



Full length article

Alterations of the immune transcriptome in resistant and susceptible hard clams (*Mercenaria mercenaria*) in response to Quahog Parasite Unknown (QPX) and temperature

Kailai Wang^a, Emmanuelle Pales Espinosa^a, Arnaud Tanguy^b, Bassem Allam^{a,*}^a School of Marine and Atmospheric Sciences, Stony Brook University, Stony Brook, NY, 11794, USA^b UPMC Université Paris 6, Station Biologique de Roscoff, 29682, Roscoff, France

ARTICLE INFO

Article history:

Received 17 September 2015

Received in revised form

3 December 2015

Accepted 6 December 2015

Available online 12 December 2015

Keywords:

Hard clam

QPX

Microarray

Gene expression

Temperature

Immunity

ABSTRACT

Quahog Parasite Unknown (QPX) is a fatal protistan parasite that causes severe losses in the hard clam (*Mercenaria mercenaria*) fisheries along the northeastern coast of the US. Field and laboratory studies of QPX disease have demonstrated a major role for water temperature and *M. mercenaria* genetic origin in disease development. Infections are more likely to occur at cold temperatures, with clam stocks originating from southern states being more susceptible than clams from northern origin where disease is enzootic. Even though the influence of temperature on QPX infection have been examined in susceptible and resistant *M. mercenaria* at physiological and cellular scales, the underlying molecular mechanisms associated with host–pathogen interactions remain largely unknown. This study was carried out to explore the molecular changes in *M. mercenaria* in response to temperature and QPX infection on the transcriptomic level, and also to compare molecular responses between susceptible and resistant clam stocks. A *M. mercenaria* oligoarray (15 K Agilent) platform was produced based on our previously generated transcriptomic data and was used to compare gene expression profiles in naive and QPX-infected susceptible (Florida stock) and resistant (Massachusetts) clams maintained at temperatures favoring disease development (13 °C) or clam healing (21 °C). In addition, transcriptomic changes reflecting focal (the site of infection, mantle) and systemic (circulating hemocytes) responses were also assessed using the oligoarray platform. Results revealed significant regulation of multiple biological pathways by temperature and QPX infection, mainly associated with immune recognition, microbial killing, protein synthesis, oxidative protection and metabolism. Alterations were widely systemic with most changes in gene expression revealed in hemocytes, highlighting the role of circulating hemocytes as the first line of defense against pathogenic stress. A large number of complement-related recognition molecules with fibrinogen or C1q domains were shown to be specially induced following QPX challenge, and the expression of these molecules was significantly higher in resistant clams as compared to susceptible ones. These highly variable immune proteins may be potent candidate molecular markers for future study of *M. mercenaria* resistance against QPX. Beyond the specific case of clam response to QPX, this study also provides insights into the primitive complement-like system in the hard clam.

© 2015 Elsevier Ltd. All rights reserved.

1. Introduction

The hard clam *Mercenaria mercenaria* is among the most commercially important bivalves in the United States. It is widely exploited along the North American Atlantic coasts from the Maritime Provinces of Canada to Florida. *M. mercenaria* is often

considered as a robust bivalve species and relatively few infectious agents were reported to cause problems in wild and aquacultured stocks. Among these, the Quahog Parasite Unknown (QPX) is a protistan parasite known to cause lethal infections and substantial losses in the hard clam industry [24,39,41,62,70,74]. QPX is considered as an opportunistic pathogen and is widely present in coastal environments [27,37,40], where it can be found associated with a variety of substrates such as seawater, sediment and marine aggregates [36,37,42,63]. Common encounters between QPX and *M. mercenaria* are therefore considered frequent but usually do not

* Corresponding author.

E-mail address: Bassem.Allam@stonybrook.edu (B. Allam).

result in disease outbreaks until other disease-favoring factors are prevalent [27,37,40]. Such factors may include the presence of highly virulent parasite variants, susceptible host strains and shifts of environmental conditions that either add to the pathogen abundance or infectivity, or lower the resistance of the host [14,15,56].

Previous studies of QPX disease have demonstrated a significant association between temperature and *M. mercenaria* genetic background and disease development. For instance, QPX infection is considered as a “cold-water disease” since the infection has never been detected in clams further south of Virginia where the water temperature is comparatively warmer. Laboratory infection trials also reported higher disease prevalence in clams held at 13 °C as compared to temperatures of 21 °C and higher, where parasite establishment was largely inhibited and signs of host healing were detected [12,14,56]. In fact, environmental factors, such as temperature, salinity and dissolved oxygen, can substantially affect the physiology of aquatic animals. Temperature, of special importance, has been demonstrated to significantly modulate host immune performance and pathogen virulence, thus changing the pattern of host–pathogen interactions and affecting the development of disease [10,48,55,56]. On the other hand, marked differences in QPX susceptibility has been observed between clams from different genetic backgrounds. Generally, clams originating from the warm southern states (e.g. Florida and South Carolina) appear to be more sensitive to QPX infection than those from relatively cold northern states (e.g. Massachusetts, New York, New Jersey), suggesting the existence of genetically controlled factors that regulate *M. mercenaria* resistance against this parasite [7,13,16,24].

The ability of a pathogen to establish infection largely depends on the capacity of the host defense system to induce an effective immune response against the invasion and the ability of the pathogen to evade or overcome the host defense. Like other invertebrates, hard clams lack the adaptive immune system and rely solely on components of their innate immune system to defend themselves against infections. As a benthic filter-feeder, hard clams are exposed to an environment highly rich in microbial pathogens, but very few microorganisms are known to infect this species. This raises fundamental questions about how *M. mercenaria* is able to protect itself against this diverse pool of opportunistic pathogens without the specific effectors of the adaptive immune system. A recent study comparing *M. mercenaria* defense response against bacteria (*Vibrio alginolyticus*) and QPX stimuli identified specific response patterns associated with QPX challenge. Interestingly, the extent of the defense response also varied considerably between resistant and susceptible clam stocks [51].

A major characteristic of clam immune response to QPX infection is the presence of granulomatous inflammation and hemocyte encapsulation of parasite cells leading often to the formation of nodules in mantle tissues [2,70]. Dead QPX cells can sometimes be observed inside the infection nodules, suggesting effective defense reactions are mounted by the host under optimal situations that can result in parasite clearance and host healing [8,11,16,22]. Interestingly, the presence of factors inhibiting QPX growth has been reported in clam plasma [54], and preliminary molecular investigations also demonstrated the modulation of several stress- and defense-related genes during QPX disease development [59]. However, the nature of these specific anti-QPX factors and the underlying mechanisms for immune response and host healing are largely unknown. Growing evidence has demonstrated that innate immune responses in invertebrates are in fact more sophisticated than previously thought [6,17,38,72]. The identification of genes associated with host-defense in *M. mercenaria* can provide insights into the diversity and evolution of innate immune mechanisms, and may also have practical implications to improve the disease

resistance for this economically and ecologically important species.

In this study, we used a high-throughput genomic approach to generate an in-depth understanding of *M. mercenaria* defense system and to identify molecular pathways and effectors involved in hard clam immune response against QPX. High throughput gene expression techniques such as microarrays have been widely adopted as powerful tools for functional genomics investigations in non-model organisms. Micro (oligo)-array is an affordable, sensitive and reproducible high-throughput platform for analyzing the expression of tens of thousands of genes simultaneously. This approach has been used for probing host–pathogen interactions in several bivalve species significantly advancing our understanding of immunological regulatory pathways and providing physiological perspectives on the environmental facilitation of infection [1,35,44,46,47,66,78]. Transcriptome profiling by microarray technique can directly compare gene expression profiles between samples of different conditions or traits (e.g. healthy vs. diseased, susceptible vs. resistant), allowing for the identification of candidate genes and underlying mechanisms involved in interested features. Our study design assessed transcriptomic changes in hard clams during QPX infection. Hemocytes and mantle tissues were inspected for gene expression difference between healthy and diseased clams, providing insights into factors involved in systemic and focal immune responses, respectively. Gene expression in response to QPX infection was also compared between resistant (MA) and susceptible (FL) clam stocks, as well as in clams held at temperatures that promote (13 °C) or inhibit (21 °C) disease development. Our goal was to explore the molecular immune mechanisms used by hard clams to fight QPX and to identify immune genes or isoforms potentially involved in *M. mercenaria* resistance to QPX infection.

2. Materials and methods

2.1. Hard clams

Adult (50–55 mm in length) aquacultured *M. mercenaria* originating from Florida (FL) and Massachusetts (MA) were used in this study. QPX-free FL clams were obtained from a commercial source (St James City, FL) and MA clams presumably infected by QPX were collected from an enzootic clamming area in Wellfleet Harbor. Disease status of subsets from each batch was checked by a specific quantitative real-time PCR assay [37] and QPX prevalence was equal to 63% in the MA clams and 0% in the Florida stock ($n = 30$ clams/batch). Clams were acclimated at 21 ± 1 °C in 150-L tanks with re-circulating water (30 ppt) upon arrival. After 1-week acclimation, half of the FL clams were challenged with QPX as described previously [15] by injecting *in vitro* cultured parasite cells into the pericardial cavity (5×10^4 cells/clam), and the other half received injection of sterile culture media as controls. Following injection, both control (FLc) and QPX challenged (FLq) FL clams, as well as the naturally infected MA clams were separately transferred to 40-L tanks and maintained at 13 and 21 °C. Temperature adjustment for the 13 °C treatment was performed within 8 days by decreasing the temperature by 1 °C per day as previously described in Refs. [15] and [56]. For each clam group (MA, FLc, FLq), a total of 60 clams were randomly assigned into 3 replicate tanks (20 clams per tank) held at each temperature condition (13 and 21 °C). All tanks were individually equipped with re-circulating filtration systems and aerated continuously. Water quality, temperature, salinity (30 ppt) and ammonia level were monitored and adjusted weekly. Clams were fed daily with commercial algae (DT's Live Phytoplankton, Sycamore, IL) and monitored twice a day for mortality.

2.2. RNA samples

After 2-month incubation at targeted temperatures, three clams were randomly sampled from each replicate tank, totaling 9 clams per clam type and per temperature condition. This timeframe was chosen because it was shown to be sufficient for QPX to initiate infection in susceptible clams or for clams to prompt healing under optimal conditions [11,15]. Clams were individually processed to collect hemolymph and mantle tissues. Hemolymph (generally 1.2–1.8 ml) was withdrawn from the adductor muscle sinus with a 1-ml syringe and centrifuged (700 g, 10 min, 4 °C) to pellet the hemocytes from the acellular fraction. Mantle biopsies and hemocyte pellets were flash-frozen in liquid nitrogen and immediately stored at –80 °C until processed for RNA extraction. Trizol reagent (MRC, Inc., Cincinnati, OH, USA) was used to isolate RNA from hemocytes and mantle tissues according to the manufacturer's protocol. RNA quality and quantity were assessed using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, WI, USA). RNA samples were pooled using the same RNA quantity from each individual to generate 3 pools per condition (tissue type, clam strain, temperature). A total of 30 RNA pools (only mantle RNA samples were assessed for MA clams) were generated and used for the downstream reactions and oligoarray hybridization (Table 1).

2.3. Oligoarray design, construction and hybridization

Our *in situ* synthesized oligoarrays were produced based on sets of expressed sequence tags (ESTs) obtained from a previous SSH libraries [59] as well as a transcriptome library generated from 454 sequencing of RNA obtained from *M. mercenaria* mantle tissues, gills, digestive gland and hemocytes (unpublished data). Sequences were annotated using the Blast2GO software (<http://www.blast2go.com/b2ghome>) with blast search against NCBI non-redundant (nr) database (blastx, E-value cut off of 10^{-5}). These included 3092 curated annotated sequences and 11,166 non-annotated sequences with a focus on sequences generated from hemocytes (7839 sequences) to emphasize the discovery of novel immune-related transcripts. All sequences were submitted to Agilent eArray application (<https://earray.chem.agilent.com/earray/>) for probe production, with 1 probe (60-mer) produced for each single submitted sequence. In parallel, a set of 595 probes designed based on annotated QPX ESTs [65] were also incorporated into this array in an attempt to target potential molecular evidence of parasite response to the host in different clam broodstocks and temperature conditions. Probes were synthesized *in situ* (14,853) along with positive and negative controls (891) using 8×15 K-feature Agilent format slides and a total of 15,744 probes were included on the oligoarray.

Cyanine dyes (Cy3 or Cy5) labeled complementary RNA (cRNA) was synthesized from 150 ng of RNA purified from mantles and hemocytes of FLC, FLq and MA clams (as described in 2.2, Table 1) using the Two-Color Microarray-Based Gene Expression Analysis Protocol (Quick Amp Labeling) according to the manufacturer's manual. Labeled cRNA was purified using Illustra CyScribe GFX

Purification Kit (GE Healthcare). cRNA quantity and quality (including dye incorporation) were determined by spectrophotometry (Nanodrop 1000 ND-1000 spectrophotometer, Thermo Scientific, Wilmington, WI, USA). Samples were considered satisfactory if cRNA concentration and incorporation efficiency exceeded 300 ng/ul and 8 pmol Cy/ug cRNA, respectively. All arrays were hybridized following a balanced block design with the same amount of cRNA (300 ng of each Cy3- and Cy5-labeled cRNA). Arrays hybridization and washes were conducted according to the kit protocol and the arrays were scanned with a GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA, USA) using the suggested Agilent scan settings.

2.4. Oligoarray data analysis

Fluorescence intensities of each spot were extracted using GenePix software and the generated intensity data were normalized using the LIMMA package in R software to remove within-array (method: global lowess) and between-array (method: quantile) non-biological variation [71]. After normalization, probes with intensities less than two-fold of background intensities were eliminated from further analysis (applied to all biological replicates for the probes). The filtered data were then submitted to statistical analysis using the Multi Experiment Viewer (MeV) program [67]. Gene expressions in hemocytes were compared between healthy (FLc) and QPX challenged (FLq) Florida clams at 13 °C and 21 °C ($n = 3$ for each treatment) to examine the molecular response induced by QPX infection and temperature. Mantle samples were examined within and between MA and FL infected clams at temperatures of 13 and 21 °C, in an attempt to identify immune genes and pathways potentially associated with *M. mercenaria* resistance against QPX and their regulation patterns at different temperature. The data analysis was based on the relative gene expression levels among compared samples, which was calculated as the ratio of intensity for a transcript in each treatment against the mean intensity of that transcript in all treatments. The criteria for final determination of differentially expressed genes were the significance by statistical testing (p -value < 0.01, t -test or ANOVA) together with one and half fold increase or decrease from the mean (up- or down-regulation). K-means clustering and hierarchical clustering were then used to cluster those significantly differentially expressed genes with similar expression profiles [73,81]. The complete dataset for *M. mercenaria* oligoarray can be found at the Gene Expression Omnibus public database (GEO) (<http://www.ncbi.nlm.nih.gov/geo/>) under the accession number GSE76182.

3. Results

The QPX infection status in clams after 2 months was described in Ref. [15]. Briefly, temperature significantly impacted disease development. Among, FL clams experimentally-injected with QPX (FLq), disease development was maximal in clams maintained at 13 °C (73% QPX disease prevalence) as opposed to those held at 21 °C (10% prevalence). Disease prevalence in naturally-infected

Table 1

Biological samples analyzed using the *M. mercenaria* oligoarray (the number of pooled RNA samples is given, each pool is made with equal quantities of RNA from 3 individual clams). NA: not assessed.

Clam source and disease status		13 °C		21 °C	
		Hemocytes	Mantle	Hemocytes	Mantle
FLc	Florida Naive	3	3	3	3
FLq	Florida Injected	3	3	3	3
MA	Massachusetts Naturally-infected	NA	3	NA	3

(MA) clams was 33% and 20% in clams held at 13 and 21 °C, respectively. Furthermore, moderate to heavy infections constituted most of the positive clams maintained at 13 °C, while none of the positive clams from the 21 °C treatment showed heavy infection, and signs of healing were only noticeable in clams held at 21 °C [15].

3.1. Differential gene expression in FL clams

The modulatory effect of temperature and QPX infection on *M. mercenaria* gene expression was investigated in hemocyte and mantle samples of FL clams maintained at 13 and 21 °C. Based on stringent criteria for the identification of significant differentially expressed (DE) genes (one-way ANOVA $p < 0.01$ in conjunction with relative fold change > 1.5), 887 and 311 DE genes were respectively identified in hemocyte and mantle samples in response to QPX challenge and temperature modulation (Supplementary file 2 and 3). The DE genes were then clustered by K-means clustering (KMC) into groups based on the expression pattern similarities in order to gain further insight into their biological functions. Within each KMC group, DE genes were further clustered using hierarchical clustering (HCL) to explore the potential functional information of novel (unannotated) DE genes, since genes clustered closely usually have linked or co-regulated expression performances thus possibly share similar function or biological importance [23].

The 887 DE genes in FL hemocyte samples were clustered into 8 groups based on the results of K-means clustering (Fig. 1A, Supplementary file 2). The cluster 1 (CL1) contained 135 DE genes that showed lower expression in QPX infected clams (FLq) held at 13 °C as compared to those held at 21 °C. Several genes from this cluster are known to play a role in mollusk immunity such as the angiopoietin-related protein, cathepsin L2 cysteine protease, complement C1q-like protein (C1q), complement C1q tumor necrosis factor-related protein 2 (C1qTNF2) and vitelline membrane outer layer protein 1. Cluster 2 (CL2) contained 129 DE genes that exhibit maximal expression in naïve FL (FLc) clams held at 21 °C. Some immune-related genes featured in this cluster include beta-glucan recognition protein, complement factor b-like protein and galectins. Cluster 3 (CL3) consisted of 26 DE genes that were found to be over-expressed in FLq hemocytes at 13 °C but under-expressed at 21 °C, with most of these being unannotated genes, except the neurogenic locus notch homolog protein 1-like, proteasome subunit beta type-6-like and ribosomal proteins. Cluster 4 (CL4) and cluster 7 (CL7) contained DE genes that were significantly up-regulated in QPX-injected clams held at either 13 °C or 21 °C, or at both temperatures (Fig. 1). Out of the 69 DE genes clustered in CL4, those encode molecules potentially involved in clam defense system were selected and presented in Fig. 1B, including immune effector proteins (cathepsin k, endo-1,3- β -glucanase, ferritin and cytochrome peroxidase) and immune pattern recognition proteins (ARPs, C1qs, galectins, tenascin and fibrinogen domain containing proteins). CL7 contains 133 DE genes that presented the highest expression pattern in FLq clams held at 21 °C (Fig. 1C). About 70% (102) of the DE genes in CL7 are unannotated genes, while those annotated DE genes were mostly pathogen pattern recognition proteins (PRRs) such as C1qs, thioester-containing protein (TEP) and sialic acid-binding lectins (Fig. 1C). Cluster 5 contains a list of 41 DE genes that were significantly down-regulated in FL clams after QPX challenge for both temperatures. This collection featured genes encoding structural proteins associated with cytoskeleton and ribosome (e.g. ribosomal proteins, actins, tubulins and ATP synthases), and one immune protein (lipopolysaccharide-induced tumor necrosis factor- α factor-like protein). Cluster 6 (CL6) contains 209 DE genes that were highly expressed at 13 °C as

compared to 21 °C. Many DE genes from this cluster represent stress-related proteins, such as heat-shock proteins, cytochrome oxidase, metallothionein, and omega glutathione s-transferase. On the contrary, cluster 8 (CL8) was characterized by DE genes (147) that are under-expressed in hemocytes from the low temperature treatment (13 °C) of both naïve and infected FL clams. Genes clustered in this group were typically those associated with growth and metabolism (eg. ribosomal proteins, latent-transforming growth factor beta-binding protein 4, short-chain collagen partial), except a few proteins involved in defense (eg. transmembrane serine 6, cytochrome p450 1a1, complement C1q subcomponent subunit b and caprin-2).

As for the mantle tissues of FL clams, a total of 311 DE genes have shown significant modulation in response to temperature and QPX infection and were clustered into 6 expression pattern groups by KMC (Fig. 2 and Supplementary file 3). Cluster 1 contained 62 DE genes that were down-regulated in mantle tissues from clams held at 13 °C as compared to 21 °C (Fig. 2). These included some structural and metabolism-related proteins (e.g. ribosomal proteins, actins, tubulins, acyl-CoA-dehydrogenase and d-beta-hydroxybutyrate mitochondrial), as well as several immune-related proteins, such as antioxidant cytochrome c, the tumor suppressor protein inhibitor of growth protein 3-like, the immune signal transducer serine threonine-protein kinase ctr1 and chitin deacetylase-like protein (Supplementary file 3, Fig. S1). In contrast, Cluster 3 (Supplementary file 3, Fig. S1) was comprised of 84 genes and highlighted DE genes that were highly expressed at 13 °C, including acyl-CoA desaturase (lipid metabolism), mucin-associated protein (biomineralization), NADH dehydrogenase subunit and heavy metal-binding protein hip (detoxification). Cluster 2 and Cluster 5 respectively included 56 and 22 DE genes that were highly expressed in FLq clams at 21 and 13 °C, respectively (Figs. 2 and 3). A considerable fraction of the annotated DE genes in Cluster 2 (Fig. 3A) belonged to the immune-related protein category; such as the tandem repeat galectin, lysozyme, polyubiquitin-c-like isoform1, C1q, and CD209 antigen-like protein. Meanwhile, only genes of the PRR C1qDCs were found in Cluster 5, whereas two QPX genes, the pyruvate dehydrogenase component x and 60s ribosomal protein, were amid the DE genes over-expressed following QPX injection at 13 °C, which could be linked to ongoing active infection in this batch. The remaining two clusters (Cluster 4 and Cluster 6, Fig. 3) included DE genes that were either down-regulated (Cluster 4) or up-regulated (Cluster 6) in infected FL clams as compared to control FL clams. Fifty-six DE genes were present in Cluster 4, of which the annotated genes were mostly associated with cell structural components (cytoskeleton, collagen and actin cytoskeleton), protein synthesis and processing (nucleotide binding, translation elongation factor, mRNA splicing), as well as several genes involved in apoptosis (Apoptosis 2 inhibitor, C1q-tumor necrosis factor 3) and detoxification processes (cytochrome p450, small heat shock protein). Out of the 28 DE genes revealed by Cluster 6, only 5 were functionally annotated, which interestingly included one QPX gene involved in polysaccharide biosynthesis and one *M. mercenaria* tandem repeat galectin gene.

3.2. Differential gene expression in MA clams

The modulation of gene expression in mantle tissues from infected MA clams in response to temperature was evaluated. A total of 563 genes were differentially expressed between 13 and 21 °C (t -test $p < 0.01$ with at least 1.5 fold change, Supplementary file 4). Among these, 217 DE genes exhibited higher expression at 13 °C, and the remaining 346 were comparatively higher in the 21 °C samples. The DE genes from this comparison were categorized by their putative functions and are summarized in Fig. 4 and

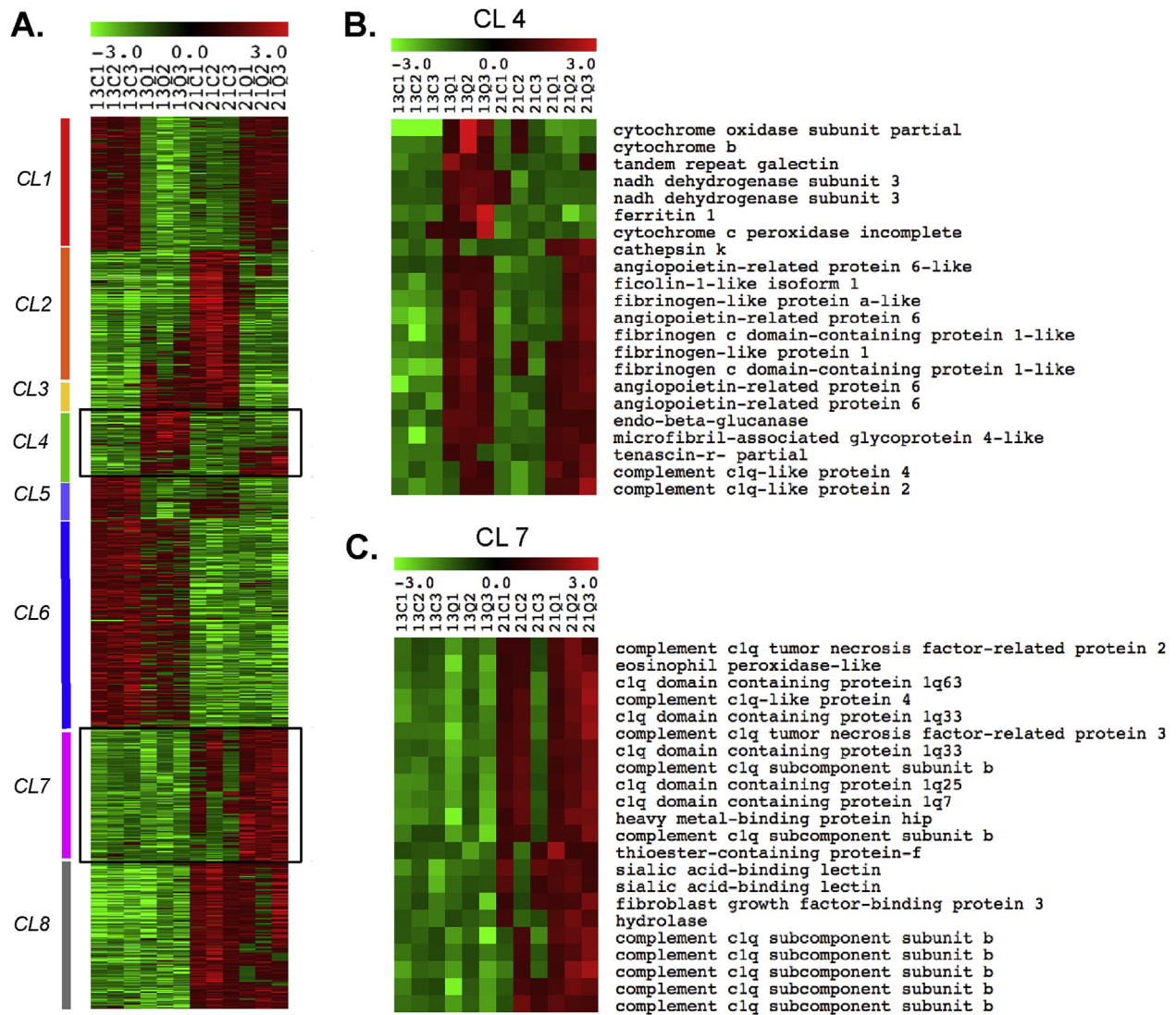


Fig. 1. Gene clusters generated by K-means clustering of the 887 differentially expressed genes in FL clam hemocytes. (A) Overview of all 8 clusters. (B) Immune related transcripts identified in cluster 4 (CL4). (C) Immune related transcripts identified in cluster 7 (CL7). Gene expression heat maps reflect relative gene expression fold changes where a gradient of red to green represents a 3-fold or greater increase in gene expression to a 3-fold or greater decrease in gene expression. Columns represent the gene expression of individual samples from each treatment with each gene displayed horizontally. Sample names give temperature treatment (13 or 21 °C), challenge status (C: control, Q: challenged with QPX), and replicate number (1–3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Supplementary file 4. DE genes that are highly expressed at 13 °C are dominated by those related to stress response and immune recognition (Fig. 4), which included HSP70, universal stress protein, metallothionein, angiopoietin-related proteins and fibrinogen domain-containing proteins (FREPs). In addition, several structural proteins were also overexpressed in mantle tissues from MA clams held at 13 °C (Fig. 4, Supplementary file 4), particularly the tubulins which are known to be associated with macrophage activity [64].

On the other hand, structural proteins overexpressed at 21 °C were mostly ribosomal proteins, with functions pertaining to mitochondria or ribosome biogenesis processes (Supplementary file 4). In parallel, genes associated with metabolic processes, signal transduction and protein synthesis were also overexpressed at 21 °C (Fig. 4), reflecting a higher metabolic rate in these clams as compared to those held at 13 °C. Interestingly, DE genes with functions related to apoptotic processes were exclusively overexpressed at 21 °C, implying a general underexpression of these genes at 13 °C (Fig. 4, Supplementary file 4).

3.3. Comparison of gene expression profiles between FL and MA clams

Gene regulation associated with QPX infection was also compared between the relatively susceptible FL and resistant MA clams [13] in order to get further insights into genes potentially related to the *M. mercenaria* resistance to QPX. Comparisons were made among mantle samples of MA and FL clams held at either 13 or 21 °C, and DE genes were identified based on the same criteria as above ($p < 0.01$ by ANOVA in conjunction with over 1.5 fold change). A total of 1569 DE genes were revealed from this comparison (Supplementary file 5). K-means and hierarchical clustering were applied to categorize expression patterns among annotated DE genes (Supplementary file 5). A total of 227 annotated DE genes were assigned into 6 K-means clusters based on the similarity of the expression patterns across samples (Supplementary file 5, Fig. 5). The first three clusters (CL1 to 3, Fig. 5) featured DE genes that displayed higher expression in MA clams than in FL clams. These 3

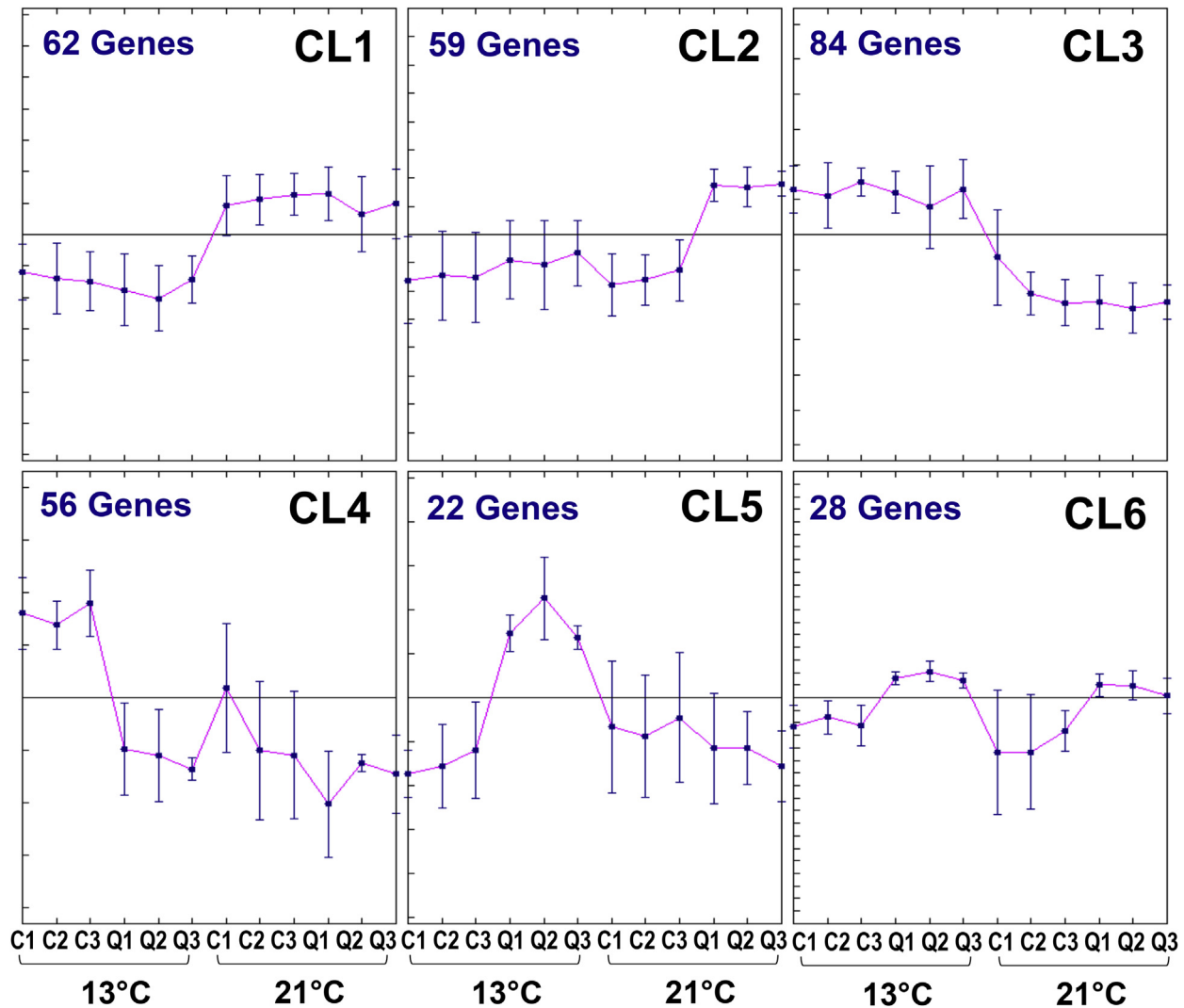


Fig. 2. Gene clusters generated by K-means clustering of the 311 differentially expressed genes in FL clam mantle samples. Gene clusters are generated based on the similarity of their gene expression profiles in the different treatments. The relative fold change of the DE gene expression within each cluster can be directly compared across samples/treatments based on their value on the Y-axis (mean \pm S.D.). Sample names are similar to those in Fig. 1.

clusters were remarkably enriched with DE genes involved in immune and stress response, particularly in pathogen recognition, such as the C1q domain containing proteins, fibrinogen related proteins and angiopoietin-related proteins (both contain fibrinogen related domains, FReD), which comprised the three main PRRs families that were overexpressed in MA clams as compared to FL clams (Fig. 5 and Supplementary file 5). Interestingly, the highest expression of these PRRs was found in MA clams held at 13 °C, suggesting a link between these immune genes and active host–pathogen interactions.

On the other hand, the remaining 3 clusters (CL 4 to 6, Fig. 5) highlighted DE genes that were overexpressed at 13 °C (Cluster 4) and 21 °C (Cluster 5) in both MA and FL clams, and those only up-regulated in FL clams at both temperatures (Cluster 6). DE genes up-regulated at 13 °C (Cluster 4) in both clam strains were enriched with functions related to immune and stress response. For example, sialic acid binding lectin, which is a pathogen binding protein, clathrins and tubulins, which are known to regulate macrophage activity, stress protein HSP70, immune enzyme serine protease and kazal-type proteinase inhibitor, as well as cytochrome b, which is involved in the ROS detoxification, were concomitantly up-

regulated, suggesting active host–pathogen interactions are taking place under this condition. Whereas DE genes involved in other biological processes were largely under-expressed at 13 °C as compared to 21 °C (Cluster 5), such as those associated with protein biosynthesis processes (ribosome biogenesis protein, eukaryotic translation initiation factor 3, transcription factor containing protein, translation elongation factor hbs1-like protein, protein folding chaperon heat shock proteins) and metabolic processes (cytochrome oxidase, ATPase inhibitor, ATP-binding protein, cytochrome c, beta-1,4-galactosyltransferase; Cluster 5, Supplementary file 5). These changes were very similar to what have been observed in FL hemocytes from clams held at 13 °C (described above), reflecting a systemic modulatory effect of temperature and QPX infection on clams regardless of strain and tissue difference. In addition, a large fraction of DE genes that are overexpressed in FL clams as compared to MA clams were related to metabolic and protein biosynthesis processes (Cluster 6, Supplementary file 5), such as ribosomal proteins, histone ribonucleoproteins, and ATP synthase. Interestingly, in term of immune-related genes, none of the PRRs that were induced upon QPX infection in MA clams (e.g. FREPs, C1q and angiopoietin) was overexpressed in infected FL clams. In

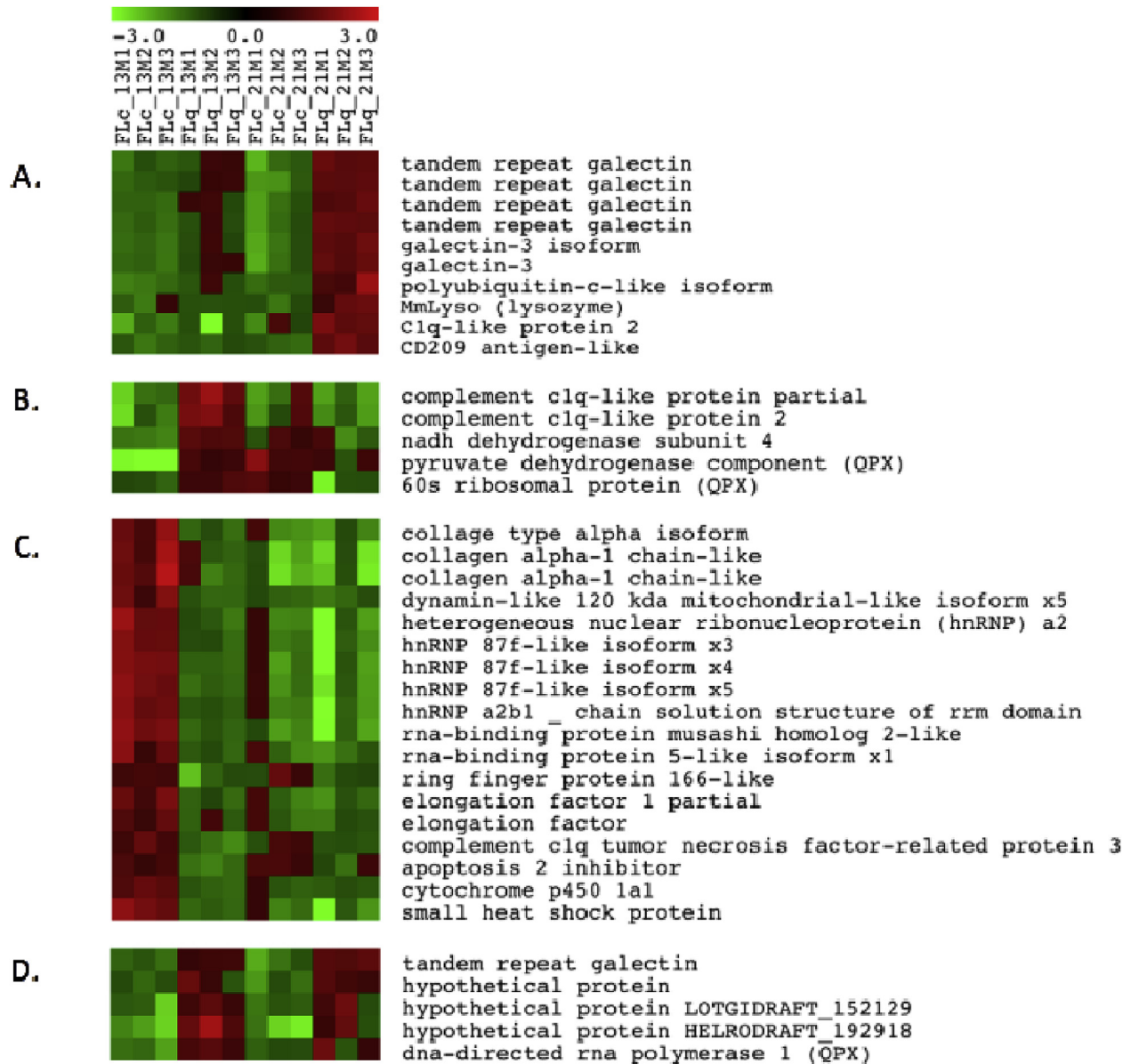


Fig. 3. Selected differentially expressed transcripts in FL clam mantles from K-means cluster 2 (A), cluster 5 (B), cluster 4 (C), cluster 6 (D). Expression heat maps reflect relative gene expression fold changes where a gradient of red to green represents a 3-fold or greater increase in gene expression to a 3-fold or greater decrease in gene expression. Columns represent the gene expression of individual samples from each treatment with each gene displayed horizontally. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

contrast, expression levels of some other defense-related genes were higher in FL clams than in MA, such as the LPS-induced TNF factor, peptidoglycan recognition protein, galactose-specific c-type lectin, proteasome subunit and several hydrolases (cathepsin 1, lipase, lysosomal cholesterol esterase), many of which were especially higher at 21 °C as compared to 13 °C. Finally, higher expression of stress proteins (superoxide dismutase, universal stress protein, omega glutathione s-transferase) was noticed in FL clams injected with QPX or exposed to 13 °C as compared to their MA counterparts suggesting that these conditions are more stressful to the former clam stock.

4. Discussion

Successful management and control of QPX disease is of great importance for the hard clam industry, since mortalities caused by

this parasite have resulted in significant economical losses [8,24,70]. Thus, understanding the pathobiology of QPX disease and host–pathogen interactions is urgently needed to set forth disease mitigation strategies and develop resistant clam stocks. Currently, our knowledge about QPX disease mostly focused on clam physiology and baseline immune processes. Information on the molecular aspects of clam response to QPX is still very limited, with a very narrow collection of common immune-related genes thought to be involved in host–pathogen interactions [50,51,56,58]. These investigations have described different clam defense strategies mounted against bacterial and QPX infections [51], and the interwoven genetic and environmental determinants associated with clam resistance [7,13,24]. Our study used high-throughput genomic tools to comprehensively assess clam response to QPX at molecular levels. The comparisons between healthy and infected clams, and between relatively resistant and susceptible stocks provided

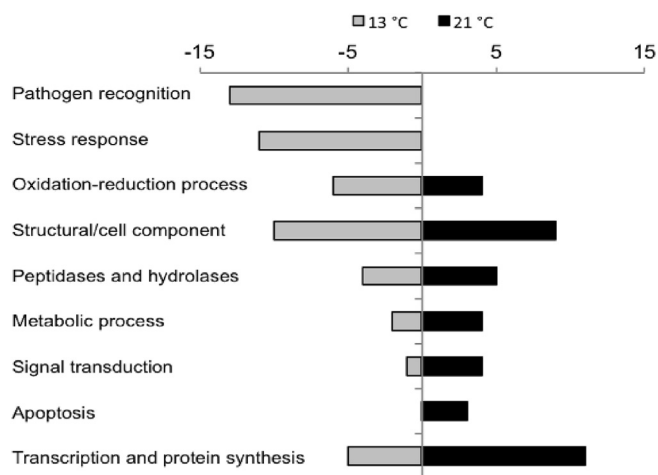


Fig. 4. Overview of annotated differentially expressed (DE) gene functions in naturally infected (MA) hard clam mantle as modulated by temperature (13 and 21 °C). The number of DE genes grouped into each functional annotation category are indicated on the horizontal axis (negative values represent for DE genes overexpressed at 13 °C and positive values represent those overexpressed at 21 °C).

insights into mechanisms involved in disease development and host-QPX interactions. At the same time, the evaluation of QPX-associated responses in mantle and hemocytes allowed for the understanding of tissue-specific defense strategies as mantle represents the main infection site [70] while hemocytes reflect an overall systemic clam response toward the invader [54].

4.1. Modulatory effects of temperature on gene expression in hemocytes

We have previously shown that temperature significantly regulates *M. mercenaria* immunity and QPX disease dynamics [15,56]. In particular, cold temperatures (13 °C) were shown to dampen cellular immunity and significantly promote the establishment of QPX disease, while warmer temperatures (21 and 27 °C) prohibit infection development and favor healing of pre-existing lesions [15,56]. Results from the current study show that temperature changes cause alterations in both constitutive and QPX-induced gene expression in FL clams. In parallel, this study shows that QPX challenge alters the expression profiles of several immune genes (at either 13 °C or 21 °C, or both; clusters CL4 and CL7, Fig. 1 and Supplementary file 2. Among these, several DE genes belonged to the fibrinogen-related protein families (FREPs) and the complement C1q domain containing proteins (C1qDCs).

The FREPs are a group of proteins that contain fibrinogen or fibrinogen-like domains. Members of the FREPs family were found largely enriched in CL4, and included the angiopoietin-related proteins, fibrinogen c domain-containing 1-like proteins, fibrinogen-like proteins, ficolin-1-like isoform and tenascin (Fig. 1B). As CL4 clustered those DE genes induced by QPX challenge especially at 13 °C, one can expect that FREPs contribute to clam defense response against ongoing QPX infections. On the other hand, C1qDCs proteins were particularly enriched in challenged clams held at 21 °C (Fig. 1C), implying the possible participation of these proteins in neutralizing QPX and preventing infection. Interestingly, levels of C1qDCs were constitutively higher in FLc clams held at 21 °C as compared to 13 °C (cluster CL7, Fig. 1C), suggesting a fundamental role of these proteins in *M. mercenaria*'s resistance to QPX.

It should be stated, however, that the exact functions of FREPs and C1qDCs have not been clearly characterized in bivalves.

Accumulating evidence supports their important roles in innate immunity as pattern recognition receptors that mediate non-self recognition [28,33,83,84]. In fact, both FREPs and C1qDCs are known to activate the complement pathway of the innate immune system in vertebrates, however their modes of action are slightly different with C1qDCs triggering the classical pathway, while FREPs activate the lectin pathway [19,26]. Up-regulation of both groups in hard clams upon QPX challenge may indicate the activation and involvement of complement proteins in *M. mercenaria* defense mechanisms. It can be further speculated that an ancestral complement system existed and may be actively involved in the neutralization of QPX in *M. mercenaria*, possibly via parasite recognition, immobilization, and tissue damage repair, which leads the way to ultimate healing. Interestingly, another complement-related protein, the thioester-containing protein (TEP), was also found overexpressed after QPX infection (Fig. 1Cure). TEP is a functional homologue to vertebrate C3, which is the central component of the complement system, and is widely recognized as an essential player in the anti-parasite defense mechanisms of insects [3,4]. The up-regulation of a TEP homologue in *M. mercenaria* after QPX stimulation provides further support to the role of the complement system in anti-QPX defense in *M. mercenaria*. Finally, other immune genes found in cluster CL4 and CL7 suggest the mobilization of diverse immune mechanisms and pathways for the purpose of parasite neutralization, with the active involvement of hydrolases (e.g. cathepsin K, endo-1,3- β -glucanase, and hydrolase), lectins (e.g. tandem repeat galectin, sialic acid-binding lectins), metal transporters (e.g. ferritin, heavy metal-binding protein hsp) and reduction/oxidation (redox) enzymes (e.g. cytochrome b peroxidase, cytochrome oxidase, eosinophil peroxidase).

The constitutive expression of the above immune-related genes was higher in control clams held at 21 °C as compared to those maintained at 13 °C, potentially linking host resistance at the former temperature to immune fitness of the host. In addition, other biological pathways such as metabolic activities, protein synthesis and anti-oxidative processes were also significantly influenced by temperature on the constitutive-level (without QPX challenge). For example, generally higher expression of ribosomal proteins and fatty acid-binding proteins were found in FLc clams at 21 °C (CL2 and CL8, Supplementary file 1), indicating a more robust protein synthesis and growth at the higher temperature. On the other hand, the production of several proteins involved in the oxidative stress response was significantly induced at 13 °C, such as the omega-glutathione s-transferase, NADH-dehydrogenase subunit 3 and metallothionein proteins (CL1 and CL6, Supplementary file 1). This suggests the activation of oxidative defense mechanisms to protect against damaging reactive oxygen species (ROS) produced in excess during exposure to sub-optimal temperatures, which is known to frequently induce hypoxia stress [32]. As reported in many marine invertebrates, the reduction of blood circulation and oxygen transport at temperatures below an animal optimal thermal range can significantly lower the oxygen supply, sometimes below tissue demand, resulting in functional hypoxia and oxidative stress [31,60,61]. This phenomenon is especially pronounced in ectothermal animals, which include all bivalve species. Thus, for *M. mercenaria* held at 13 °C, higher levels of oxidative defense activities need to be maintained in order to avoid oxidative damage, which may consume a considerable portion of energy that otherwise could have been used for other purposes such as growth and immunity. This might partially account for the overall lower host defense levels at 13 °C as compared to 21 °C, and may also explain the dubbing of QPX disease as a "cold water disease" [15]; [56], particularly since parasite growth *in vitro* is in fact optimal at 21 °C [57] and that disease onset is largely dependent upon clam immune performances [56].

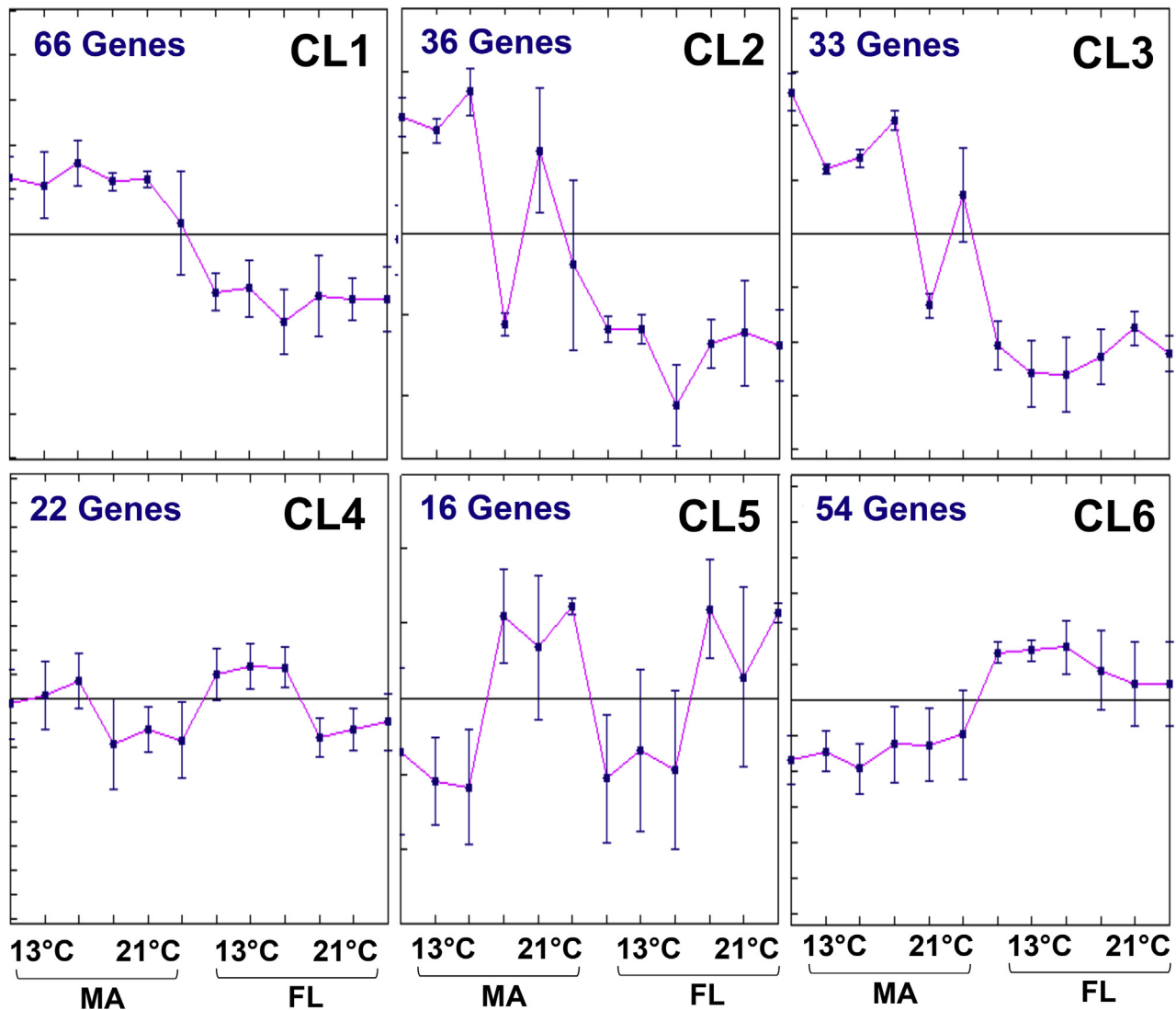


Fig. 5. Gene clusters generated by K-means clustering of the annotated genes differentially expressed in mantle tissues between QPX infected FL and MA clams at 13 °C or 21 °C. Gene clusters are generated based on the similarity of their gene expression profiles in the different treatments. The relative fold change of the DE gene expression within each cluster can be directly compared across samples/treatments based on their value on the Y-axis (mean \pm S.D.).

4.2. Modulatory effects of temperature on gene expression in mantle tissue of FL clams

QPX lesions are most often found inside clam mantle tissues, sometimes leading to the development of nodules. The formation of nodules materializes intensive interactions between host defense system and the invading parasite. In this context, mantle-related host factors likely contribute to the inflammatory response and encapsulation of QPX cells at the lesion sites [2,70]. Interestingly, tissue extracts from *M. mercenaria* mantle were shown to significantly inhibit QPX growth *in vitro*, even though the nature of these antimicrobial factors remains unknown [53]. Gene expression profiles of mantle tissues were determined in FL clams in response to temperature and QPX challenge and allowed the identification of candidate anti-QPX factors (Fig. 2). For example, the potent antimicrobial protein lysozyme was overexpressed in mantle from challenged clams held at 21 °C as compared to all other treatments (Supplementary file 3) suggesting a possible role of this enzyme in disease resistance. A similar role of lysozyme was suggested in the resistance of the clam *Meretrix meretrix* to

bacterial infections [82]. Several other immune-related genes were also identified in challenged clams held at either or both temperatures. Similar to the observations in FL clam hemocytes, the overexpressed DE genes induced by QPX challenge mainly included those complement C1q like proteins, tandem repeat galectin and galectin-3, all of which are PRRs involved in pathogen recognition and neutralization [34,75,77,80]. This overrepresented induction of PRRs in mantle suggests that parasite detection and targeting might be a key process for QPX neutralization. The induction of complement C1qDCs in both mantle and hemocytes following QPX stimulation corroborates the idea that the complement-like system may play a central role in bivalve defense against protozoan parasites, despite the fact that the functionality of such pathway has not been extensively characterized in most bivalve species [35,76]. On the other hand, the crucial role of galectins has been recently confirmed in bivalves as these were shown to mediate hemocyte recognition and binding of the protozoan parasites *Perkinsus marinus* in the oyster *Crassostrea virginica* and *Perkinsus olseni* in Manila clam *Ruditapes philippinarum* [34,75]. In our study, the up-regulation of multiple galectins and tandem-repeated galectins upon QPX

infection also implies their contribution to the protection against protozoan infections, possibly facilitating parasite recognition. Not surprisingly, higher induction levels of these galectins were also noticed at 21 °C as compared to 13 °C (cluster 2), which again suggests a positive relationship between suitable environmental conditions and better immune performances in *M. mercenaria*. Interestingly, one QPX gene involved in polysaccharide biosynthesis showed up as DE gene in infected FL clam mantle tissue (cluster 6), which could reflect the intensity of the parasite proliferation at the temperature that favored disease establishment.

On the other hand, FL mantle samples constitutively exhibited a set of expression profiles very similar to those observed in hemocytes (Fig. S1). For example, the DE genes up-regulated at 21 °C have annotated functions generally related to protein synthesis (e.g. ribosomal proteins, small heat shock protein, alpha-B crystallin), cellular components (β -actins, laminin receptor) and metabolism (chitin deacetylase-like, cytochrome, protein kinases). Whereas at 13 °C, interestingly, the up-regulated DE genes were largely associated with cell respiration (e.g. nadh dehydrogenase subunit 1 and 3) and fatty acid desaturation (e.g. acyl-desaturase, such as, acyl delta desaturase), suggesting that these processes are involved in the biological adjustments of *M. mercenaria* in response to cold environment.

The total number of DE genes regulated by temperature and QPX infection in FL clam mantle is 311 (Fig. 2), which is a considerably smaller than that of hemocyte (877). This directly reflects the fact that hemocytes are more sensible and responsive to stimuli, possibly because they play a central role as sentinels that monitor and react immediately to changes and danger signals. Hemocytes are the primary immune cells in invertebrates, and alterations of host immune response are readily reflected by their gene expression profiles. Moreover, bivalve hemocytes also function in many other processes besides immune protection, such as tissue repair, shell production and nutrition [20], so temperature and QPX impacts imposed on all other processes can also be recorded in hemocytes. On the other hand, the makeup and functions of clam mantle tissues are more stable and homogeneous as compared to hemocytes, and this property can also be reflected in gene expression data. However, given the fact that bivalves have open circulatory system with hemocytes wandering in all tissue, gene expression changes monitored in mantle could encompass the response of circulating hemocytes in addition to constitutive mantle cells. Nevertheless, considering the typical localization of QPX lesions in *M. mercenaria*, changes induced inside mantle tissues provide insights into the molecular mechanisms at play during *in situ* host–pathogen interactions, regardless of their contributors.

4.3. Comparisons of gene expressions in FL and MA clams in response to temperature and QPX infection

Variation in resistance to QPX disease has been broadly reported in hard clams from different geographic and genetic origins. In general, better QPX resistance and host survival are more often associated with *M. mercenaria* stocks originating from northern states (Massachusetts, New York and New Jersey), while higher susceptibility and mortality are found in southern stocks (South Carolina, Florida) [7,16,24,69]. The reasons leading to the above observations of difference in resistance have been previously ascribed to 1) the poor adaptation of southern strains to the enzootic northern cold waters and 2) variations in genetic background among clam stocks that result in different abilities of the immune system to mount effective defense against the invading parasite [7,24]. However, results from the field and laboratory observations of [13,16] have played down the cause of “poor acclimation” from being a main disease-aggravating factor in southern

clam strains, leaving the factor of genetic variations, likely driven by the selective mortality caused by enzootic QPX in northern locales, to act as the most important determinant for hard clam susceptibility to QPX disease. In our study, significant difference in molecular response to QPX infection was observed between the susceptible (FL) and resistant (MA) clam strains, providing additional support to the argument that clams ability to resist QPX infection is closely associated with their genetic makeup. The comparison of expression profiles among defense-related genes before and after QPX induction between the two strains give further insights into gene candidates potentially regulating this trait.

As many as 1569 DE genes were found between challenged clams from both stocks held at both experimental temperatures (Supplementary file 5), highlighting a divergence in molecular responses between FL and MA clams to QPX infection. This dissimilarity in gene regulation might be related to myriad of biological processes, including pathways regulating both immune response and metabolism, and the observed difference in QPX resistance between the host strains might result from the collective effect of all processes involved. Interestingly, a large portion of the DE genes were up-regulated in MA clams when compared to FL clams (Fig. 5 and Supplementary file 5), especially those with putative functions related to immune response (e.g. PRRs, C1q and FReDs containing proteins). This suggests that the rather resistant MA clams can react to the parasite infection more strongly than susceptible FL clams. Furthermore, higher expression levels of these DE genes were observed in MA clams held at 13 °C as compared to those maintained at 21 °C, and also corresponded positively with both infection severity and host resistance. Therefore, it seems that MA clams can regulate the level of their immune proteins to tailor the severity of ongoing QPX infections, with higher expression levels associated with heavy infections. On the other hand, FL clams overexpressed DE genes that were related to metabolic and protein biosynthesis (Cluster 6, Supplementary file 5), which may be related to fast growth typically observed in southern aquacultured strains. Interestingly, the defense-related genes up-regulated in FL clams upon infection did not overlap with those induced in MA clams by QPX, which may be linked to the susceptibility of the former clams. This may also suggest the attempt of FL clams to possibly compensate for their inability to produce effective anti-QPX factors by triggering the up-regulations of universal defense-related proteins, and this compensatory overexpression of immune molecules were more efficient and pronounced at their optimal temperature (21 °C). Such strategy is energetically-savvy and might be considered as evidence that resistant MA clams have gone through selection processes that endowed them with better adaptations to survive in QPX enzootic areas [27,40,63]. The fact that QPX is widely distributed in northern cold waters has led to speculations that certain levels of selective mortality have taken place in the local clam stocks due to frequent QPX encounter, which may have increased the overall resistance in surviving animals [8,27]. Whereas the southern clam stocks may never have been exposed to the selection pressure imposed by this parasite since QPX has never been reported in waters further south than Virginia [27].

The over-expressed immune genes associated with MA clams were mostly those specifically induced by QPX as shown from the comparison between FLc and FLq clams discussed above, such as the C1q and FReD domain containing proteins (Fig. 6). Efficient targeting of foreign invaders is a key step for the activation of anti-parasite defense mechanisms and often result in the prompt neutralization of the invader. Higher resistance to QPX observed in MA clams could partly result from higher expression of these QPX-responsive PRRs, which were speculated to be the primary recognition proteins for QPX-specific molecular structures (see above).

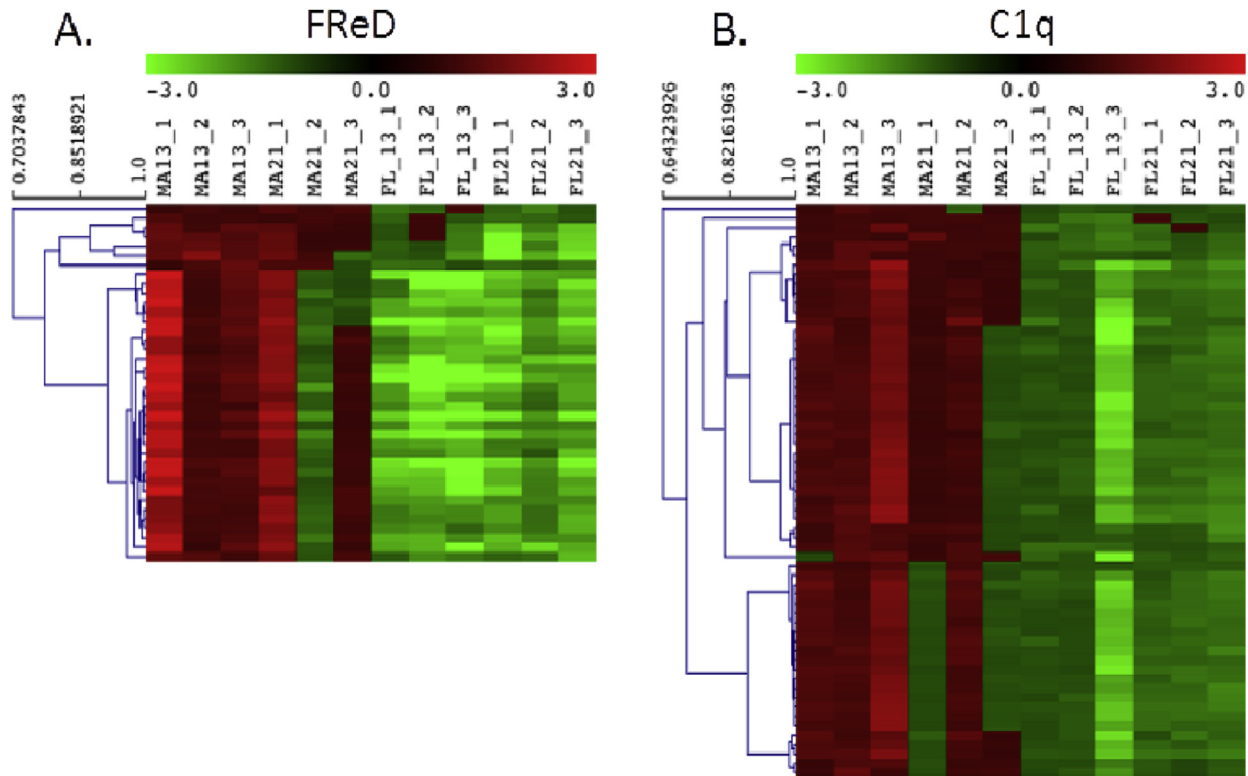


Fig. 6. Subsets of differentially expressed genes identified between QPX infected FL and MA clam mantles containing (A) fibrinogen-related domains (FReD) and (B) complement C1q domains. Gene expression heat maps reflect relative gene expression fold changes where a gradient of red to green represents a 3-fold or greater increase in gene expression to a 3-fold or greater decrease in gene expression. Columns represent the gene expression of individual samples from each treatment with each gene displayed horizontally. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

On the contrary, the broad under-expression of these proteins in FL clams suggests apparent incompetency of susceptible clams to mount sufficient defense response against QPX. In addition, expression level for these DE PRR genes appears to be correlated with infection intensity. For instance, the highest over-expression was generally measured in MA clams held at 13 °C, the temperature that causes the most severe QPX infections; and the up-regulation was not uniform in MA samples at 21 °C (Fig. 5). This last observation may come from the fact that MA clams held at 21 °C displayed a range of infection severities with some clams displaying complete signs of healing [14].

A great number of DE sequences containing C1q domain and FReD have been noticed (Fig. 6), possibly reflecting the high sequence diversification in these two gene families. In fact, beside *M. mercenaria*, members of these two gene families have been shown to display extensive sequence diversity in many molluscan species [29,30,35,45,76,84,85]. The broad sequence variability in these genes usually translates into immune molecules with highly diversified pathogen recognition domain structures [9,45,88]. Mechanisms leading to this diversification may include recombinatorial diversification, alternative splicing and somatic diversification through gene conversions and point mutations [30,88]. The somatic generation of polymorphism and diversification of these putative immune receptor sequences is important for the host to maintain a dynamic and rich repertoire of putative recognition molecules so that response against a variety of pathogen epitopes could be promptly mounted. In this study, the differential expression of diverse forms of C1qDCs and FREPs between MA and FL clams might suggest high responsiveness of these molecules against QPX, particularly when taking into consideration that the overexpression was mostly associated with the more resistant clam

stocks held at the condition favoring disease development (MA clams, 13 °C). The regulation of these genes likely help MA clams fight QPX, but can also contribute to the elimination of putative secondary pathogens.

It is widely recognized that invertebrates do not have acquired immunity, and their innate immune system exhibits less diversity of receptor repertoire leading to reduced specificity. Their ability to detect parasites exclusively relies on invariable germline-encoded immune receptors and effectors that interact with universal microbial antigens [43]. However, increasing evidence suggests the presence of sophisticated recognition systems in some invertebrate species, including echinoderms (sea urchin), insects (*Drosophila melanogaster* and *Anopheles gambiae*), crustaceans and mollusks (*Biomphalaria glabrata*) [5,21,49,79,87]. In addition, remarkably different immune responses were observed in *M. mercenaria* following QPX and bacterial challenge, suggesting the involvement of different immune pathways for the discrimination and elimination of different pathogen types [52]. Together, these observations imply the existence of a form of specific immunity in invertebrates, which has been suggested to be linked to those highly diversified immune molecules generated via various types of somatic diversification [45]. This broad reservoir of recognition molecules also serves as the source for the development of host adaptation to parasite-driven selective pressures [9,18,85,86]. For example, the *B. glabrata* FREPs are a group of highly variable receptors that precipitate variable antigens of trematode parasites [45]. Exceptional somatic diversifications exist among these molecules, creating quite individualized FREP pools that vary from one snail to another. Therefore, the recognition capacity has been dramatically enlarged in a random way, however individuals possessing the receptor variants that are capable of recognizing

specific antigens would be favored in an environment where they are exposed to corresponding pathogens, allowing these snails to survive the selective pressure [45]. The higher expression levels of C1qDCs and FREPs associated with QPX-resistant MA clams are very likely the result of QPX-derived selection process via similar mechanisms.

Overall, more and more studies advocate that invertebrate innate immunity has considerable specificity and is capable of discriminating between pathogens. Recent identification of several components of the lectin-based complement pathway from ascidians reveals that the primitive complement system is one of the most highly organized innate immune systems in invertebrates [25,26]. In fact, the complement system plays a pivotal role in innate immunity before the evolution of an adaptive immune system in vertebrates and is widely thought to act as an evolutionarily transitional mechanism that links innate immunity to acquired immunity [25,68]. Since both the FREPs and C1q proteins are important components initiating the complement system, a primitive complement-like system capable of providing tailored immune protection against various pathogens is speculated to also exist in *M. mercenaria*.

Although the expression of *M. mercenaria* C1qDCs and FREPs increases in response to QPX challenge, the nature of the QPX ligands that these receptors recognize are unknown. In addition, the specific roles of these molecules in *M. mercenaria* immunity remain mysterious. Future studies should focus on addressing the specific role of these molecules in hard clam immunity against QPX infection, and their mechanisms of interaction with various parasite antigens. In-depth understanding of these questions should shed light on the properties of anti-QPX factors present in *M. mercenaria*. It may lead to the discovery of promising molecular candidates for marker-assisted selection of disease resistant hard clam broodstocks to better control QPX disease and minimize losses caused by this parasite.

5. Conclusions

This study represents the first attempt to investigate the molecular immune response of the hard clam *M. mercenaria* using high-throughput techniques. The first *M. mercenaria* oligoarray was designed and used to explore transcriptomic changes in clams during QPX infection. Gene expression profiles were compared between naïve and QPX-challenged clams at temperatures known to affect infection establishment in order to gain an understanding of molecular mechanisms of host response at conditions known to favor disease establishment or healing. A large set of defense-related genes was regulated in infected clams, including genes involved in microbe recognition, pathogen killing, metabolism and stress response. The results suggest that the modulation of disease development by temperature is mainly through alteration of the extent of constitutive and QPX-inducible immune responses. Comparison of gene expression profiles between susceptible and resistant clam broodstocks identified molecular candidates that could mediate clam resistance against QPX. Special interest was placed upon the key families of highly diversified recognition molecules, such as C1qDCs and FREPs, which have not only been significantly induced after the parasite challenge but also displayed higher expression in resistant clams as compared to the susceptible stock. The findings underscore the role of these receptors in QPX recognition and possibly mediation of subsequent parasite elimination via the initiation of a primitive complement-like system. However, further investigations are needed to characterize the nature of these molecular components and probe their specific role during *M. mercenaria*-QPX interactions, with perspectives on their molecular functions, diversification mechanisms and interactions

with various pathogen epitopes.

Acknowledgments

This research was supported by projects R/XG-19 (to BA, EPE and AT) and R/FBM-34 (to BA and EPE), funded under award NA07OAR4170010 from the National Sea Grant College Program of NOAA to the Research Foundation of State University of New York on behalf of New York Sea Grant. The study was also partially supported by project IOS 1050596 (to BA and EPE) supported by the National Science Foundation. The statements, findings, conclusions, views and recommendations are those of the authors and do not necessarily reflect the views of any of those organizations.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.fsi.2015.12.006>.

References

- [1] B. Allam, E. Pales Espinosa, A. Tanguy, F. Jeffroy, C. Le Bris, C. Paillard, Transcriptional changes in Manila clam (*Ruditapes philippinarum*) in response to brown ring disease, *Fish Shellfish Immunol.* 41 (2014) 2–11.
- [2] R.S. Anderson, B.S. Kraus, S.E. McGladdery, K.S. Reece, N.A. Stokes, A thraustochytrid protist isolated from *Mercenaria mercenaria*: molecular characterization and host defense responses, *Fish Shellfish Immunol.* 15 (2003) 183–194.
- [3] S. Blandin, S.H. Shiao, L.F. Moita, C.J. Janse, A.P. Waters, F.C. Kafatos, E.A. Levashina, Complement-like protein TEP1 is a determinant of vectorial capacity in the malaria vector *Anopheles gambiae*, *Cell* 116 (2004) 661–670.
- [4] S.A. Blandin, E. Marois, E.A. Levashina, Antimalarial responses in *Anopheles gambiae*: from a complement-like protein to a complement-like pathway, *Cell Host Microbe* 3 (2008) 364–374.
- [5] D. Brites, S. McTaggart, K. Morris, J. Anderson, K. Thomas, I. Colson, T. Fabbro, T.J. Little, D. Ebert, L. Du Pasquier, The Dscam homologue of the crustacean *Daphnia* is diversified by alternative splicing like in insects, *Mol. Biol. Evol.* 25 (2008) 1429–1439.
- [6] K. Buchmann, Evolution of innate immunity: clues from invertebrates via fish to mammals, *Front. Immunol.* 5 (2014).
- [7] L.M.R. Calvo, S.E. Ford, J.N. Kraeuter, D.F. Leavitt, R. Smolowitz, E.M. Bureson, Influence of host genetic origin and geographic location on QPX disease in Northern quahogs (=hard clams), *Mercenaria mercenaria*, *J. Shellfish Res.* 26 (2007) 109–119.
- [8] L.M.R. Calvo, J.G. Walker, E.M. Bureson, Prevalence and distribution of QPX, Quahog Parasite Unknown, in hard clams *Mercenaria mercenaria* in Virginia, *USA. Dis. Aquat. Org.* 33 (1998) 209–219.
- [9] L. Cerenius, K. Soderhall, Variable immune molecules in invertebrates, *J. Exp. Biol.* 216 (2013), 4313–4319.
- [10] F.L.E. Chu, J.F. LaPeyre, Development of disease caused by the parasite, *Perkinsus marinus* and defense-related Hemolymph factors in 3 populations of Oysters from the Chesapeake Bay, USA, *J. Shellfish Res.* 12 (1993) 21–27.
- [11] S.F. Dahl, B. Allam, Laboratory transmission studies of QPX disease in the northern quahog (=hard clam): development of an infection procedure, *J. Shellfish Res.* 26 (2007) 383–389.
- [12] S.F. Dahl, B. Allam, QPX disease progress in cultured and wild type hard clams in New York waters, *J. Shellfish Res.* 27 (2008), 1000–1000.
- [13] S.F. Dahl, M. Perrigault, B. Allam, Laboratory transmission studies of QPX disease in the hard clam: Interactions between different host strains and pathogen isolates, *Aquaculture* 280 (2008) 64–70.
- [14] S.F. Dahl, M. Perrigault, Q. Liu, J.L. Collier, D.A. Barnes, B. Allam, Effects of temperature on hard clam (*Mercenaria mercenaria*) immunity and QPX (Quahog Parasite Unknown) disease development: I. Dynamics of QPX disease, *J. Invertebr. Pathol.* 106 (2011a) 314–321.
- [15] S.F. Dahl, M. Perrigault, Q.Q. Liu, J.L. Collier, D.A. Barnes, B. Allam, Effects of temperature on hard clam (*Mercenaria mercenaria*) immunity and QPX (Quahog Parasite Unknown) disease development: I. Dynamics of QPX disease, *J. Invertebr. Pathol.* 106 (2011b) 314–321.
- [16] S.F. Dahl, J. Thiel, B. Allam, Field performance and Qpx disease progress in cultured and wild-type strains of *Mercenaria mercenaria* in New York waters, *J. Shellfish Res.* 29 (2010) 83–90.
- [17] S.M. Degnan, The surprisingly complex immune gene repertoire of a simple sponge, exemplified by the NLR genes: a capacity for specificity? *Dev. Comp. Immunol.* 48 (2015) 269–274.
- [18] N.M. Dheilly, D. Duval, G. Mouahid, R. Emans, J.-F. Allienne, R. Galinier, C. Genthon, E. Dubois, L. Du Pasquier, C.M. Adema, C. Grunau, G. Mitta, B. Gourbal, A family of variable immunoglobulin and lectin domain containing molecules in the snail *Biomphalaria glabrata*, *Dev. Comp. Immunol.* 48 (2015)

- 234–243.
- [19] A.W. Dodds, M. Matsushita, The phylogeny of the complement system and the origins of the classical pathway, *Immunobiology* 212 (2007) 233–243.
 - [20] L. Donaghy, C. Lambert, K.S. Choi, P. Soudant, Hemocytes of the carpet shell clam (*Ruditapes decussatus*) and the Manila clam (*Ruditapes philippinarum*): current knowledge and future prospects, *Aquaculture* 297 (2009) 10–24.
 - [21] Y. Dong, H.E. Taylor, G. Dimopoulos, AgDscam, a hypervariable immunoglobulin domain-containing receptor of the *Anopheles gambiae* innate immune system, *PLoS Biol.* 4 (2006) e229.
 - [22] A.D.M. Dove, P.R. Bowser, R.M. Cerrato, Histological analysis of an outbreak of QPX disease in wild hard clams *Mercenaria mercenaria* in New York, *J. Aquat. Animal Health* 16 (2004) 246–250.
 - [23] M.B. Eisen, P.T. Spellman, P.O. Brown, D. Botstein, Cluster analysis and display of genome-wide expression patterns, *Proc. Natl. Acad. Sci. U. S. A.* 95 (1998) 14863–14868.
 - [24] S.E. Ford, J.N. Kraeuter, R.D. Barber, G. Mathis, Aquaculture-associated factors in QPX disease of hard clams: density and seed source, *Aquaculture* 208 (2002) 23–38.
 - [25] T. Fujita, Evolution of the lectin–complement pathway and its role in innate immunity, *Nat. Rev. Immunol.* 2 (2002) 346–353.
 - [26] T. Fujita, M. Matsushita, Y. Endo, The lectin–complement pathway – its role in innate immunity and evolution, *Immunol. Rev.* 198 (2004) 185–202.
 - [27] R.J. Gast, D.M. Moran, C. Audemard, M.M. Lyons, J. DeFavari, K.S. Reece, D. Leavitt, R. Smolowitz, Environmental distribution and persistence of Quahog Parasite Unknown (QPX), *Dis. Aquat. Org.* 81 (2008) 219–229.
 - [28] M. Gerdel, C. Manfrin, G. De Moro, A. Figueras, B. Novoa, P. Venier, A. Pallavicini, The C1q domain containing proteins of the Mediterranean mussel *Mytilus galloprovincialis*: a widespread and diverse family of immune-related molecules, *Dev. Comp. Immunol.* 35 (2011) 635–643.
 - [29] R. Ghai, P. Waters, L.T. Roumenina, M. Gadjeva, M.S. Kojouharova, K.B. Reid, R.B. Sim, U. Kishore, C1q and its growing family, *Immunobiology* 212 (2007) 253–266.
 - [30] P.C. Hanington, M.A. Forys, E.S. Loker, A somatically diversified defense factor, FREP3, is a determinant of snail resistance to schistosome infection, *PLoS Negl. Trop. Dis.* 6 (2012) e1591.
 - [31] K. Heise, M.S. Estevez, S. Puntarulo, M. Galleano, M. Nikinmaa, H.O. Pörtner, D. Abele, Effects of seasonal and latitudinal cold on oxidative stress parameters and activation of hypoxia inducible factor (HIF-1) in zoarcid fish, *J. Comp. Physiol. B* 177 (2007) 765–777.
 - [32] K. Heise, S. Puntarulo, M. Nikinmaa, M. Lucassen, H.-O. Pörtner, D. Abele, Oxidative stress and HIF-1 DNA binding during stressful cold exposure and recovery in the North Sea eelpout (*Zoarces viviparus*), *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* 143 (2006) 494–503.
 - [33] L.A. Hertel, C.M. Adema, E.S. Loker, Differential expression of FREP genes in two strains of *Biomphalaria glabrata* following exposure to the digenetic trematodes *Schistosoma mansoni* and *Echinostoma paraensei*, *Dev. Comp. Immunol.* 29 (2005) 295–303.
 - [34] J.Y. Kim, Y.M. Kim, S.K. Cho, K.S. Choi, M. Cho, Noble tandem-repeat galectin of Manila clam *Ruditapes philippinarum* is induced upon infection with the protozoan parasite *Perkinsus olseni*, *Dev. Comp. Immunol.* 32 (2008) 1131–1141.
 - [35] R.B. Leite, M. Milan, A. Coppe, S. Bortoluzzi, A. dos Anjos, R. Reinhardt, C. Saavedra, T. Patarnello, M. Canceled, L. Bargelloni, mRNA-Seq and microarray development for the Grooved carpet shell clam, *Ruditapes decussatus*: a functional approach to unravel host–parasite interaction, *BMC Genomics* 14 (2013) 741.
 - [36] Q. Liu, J.L. Collier, S. Pawagi, D. Barnes, B. Allam, Quahog parasite unknown (QPX) dynamics in wild hard clams from Raritan Bay, New York, *J. Shellfish Res.* 27 (2008), 1024–1024.
 - [37] Q.Q. Liu, B. Allam, J.L. Collier, Quantitative real-time PCR assay for QPX (*Thraustochytridae*), a parasite of the hard clam (*Mercenaria mercenaria*), *Appl. Environ. Microbiol.* 75 (2009) 4913–4918.
 - [38] E.S. Loker, C.M. Adema, S.M. Zhang, T.B. Kepler, Invertebrate immune systems—not homogeneous, not simple, not well understood, *Immunol. Rev.* 198 (2004) 10–24.
 - [39] M.M. Lyons, R. Smolowitz, M. Gomez-Chiarri, J.E. Ward, Epizootiology of Quahog Parasite Unknown (QPX) disease in northern quahogs (=hard clams) *Mercenaria mercenaria*, *J. Shellfish Res.* 26 (2007) 371–381.
 - [40] M.M. Lyons, J.E. Ward, R. Smolowitz, K.R. Uhlinger, R.J. Gast, Lethal marine snow: pathogen of bivalve mollusc concealed in marine aggregates, *Limnol. Oceanogr.* 50 (2005) 1983–1988.
 - [41] P.A.Y. Maas, S.J. Kleinschuster, M.J. Dykstra, R. Smolowitz, J. Parent, Molecular characterization of QPX (Quahog Parasite Unknown), a pathogen of *Mercenaria mercenaria*, *J. Shellfish Res.* 18 (1999) 561–567.
 - [42] G.S. MacCallum, S.E. McGladdery, Quahog Parasite Unknown (QPX) in the northern quahog *Mercenaria mercenaria* (Linnaeus, 1758) and *M. mercenaria* var. *notata* from Atlantic Canada, survey results from three maritime provinces, *J. Shellfish Res.* 19 (2000) 43–50.
 - [43] R. Medzhitov, C.A. Janeway Jr., Innate immunity: the virtues of a nonclonal system of recognition, *Cell* 91 (1997) 295–298.
 - [44] M. Milan, A. Coppe, R. Reinhardt, L.M. Canceled, R.B. Leite, C. Saavedra, C. Ciofi, G. Chelazzi, T. Patarnello, S. Bortoluzzi, L. Bargelloni, Transcriptome sequencing and microarray development for the Manila clam, *Ruditapes philippinarum*: genomic tools for environmental monitoring, *BMC Genomics* 12 (2011) 234.
 - [45] Y. Mone, B. Gourbal, D. Duval, L. Du Pasquier, S. Kieffer-Jaquinod, G. Mitta, A large repertoire of parasite epitopes matched by a large repertoire of host immune receptors in an invertebrate host/parasite model, *PLoS Negl. Trop. Dis.* 4 (2010).
 - [46] R. Moreira, M. Milan, P. Balseiro, A. Romero, M. Babbucci, A. Figueras, L. Bargelloni, B. Novoa, Gene expression profile analysis of Manila clam (*Ruditapes philippinarum*) hemocytes after a *Vibrio alginolyticus* challenge using an immune-enriched oligo-microarray, *Bmc Genomics* 15 (2014) 267.
 - [47] G. Nunez-Acuna, A.E. Aballay, H. Hegaret, A.P. Astuya, C. Gallardo-Escarate, Transcriptional responses of *Mytilus chilensis* exposed in vivo to saxitoxin (STX), *J. Molluscan Stud.* 79 (2013) 323–331.
 - [48] C. Paillard, B. Allam, R. Oubella, Effect of temperature on defense parameters in Manila clam *Ruditapes philippinarum* challenged with *Vibrio tapetis*, *Dis. Aquat. Org.* 59 (2004) 249–262.
 - [49] Z. Pancer, Dynamic expression of multiple scavenger receptor cysteine-rich genes in coelomocytes of the purple sea urchin, *Proc. Natl. Acad. Sci. U. S. A.* 97 (2000) 13156–13161.
 - [50] M. Perrigault, B. Allam, Cytotoxicity of quahog parasite unknown (QPX) toward hard clam (*Mercenaria mercenaria*) haemocytes and interactions between different pathogen isolates and host strains, *Parasitology* 136 (2009) 1281–1289.
 - [51] M. Perrigault, B. Allam, Differential immune response in the hard clam (*mercenaria mercenaria*) against bacteria and the protistan pathogen QPX (quahog parasite unknown), *Fish Shellfish Immunol.* 32 (2012a) 1124–1134.
 - [52] M. Perrigault, B. Allam, Differential immune response in the hard clam (*mercenaria mercenaria*) against bacteria and the protistan pathogen QPX (quahog parasite unknown), *Fish Shellfish Immunol.* 32 (2012b) 1124–1134.
 - [53] M. Perrigault, D.M. Bugge, C.C. Hao, B. Allam, Modulatory effects of hard clam (*Mercenaria mercenaria*) tissue extracts on the in vitro growth of its pathogen QPX, *J. Invertebr. Pathol.* 100 (2009a) 1–8.
 - [54] M. Perrigault, C.H. Chen, D.M. Bugge, B. Allam, Investigations of anti-QPX factors in plasma from susceptible and resistant hard clam stocks, *J. Shellfish Res.* 27 (2008) 1039–1040.
 - [55] M. Perrigault, S.F. Dahl, E.P. Espinosa, B. Allam, Effects of salinity on hard clam (*Mercenaria mercenaria*) defense parameters and QPX disease dynamics, *J. Invertebr. Pathol.* 110 (2012) 73–82.
 - [56] M. Perrigault, S.F. Dahl, E.P. Espinosa, L. Gambino, B. Allam, Effects of temperature on hard clam (*Mercenaria mercenaria*) immunity and QPX (Quahog Parasite Unknown) disease development: II. Defense parameters, *J. Invertebr. Pathol.* 106 (2011) 322–332.
 - [57] M. Perrigault, S.F. Dahl, Q.Q. Liu, J.L. Collier, D. Barnes, B. Allam, Effects of environmental factors on *Mercenaria mercenaria* Immunity, Qpx survival and host–pathogen interactions, *J. Shellfish Res.* 28 (2009b), 720–720.
 - [58] M. Perrigault, A. Tanguy, B. Allam, Identification and expression of differentially expressed genes in the hard clam, *Mercenaria mercenaria*, in response to quahog parasite unknown (QPX), *Bmc Genomics* 10 (2009c) 377.
 - [59] M. Perrigault, A. Tanguy, B. Allam, Identification and expression of differentially expressed genes in the hard clam, *Mercenaria mercenaria*, in response to quahog parasite unknown (QPX), *Bmc Genomics* 10 (2009d).
 - [60] H.O. Pörtner, Climate variations and the physiological basis of temperature dependent biogeography: systemic to molecular hierarchy of thermal tolerance in animals, *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* 132 (2002) 739–761.
 - [61] H.O. Pörtner, B. Berdal, R. Blust, O. Brix, A. Colosimo, B. De Wachter, A. Giuliani, T. Johansen, T. Fischer, R. Knust, G. Lannig, G. Naevdal, A. Nedenes, G. Nyhammer, F.J. Sartoris, I. Serendero, P. Sirabella, S. Thorkildsen, M. Zakkartsev, Climate induced temperature effects on growth performance, fecundity and recruitment in marine fish: developing a hypothesis for cause and effect relationships in Atlantic cod (*Gadus morhua*) and common eelpout (*Zoarces viviparus*), *Cont. Shelf Res.* 21 (2001) 1975–1997.
 - [62] M.A. Ragan, G.S. MacCallum, C.A. Murphy, J.J. Cannone, R.R. Gutell, S.E. McGladdery, Protistan parasite QPX of hard-shell clam *Mercenaria mercenaria* is a member of Labyrinthulomycota, *Dis. Aquat. Org.* 42 (2000) 185–190.
 - [63] L.M. Ragone Calvo, J.G. Walker, E.M. Bureson, Prevalence and distribution of QPX, Quahog Parasite Unknown, in hard clams *Mercenaria mercenaria* in Virginia, USA, *Dis. Aquat. Org.* 33 (1998) 209–219.
 - [64] J.M. Robinson, D.D. Vandre, Stimulus-dependent alterations in macrophage microtubules: increased tubulin polymerization and detyrosination, *J. Cell Sci.* 108 (Pt 2) (1995) 645–655.
 - [65] E. Rubin, A. Tanguy, M. Perrigault, B. Allam, Virulence-Related Genes in Qpx, the Thraustochytrid parasite of the hard clam *Mercenaria mercenaria*, *J. Shellfish Res.* 30 (2011), 550–550.
 - [66] E. Rubin, A. Tanguy, M. Perrigault, E. Pales Espinosa, B. Allam, Characterization of the transcriptome and temperature-induced differential gene expression in QPX, the thraustochytrid parasite of hard clams, *BMC Genomics* 15 (2014) 245.
 - [67] A.I. Saeed, V. Sharov, J. White, J. Li, W. Liang, N. Bhagabati, J. Braisted, M. Klapa, T. Currier, M. Thiagarajan, A. Sturn, M. Snuffin, A. Rezantsev, D. Popov, A. Ryltsov, E. Kostukovich, I. Borisovsky, Z. Liu, A. Vinsavich, V. Trush, J. Quackenbush, TM4: a free, open-source system for microarray data management and analysis, *Biotechniques* 34 (2003) 374–378.
 - [68] H. Sekine, A. Kenjo, K. Azumi, G. Ohi, M. Takahashi, R. Kasukawa, N. Ichikawa, M. Nakata, T. Mizuochi, M. Matsushita, Y. Endo, T. Fujita, An ancient lectin-dependent complement system in an ascidian: novel lectin isolated from

- the plasma of the solitary ascidian, *Halocynthia roretzi*, J. Immunol. 167 (2001) 4504–4510.
- [69] R. Smolowitz, J. Defaveri, W. Walton, D. Murphy, D. Leavitt, The interaction of temperature and hard clam (*Mercenaria mercenaria*) strain on the occurrence of QPX disease in the laboratory and in the field, J. Shellfish Res. 27 (2008) 1052–1053.
- [70] R. Smolowitz, D. Leavitt, F. Perkins, Observations of a protistan disease similar to QPX in *Mercenaria mercenaria* (hard clams) from the coast of Massachusetts, J. Invertebr. Pathol. 71 (1998) 9–25.
- [71] G.K. Smyth, T. Speed, Normalization of cDNA microarray data, Methods 31 (2003) 265–273.
- [72] K. Soderhall, Invertebrate Immunity. Advances in Experimental Medicine and Biology, Landes Bioscience and Springer Science+Business Media, LLC, Boston, MA, 2010 pp. xxiv, 316 pp.
- [73] A. Soukas, P. Cohen, N.D. Socci, J.M. Friedman, Leptin-specific patterns of gene expression in white adipose tissue, Genes Dev. 14 (2000) 963–980.
- [74] N.A. Stokes, L.M.R. Calvo, K.S. Reece, E.M. Burrenson, Molecular diagnostics, field validation, and phylogenetic analysis of Quahog Parasite Unknown (QPX), a pathogen of the hard clam *Mercenaria mercenaria*, Dis. Aquat. Org. 52 (2002) 233–247.
- [75] S. Tasumi, G.R. Vasta, a galectin of unique domain organization from hemocytes of the Eastern Oyster (*Crassostrea virginica*) is a receptor for the protistan parasite *Perkinsus marinus*, J. Immunol. 179 (2007) 3086–3098.
- [76] P. Venier, L. Varotto, U. Rosani, C. Millino, B. Celegato, F. Bernante, G. Lanfranchi, B. Novoa, P. Roch, A. Figueras, A. Pallavicini, Insights into the innate immunity of the Mediterranean mussel *Mytilus galloprovincialis*, BMC Genomics 12 (2011) 69.
- [77] L. Wang, L. Wang, P. Kong, J. Yang, H. Zhang, M. Wang, Z. Zhou, L. Qiu, L. Song, A novel C1qDC protein acting as pattern recognition receptor in scallop *Argopecten irradians*, Fish Shellfish Immunol. 33 (2012) 427–435.
- [78] S. Wang, E. Peatman, H. Liu, D. Bushek, S.E. Ford, H. Kucuktas, J. Quilang, P. Li, R. Wallace, Y. Wang, Microarray analysis of gene expression in eastern oyster (*Crassostrea virginica*) reveals a novel combination of antimicrobial and oxidative stress host responses after dermo (*Perkinsus marinus*) challenge, Fish Shellfish Immunol. 29 (2010) 921–929.
- [79] F.L. Watson, R. Puttmann-Holgado, F. Thomas, D.L. Lamar, M. Hughes, M. Kondo, V.I. Rebel, D. Schmucker, Extensive diversity of Ig-superfamily proteins in the immune system of insects, Science 309 (2005) 1874–1878.
- [80] S.-y. Xu, L. Wang, L. Wang, H. Zhang, Z. Zhou, V.S. Siva, L. Song, A C1q domain containing protein from Scallop *Chlamys farreri* serving as pattern recognition receptor with heat-aggregated IgG binding activity, PLoS ONE 7 (2012) e43289.
- [81] K.Y. Yeung, D.R. Haynor, W.L. Ruzzo, Validating clustering for gene expression data, Bioinformatics 17 (2001) 309–318.
- [82] X. Yue, H. Wang, X. Huang, C. Wang, X. Chai, C. Wang, B. Liu, Single nucleotide polymorphisms in i-type lysozyme gene and their correlation with vibrio-resistance and growth of clam *Meretrix meretrix* based on the selected resistance stocks, Fish Shellfish Immunol. 33 (2012) 559–568.
- [83] H. Zhang, L. Song, C. Li, J. Zhao, H. Wang, L. Qiu, D. Ni, Y. Zhang, A novel C1q-domain-containing protein from Zhikong scallop *Chlamys farreri* with lipopolysaccharide binding activity, Fish Shellfish Immunol. 25 (2008) 281–289.
- [84] H. Zhang, L. Wang, L. Song, X. Song, B. Wang, C. Mu, Y. Zhang, A fibrinogen-related protein from bay scallop *Argopecten irradians* involved in innate immunity as pattern recognition receptor, Fish Shellfish Immunol. 26 (2009) 56–64.
- [85] L. Zhang, L. Li, Y. Zhu, G. Zhang, X. Guo, Transcriptome analysis reveals a rich gene set related to innate immunity in the Eastern Oyster (*Crassostrea virginica*), Mar. Biotechnol. 16 (2013) 17–33.
- [86] S.-M. Zhang, E.S. Loker, The FREP gene family in the snail *Biomphalaria glabrata*: additional members, and evidence consistent with alternative splicing and FREP retrosequences, Dev. Comp. Immunol. 27 (2003) 175–187.
- [87] S.M. Zhang, Diversification of Ig Superfamily Genes in an Invertebrate, Science 305 (2004) 251–254.
- [88] S.M. Zhang, E.S. Loker, Representation of an immune responsive gene family encoding fibrinogen-related proteins in the freshwater mollusc *Biomphalaria glabrata*, an intermediate host for *Schistosoma mansoni*, Gene 341 (2004) 255–266.