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

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ABSTRACT

Quahog Parasite Unknown (QPX) causes disease and mortality in hard clams, *Mercenaria mercenaria*. Seasonality of QPX disease prevalence in the field and changes in QPX growth and survival *in vitro* suggest a role of temperature in the hard clam-QPX interaction and disease development. This study specifically examined the effect of temperature on QPX disease development and dynamics. Naturally and experimentally infected clams were separately maintained in the laboratory at 13 °C, 21 °C, or 27 °C for 4 months. Following this initial treatment, temperature was adjusted to 21 °C for 5 additional months to simulate seasonal changes of temperature in the field and to investigate the effect of temperature variations on QPX disease dynamics. Mortality was continuously monitored during the experiment and clams were sampled at 2, 4 and 9 months for the assessment of QPX disease prevalence and intensity using our standard histological and quantitative PCR techniques. Results demonstrated significantly higher QPX disease prevalence and intensity, as well as higher mortality, in naturally-infected clams maintained at 13 °C as compared to those held at 21 °C or 27 °C. Similarly, disease development was significantly higher in experimentally infected clams maintained at the colder temperature (70% prevalence after 4 months) as compared to those maintained under warmer conditions (<10%). Additionally, our results demonstrated an improvement in the condition of clams initially maintained at 13 °C for 4 months after transfer to 21 °C for 5 additional months, with a significant reduction of QPX prevalence (down to 19%). Interestingly, disease development or healing in clams maintained at different temperatures exhibited a strong relationship with clam defense status (*jointly submitted paper*) and highlighted the impact of temperature on clam activity and QPX disease dynamics. These findings should be taken into account for the timing of activities involving the monitoring, movement (e.g. relays, transplants) or grow out (e.g. commercial culture, municipal enhancement) of hard clams in enzootic areas.

1. Introduction

QPX (Quahog Parasite X = Unknown) causes disease and mortality in hard clams, *Mercenaria mercenaria*. This protistan parasite has been detected in hard clams (a.k.a. quahogs) along the eastern coast of North America from Atlantic Canada down to Virginia, USA. (Dove et al., 2004; MacCallum and McGladdery, 2000; Ragone Calvo et al., 1998; Smolowitz et al., 1998). Histological observation of QPX-infected tissues revealed that some clams are able to mount an effective defense reaction characterized by a significant granulomatous inflammatory response leading to the healing of infected individuals and death of parasite cells (Dahl and Allam, 2007; Dove et al., 2004; Ragone Calvo et al., 1998). Variation in susceptibility to QPX has been demonstrated among different clam populations and is characterized by higher susceptibility of southern broodstocks

compared to northern broodstocks, yet QPX disease has never been detected in clams south of Virginia (Dahl et al., 2008; Dahl et al., 2010; Ford et al., 2002; Ragone Calvo et al., 2007). These observations suggest the existence of factors limiting QPX disease development south of Virginia despite the presence of relatively susceptible clams there.

Previous studies have noted a significant impact of temperature on *in vitro* proliferation of the parasite, with optimum growth between 20 °C and 23 °C (Perrigault et al., 2010). Similarly, field surveys demonstrated seasonality of QPX infections with peaks in disease prevalence and clam mortality during summer in New York and Massachusetts (Liu et al., 2008; Smolowitz et al., 1998). In addition, our previous investigations showed significant healing and recovery in naturally-infected clams maintained for 10 months at 21 °C (Dahl and Allam, 2007). This evidence suggests a major effect of environmental parameters, especially temperature, on QPX disease dynamics. Seasonality and modulatory effects of temperature on epizootics caused by bacterial and protistan pathogens are well documented in bivalves (Carnegie et al., 2008; Ford et al.,

1999; Paillard, 2004; Travers et al., 2008; Villalba et al., 2004). However, no previous investigations specifically evaluated the effect of environmental parameters on QPX disease development in hard clams.

This study aimed at elucidating the effect of temperature on QPX disease development and resulting mortalities. Naturally and experimentally infected clams were exposed to three environmentally relevant temperatures (13 °C, 21 °C and 27 °C) for 4 months. These temperatures represent the range observed in shallow and deep clamming waters in New York 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). Following this initial treatment, clams were transferred to 21 °C for 5 additional months to simulate seasonal changes of temperature in the field and investigate the effect of temperature variations on QPX disease dynamics. QPX disease prevalence and intensity were assessed throughout the experiment by using our standard histological (Dahl and Allam, 2007) and quantitative PCR (QPCR) (Liu et al., 2009) methods. Results showed a major effect of temperature on QPX disease development and resulting mortality. These findings are discussed in light of our current knowledge of the effect of temperature on hard clam immunity (Perrigault et al., 2011), highlighting the modulatory influence of temperature on host–pathogen interactions.

2. Materials and methods

2.1. M. mercenaria

Eight hundred naïve *M. mercenaria* (30–35 mm in length) originating from Florida (FL) were obtained from a commercial source (Farm Raised Clams, St James City, FL). Four hundred presumably infected clams (40–50 mm in length) were obtained during the fall from an enzootic clamming area in Massachusetts (MA). Clams were acclimated for 1 week in 150-l tanks with re-circulating water (28–30 ppt) at 21 ± 1 °C and fed daily with commercial algae (DT's Live Phytoplankton, Sycamore, IL). Clams (30 animals from each population) were sampled, dissected and processed for histology and quantitative PCR (Section 2.6) to determine initial QPX prevalence and intensity. QPX was not detected by either histology or QPCR in FL clams. Histopathological observations of tissues from MA clams indicated active QPX lesions and QPCR revealed a 63.3% prevalence of QPX DNA among MA clams at the beginning of the experiment.

2.2. QPX

QPX strain NY0313808BC7 was isolated from nodules of an infected New York clam (Qian et al., 2007) and subcultured in muscle tissue homogenates (MTH) from *M. mercenaria* according to Perrigault et al. (2009). QPX cultures were initiated in 25-cm² flasks containing MTH at 1000 µg ml⁻¹ protein and incubated at 23 °C for 2 weeks (Perrigault et al., 2009). MTH culture medium was also incubated in the same conditions to provide the suspension injected in control clams (Section 2.4). Neubauer chamber and the FDA technique (Buggé and Allam, 2005) were used to monitor the growth and determine the concentration of QPX cells.

2.3. Temperature treatment

Following the 1-week acclimation, clams were distributed in 40-l tanks (20 clams per tank) with a total of 36 tanks for the FL clams and 18 tanks for the MA clams. All tanks were aerated and individually equipped with re-circulating water (30 ppt) filtration systems. Three groups composed of 18 tanks each (6 controls FL

[FL-c], 6 QPX-challenged FL [FL-q] and 6 MA [MA]) were kept at 13, 21 or 27 °C. Temperatures were selected based on data logged in clamming areas in New York (and other northeastern states) from spring through fall (Dahl et al., 2010 and Allam, unpublished). Temperature adjustments were performed within 8 days by increasing or decreasing the temperature by 1 °C per day as appropriate for each treatment. Treatments at 13 and 27 °C were controlled by water baths equipped with heaters or connected to chillers. Water quality, salinity and ammonia level were monitored weekly and adjusted if necessary. Water temperature was monitored daily over the entire 9-month duration of the experiment. Clams were fed daily with commercial algae and monitored twice a day for mortality.

2.4. QPX challenge of Florida clams

After 1 week of acclimation at each of the three experimental temperatures, FL clams were challenged by injecting sterile culture medium (MTH, control clams, FL-c) or 5 × 10⁴ QPX cells (FL-q) into the pericardial cavity according to Dahl and Allam (2007). Once injected, clams were kept out of the water for 1.5 h before being transferred back to their respective tanks.

2.5. Sampling of naturally and experimentally infected clams

Naturally and experimentally QPX-infected clams (30 clams per treatment) were randomly sampled (5 clams tank⁻¹) after 2 and 4 months, totaling 540 clams individually processed during the initial temperature exposure experiment. The remaining clams were then condensed into two replicate tanks to obtain clam densities comparable to those existing at the start of the trial (20–30 clams tank⁻¹ dependent on prior mortalities). Tanks initially submitted to 13 °C and 27 °C were incrementally brought to 21 °C within a period of 8 days (1 °C day⁻¹). All tanks were then maintained at 21 °C for an additional 5 months before the final sampling was made (30 clams from each treatment, totaling 270 clams, after 9 months for the whole experiment). This sampling scheme was chosen based on results from our previous laboratory trials showing this time period to be sufficient to observe significant disease progress (Dahl and Allam, 2007; Dahl et al., 2008).

2.6. Histopathology and quantitative PCR

All sampled clams were processed for histopathology following the general procedures described previously (Dahl and Allam, 2007; Dahl et al., 2010). A transverse slice of tissue roughly between 3 and 5 mm in thickness through the central region of the meat was made in an attempt to include visceral organs, as well as gill and mantle tissues. A particular effort was made to include tissue from the base of the siphon, where infections are common (Smolowitz et al., 2001). Tissue sections were placed in histocassettes, embedded in paraffin, sectioned (5–6 µm in thickness), and mounted on histology slides. Stained (Harris's hematoxylin for 2 min and Eosin Y for 1 min) slides were examined by light microscopy for presence of QPX. When QPX cells were discovered, the tissue(s) infected and the infection intensities were determined based on the number of QPX cells present on the histological section, and were recorded as follows: light (⩽10 QPX cells on the section), moderate (11–100 QPX cells), heavy (101–1000 QPX cells), or severe (>1000 QPX cells) (Dahl et al., 2010). Histological presence of old lesions and degrading QPX cells associated with the healing processes of hard clams were also recorded (Dahl and Allam, 2007).

Quantification of QPX cells in clam tissues by QPCR was performed on a random subset of clams processed for histopathology at 2 and 4 months. Following the collection of histopathology samples, remaining mantle and siphon tissues from 10 clams per

treatment (out of 30 for histopathology) were collected and processed individually for QPCR according to our previously described protocol (Liu et al., 2009). Disease intensity among positive clams was ranked based on the number of equivalent parasite cells in 0.1 g tissue as: light (1–10 QPX cells), moderate (11–100), heavy (101–1000) and severe (>1000 cells).

2.7. Statistical analyses

Mortality data, consisting of time of death (i.e. day of experiment) for individual clams, were compared by survival analysis through SigmaStat for Windows version 3.10 (Systat Software, Inc). Kaplan–Meier survival analysis was employed, which includes both failures (death) and censored values (Kleinbaum and Klein, 2005). 'Censored' means the values have been lost from view of the study. This compensates for the removal of clams at set points in time for infection diagnosis. A LogRank test was then conducted to determine whether survival curves were significantly different (Kleinbaum and Klein, 2005). The Holm–Sidak test was used for multiple comparison procedures to determine which pairs of curves were different. It applies a sequential adjustment of critical values that compensates for the number of comparison tests (Glantz, 2005).

Disease prevalence data for naturally and experimentally infected clams from each sampling date were separately tested for significant differences according to each temperature treatment. Counts of QPX-infected and uninfected individuals from each sample (histology or QPCR) were arranged in a 2-way, row-by-column contingency table and tested for independence of variables by means of the G-test through BIOMstat (Statistical Analysis for Biologists, version 3.3, Applied Biostatistics, Inc., Sokal and Rohlf, 1995). The first variable was original temperature treatment: 13, 21, or 27 °C. The second variable was infection, with one class for infected and one class for uninfected clams. Additionally, counts of individuals with and without signs of healing and remission at the end of 9 months were tested in the same manner. All counts were pooled from replicate samples of the same treatment group and time period. William's correction for G (which results in a more conservative p value) was determined to obtain a better approximation to the Chi-square distribution as recommended by Sokal and Rohlf (1995). BIOMstat additionally carries out Gabriel's simultaneous test procedure which identifies all maximal non-significant sets of rows and columns (i.e. a set that becomes significantly heterogeneous if any other row or column is added). All results were considered significant at an overall level of $\alpha = 0.05$.

3. Results

3.1. Mortality

3.1.1. Control and experimentally infected clams from Florida (FL)

During the initial 4-month exposure to different temperatures, mortality was very low (<4%) across all temperature treatments for both control and QPX-challenged clams (Data not shown). Similarly, the 5-month extension of the experiment (after treatments were converged to 21 °C) resulted in minimal (<6%) to no mortality in control clams initially maintained at 13 °C or 21 °C whereas mortality in QPX-challenged clams reached 16% at 13 °C and 21% at 21 °C (Data not shown). High mortalities were observed during the extension period in clams initially held at 27 °C, with 36% and 41% mortality for control and challenged clams, respectively.

3.1.2. Naturally infected Massachusetts (MA) clams

Clams maintained at 13 °C exhibited a steady increase in mortality over the first 4 months (Fig. 1A). After 4 months of temperature exposure, cumulative mortality was significantly higher (p = 0.01) in the coldest treatment (19%) as compared to clams held at 21 °C (6%) or 27 °C (8%). Mortalities continued during the 5-month extension period at 21 °C for clams initially maintained at 13 °C and 21 °C (Fig. 1B) but cumulative mortality leveled off after 1 month under the new temperature condition at 10% for the 13 °C group and 8%, for the 21 °C group. Clams initially maintained at 27 °C for 4 months exhibited a very low level of mortality during the 5-month extension at 21 °C (Fig. 1B).

3.2. QPX disease development

3.2.1. Disease prevalence by histology

QPX was not detected in any control (FL-c) clam. Histological observations of both experimentally and naturally-infected clams exhibited similar trends according to temperature treatment (Fig. 2). Experimentally infected clams (FL-q) maintained at 13 °C displayed significantly higher QPX prevalence (p < 0.001), 73% at 2 months and 70% at 4 months, as compared to challenged clams maintained at 21 °C or 27 °C (≤10%, Fig. 2A). Disease prevalence in FL-q clams remained steady in all temperature treatments during the initial 4-month experiment but a general decrease in prevalence was subsequently observed after all clams were converged to 21 °C for the 5-month extension, particularly in clams initially maintained at 13 °C (19% at 9 months, Fig. 2A). The decrease in disease prevalence during the extension period coincided with an increase in the proportion of clams displaying healing signs among individuals initially submitted to 13 °C and 21 °C. On the other hand, the proportion of healing clams peaked at 4 months in clams maintained at 27 °C, and no clams in this batch displayed disease or healing signs at the end of the 5-month extension period.

QPX prevalence in naturally infected (MA) clams was also significantly modulated by temperature (Fig. 2B). Two months after the beginning of the experiment, disease prevalence was 23%, 10% and 0% in MA clams submitted to 13 °C, 21 °C and 27 °C, respectively (significant difference between 13 °C and 27 °C;
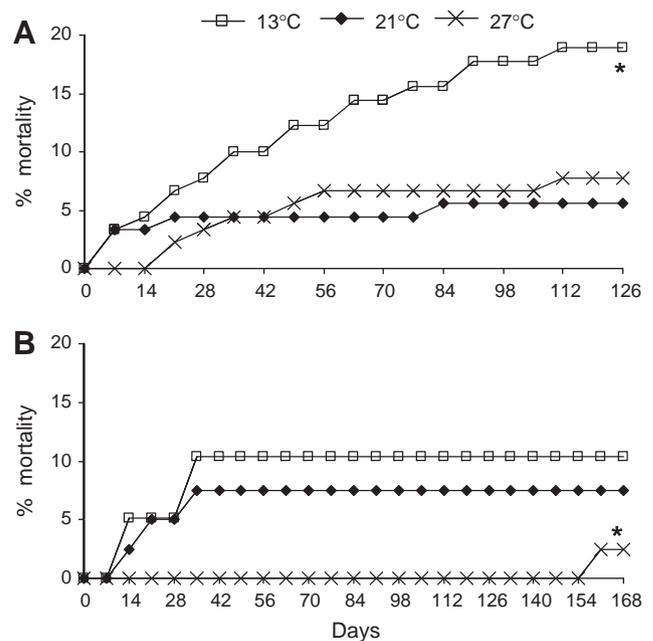
Fig. 1. Cumulative mortality in naturally-infected clams submitted to 13 °C, 21 °C and 27 °C for 4 months (A), after which all treatments were maintained at 21 °C (B) (day 0 in B represents the beginning of the 5 month extension). *Designates significant difference between this and the other 2 treatments (LogRank with Holm-Sidak procedure, p ⩽ 0.01).
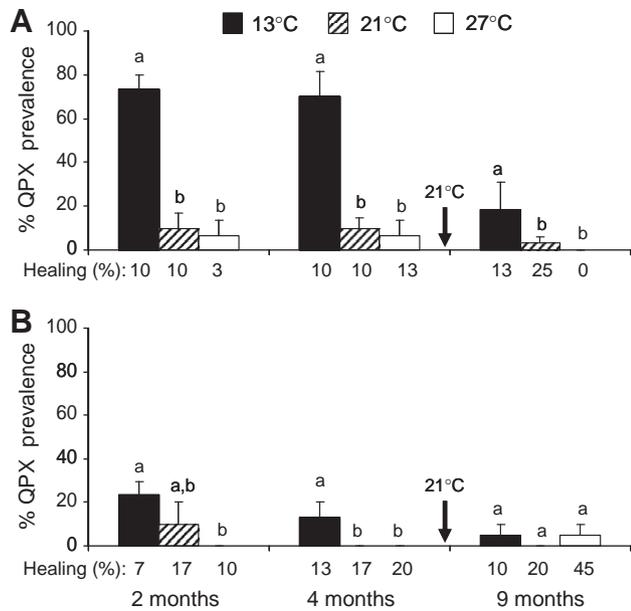
Fig. 2. QPX prevalence expressed as mean and standard error (6 tanks/treatment and 5 clams sampled from each tank at each sampling time) determined by histology in (A) experimentally (FL-q) and (B) naturally (MA) infected clams maintained at different temperatures. All clams were transferred to 21 °C after the 4-month sampling (arrow). Values along the x-axis indicate percentage of clams presenting healing signs. Different letters (a and b) designate significant differences between treatments (p < 0.05, G-test with Gabriel's procedure).

Fig. 3. QPX infection intensity determined by histology in (A) experimentally (FL-q) and (B) naturally (MA) infected clams maintained at 13 °C, 21 °C and 27 °C. Number of infected clams is indicated on the x-axis. All clams were transferred to 21 °C after the 4-month sampling.

p < 0.01). A more pronounced difference was noticeable after 4 months with 13% disease prevalence in clams maintained at 13 °C as opposed to 0% in those held at 21 °C or 27 °C (p < 0.05, Fig. 2B). However, a gradual decrease in disease prevalence was observed in MA clams over the entire 9-month period and was associated with an overall increase in the percentage of clams displaying healing signs among all treatments, particularly among those initially maintained at 27 °C (p < 0.01, Fig. 2B).

### 3.2.2. Disease intensity by histology

High QPX prevalence observed in FL-q and MA clams submitted to 13 °C was associated with higher disease intensity in these groups as compared to clams maintained at the warmer temperatures (Fig. 3). FL-q clams maintained at 13 °C exhibited mostly light infections at 2 months which progressively evolved to more serious infections at 4 months (Fig. 3A). Similarly, moderate and heavy infections constituted most (>75%) of the positive MA clams maintained at 13 °C for 2 or 4 months, in contrast to the warmer treatments where low numbers of QPX-positive clams were observed and none of them presented heavy infection (Fig. 3B).

3.2.3. Histological analysis of moribund clams

Five moribund clams were collected for histology among experimentally infected (FL-q) individuals during the initial 4-month experiment (Fig. 4A). These included three individuals from the 13 °C treatment (2 negative and 1 displaying a moderate infection) and two individuals from the 21 °C treatment (1 moderately and 1 heavily infected). Disease prevalence was significantly higher among moribund clams collected during the 5-month extension from the 13 °C treatment (100% prevalence) as compared to FL-q clams initially maintained at 21 °C (55%) or 27 °C (11%) (p < 0.001, Fig. 4A). The difference between the latter 2 groups was also significant (p = 0.01). Very low QPX prevalence among moribund FL-q clams from the 27 °C group during the 5-month extension at 21 °C contrasted with the high mortality observed in
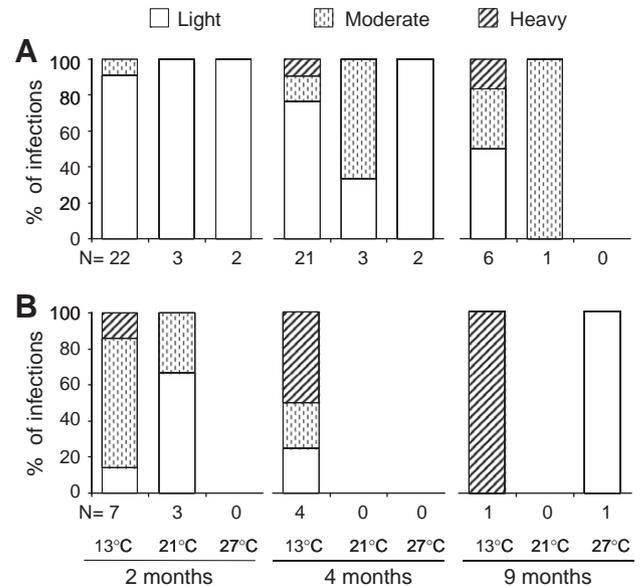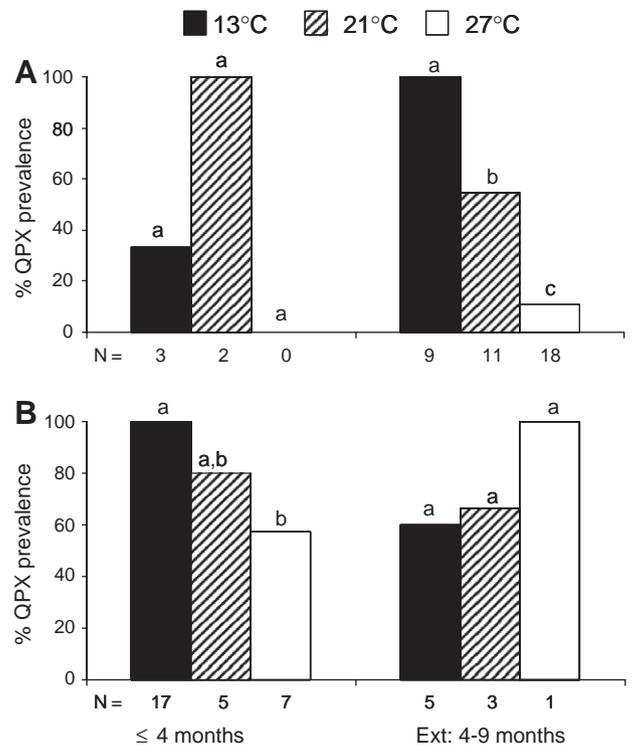
Fig. 4. QPX prevalence determined by histology in moribund clams from (A) experimentally challenged (FL-q) and (B) naturally infected (MA) clams, collected during the initial temperature exposure (≤4 months) and during the 5-month extension at 21 °C (4–9 months). Number of moribund clams processed is indicated along the x-axis. Different letters (a, b and c) designate significant differences between treatments (p < 0.05, G-test with Gabriel's procedure).

this batch (as well as in non-infected controls from the 27 °C group, Section 3.1.1), suggesting that mortality at this temperature was not related to QPX. In naturally-infected clams, disease prevalence was maximal (100%) at the end of the initial 4-month exposure among moribund MA clams from the 13 °C treatment, followed

by 80% positive in the 21 °C group and 57% in the 27 °C treatment ($p < 0.05$, Fig. 4B). Severe infections were only observed in clams maintained at 13 °C (4 out of 17 clams or 24%) while mostly moderate infections were detected in those held at 27 °C. However, trends were not as clear during the 5-month extension (no significant difference between treatments) although mortality was relatively low during that period and a very limited number of moribund clams were processed for histology (Fig. 4B). Overall, there was a substantial decrease in intensity among positive clams collected during the extension period and mostly light and moderate infections were detected.

3.2.4. Determination of QPX cell numbers in clam tissues by QPCR

QPCR analyses were performed on a subset (10 out 30) of clams processed for histopathology after 2 and 4 months. These analyses revealed similar trends as histological observations. For instance, a higher percentage of QPX-positive clams was observed at lower temperature for both experimentally and naturally-infected clams, with a significant difference at 4 months of FL-q initially submitted to 13 °C compared to the other 2 treatments ($p < 0.01$, Fig. 5A). Interestingly, QPX prevalence in clams from the 13 °C treatments were similar, apart from the diagnostic technique (histology or by QPCR), whereas higher prevalences were regularly observed by QPCR as compared to histological analysis among clams maintained at 21 °C or 27 °C (Figs. 2 and 5).

Determination of the number of QPX cells in clam tissues by QPCR also revealed some similarities with the histological assessment of disease intensity. For instance, the heaviest infections were detected by QPCR in the 13 °C group, which includes most severe cases observed histologically in the naturally infected MA clams (Fig. 6B). Similarly, the least intense infections were observed in the 27 °C group. Additionally, an increase in QPX cell numbers over time was seen only among clams maintained at 13 °C whereas both FL-q and MA clams submitted to 21 °C and
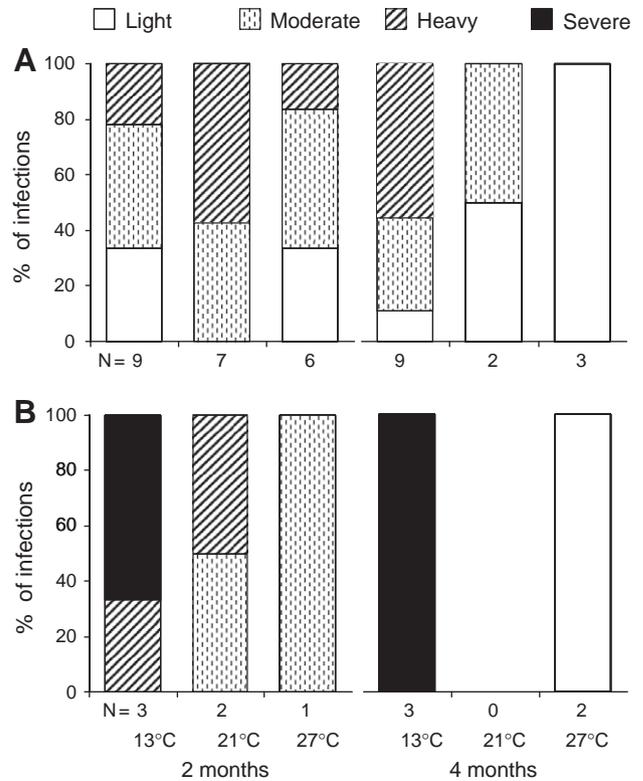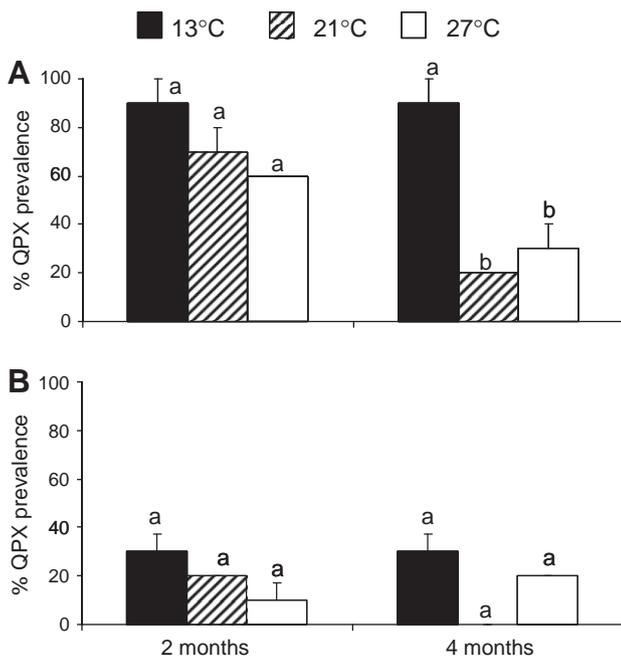
Fig. 6. QPX disease intensity (based on QPX cell numbers in clam tissues, see Section 2.6) determined by quantitative PCR in (A) experimentally (FL-q) and (B) naturally (MA) infected clams maintained at 13 °C, 21 °C and 27 °C. Number of infected clams is indicated on the x-axis.

27 °C exhibited a decrease in the number of QPX cells in tissues over the initial 4-month experiment (Fig. 6).

4. Discussion

The aim of this study was to investigate the effect of temperature on QPX disease development and associated mortalities in *M. mercenaria* naturally or experimentally infected by QPX. We also evaluated the evolution of the disease in response to temperature variations roughly simulating seasonal changes. Our results demonstrated a clear impact of temperature on QPX disease development and dynamics in both naturally and experimentally infected clams. This study, in conjunction with our data on the impact of temperature on immune parameters (Perrigault et al., 2011), provides a comprehensive assessment of the effect of this primary environmental factor on host–pathogen interactions.

QPX disease prevalence and intensity were strongly modulated by temperature. Clams submitted to 13 °C for 4 months exhibited significantly higher QPX prevalence by histological analysis than those maintained at 21 °C or 27 °C and these trends were corroborated by results of the QPCR assay. Interestingly, the difference in QPX prevalence between the 21 °C and 27 °C groups was less dramatic. Temperature is well known to influence clam physiology (Grizzle, 2001) as well as the development of infectious diseases in other bivalve species (Carnegie et al., 2008; Chu and La Peyre, 1993; Ford and Haskin, 1982; Paillard et al., 2004) although the modulatory mechanisms are not always clear. Higher QPX disease prevalence and intensity among clams maintained at low temperature (13 °C) could result from better performance of the parasite at this temperature, an immunodepression of clams under this condition, or both. Our previous *in vitro* investigations demon-

Fig. 5. QPX prevalence expressed as mean and standard error (2 tanks/treatment and 5 clams sampled from each tank at each sampling time) determined by quantitative PCR in (A) experimentally (FL-q) and (B) naturally (MA) infected clams maintained at 13 °C, 21 °C and 27 °C. Different letters (a and b) designate significant differences between treatments ($p < 0.05$, G-test with Gabriel's procedure).

strated that QPX grows optimally between 20 and 23 °C (Perrigault et al., 2010), and growth is noticeably reduced below this range reaching about 60% of maximal growth at 13 °C. Despite the apparent suboptimal condition for the parasite at 13 °C, disease development was higher in hard clams maintained at this temperature as compared to those maintained at 21 °C and 27 °C. Interestingly, previous investigations suggested 20–24 °C to represent the optimal range for hard clam while 13 °C is noticeably suboptimal. For example, pumping rates in *M. mercenaria* increase at a greater rate when temperatures are 20 °C and above, and rates are maximal at 24–26 °C and start to decline rapidly at temperatures above 27 °C (Hamwi, 1969). Clearance rate and oxygen consumption in hard clams also increase with increasing temperature although oxygen consumption rates increase faster at temperatures over 20 °C and surpass clearance rates at 25 °C and above (Hibbert, 1977). Similarly, shell growth is greatest between 20 and 24 °C (Ansell, 1968). Viewed collectively, it appears that the exposure to 21 °C in the current study was the most optimal temperature for clam activity among the three tested temperatures. Alternatively, 13 °C represented a suboptimal temperature and was stressful as witnessed by alterations of hemolymph parameters described in the companion paper (Perrigault et al., 2011). Likewise, the 27 °C treatment appears to be near a physiological tolerance limit and might become stressful for clams over long periods of time. As a matter of fact, stress and exhaustion may have been at the origin of clam mortalities observed during the 5-month extension in Florida clams (both challenged and controls) initially maintained at 27 °C. On the other hand, Perrigault et al., (2011) demonstrated significant immunodepression in FL-c clams maintained at 13 °C for 2 and 4 months compared to those held at 21 °C and 27 °C (same clams used in the current study). The same study also showed significant alteration of clam immune response to QPX among naturally and experimentally infected clams maintained at the lower temperature. Conversely, low QPX prevalence and intensity and high levels of healing were noted in clams submitted to 21 °C and 27 °C. Failure of QPX to induce infection in clams maintained at 27 °C is not surprising since this temperature is detrimental to QPX *in vitro* (Perrigault et al., 2010). More interesting is the failure of QPX to induce infections among clams maintained at 21 °C. This finding is particularly relevant to our parallel study showing enhancement of immune response against QPX among naturally and experimentally infected clams maintained at 21 °C (Perrigault et al., 2011). Therefore, it appears that low QPX prevalence in clams maintained at 21 °C is a result of an effective immune response in clams to the presence of QPX, while the inability of QPX to establish infection in clams maintained at 27 °C derives from the deleterious effect of high temperature on the parasite itself, independent of clam immune response which was minimal among QPX-challenged clams held at this highest temperature (Perrigault et al., 2011).

Determination of QPX prevalence in clams by histological or QPCR methods exhibited similar trends, highlighting the comparability of the two methods. Prevalence as well as number of QPX cells present in clam tissues determined by QPCR were higher than those obtained from histological analysis. These findings are not surprising considering the greater degree of sensitivity of our QPCR over standard histological procedures (Liu et al., 2009). Interestingly, differences in disease prevalence determined by the two techniques were more pronounced at 21 °C and 27 °C than at 13 °C, which can be related to the disease status of the tested clams. For instance, histological observations only reported active QPX lesions containing live parasite cells, which were significantly more abundant in clams submitted to 13 °C, whereas healing processes were more common among clams maintained at higher temperatures. Determination of QPX prevalence and intensity by molecular tools cannot discriminate between active and "inactive"

QPX lesions. This limitation has been discussed for other PCR-based methods (Burreson, 2008). It is likely that the higher QPX prevalence detected by QPCR in clams submitted to 21 °C and 27 °C resulted from the presence of very few isolated QPX cells or dying and recently dead parasites. Fewer isolated parasite cells may be missed by histology. When only dead and degrading QPX cells were histologically observed, they were ascribed to the healing process and were not included as positive for active infection.

Differences in disease dynamics were noted between the two clam populations and were likely attributable to the stage of infection at the start of the trials. The naturally-infected clams had well-established infections at the start of the experiment and were therefore more likely to achieve advanced disease stages within the initial 4 months, which could explain the observations of higher mortality and greater disease prevalence and intensity among moribund clams. As a matter of fact, while QPX prevalence in FL-q clams tended to be constant (with intensity increasing over time) during the initial 4-month exposure to different temperatures, prevalence dropped in MA clams in all treatments over time as a result, at least in part, of the death of the most severely infected individuals. Also, greater numbers of moribund clams were collected within the first 4 months in naturally-infected clams whereas mortality associated with QPX occurred mostly during the extension period in experimentally infected clams.

Transfer of all clams to 21 °C for 5 additional months resulted in a noticeable decrease of QPX prevalence in all treatments, particularly in those initially held at 13 °C, confirming the ability of clams to mount an effective response against the infection under favorable temperature conditions (21 °C). This observation is important since it demonstrates the dynamic impact of temperature on QPX disease, favoring disease development at lower temperatures but supporting elimination of the parasite and clam healing at higher temperatures. Clearly, temperature affected both QPX establishment and disease development in clam tissues and also the ability of clams to mount an effective immune response against QPX (Perrigault et al., 2011).

Previous field reports have found the highest disease prevalence and mortality during summer months or early fall. A seasonal survey of hard clams in Atlantic Canada found the highest QPX prevalence in August samples (MacCallum and McGladdery, 2000). Similarly, multi-year monitoring of clams in New York waters showed that QPX prevalence generally peaks during summer months (Liu et al., 2008, Allam, unpublished). The highest QPX associated mortalities also occurred during the late summer and early fall in Massachusetts (Smolowitz et al., 1998) while the first major mortality event took place in New York during July (Dove et al., 2004). The apparent discrepancy between field observations and our lab experiments (of higher disease development at 13 °C as compared to warmer summer-like temperatures) could be explained by two main factors. First, QPX disease is a relatively slow and chronic infection and results from the current study and prior investigations show that several months are needed for the parasite to establish infections and progress to overt disease (Dahl and Allam, 2007; Dahl et al., 2008). Similar findings were also made in oysters infected with the mesophilic alveolate *Perkinsus marinus* by Ford and Smolowitz (2007), who demonstrated a lag of more than 3 months between optimal water temperature and maximal disease prevalence. In addition, mortality is an end point for the disease process and the observed mortality peak in summer implies that infection had to be established earlier in the year. Interestingly, there was a noticeable pulse in mortality levels associated with QPX disease after naturally and experimentally infected clams were moved from 13 to 21 °C suggesting that the temperature change was detrimental to heavily infected clams. This is not surprising because severely infected clams are less able to cope with enhanced metabolic demands under higher tempera-

tures, leading to exhaustion of the host and high mortality. Increasing metabolic demands during summer have been recognized as an aggravating factor for infectious diseases in several marine mollusks, including abalone (Travers et al., 2008) and oysters (Li et al., 2009; Samain et al., 2007; Sauvage et al., 2009). Overall, a summer pulse in QPX-associated mortality may reflect processes underway in the field during both spring and summer, with major disease development during mid to late spring (with water temperatures ~13 °C) and maximal mortality at higher temperatures when metabolic demands on clams increase. Alternatively, previous studies have highlighted intraspecific variations in QPX resistance among different clams from the same broodstock (Perrigault et al., 2009). Increasing temperature during summer time can be beneficial to lightly infected and/or more resistant clams that are still capable of mounting a response against the infection, leading to remission as observed here during the 5-month extension in challenged FL clams initially maintained at 13 °C where mortality alone cannot explain the significant drop in disease prevalence. A survey conducted in Virginia, which is the most southern extent (and warmest area) of the known range of QPX disease in clams, found the highest QPX prevalence and intensities in May and November associated with active division stages of the parasite within tissues, but not during the hot summer months (Ragone Calvo et al., 1998).

In conclusion, this study demonstrated a strong effect of temperature on QPX disease development and resulting mortalities. The results showed highest disease development at 13 °C as compared to 21 °C or 27 °C, confirming QPX as a "cold-water" infection. These findings have important implications for the management of aquacultured and wild clams, as well as for the adjustment of monitoring efforts. For example, the timing of large scale clam movements (as in transplant operations or seed deployment) can be selected to reduce the risk of disease outbreaks. Because some clams have the ability to heal under optimal temperature conditions, a potential disease mitigation strategy for clam stocks sustaining relatively low QPX levels is transfer of clams to shallow warmer embayments (within the same geographic area to avoid parasite spread), with generally higher temperatures to foster remission. The ambient water temperature can bolster immune response capacity but caution is still warranted against use of hard clam stocks with high susceptibility in enzootic areas, as they can acquire infections even during the warmest times of the year (Dahl et al., 2010), confirming the suggestion that QPX disease results from "unfavorable genotype–environment interactions" made by Ford et al. (2002). Finally, our findings of better resistance of clams toward the infection at high temperature provide a small consolation for the devastating impact of climate change in the decades to come, since QPX disease outbreaks may become less frequent in the most southern areas currently impacted by the disease as ocean temperature continues to increase.

Acknowledgments

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