. Overall, our results show that salinity modulates clam immunity and the progress of QPX disease although its impact appears secondary as compared to findings we reported earlier for temperature.



## 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 infectious agents (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 anti-microbial peptides (such as defensins) and hydrolytic enzymes (such as lysozyme and peptidases) which are also involved in host responses because of their various anti-microbial properties (Allam et al., 2000; 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 (Perrigault et al., 2009a). On the other hand, extracellular products (ECPs) secreted by QPX were shown to significantly alter clam hemocytes (Perrigault and Allam, 2009). Interestingly, both anti-QPX activity in clam plasma

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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 following QPX challenge (Perrigault et al., 2009b). 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., 2011; Perrigault et al., 2011). 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 (34 psu) (Perrigault et al., 2010). 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 exposure (4 months) to low (17 psu) and high (30 psu) salinities on disease development as well as constitutive and QPX-induced defense 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.

## 2. Materials and methods

### 2.1. M. mercenaria

Five hundred and ten naïve *M. mercenaria* (40–50 mm in length) were obtained from a commercial grow out site (30 psu, 22 °C) in Florida (FL). Three hundred clams (40–50 mm in length) were collected from an enzootic clamming area in Massachusetts (MA, 30 psu, 16 °C). Clams were acclimated for 1 week in 150-L tanks with re-circulating water (28–30 psu) at 17 ± 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 (Dahl and Allam, 2007) and quantitative PCR techniques (Liu et al., 2009). No detectable QPX infection was observed in FL clams by histology or quantitative PCR. Histopathological analysis of MA clams indicated 37% prevalence of active QPX lesions in clam tissues while quantitative PCR revealed a 63% prevalence of QPX DNA 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, 30 psu) from *M. mercenaria* according to Perrigault et al. (2009a). 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 (17 °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 or 30 psu. These salinities represent the range observed in New York clamming waters and were shown to differentially affect the *in vitro* performance of QPX (Perrigault et al., 2010). Salinity adjustment for the 17 psu treatment was performed by adding freshwater to decrease the salinity (1 unit per day). All tanks were maintained at 17 °C which was selected according to Dahl et al. (2011) as an intermediate between conditions providing disease development (13 °C) and host healing (21 °C). 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 $5 \times 10^4$ 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, 30 psu). Once injected, clams were maintained out of the water for 3 h and then returned to their respective tanks.

### 2.5. Defense parameters

Before QPX challenge ($t_0$, after 2 weeks at the two 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 1.2–1.8 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) in ice-cold filtered artificial seawater (FASW, 30 psu) and used for assessment of clam parameters according to Perrigault et al. (2011). Total (THC) and differential (DHC) hemocyte counts as well as percentage of dead cells (PDCs) were assessed on a FACSCalibur flow cytometer (Becton Dickinson Biosciences) equipped with a 488-nm laser by counting 10,000 events. Reactive oxygen species (ROSs) production was measured before (unstimulated or native) and after stimulation (5 min post-stimulation) of hemocytes with zymosan A (Perrigault et al., 2011). 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* (Perrigault et al., 2011). Similarly, our previously described neutral red uptake (NRU) assay was applied to assess *in vitro* resistance of clam

hemocytes (not performed at $t_0$) to QPX extracellular products (Perrigault and Allam, 2009). Plasma from undiluted hemolymph was also recovered by centrifugation (700×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 (Perrigault et al., 2011). Anti-QPX activity in plasma of clams (not performed at $t_0$) was measured using a previously described *in vitro* growth inhibition assay (Perrigault et al., 2009a).

### 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 (5–6 μm in thickness), mounted on histology slides and stained (Harris's hematoxylin and Eosin Y). QPX intensity was assessed and scored as follows: light (⩽10 QPX cells on the section), moderate (11–100 QPX cells), or heavy (>100 QPX cells). Histological presence of old lesions and degrading QPX cells associated with the healing processes of hard clams were also recorded (Dahl and Allam, 2007).

### 2.7. Statistical analyses

Mortality data were compared using Kaplan–Meier survival analysis with LogRank significance test through SigmaStat (Systat Software, Inc., San Jose, California, USA) (Dahl et al., 2011; Kleinbaum and Klein, 2005). All hemolymph parameters 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. Multivariate analyses were performed using Principal Component Analysis (PCA) to analyze relationships between variables and evaluate overall effects of salinity and QPX on clam immunity since analyses based on individual hemolymph parameters may not be appropriate to unravel compensatory immune responses. 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 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. For instance, exposure of naïve *M. mercenaria* (FL-c) to low salinity induced higher mortalities (8% after 4 months) when compared to control clams maintained at 30 psu (0.7%, $p < 0.01$; LogRank test). Mortality in clams exposed to 17 psu was not significantly greater, however, following QPX challenge (9% after 4 months). Conversely, mortality was significantly higher in FL-q clams maintained at 30 psu (5%) as compared to controls (FL-c) maintained at the same salinity ($p = 0.03$). Microscopic observations did not detect any active QPX infection in clams sampled after 2 and 4 months as only dead QPX cells were occasionally seen in vascular tissues of challenged (FL-q) clams.

Salinity also modulated mortality levels in naturally infected (MA) clams. For instance, mortality was significantly higher in MA clams submitted to 30 psu (17%) as compared to those held at 17 psu (3.6%, $p = 0.029$, Fig. 1A). QPX disease prevalence was also 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. 1B). QPX prevalence among moribund clams collected throughout the experiment was also higher in clams maintained at 30 psu (91%) as compared to those maintained at 17 psu (67%). Healing signs (e.g. old lesions in clam tissues) were noted in 7–13% of naturally infected clams during the 4-month experiment but were statistically not different between both salinity treatments (Fig. 1B).

### 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 1 and 2, Figs. 2A and 3A). Total hemocyte counts (THCs) was significantly modulated by the salinity and tended to be higher in clams exposed to the lower salinity (Tables 1 and 2). Differential hemocyte counts of clams maintained at 17 psu also showed a 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

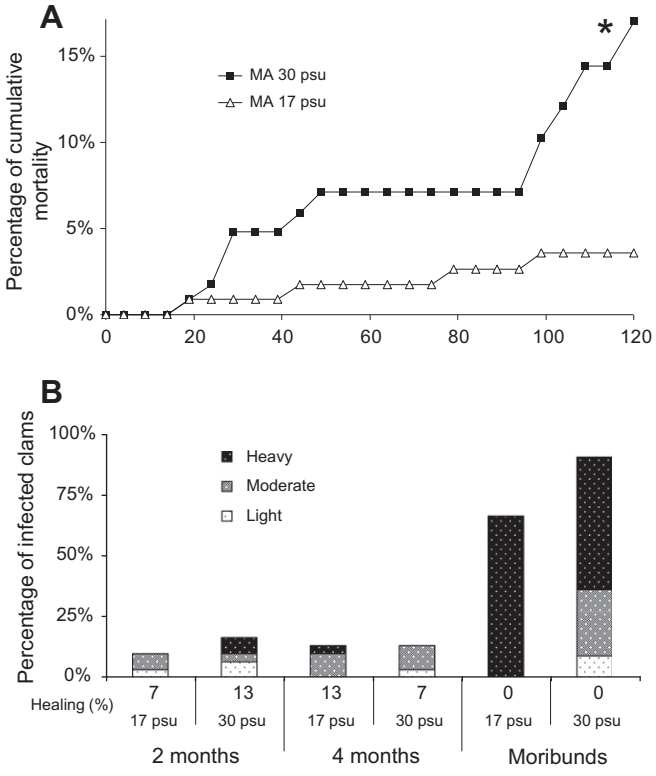


**Fig. 1.** Cumulative mortality [A] and QPX disease prevalence and intensity [B] in naturally-infected clams (MA) maintained at 17 and 30 psu for 4 months. ∗ in [A] designates significant difference between both salinity treatments ($p = 0.029$, LogRank test). In [B], QPX disease prevalence and intensity were determined in 30 clams per treatment excluding moribund clams where $n = 4$ and 10 clams for 17 psu and 30 psu, respectively. Values along the x-axis in [B] indicate percentage of clams presenting healing signs.

**Table 1**
Effects of salinity and QPX challenge on Florida *M. mercenaria* cellular and humoral parameters over the entire experiment (two 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 |
| Zymosan-stimulated ROS | ** | * | *** | * | ns | * |
| Phagocytosis | ** | *** | *** | *** | *** | ns |
| Protein concentration | * | *** | ns | ns | ** | ns |
| Lysozyme | ns | ns | ns | ns | ns | ns |
| Anti-QPX activities in plasma | ns | ns | ns | ns | ns | ** |
| Hemocyte resistance to QPX ECP | ** | ns | * | ** | * | *** |

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

**Table 2**
Cellular and humoral parameters in *M. mercenaria* (FL) 2 weeks after acclimation to 17 and 30 psu ($t_0$) and in control (FL-c) and QPX-challenged (FL-q) clams maintained at 17 psu or 30 psu for 2 and 4 months.

| | $t_0$ – 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 $ml^{-1} \times 10^5$) | 12.2 | 9.5 | 5.2 | 5.3 | 4.0 | 6.5[b] | 8.5 | 6.5 | 7.3 | 7.1 |
| % Granulocytes | 48.6 | 50.7 | 42.9 | 51.2 | 46.5 | 57.5[a,b] | 44.3 | 51.1 | 52.8[a] | 58.14 |
| PDC (%) | 13.9 | 7.8[a] | 12.3 | 13.1 | 6.2[a] | 11.3[b] | 10.4 | 10.8 | 9.4 | 8.5[a] |
| Unstimulated ROS production (fluorescence units/$10^4$ hemocytes) | 1.8 | 2.3[a] | 3.4 | 3.4 | 5.7[a] | 2.3[a,b] | 3.4 | 3.6 | 3.0 | 2.4 |
| Zymosan-stimulated ROS production (fluorescence units/$10^4$ hemocytes) | 2.6 | 3.0 | 3.4 | 4.9 | 8.5[a] | 3.4[b] | 3.9 | 2.2 | 3.0 | 3.1 |
| Phagocytosis (fluorescence units/$10^4$ hemocytes) | 2.0 | 1.6 | 1.5 | 2.6 | 7.1[a] | 4.2[b] | 2.0 | 1.9 | 1.9 | 2.5 |
| Protein concentration ($\mu g\ ml^{-1}$) | 411.5 | 405.5 | 313.1 | 310.7 | 433[a] | 446.6[a] | 300.4 | 330.9 | 346.9 | 394.1[a] |
| Lysozyme ($\mu g\ ml^{-1}$) | 7.6 | 7.1[a] | 8.9 | 8.9 | 8.8 | 9.1 | 8.9 | 9.0 | 8.9 | 8.8[a] |

[a] Significance at $p < 0.05$ between salinity treatments within the same clam group (FL, FL-c or Fl-q) and sampling time (2 weeks, 2 or 4 months).
[b] Significant differences at $p < 0.05$ between FL-c and FL-q within the same salinity treatment and sampling time. (Mean, $n = 30$ clams per data point.)

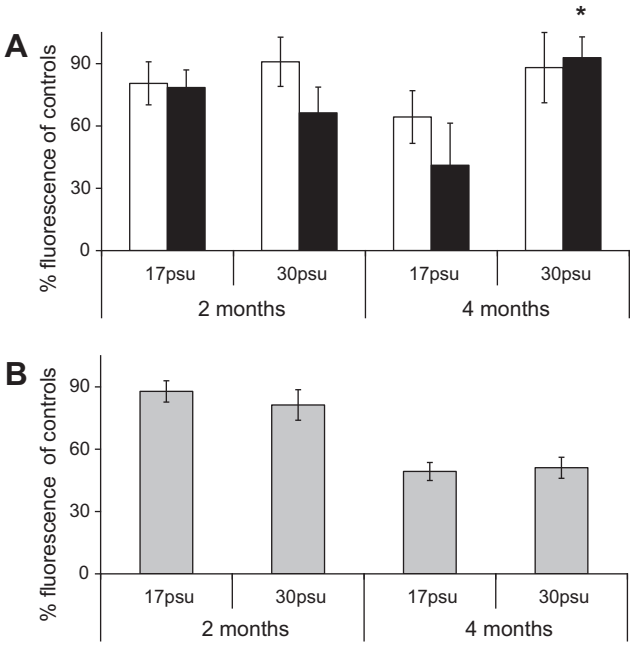


**Fig. 2.** Mean (±SEM, $n = 30$ clams per condition) of anti-QPX activity in plasma from [A] control (□) and QPX challenged (■) FL clams and [B] naturally-infected MA clams after 2 and 4 months at 17 and 30 psu (higher readings represent lower activities). * denotes significant differences (Holm-Sidak post-hoc test, $p < 0.05$) among challenged clams (FL-q) maintained for 4 months at 17 or 30 psu.

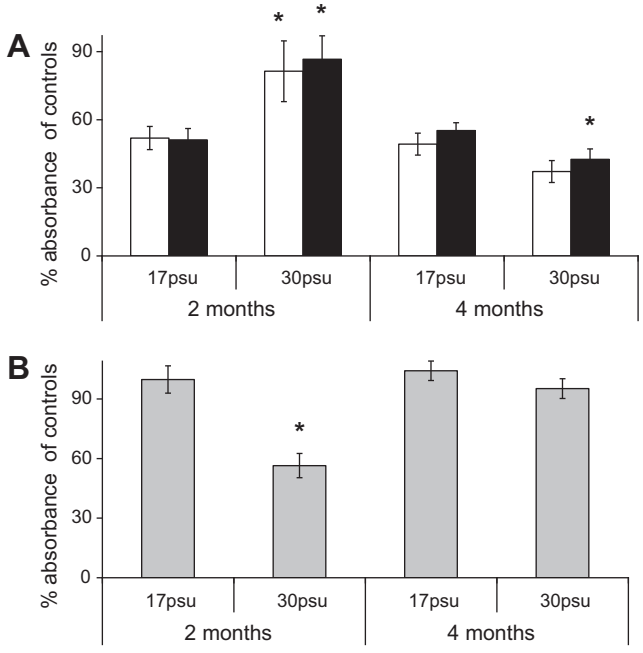


**Fig. 3.** Mean (±SEM, $n = 30$ clams per conditions) of hemocyte resistance to QPX ECPs in [A] control (□) and QPX challenged (■) FL clams and [B] naturally-infected MA clams after 2 and 4 months at 17 and 30 psu (higher readings represent higher resistance of hemocytes). Symbols (*) denote significant 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).

equivalent and constant over the 4 month experiment. Percentage of dead cells (PDCs) was systematically higher in naïve clams maintained at 17 psu as compared to those held at 30 psu and differences were highly significant ($p < 0.001$, Table 2) after 2 weeks and 2 months. Reactive oxygen species (ROSs) production in naïve clams was also modulated by salinity but only during the first two sampling points. Significantly lower unstimulated ROS production was observed in clams after 2 weeks and 2 months of exposure to 17 psu ($p < 0.032$, Table 2). Similarly, the production of ROS following stimulation with zymosan A was also lower in clams exposed to 17 psu as compared to those held at 30 psu with a significant difference observed at 2 months ($p < 0.001$, Table 2). Phagocytosis activity was also 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 ($p < 0.001$, Tables 1 and 2). 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 2). Conversely, lysozyme activity was not modulated by the salinity overall (Table 1) although higher activity was observed in FL-c clams maintained at 17 psu for 2 weeks as compared to the higher salinity treatment ($p = 0.005$, Table 2) and this trend disappears in subsequent samplings.

Anti-QPX activity of plasma was not modulated in unchallenged (FL-c) clams in response to salinity (Table 1 and Fig. 2A). Results from the neutral red uptake assay showed that hemocyte resistance to QPX ECPs was temporarily modulated by salinity since significantly higher resistance was detected in the 30 psu treatment only after 2 months (Fig. 3A).

### 3.2.2. Effects of salinity on clam defenses in experimentally-challenged clams (FL-q)

Salinity treatment also induced significant modifications of hemolymph parameters in experimentally challenged clams (FL-q) (Tables 1 and 2, Figs. 2A and 3A) and results highlighted the interactions between salinity treatments and QPX challenge. 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 (Table 2, $p < 0.001$). This was associated to an increase in the percentage of granulocytes in FL-q clams maintained at 30 psu as compared to their FL-c controls or to FL-q clams held at 17 psu (Table 2). QPX challenge also caused a significant increase in PDC among FL-q clams maintained at 30 psu for 2 months (11.3%, Table 2) 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. PDC levels were generally lower in FL-q clams maintained at 30 psu compared to those held at 17 psu (Table 2, $p < 0.001$ at 4 months) and these trends were confirmed in 2-way ANOVA results (Table 1). ROS production by hemocytes from FL clams was highly modulated by both QPX challenge and salinity treatment (Tables 1 and 2). Interestingly, unstimulated 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 2, $p < 0.001$). Additionally, decreasing salinity caused an increase in unstimulated ROS production after 2 months in FL-q clams (Table 2, $p = 0.001$). 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 2, $p < 0.001$). Phagocytic activity of hemocytes exhibited inverted trends with higher levels at 30 psu compared to 17 psu (Table 2). Interestingly, significantly lower phagocytic 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 salinity ($p = 0.01$) whereas the effect of QPX challenge was not detectable after 4 months. Protein concentration in plasma was significantly higher in FL-q clams maintained at 30 psu compared to those held at 17 psu ($p < 0.023$, Table 2). Lysozyme activity was not significantly modulated by salinity except for FL-q clams sampled after 4 months where levels were lower at 30 psu as compared to 17 psu ($p = 0.007$). Experimental challenge did not cause any significant alteration in protein concentration in plasma or lysozyme activity over the entire duration of the study (Table 2).

Anti-QPX activity in plasma was significantly lower in FL-q clams maintained for 4 months at 30 psu as compared to those held at 17 psu ($p = 0.019$, Fig. 2A). A significant shift was detected over time in hemocyte resistance to QPX ECP (Fig. 3A) with inverted trends between 2 and 4 months. 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 the former group after 4 months ($p = 0.047$). Interestingly, hemocyte resistance to QPX ECPs decreased over time in FL-q clams maintained at 30 psu but remained constant in clams maintained at 17 psu. Overall, experimental challenge did not cause any consistent 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. 2A and 3A).

### 3.2.3. Effects of salinity on clam defenses in naturally-infected clams (MA)

Salinity treatment also caused significant modifications of several hemolymph parameters in naturally infected (MA) clams (Tables 3 and 4, Figs. 2B and 3B). THC tended to be higher in clams maintained at 30 psu as compared to 17 psu although differences were not statistically significant (Table 4). Alternatively, significantly higher percentage of granulocytes were measured in MA clams maintained at 30 psu compared to those held at 17 psu for 2 and 4 months ($p < 0.005$, Table 4) in agreement with results obtained in FL-q clams. Similarly, PDC levels in MA clams followed the same trend described above in FL-q clams with lower levels at 30 psu compared to 17 psu ($p < 0.019$, Table 4). Decreasing salinity also caused a significant increase in unstimulated ROS production after 2 and 4 months in MA clams ($p < 0.016$, Table 4) and zymosan-activated ROS production generally followed similar trends with lower activity at 30 psu although differences were not statistically significant (Table 4). As in FL-q clams, protein concentration in plasma was significantly higher in MA clams maintained at 30 psu compared to those held at 17 psu ($p < 0.02$, Table 4). Lysozyme activity was not significantly modulated by salinity.

Anti-QPX activity was similar in plasma from MA clams maintained at 17 or 30 psu (Fig. 2B). In contrast, hemocyte resistance to QPX ECP was strongly modulated by salinity and was lower in

**Table 3**
Effects of salinity on cellular and humoral parameters of QPX infected clams from MA over the entire experiment.

| | Time | PSU | Time/PSU |
|---|---|---|---|
| THC | ** | ns | ns |
| % Granulocytes | ns | *** | ns |
| PDC | ns | *** | ns |
| Unstimulated ROS | ns | *** | ns |
| Zymosan-stimulated ROS | ** | ns | ns |
| Protein concentration | ns | *** | ns |
| Lysozyme | ns | ns | ns |
| Anti-QPX activity in plasma | *** | ns | ns |
| Hemocyte resistance to QPX ECP | * | ** | ns |

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

**Table 4**
Hemocyte and plasma parameters of MA *M. mercenaria* naturally infected by QPX and maintained at 17 psu or 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[a] | 44.5 | 66.0[a] |
| PDC (%) | 6.3 | 4.9[a] | 7.6 | 4.4[a] |
| Unstimulated ROS production (fluorescence units/$10^4$ hemocytes) | 2.4 | 1.1[a] | 1.9 | 1.5[a] |
| Zymosan-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[a] | 349.8 | 451.4[a] |
| Lysozyme ($\mu g\ ml^{-1}$) | 6.1 | 6.4 | 6.7 | 4.6 |

[a] Significant differences ($p < 0.05$, ANOVA) between salinity treatments for the same sampling time (2 or 4 months). (Mean, $n = 30$ clams per data point.)

MA clams maintained at 30 psu for 2 months as compared to their 17 psu counterparts ($p < 0.001$, Fig. 3B). Interestingly, hemocyte resistance to QPX ECPs increased over time in MA clams held at 30 psu while it remained constant during the 4-month experiment in clams maintained at 17 psu (Fig. 3B).

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

Principal Component Analysis was performed on hemolymph parameters combined from all FL (Fig. 4) and MA clams (data not shown) sampled at 2 and 4 months to assess the overall effect of salinity and QPX challenge on clam immune status. This multivariate approach offset possible problems related to the interpretation and meanings of changes in single immune parameters. 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 (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 (Fig. 5). For instance, PC1 values were significantly different between FL-c clams maintained at 17 psu and those maintained at 30 psu for the 2-month sampling and for both sampling times combined ($p = 0.0012$, Fig. 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. 5A). 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. 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. 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. 5B and D).

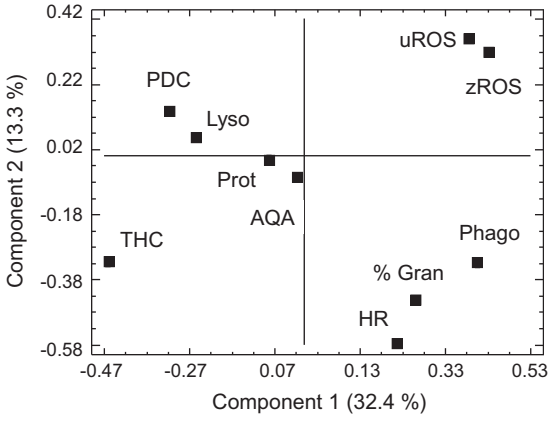


**Fig. 4.** Principal Component Analysis (PCA) plot of hemolymph parameters (2 and 4 months) in FL clams abbreviated as follows, PDC: percentage of dead cells, uROS: unstimulated ROS production, zROS: zymosan-stimulated ROS production after 5 min, THC: total hemocyte count, % Gran: percentage of granulocytes, Phago: phagocytosis, Prot: protein concentration in plasma, Lyso: lysozyme activity, AQA: anti-QPX activity of clam plasma, HR: hemocyte resistance to cytotoxicity of QPX ECP.

## 4. Discussion

The aim of this study was to investigate the effect of salinity on defense parameters (both cellular and humoral) of naïve 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 the impact of salinity on defense factors in naïve clams, QPX disease dynamics and clam response to the parasite. QPX is a relatively slow and chronic disease (Dahl and Allam, 2007; Dahl et al., 2011). The duration of the experiment (4 months) and temperature conditions (17 °C) were selected to enhance the differential effect of salinity treatments on clam defense parameters and the response to QPX challenge according to results from previous studies (Dahl and Allam, 2007; Perrigault et al., 2011; Perrigault et al., 2009b).

### 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 1 and 2). Although mortality levels among FL clams were overall low, they tended to be higher in naïve 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

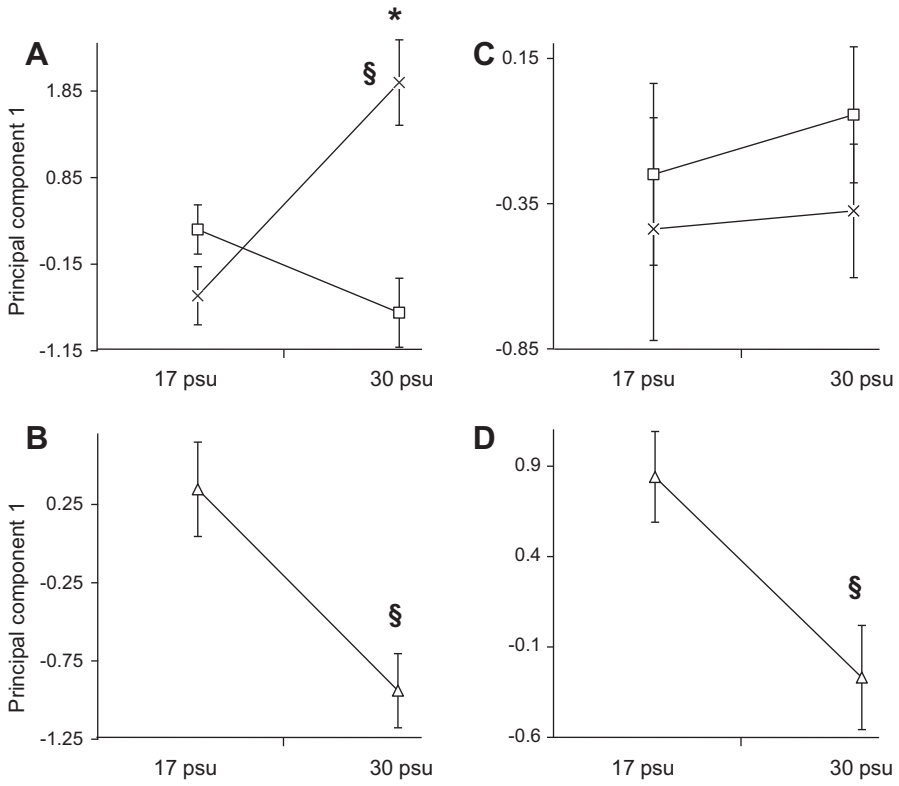


**Fig. 5.** Mean plots of Component 1 from Principal Component Analysis of immune parameters in [A and C] unchallenged (×) and QPX challenged (□) FL clams and [B and D] MA clams maintained at 17 and 30 psu. Components were extracted after 2 [A and B] and 4 [C and D] months of salinity exposure. Symbols (§) denote significant differences between salinity treatments and (∗) indicate significant differences between FL-c and FL-q at 30 psu ($p < 0.05$, ANOVA).

mortality in FL-c clams maintained at 30 psu. Additionally, decreasing salinities are usually associated with 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 hemolymph 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. 5A). Clams exposed to lower salinity generally had higher THC, PDC, and lysozyme activity as well as low phagocytosis activity and ROS production (Table 2). 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., 2004; 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., 1993; Pipe and Coles, 1995). Additionally, THC increase at low salinity was associated with a significant increase of PDC (Tables 1 and 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 1 and 2), in agreement with prior findings of higher ROS production with higher salinity in abalone (Cheng et al., 2004) 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 2). 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., 1997; Cheng et al., 2004; Gagnaire et al., 2006; Matozzo et al., 2007; Reid et al., 2003; Wang et al., 2008) which demonstrated variation of phagocytic activity after acute 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 2). 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, clam mortality and immune responses to the parasite in naturally infected and experimentally challenged *M. mercenaria* (Tables 1–4, Fig. 1). Mortality was 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 17% and 3.6% respectively. Disease prevalence was also higher among MA moribund clams maintained at 30 psu (91%) as compared to those held at 17 psu (67%). These findings contrast with relatively low and somehow similar disease prevalence detected in MA clams sampled at 2 and 4 months from both salinity treatments (Fig. 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 two different processes: (1) mortality of diseased clams; this is particularly true among clams held at 30 psu that displayed high mortality (17%) and disease prevalence among moribund clams (91%) and (2) healing of infected clams, as detected in clams maintained at both salinities.

Salinity significantly affected defense factors in clams naturally and experimentally infected with QPX (Tables 1 and 3). 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). Interestingly, PC1 changes in response to salinity treatment displayed similar trends among naturally and experimentally infected clams at 2 months (Fig. 5A and B), but differences between 17 psu and 30 psu were significant only in MA clams. Furthermore, 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. 5A) and at both sampling times combined (data not shown) 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 2). These results are in agreement with our prior report of changes in phagocytosis and ROS production in naturally-infected clams or following experimental challenge with QPX (Perrigault et al., 2011). QPX challenge induced a significant increase in THC among FL-q 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 2). 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., 2004; Ford et al., 1993). 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 no 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. As a matter of fact, hemocyte resistance to QPX ECPs was significantly higher in FL-q clams maintained for 2 months at 30 psu as compared to their 17 psu counterparts and trends were 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 inflammatory 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 time frames using the same technique (Dahl and Allam, 2007; Dahl et al., 2011). 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. 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, an effect of salinity on disease development and resulting mortality and defense response were identified in MA clams. Together, these results suggest that at 17 °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. This interpretation agrees well with findings by Perrigault et al. (2010) that demonstrated significant inhibition 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. virginica* 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 the elimination of the parasite. The impact of salinity on clam response against QPX was obvious in naturally infected clams (Fig. 5B and D).

In conclusion, this study demonstrates a significant impact of salinity on clam immunity and QPX disease progression 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 (Perrigault et al., 2011). The fact that our current experiments were performed at 17 °C appeared to limit 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, the impact of salinity on disease development appears secondary as compared to that we reported earlier for temperature.

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