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

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Abstract

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

Keywords: Hard clam; QPX; Heat shock; Immunity; Stress; Air exposure

1. Introduction

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

Previous studies reported that the ability of clams to resist QPX infection is largely influenced by environmental factors, such as temperature and salinity, which significantly alter the host-parasite interactions by affecting the host immune performance and the fitness of the parasite (Perrigault et al., 2010; Perrigault et al., 2011; Wang et al., 2016b). Among all the investigated environmental factors, temperature seems to play a predominant role in regulating the development of QPX disease (Dahl et al., 2011). In the field, QPX disease has never been detected in clams south of Virginia where water temperature is comparatively warmer despite the fact that clam broodstocks originating from southern states (Florida and South Carolina) are more susceptible to QPX than northern stocks (Calvo et al., 2007; Dahl et al., 2008; Dahl et al., 2010; Ford et al., 2002). In addition, results of previous laboratory investigations showed that naturally-infected clams exposed to 13 °C exhibit significantly higher disease-related mortality than their counterparts exposed to 21 or 27 °C, whereas the clams submitted to the latter two conditions displayed signs of healing and recovery from QPX infection (Dahl and Allam, 2007;

Dahl et al., 2011; Perrigault et al., 2011). Further, this healing process was associated with efficient defense response in clams maintained at or above 21 °C featured by significant increase in hemocyte resistance to the cytotoxicity of QPX extracellular products and induction of higher anti-QPX activity in plasma as compared to clams held at 13 °C (Perrigault et al., 2011). Finally, *in vitro* studies showed significant decrease in QPX growth and survival at temperatures exceeding 23 ºC (Perrigault et al., 2010). The ensemble of these observations underlines a major effect of temperature on disease dynamics and supports the categorization of QPX infection as a "cold water disease" (Perrigault et al., 2011).

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

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

(2007) also demonstrated that a non-lethal heat shock significantly increased the expression of HSP 70, leading to higher survival in shrimp larvae subsequently challenged with pathogenic bacteria. The mechanism of HSPs induction leading to improved resistance against infection is not thoroughly understood, although it has been demonstrated that HSPs contribute to the host immune response, serving as signaling molecules that initiate the inflammatory cascade or binding and forming complexes with non-self proteins to enhance the recognition and opsonization of foreign entities (Roberts et al., 2010).

The main objective of this study is to evaluate the effect of "heat shock" treatments (acute short-term exposure to high temperature) on the dynamic of pre-established QPX infections. The design of this experiment is based on previous findings that high temperature reduces the establishment of QPX infection and promotes the host-healing process. We hypothesized that heat shock treatments would have the potential to limit the proliferation of the parasite and stimulate the immunity of the host allowing for better resistance and recovery of infected clams. The ultimate aim of this work is to evaluate and develop potential field-applicable strategies for QPX disease control and reduce the impact of this disease on the clamming industry.

2. Materials and Methods

2.1 Hard clams

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

2.2 Heat shock treatments

Following the 2-week acclimation, clams were randomly assigned to one of 10 treatments (Table 1). The combination of different exposure times and temperatures employed during the treatments was intended to help determine the minimal exposure temperature and duration needed to significantly reduce QPX infection and enhance the host healing process. During the treatments, clams were taken out of the water and were maintained either in incubators (21, 27, 32 and 37 °C) or at room temperature (18 °C) to achieve the targeted temperatures. For the accuracy of temperature measurement during the heat shock treatment, the internal temperatures of clams (the actual temperature of clam meat inside the shells) were measured and recorded using hypodermic thermocouple probes (HYP-2 probes connected with HH147U electronic data loggers (Omega Engineering, Stamford, Connecticut, USA) that were carefully inserted inside a

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

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

2.3 Anti-QPX activity

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

187 *2.4 Total RNA isolation and cDNA synthesis*

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

199 *2.5 Real-time PCR analysis of relative expression of stress-related genes*

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

217 *2.6 QPX diagnosis*

218 QPX disease status of individual clams was determined in mantle tissues using the standard
219 qPCR diagnosis protocol (Liu et al., 2009). Changes in QPX prevalence and intensity following
220 heat shock treatments were compared to evaluate the effect of each treatment on QPX infection
221 and disease development. QPX prevalence was calculated as the percentage of QPX positive
222 clams in all sampled clams in each treatment and the QPX intensity was assessed as the number

of QPX cells per gram clam tissue and was categorized into the following ranks: light (≤ 10 QPX cells per gram of clam tissue), moderate (11-100 QPX cells), heavy (101-1,000 QPX cells), or severe (> 1,000 QPX cells).

2.7 Statistics

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

3. Results

3.1 Disease prevalence

In general, heat shock treatments with different temperatures and exposure times resulted in various impacts on QPX prevalence. Compared to the initial QPX prevalence (36.6%) at the beginning of the experiment, disease prevalence remained relatively unchanged in untreated control clams kept in seawater at 18ºC (36%) while a general decrease in disease prevalence was found in most of the heat shock treatments, except in the 27 °C 8h and 32°C 4h treatments, where disease prevalence showed a slight yet non-significant increase to 38.1% and 40.0%, respectively (Fig. 1A). The only treatment that displayed a significant decrease in disease prevalence was the 27°C 2h treatment (Binomial exact test, $P<0.01$), which exhibited 10% prevalence after the 10-week recovery (a 72% reduction as compared to untreated controls). Comparatively, a large disease reduction was also seen in the 32°C 2h treatment (23% prevalence or 36% reduction), although this change was not statistically significant. Among clams subjected to the same temperature, various exposure durations resulted in different recovery performances. For instance, in clams treated with 27 °C, the most effective heat shock exposure time was 2 hours and extending the exposure time to 4, 8 and 18 h did not further decrease the disease prevalence but had no or even adverse effects that resulted in a slight increase in prevalence. Similarly, in the 32 °C treatments, the short exposure (2 h) also led to more noticeable disease

reduction than its longer duration counterpart (4 h). The 2-hour treatment at 37 °C seems to be the least effective in terms of disease reduction among the three 2-hour treatments (27, 32, 37 °C) where the higher the treating temperature, the less disease mitigation effect resulted. On the other hand, for the clams subjected to 21°C treatments, longer exposure time (18 h, 27% prevalence) seems to be more effective in decreasing QPX infection than the shorter one (8 h, 35% prevalence). Compared to the untreated control group, the 18 h of air exposure at 18 °C resulted in very minor change in QPX prevalence.

3.2 Disease intensity

QPX disease intensity, expressed as average parasite cell counts per gram of clam tissues in each treatment, is shown in Fig. 1A. In general, changes in disease intensity 10 weeks after heat shock treatments followed similar trends as disease prevalence with the lowest QPX infection intensity found in the 27 °C for 2h treatment however the difference was not statistically significant and therefore no clear trend can be drawn. For other treatments at 27 °C but with longer duration, the QPX intensity tends to increase with the exposure time. The highest QPX intensity among all treatments was found in the 27 °C 18h treatment, suggesting an adverse effect of excess heat exposure in air on disease control efficiency. Among the two 32 °C treatments, a 2 hours exposure resulted in slightly lower QPX cell counts as compared to the 4 hours treatment, in agreement with the prevalence data. Disease intensity was also relatively low in the 37 °C 2h treatment, but noticeably high clam mortality was observed in this group (discussed below; Fig. 1B).

3.3 Mortality

Throughout the 10-week period, dead and moribund clams were collected and analyzed for disease status by qPCR (Fig. 1B). Mortality was generally low in most treatments (2 to 4 clams or 5 to 10% mortality) and tended to increase with higher temperatures and/or extended exposure times, reaching 50% (20 dead clams) in the 37ºC 2h treatment. Diagnostic results indicated that not all cases of mortality were associated with QPX infection, suggesting that some mortality may have resulted from stressful experimental conditions. Overall, all dead clams from the low temperature or short exposure time treatments displayed moderate to severe QPX infections. This was the case for both of the 18 °C treatments, for 21 °C 8 h treatment, as well as the 2-hour exposures at 27 °C and 32 °C. On the other hand, an increasing proportion of uninfected clam was detected among dead and moribund clams from treatments using high temperatures or longer exposure times or a combination of both. For example, higher percentage of uninfected clams was found among dead clams from the 27 °C 18 h treatment as compared to the 4 and 8h treatments at the same temperature, and the percentage increased at higher temperature (32 °C 4h vs. 27 °C 4h). At 37 °C, half of the dead clams were negative for QPX, and light infections dominated the positive clams.

3.4 Expression of stress-related genes

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

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

The expression of the antioxidant GPx seems to be generally associated with the duration of air exposure rather than the temperature level of each treatment. For instance, the expression of GPx was significantly up regulated at Day 1 in clams submitted to an air exposure for 18 h at a temperature (18ºC) as compared to controls which remained submerged in seawater (Fig. 2C). Shorter exposure times (2 h, 4 h and 8 h) did not trigger substantial modulation of GPx in most of the temperature treatments (except the most extreme high temperature treatment 37 °C 2h). However, as the exposure time extended, GPx expression considerably increased. Higher expression of GPx was also associated with higher temperatures in clams submitted to the same long-term exposure (18 h). The expression of GPx dropped to normal levels after 7 days of recovery in treatments using temperatures at or below 27 °C, however, the GPx remained

significantly highly expressed in the 32 °C 2 h and 37 °C 2h treatments.

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

Discriminant analysis (DA) using expression levels of all tested stress-related genes was performed to provide an integrative assessment of the stress response following each heat shock treatment (Fig. 3). Results show significant impact of heat shock treatments on overall stress, which varied with treatment conditions and post treatment recovery time. At day 1 (Fig. 3A), treatment effect on the expression of stress genes was clearly discriminated on function 1 (91.3% total variance explained, Eigenvalue = 10.874, Wilks Lambda = 0.036, $p < 0.001$), with the most pronounced separation found in treatments with most extreme conditions. For example, centroid of 37 °C 2h treatment was remarkably separated from all other treatments and positioned furthest from the control centroid on the DA scatter plot. Centroids of the 32 °C treatments (2h and 4h) also exhibited marked separation on function 1, however with less distance from controls as compared to the 37 °C treatment. The treatment of 27 °C 18h had its group centroid modestly yet clearly separated, whereas the control and remaining treatments were tightly clustered together. On the other hand, group centroids were less separated at day 7 (Fig. 3B) as compared to day 1 by the discriminant functions (function 1 explained 77.5 % total variance, Eigenvalue = 0.997, Wilks Lambda = 0.383, $p < 0.001$). Only the 37 °C treatment was noticeably separated, and the 2 32 °C treatments were only slightly divergent from the cluster formed by the control and all other treatments. This shift of patterns indicated a dissipation of stress from day 1 to day 7 for most treatments (excluding the most extreme temperature) due to recovery.

3.5 Anti-QPX activity

The anti-QPX activity (AQA) of clam plasma after 1 and 7 days post heat shock are shown in Fig. 4. The data are expressed as percentage of suppression of QPX growth in tested plasma as compared to control cultures (plasma substituted with QPX growth in FSW). In general, the AQA across all experimental treatments at Day 1 (Fig. 4A) were comparable or slightly lower than that of the control treatment clams, suggesting a decrease in plasma ability to neutralize the parasite caused by possible stress in clams exposed to high temperature and/or hypoxia due to air exposure. At Day 7 (Fig. 4B), the AQA level generally recovered in most of the heat shock

369 treatment groups to a level equaling to or slightly higher than the control, whereas the AQA of
370 controls remained almost unchanged as compared to Day 1. However, no significant difference
371 in AQA was found between experimental groups and control clams. Interestingly, the highest
372 AQA was found in the 27 °C 2 and 4 h treatments, which was respectively 9% and 12% higher
373 than that measured in controls, corresponding well with the lower QPX prevalence observed in
374 the first and with lower disease intensity in both treatments (Fig. 1). Among treatments at each
375 temperature level, longer exposure time led to a slight decrease in AQA, even though the
376 difference was not significant.

377

378 **4. Discussion**

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

390 In this context, exposing infected clams to thermal stress might have resulted in enhanced
391 resistance or protection against QPX. Observations of cross-tolerance have been described in
392 many aquatic organisms, including fish, crustaceans and bivalves. For example, heat exposure
393 was able to increase the resistance of flounder cells against exposure to toxic chemicals (Brown
394 et al., 1992). Similarly, thermal shocks (15 min at 26 °C) conferred protection that allowed for
395 higher survival against subsequent osmotic shocks in salmon smolts (DuBeau et al., 1998). Heat
396 shocks followed by 4 to 48 h recovery enhanced the capability of tide pool sculpin to cope with
397 both osmotic and hypoxic stress and significantly increased their survival rate (Todgham et al.,
398 2005). In crustaceans, it has been reported that a sub-lethal heat shock at 40 °C for 1 h provided
399 brine shrimp larvae with higher thermotolerance for extended heat exposures the following days
400 (Miller and McLennan, 1988). Thermal stress also enhanced the resistance of brine shrimp larvae
401 to *Vibrio campbellii* and *Vibrio proteolyticus* infections, significantly increasing their survival in
402 the presence of pathogenic bacteria as compared to non-stressed animals (Sung et al., 2008; Yik
403 Sung et al., 2007). Pacific oysters submitted to 1 h thermal stress at 37 °C acquired
404 thermotolerance to survive a subsequent lethal high temperature exposure at 44 °C (Clegg et al.,
405 1998). Similarly, exposure to a 3 h sublethal heat shock conferred tolerance to subsequent lethal

406 heat treatment (35°C) in juvenile northern bay scallops, and this thermotolerance persisted for at
407 least 7 days (Brun et al., 2009).

408 It is widely recognized that HSPs overproduction in response to physiological perturbations
409 during thermal stress is critical for the acquired cross-tolerance against other environmental and
410 biotic stressors in aquatic organisms (Aleng et al., 2015; Rahman et al., 2004; Sun et al., 2002;
411 Todgham et al., 2005; Zugel and Kaufmann, 1999). For example, the accretion of HSP 70 after
412 short-term hyperthermic stress correlates with the attenuation of gill-associated virus (GAV)
413 replication in the black tiger prawn (de la Vega et al., 2006). Similarly, the enhanced resistance of
414 gnotobiotic brine shrimp larvae to *V. campbellii* and *V. proteolyticus* following thermal stress
415 (discussed above) was associated with HSP 70 accumulation (Sung et al., 2008; Sung et al.,
416 2007). Moreover, reduced mortality and lower bacterial loads after *V. campbellii* challenge were
417 only observed among shrimp larvae with enriched HSP 70 levels (Sung et al., 2008). In addition,
418 non-lethal heat shock induced HSP 70 in the Asian green mussel *Perna viridis* and promoted
419 thermotolerance and resistance against *Vibrio alginolyticus* (Aleng et al., 2015). Although the
420 exact mechanisms behind the cross-tolerance between heat shock and pathogen resistance have
421 not been described, several possible explanations were proposed. High HSP production
422 (particularly HSP 70) as a result of non-lethal thermal stress may stabilize cells against injury
423 due to pathogen infestation, promote the proper folding of host immune proteins, re-fold proteins
424 damaged by pathogens and stimulate the innate immune response (Sung, 2011). Heat shock may
425 also induce the expression of a collection of immune-related genes resulting in the activation of
426 immune pathways. For example, the prophenoloxidase cascade system was shown to be
427 stimulated by heat shock in the shrimp *Litopenaeus vannamei* leading to an increase in host cell
428 adhesion, encapsulation and phagocytosis of invading microbes (Loc et al., 2013; Pan et al.,
429 2008). In fact, boosting host HSP levels has been increasingly used to enhance disease resistance
430 in many aquacultured species (Roberts et al., 2010; Sung, 2014). Even though most of these
431 studies have established a relationship between disease resistance and HSP overproduction at the
432 protein level, recent studies have also linked HSP genes expression to enhanced immunity in
433 marine invertebrates (Cellura et al., 2006; Qian et al., 2012; Rungrassamee et al., 2010; Zhou et
434 al., 2010). In our study, significant increase of HSP 70 and HSP 25 gene expression was induced
435 in clams subjected to heat shock treatment (Fig. 2A and 2B), supporting our speculation that the
436 up-regulation of HSP genes may contribute to an increase in host resistance and/or remission
437 from QPX disease.

438 While well-dosed heat shock presents great potential to enhance host resistance against
439 infections, extreme heat exposure results in adverse effects due to overwhelming stress. Severe
440 hyperthermic stress is known to disrupt the normal physiological processes, decrease growth, and
441 weaken the immune response, sometimes even leading to mortality under severe conditions
442 (Roberts et al., 2010). This could be the case in clams submitted to 37 °C where high mortality

(50%) was recorded among uninfected or lightly infected clams, suggesting that the heat stress itself was extreme enough to induce mortality. Moreover, clam exposure to 32 and 37 °C for 2h did not reduce QPX disease as much as 27 °C. In addition, prolonged exposure periods at the same heat shock temperatures also seemed to undermine the effectiveness of the cross-tolerance against QPX. Extended air exposure could result in oxidative stress which can significantly constrain the innate immune responses by reducing the activity of immune cells and suppressing or delaying the expression of important immune-related genes, thus increasing the susceptibility to infectious diseases (Chen et al., 2007a; Chen et al., 2007b; Cheng et al., 2004; Pampanin et al., 2002). High temperatures may further aggravate the stress caused by the lack of oxygen thus attenuating the benefits of the heat treatment on QPX disease. In fact, the impact of combined thermal and air exposure stress is well reflected by the expression of the stress-related genes GPx and USP (Fig. 2 C and D). The expression of GPx peaked in treatments with the longest (18 h) air exposures and or the highest temperature (37 °C), and the overall induction of USP at Day 1 dissipated at Day 7 in most treatments except the 18 °C 18 h air exposure and 37 °C 2h heat shock. Air exposure is often associated with the generation of large amounts of reactive oxygen species (ROS) in bivalves (Pampanin et al., 2002) and the afterward re-oxygenation during recovery is known to cause excessive oxidants production leading to oxidative stress (Matozzo et al., 2005; Pampanin et al., 2002; Santovito et al., 2005). This oxidative stress is known to be exacerbated by high temperature (Chen et al., 2007b). GPx is considered one of most readily mobilizable antioxidants that protect cells by buffering against a sudden increase in the generation of radical oxygen species (Santovito et al., 2005), so the significant induction of GPx expression indicated oxidative stress associated with extended (18 h) air exposure or extreme heat (37 °C, Fig. 2C). On the other hand, USP is a member of a group of proteins that respond to a variety of stressors, including heat, starvation, infections and oxidative stress (Kvint et al., 2003). The expression patterns of USP in response to the heat shock treatment could be linked to the compounded stress deriving from both hyperthermia and air exposure stress (Fig. 2D).

Members of the HSP family are widely used as indicators for thermal stress. The temperature change and heat exposure time required to induce heat shock and modulate HSP synthesis are known to be affected by the acclimation temperature, the heat tolerance of the organisms and the environmental conditions under which the organisms normally grow (Sung, 2014). Thermal shock at 32 °C or above was shown to effectively induce HSP 70 production in several oyster species that were previously acclimated at 12 to 18 °C (Clegg et al., 1998; Encomio and Chu, 2005). For example, in the European flat oyster, heat exposure stimulates HSP 70 synthesis with maximum levels observed in the gills between 2-3 h of post-stress recovery at 18 °C (Piano et al., 2005; Piano et al., 2004). A sub-lethal heat shock for 3 h stimulates HSP 70 and HSP 40 production in bay scallops, with the HSP 40 response being less vigorous and decreasing to pre-stress values by 8 days, whereas HSP 70 was maintained for 14

480 days (Brun et al., 2009). In our study, clams responded promptly to temperature elevation
481 resulting in significant up-regulation of HPS 70 and HSP 25 gene expression at or above 27 °C.
482 However, HSP gene expression levels decreased to baseline values after 7 days of recovery at 18
483 °C. This could reflect that the normal cell activity was gradually restored and suggests that the
484 overproduction of HSP 70 proteins, which is energetically costly (Hoekstra and Montooth, 2013;
485 Krebs and Loeschcke, 1994), is not required anymore to provide protection. This HSP 70
486 regulatory pattern is in agreement with the remarkable ability of hard clams to tolerate a wide
487 range of environmental conditions (Grizzle et al., 2001), in particular a wide thermal range. For
488 instance, hard clams populate both intertidal and subtidal habitats from Canada to Florida. The
489 reported temperature tolerance range for the species is 1 to 34 °C with the optimal range from 16
490 to 27 °C (Malouf and Bricelj, 1989). In addition, hard clams have greater tolerance to low
491 dissolved oxygen (DO) as compared to other bivalves, as they fare well with DO level as low as
492 0.9 mg/L at 16 to 19 °C (Malouf and Bricelj, 1989). The timely modulation of stress-related
493 proteins, especially HSP, may comprise a significant part of the mechanisms that allow hard
494 clams to successfully counterbalance detrimental stimuli and gain adaptability to a variety of
495 environmental conditions.

496 It is not too surprising that the best reduction in QPX was observed in the clams from the 27
497 °C 2h treatment, a condition that appears much milder than what have been reported to induce
498 cross-protection in other aquatic animals. This temperature (27 °C) is the upper limit of the
499 optimum range for hard clams and different levels of physiological impairments result above this
500 limit (Malouf and Bricelj, 1989). For example, hard clams cease pumping and feeding at
501 temperatures above 31 °C (Malouf and Bricelj, 1989). Clams used in this study were grown in
502 Massachusetts where the yearly water temperature generally fluctuates between 2 and 23°C, and
503 the QPX infected clams were collected from the field during winter (4 to 5ºC) and were
504 acclimated for 2 weeks at 18 °C after collection. This initial acclimation may have primed the
505 heat-shock response in clams. Therefore, the subsequent exposure to 27 °C represented an
506 additional temperature rise of 10 °C, which appears adequate to stimulate significant up-
507 regulation of HSP 70 and other stress response genes. Although certain thermotolerance could be
508 attained progressively with prolonged heat shock time (as reflected by gene expression of HSP
509 70), the compounded stress resulting from both heat and air exposure during the thermal
510 incubation may have induced synergistic effects between both stressors that escalated the overall
511 stress to higher levels. The overall stress levels were comprehensively reflected by the DA
512 scatter plot (Fig. 3) which highlighted increased level of stress as a synergistic result from both
513 heat level and air exposure time. Severe stress may compromise the possible beneficial effect of
514 mild heat shock treatments on disease recovery. From an energy expenditure perspective, stress

response may undermine immune competency in clams exposed to very high temperatures, in agreement with the energy trade-off concept described in the stress model developed by Moberg (2000). Under stressful conditions, animals must coordinate their competing energy demands for combating stress and maintaining other functions. In this context, response to mild stress requires little energy that can be easily met by reserves, resulting in minimal impact on other physiological processes. However, increasing stress severity and/or duration requires higher energy demands that are hardly met by reserves alone. Under this situation, extra resources must be allocated to stress response causing a reduction in energy available to other biological processes such as growth, reproduction and immunity, ultimately increasing the chances of infection and mortality (Segerstrom, 2007). In the current study, a heat shock treatment at 27 °C for 2 h appears to provide adequate induction of stress-related proteins to shield off clams from damage, without causing overwhelming stress to impair immune functions, resulting in the most significant cross-protection against QPX disease.

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

It is noteworthy to mention, however, that our evaluation of heat shock effect was mainly focused on clam parameters, whereas its impact on the physiology of QPX cells present in clam tissues was not investigated. It is likely that our heat shock treatments also caused stress in QPX cells since the optimum temperature for QPX proliferation is between 20 and 23 °C and higher temperatures reduce the viability and growth of parasite cells *in vitro* (Perrigault et al., 2010). On

551 the other hand, it is also possible that the increasing levels (intensity and/or duration) of thermal
552 exposure resulted in a differentially stressful condition to both the host and the parasite, possibly
553 providing an advantageous opportunity for QPX to thrive. Such a scenario supports our
554 observations of limited reduction of QPX prevalence in clams submitted to 32 and 37 °C for 2 h
555 as compared to the 27 °C treatments.

556 Overall, findings from our current study could have implications for the improvement of
557 aquaculture operations and QPX disease management in hard clams. The development of non-
558 traumatic methods for enhancing disease resistance in aquaculture has been increasingly focused
559 on boosting HSP levels in economically-important crops. Methods that have been suggested to
560 increase HSPs levels in fish and shrimp include heat exposure, exogenous HSPs supplement, and
561 oral or water administration of HSP stimulants, as reviewed in Sung (2014) and Roberts et al.
562 (2010). Given the fact that QPX disease development is largely suppressed by warm
563 temperatures (Dahl et al., 2011; Perrigault et al., 2011) and our observation that brief heat shock
564 exposures can potentially reduce the disease, we propose that some easily achievable heat shock
565 procedures could be designed and incorporated into the current QPX disease management
566 practices to enhance clam resistance to the infection, promote the healing process and minimize
567 the risk of loss due to disease outbreaks. Such strategies naturally lend themselves to production
568 practices that involve clam handling, such as the hard clam transplant program run by New York
569 State. This program allows the transport of clams from production areas to depuration sites in
570 non-refrigerated vehicles during summer, where the heat exposure time and temperature
571 conditions (27~32°C for 2~4h) can be readily achieved during transport. More research is needed
572 to further explore these promising strategies and to better understand the mechanisms favoring
573 disease reduction with the aim of developing guidelines for applying the most appropriate heat
574 shock treatments (both in exposure temperatures and periods) as a complementary measure for
575 QPX disease control in hard clams.

576

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Table 1 Experimental design for laboratory heat shock treatments. All exposures were made in air excluding a control treatment where clams remained in seawater at 18 ℃ (not shown). A " X " indicates that this treatment was implemented. Two replicate tanks (20 clams/tank) were made for each treatment.

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

Table 2 Primer sequences for the tested genes

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

Figure legends

Figure 1. QPX disease status in experimental clams. (A) QPX prevalence (bars, left y-axis) and intensity (line, right y-axis) after 10-week incubation at 18 °C following the heat shock treatments. “*” denotes significant reduction in QPX prevalence as compared to untreated controls (Binomial exact test, $P<0.01$). (B) Proportions of QPX infection stages in moribund clams from each treatment collected throughout the 10-week experiment. Numbers of moribund clams (and percent mortality) are indicated along the x-axis.

Figure 2. Expression of stress-related genes in clams sampled 1 and 7 days after heat shock.

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

Figure 3. Discriminant analysis of all stress-related genes expression at day 1 (A) and day 7 (B) after heat shock treatments. Different treatment groups are indicated by different symbols and positions of group centroids for each treatment are indicated by the star symbol.

Figure 4. Anti-QPX activity in plasma expressed as percent growth inhibition rate (mean ± SE) from clams sampled at Day 1 (A) and Day 7 (B). Different letters indicate significant difference across treatments (ANOVA, $P < 0.05$).

Disease reduction effects