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

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ABSTRACT

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

1. Introduction

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

Bivalves are characterized by a non-specific defense system based on both cellular (phagocytosis, respiratory burst, etc.) and humoral (lectins, lysosomal enzymes, antimicrobial factors, etc.) activities. Since bivalves have an open circulatory system, antimicrobial constituents in plasma and hemocytes are present in all tissues. Prior studies investigated the effects of bivalve plasma on pathogens (Anderson and Beaven, 2001a,b). Gauthier and Vasta (2002) observed an inhibition of *Perkinsus marinus*, a pathogen of the eastern oyster (*Crassostrea virginica*), in the presence of plasma from uninfected oysters. This inhibition increased when plasma from *P. marinus*-infected oysters was used. Similarly, Anderson et al. (2003) demonstrated the presence of QPX inhibitory agents in *M. mercenaria* plasma at moderate protein concentrations (10–50 μg ml^{-1}) when mucoid secretions of QPX were removed by washing. Inhibitory activities of the plasma disappeared when unwashed QPX cells were used (Anderson et al., 2003), suggesting protective functions of pathogen secretions. Prior studies also investigated the effects of bivalve tissue extracts on pathogen proliferation. For instance, supplementation of *P. marinus* cultures with oyster tissue homogenates modulated cell proliferation and protease expressions, suggesting the presence of molecular signaling in oysters that regulates *P. marinus* proliferation (Earnhart et al., 2004; Brown et al., 2005). However, few studies focused on the comparison of antimicrobial activities in specific tissues of marine bivalves. Haug et al. (2004) tested antibacterial activities of different tissues (gill, mantle, foot, muscle) including plasma and hemocytes from the horse mussel *Modiolus modiolus* over four

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different bacterial strains. They demonstrated the presence of specific antimicrobial activities in gill, mantle, foot and muscle as compared to plasma and hemocytes. Given the typical localization of QPX infections in *M. mercenaria*, we hypothesized that various clam tissues modulate QPX growth differently. Siphon tissue is of particular interest for these investigations because it has been hypothesized to represent the portal of entry for QPX as the clam filters seawater from its surrounding environment (Lyons et al., 2005).

In this study, the effects of plasma and extracts of various clam tissues on QPX growth were investigated. Plasma from clams originating from New York and Florida were also compared in an attempt to link differences in susceptibility toward the disease *in vivo* (Calvo et al., 2007; Ford et al., 2002; Dahl et al., 2008) to variations in QPX modulating activities *in vitro*.

2. Materials and methods

2.1. Hard clam plasma and tissue homogenates

Naïve *M. mercenaria* originating from New York (NY) were obtained from Frank M. Flowers Oyster Company (Oyster Bay, NY). Clams originating from Florida (FL) were also obtained from a commercial source (Farm Raised Clams, St. James City, FL). Clams from experimental stocks showed no QPX infections. Clams were acclimated at least 1 week in the laboratory, held in 150-L tanks with re-circulating water (28–30 ppt) and fed daily with commercial algae (DT's Live Phytoplankton, Sycamore, IL). Hemolymph samples were individually withdrawn with a 1 ml-syringe from adductor muscle and held on ice. Plasma was recovered by centrifugation (700*g*, 10 min, 4 °C) and the supernatant was sterilized by filtration (0.22 μm pore size). Different clam tissues were dissected and used individually or pooled according to each experiment. Tissues were suspended in filter-sterilized artificial seawater (FASW; 1/10, w/v) and homogenized with a tissue homogenizer (Kinematica AG, Switzerland). Homogenates were centrifuged at 5000*g* for 15 min and the resulting supernatant was sterilized by filtration through 0.22 μm syringe filters. Fifty microliters of filtered plasma and homogenates was allocated to protein analysis using the BCA protein assay kit (Pierce, Rockford, IL, USA).

2.2. QPX

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

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

2.3. Effects of M. mercenaria plasma on QPX growth

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

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

2.4. Effects of clam extracts on QPX growth

Gill, mantle and adductor muscle tissues from six clams (NY) were dissected, separately pooled, suspended in FASW and homogenized as described above. Protein concentrations of the different tissue homogenates were normalized by dilution with FASW to 2000 μg ml^{-1}. Controls were composed of MEM diluted with FASW to 2000 μg protein ml^{-1}. Assays were initiated in 4-ml sterile culture tubes by adding 100 μl of QPX suspension in FASW to 1.5 ml of the different treatments and controls (in triplicate). Tubes were incubated on a rotating shaker at room temperature (21 ± 1 °C) and QPX growth was measured at t_0 and on days 4, 8 and 15 by the FDA technique. A second assay used these same homogenates as a supplement to MEM to account for potential nutrient limitation that may result when tissue homogenates are used alone as a growth medium. Assay tubes containing 750 μl MEM were added with an equal volume of the appropriate tissue homogenate (in triplicate). One control consisted of 1.5 ml MEM and a second control contained 1.5 ml FASW-diluted MEM (vol:vol). The assay was carried out as above, and biovolume was measured at t_0 and on days 3, 8 and 15.

In a second set of experiments, mantle tissues from 12 NY clams were further dissected into more distinct regions: siphon, edge of mantle, and central part of the mantle. This processing was made in light of available information concerning the microscopic detection of QPX in certain areas of the mantle, particularly siphon and the edge of the mantle, and based on structural differences between siphon (mostly muscular), the edge of the mantle (mix of muscular and connective tissues) and the central part of the mantle (mostly connective). Tissue homogenates were divided into three pools (4 clams pool^{-1}) and protein concentrations were normalized with FASW at 2000 μg ml^{-1}. QPX suspension was added to each pool and parasite growth was assessed as described above at t_0 and on days 3, 8 and 15. MEM adjusted with FASW at the same protein concentration (2000 μg ml^{-1}) was used as control.

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

(MEM diluted with FASW to 2000 μg ml^{-1}). QPX growth was measured at t_0 and on days 2, 8 and 15.

2.5. Dose effect of tissues homogenates

Gill, mantle and adductor muscle tissues from five New York clams were pooled and protein concentrations of tissue homogenates were adjusted with FASW to obtain protein ranges between 100 and 2500 μg ml^{-1}. As previously, assays were performed with and without MEM supplementation to ascertain that growth inhibition was not a simple result of trophic limitations of culture media. Briefly, one series was composed of 2 ml of tissue homogenates and another series was performed by adding 1 ml of MEM to 1 ml of each tissue homogenate dilution. Controls were composed of MEM and MEM diluted in FASW (vol:vol) for unsupplemented and MEM-supplemented assays, respectively. The FDA technique was used to measure QPX growth at t_0 and on days 4, 7 and 14.

2.6. Statistical analysis

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

3. Results

3.1. Effects of M. mercenaria plasma on QPX growth

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

Results from the second series of experiments, which used plasma from individual clams normalized to 100 μg protein ml^{-1}, confirmed the findings obtained above. For instance, averaged biovolume data showed that inhibition was significantly higher in plasma from NY clams when compared to FL clams with a reduction of 65% and 26% of parasite growth compared to controls, respectively ($p = 0.006$, Fig. 1). However, results also demonstrated major variations in QPX biovolume using plasma from individual clams within each population (Fig. 1). Some plasma samples efficiently inhibited QPX growth whereas others enhanced parasite proliferation. Fluorescence signals were stronger than the controls (grown in diluted culture media) using plasma samples from 3 FL clams but none from the NY clams.

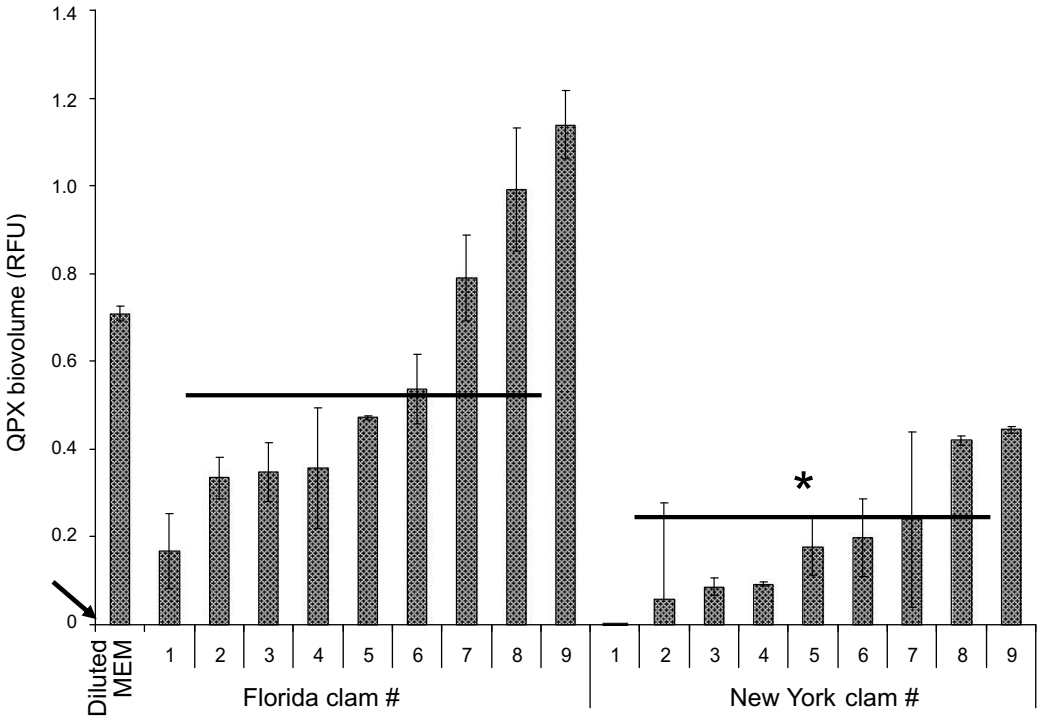

Fig. 1. QPX biovolume (relative fluorescent units) after 4 days in cultures made with plasma from nine clams belonging to two populations of *M. mercenaria*. Protein concentrations were adjusted to 100 μg ml^{-1} in FASW and supplemented to the culture medium (vol:vol). Plasma was substituted with FASW in the MEM control (Diluted MEM). The arrow on the *Y* axis represents QPX biovolume added to each culture at t_0. The lines represent average QPX biovolume in the plasma for each clam population. * denotes significant differences between both populations (Student's *t*-test, $p = 0.006$). All data are presented as Means ± SEM (two replicates).

3.2. Effects of clam extracts on QPX growth

Incubation of QPX with *M. mercenaria* tissue homogenates yielded different growth results in various tissues (Fig. 2a). QPX biovolume increased in adductor muscle homogenates and surpassed the biovolume of QPX grown in culture media from Day 4 until the end of the experiment. There was no increase in QPX biovolume in gill or mantle tissue homogentates (Fig. 2a). The second assay, in which QPX cells were incubated in culture media supplemented with clam tissue homogenates, indicated similar trends with a limited increase in QPX biovolume in cultures supplemented with gill and mantle homogenates (Fig. 2b). On the other hand, supplementation of culture media with adductor muscle homogenates resulted in significantly higher QPX biovolumes than in undiluted or diluted MEM (ANOVA, $p = 0.01$).

In the second set of experiments that compared different parts of the mantle, QPX growth was completely inhibited in pools made with homogenates from the central part and the edge of the mantle (data not shown but trends were similar to those reported in Fig. 2a). Two pools made with siphon tissue homogenates were also inhibitory, while the third pool significantly enhanced QPX growth, as compared to control preparations grown in culture media (Fig. 3, ANOVA, $p = 0.001$).

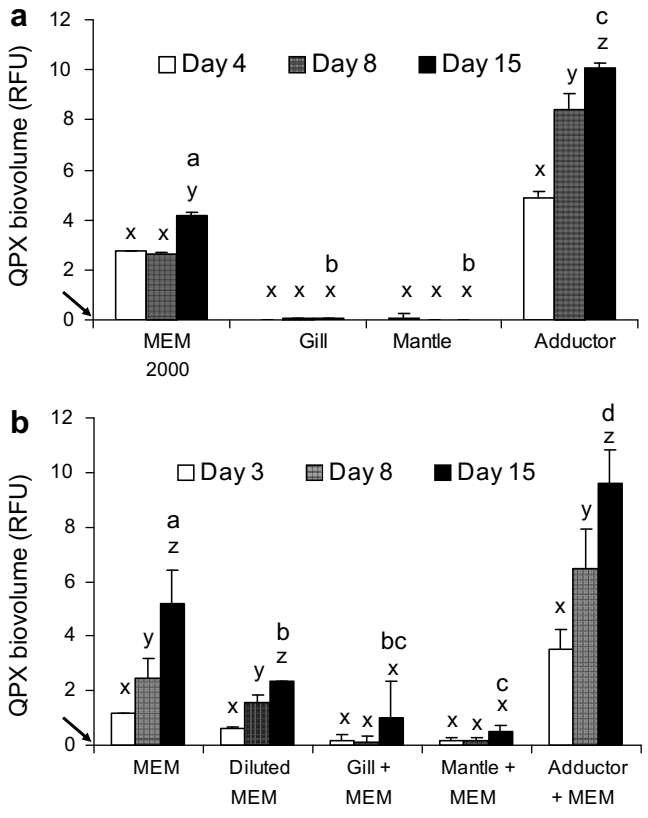

Fig. 2. QPX biovolume (relative fluorescent units, Mean ± SEM, $n = 3$ replicates) in cultures made with different tissue extracts. Extracts were adjusted at 2000 μg protein ml^{-1} in FASW and used as sole source of nutrient [a] or supplemented (vol/vol) to MEM [b]. Controls were composed of culture medium adjusted to 2000 μg protein.ml^{-1} with FASW (MEM 2000), undiluted culture medium (MEM) and diluted (vol:vol) culture medium in FASW (Diluted MEM). The arrows on the *Y* axis represent QPX biovolume added to each culture at t_0. Letters indicate which experimental treatments shared statistically equivalent (same letters) or different (different letters) QPX biovolume means among cultures in different experimental media at Day 15 (a, b, c, d), or among measurements taken at different times within each culture (x, y, z).

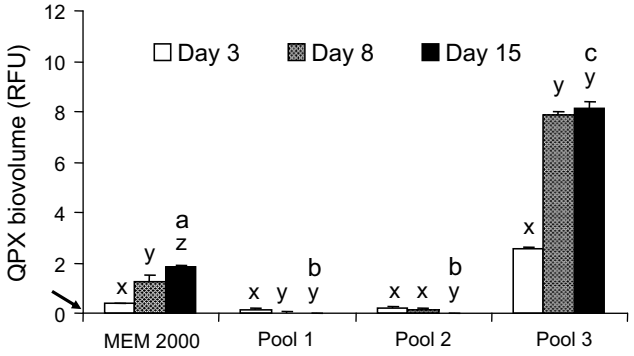

Fig. 3. QPX biovolume (relative fluorescent units, Mean ± SEM, $n = 3$ replicates) in extracts made with three pools of siphon tissues. Extracts and MEM control (MEM 2000) were normalized to 2000 μg protein ml^{-1} with FASW. The arrow on the *Y* axis represents QPX biovolume added to each culture at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

Trends observed among pools of tissue homogenates were confirmed in the third set of experiments that used tissues from individual clams. QPX growth was maximal in homogenates made with foot and adductor muscle tissues, while significant inhibition of QPX growth was noted when homogenates from gill, and central part and edge of mantle were used (Fig. 4a). A repeated measure ANOVA comparing the growth of QPX in homogenates of different tissues collected from the same clam showed the following significant trends: muscle = foot > siphon > edge of mantle > gill = central part of mantle. Interestingly, homogenates made with siphon tissues displayed high inter-individual variability: siphon obtained from one clam displayed significantly higher QPX growth than other siphon homogenate samples (Fig. 4b).

3.3. Dose effect of tissue homogenates

Incubation of QPX cells in serially-diluted muscle homogenates showed a clear dose effect of clam extracts at Day 14 (Fig. 5a and b). For instance, QPX growth was maximal at 1000 μg ml^{-1} of protein using muscle homogenates for both MEM-supplemented and unsupplemented assays (ANOVA, $p = 0.001$). QPX growth was relatively limited when diluted homogenates (100 and 500 μg protein ml^{-1}) were used as sole source of nutrients since parasite biovolumes in these preparations peaked at Day 7 before decreasing at Day 14 (Fig. 5a). The decrease at Day 14 disappeared when muscle homogenates were supplemented to MEM (Fig. 5b). Significant decrease in parasite proliferation at Day 14 was observed at high protein concentrations (2000 and 2500 μg.ml^{-1}) for MEM-supplemented and unsupplemented cultures when compared with values obtained at 1000 μg protein ml^{-1} of muscle homogenates (ANOVA, $p = 0.016$).

Inhibitory effects of gill homogenates on QPX growth were also dose-dependent (Fig. 6). Concentrations of gill homogenates as low as 100 μg protein ml^{-1} were inhibitory for QPX growth when added to MEM (ANOVA, $p = 0.001$), but this inhibition was only temporary when compared to parasite growth in MEM controls (measured 4 and 7 days after incubation). After 14 days, significant increase in parasite biovolume, compared to the control, was observed at protein concentrations of 100 μg ml^{-1} of gill homogenates (ANOVA, $p = 0.001$). QPX inhibition was total at 1000 μg ml^{-1} and 2500 μg protein ml^{-1} over the entire length of the experiment. Dose-dependent inhibitory effects of mantle homogenates (data not shown) were similar to those obtained from gill tissue homogenates. No QPX growth was observed when gill and

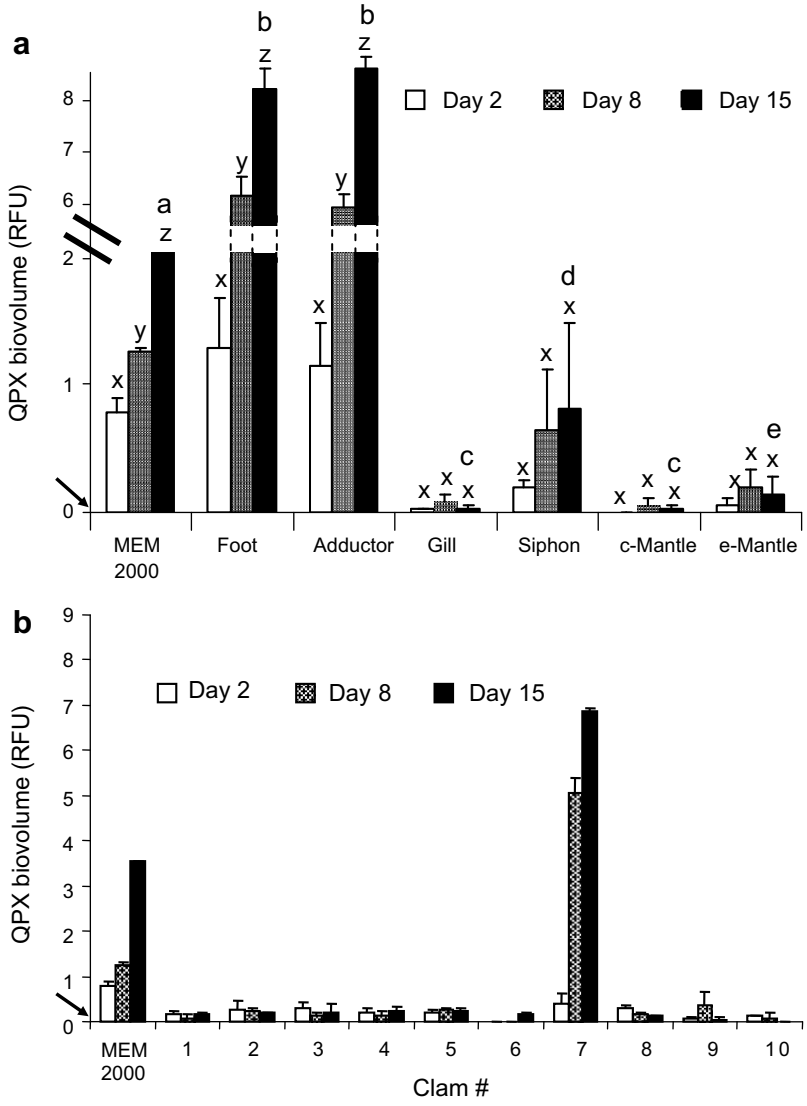

Fig. 4. [a] QPX biovolume (relative fluorescent units, Mean ± SEM, n = 10 clams) in cultures made with different tissue extracts. c-Mantle and e-Mantle designate the central part and the edge of the mantle, respectively. Tissue extracts and MEM control (MEM 2000) were normalized at 2000 μg protein ml^{-1} with FASW. [b] QPX biovolume in cultures made with siphon tissue homogenates (same data as in [a] shown individually). The arrows on the Y axis represent QPX biovolume added to each culture at t_0. Letters indicate which experimental treatments shared statistically equivalent (same letters) or different (different letters) QPX biovolume means among cultures in different experimental media at Day 15 (a, b, c, d, e), or among measurements taken at different times within each culture (x, y, z).

mantle homogenates were used without supplementation of MEM (data not shown), including for QPX cells incubated in diluted gill and mantle homogenates with protein concentrations as low as 100 μg ml^{-1}.

4. Discussion

In bivalves, plasma is known to contain a wide array of antimicrobial compounds (Anderson and Beaven, 2001a; Bachère et al., 2004; Hubert et al., 1996; Tincu and Taylor, 2004). Anderson et al. (2003) demonstrated the presence of inhibitory compounds against QPX in the plasma of *M. mercenaria* originating from Virginia. In the same way, this study demonstrated a strong inhibition of QPX growth by plasma of *M. mercenaria* from New York and Florida. A reduction of up to 92% of the parasite biovolume, compared to the controls, was observed in cultures made in MEM-supplemented with plasma. Moreover, results showed that plasma from NY clams inhibited QPX growth more efficiently than plasma from FL clams. These *in vitro* results coincide with results of disease transmission studies provided by Dahl et al. (2008), which showed significantly higher resistance toward QPX in NY clams than in FL clams (same broodstock as those used in this study). Similar results showing higher resistance toward QPX disease in northern strains of clams as compared to southern strains have also been described in previous studies (Calvo et al., 2003, 2007; Ford et al., 2002), and are thought to be an outcome of the selection process resulting from clam mortalities due to prior exposures to QPX among northern stocks.

Protein concentrations were also significantly higher in plasma from NY clams compared to FL clams. However, there was no correlation between protein levels and inhibitory activity of plasma on QPX growth and higher inhibition levels were also detected when normalized plasma samples were used (Fig. 1). Differences in the diversity of plasma proteins, as well as the involvement of non-protein compounds, could explain differences in QPX inhibition of plasma between NY and FL clams. Overall, it is unclear whether or not resistance of clams to QPX disease *in vivo* is linked to the inhibitory activity of plasma on QPX growth *in vitro*, but it is

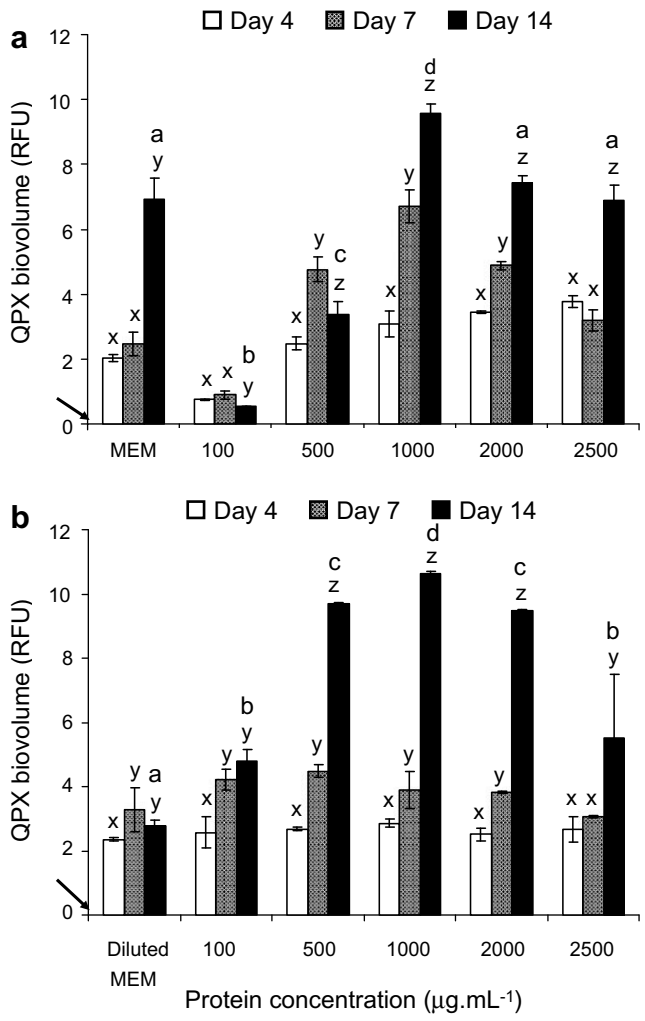

Fig. 5. [a] QPX biovolume (relative fluorescent units, Mean ± SEM, $n = 2$ replicates) in cultures made with different dilutions of adductor muscle extracts. Control cultures were grown in undiluted MEM. In [b], muscle extracts were supplemented to MEM (vol/vol) before the initiation of the cultures and FASW was substituted to muscle extracts in control (Diluted MEM). Values on the *X* axis indicate the final concentration of proteins from muscle extracts in each condition. The arrows on the *Y* axis represent QPX biovolume added to each culture at t_0. Letters indicate which experimental treatments shared statistically equivalent (same letters) or different (different letters) QPX biovolume means among cultures in different experimental media at Day 14 (a, b, c, d), or among measurements taken at different times within each culture (x, y, z).

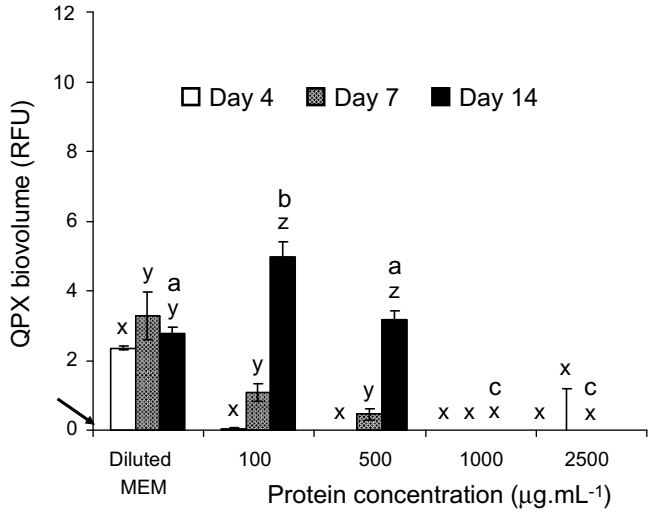

Fig. 6. QPX biovolume (relative fluorescent units, Mean ± SEM, $n = 2$ replicates) in cultures made with different dilutions of gill extracts supplemented with MEM (vol:vol). Tissue extracts were substituted with FASW in control (Diluted MEM). Values on the *X* axis indicate the final concentration of proteins from gill extracts in each condition. The arrow on the *Y* axis represents QPX biovolume added to each culture at t_0. Letters indicate which experimental treatments shared statistically equivalent (same letters) or different (different letters) QPX biovolume means among cultures in different experimental media at Day 14 (a, b, c), or among measurements taken at different times within each culture (x, y, z).

intriguing that both were higher in NY clams. Interestingly, further comparison of QPX inhibitory factors in plasma from individual clams demonstrated also a strong variability within each clam population. Some clam plasma efficiently inhibited parasite's growth whereas others had no effect or even stimulated QPX growth compared to the controls. Specific effects of active compounds in the plasma on QPX remain unknown. In some cases, QPX biovolume was below levels inoculated at Day 0, suggesting killing activities of plasma compounds whereas in other cases, only static effects were observed.

The present study also demonstrated the modulatory effects of clam tissue extracts on QPX growth *in vitro*. Our approach based on tissue homogenates could be compared to the microenvironment created by QPX activity within host tissues. For instance, QPX is known to disrupt host tissues and to lyse their cells, releasing cell contents, as demonstrated by histological observations of infected clam tissues (Smolowitz et al., 1998; Dove et al., 2004). Our *in vitro* results showed that gill and mantle homogenates significantly inhibited QPX growth, while homogenates made with foot and adductor muscle tissues enhanced parasite growth compared to the standard culture medium (MEM).

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

Our results with adductor muscle extracts showed a clear difference in growth trends between samples incubated in very low (100 µg ml^{-1} for example) and high (2000 and 2500 µg ml^{-1}) protein concentrations (Fig. 5a). In both cases, QPX biovolume was below maximal values measured in preparations made with 1000 µg ml^{-1} protein but temporal evolution of QPX biovolume demonstrated trophic exhaustion of the medium after 7 days at very low protein levels. The inhibitory effects of muscle homogenates above 1000 µg protein ml^{-1} demonstrated the presence of compounds limiting QPX growth, but at much lower levels than gill and mantle tissue extracts.

Overall, these results suggest the existence of a balance between enhancing and inhibitory factors in tissue extracts. In the muscle homogenates, increasing protein concentration could raise the concentration of inhibitory factors, leading to a decrease in QPX growth at protein concentrations exceeding 1000 µg protein ml^{-1}. Anderson et al. (2003) noticed that low to moderate concentrations of proteins from *M. mercenaria* plasma also enhanced QPX growth. The existence of both types (enhancing and inhibitory) of factors in clam tissues could also explain the evolution of QPX growth in gill (Fig. 6) and mantle (not shown but similar to gills) homogenates. Homogenates from these tissues possess an efficient activity against QPX growth at low protein concentration but after 7 days, these factors are no longer active at low protein concentrations.

Such loss of activity may result from degradation of inhibitory factors due to parasite's activity and the production of extracellular proteases (Anderson et al., 2006), but also possibly as a result of spontaneous degradation of inhibitory factors in the homogenates under the effect of proteolytic enzymes released from disrupted clam cells during tissue homogenization.

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

Interestingly, QPX inhibitory activity was not homogeneous in tissue extracts made of different parts of the mantle. The central part of the mantle exhibited the most inhibitory activity, followed by the edge of the mantle and finally siphon tissue homogenates. This is likely the result of differences in tissue composition among these different parts, with the siphon being mostly composed of muscular tissues. These findings are particularly relevant since in naturally-infected clams, QPX lesions are particularly common in siphon (Smolowitz et al., 1998) and more rarely in the central part of the mantle or in the gill, leading some scientists to speculate that siphon specifically represents the most important portal of entry for QPX into its host (Lyons et al., 2005). Thus, despite major limitations related to the fact that our *in vitro* experiments may not reflect parasite's behavior *in vivo*, we can speculate that QPX establishment in siphon and the edge of mantle may be the result of lower inhibitory activity in these tissues and not necessarily specific acquisition of the parasite in these areas, as compared to other parts of the pallial organs (central part of the mantle and gills). The large variability of inhibitory factors in homogenates of siphon tissues (Figs. 3 and 4) supports our speculation and suggests that some clams appear to be deficient in these factors, allowing parasite's proliferation. These deficiencies might be linked to the sensitivity of some clams to the infection *in vivo*, where a relatively small fraction of clams is typically affected in enzootic areas.

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

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

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