Version of Record: https://www.sciencedirect.com/science/article/pii/S1050464815302631
Manuscript_e537702c1352408ae85d06f9425c2431

1
2
3
Alterations of the immune transcriptome in resistant and susceptible hard clams (*Mercenaria mercenaria*) in response to Quahog Parasite Unknown (QPX) and temperature

4
5
6
Kailai Wang[1], Emmanuelle Pales Espinosa[1], Arnaud Tanguy[2], Bassem Allam[1§],

7

8
9
[1] *School of Marine and Atmospheric Sciences, Stony Brook University, Stony Brook, NY 11794, USA*

10

11
[2] *UPMC Université Paris 6, Station Biologique de Roscoff, 29682 Roscoff, France*

12
13
14
15
16
17
18
[§]Corresponding author. Tel.: +1 631 632 8745; Fax: +1 631 632 8915
E-mail address: Bassem.Allam@stonybrook.edu

19
20
21

© 2015 published by Elsevier. This manuscript is made available under the Elsevier user license
https://www.elsevier.com/open-access/userlicense/1.0/

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 (15K 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.

Keywords:

Hard clam; QPX; Microarray; Gene expression; Temperature; Immunity

58 **1. Introduction**

59 The hard clam *Mercernaria mercenaria* is among the most commercially important
60 bivalves in the United States. It is widely exploited along the North American Atlantic coasts
61 from the Maritime Provinces of Canada to Florida. *M. mercenaria* is often considered as a
62 robust bivalve species and relatively few infectious agents were reported to cause problems in
63 wild and aquacultured stocks. Among these, the Quahog Parasite Unknown (QPX) is a
64 protistan parasite known to cause lethal infections and substantial losses in the hard clam
65 industry (Ford et al., 2002; Lyons et al., 2007; Maas et al., 1999; Ragan et al., 2000;
66 Smolowitz et al., 1998; Stokes et al., 2002). QPX is considered as an opportunistic pathogen
67 and is widely present in coastal environments (Gast et al., 2008; Liu et al., 2009; Lyons et al.,
68 2005), where it can be found associated with a variety of substrates such as seawater,
69 sediment and marine aggregates (Liu et al., 2008; Liu et al., 2009; MacCallum and
70 McGladdery, 2000; Ragone Calvo et al., 1998). Common encounters between QPX and *M.*
71 *mercenaria* are therefore considered frequent but usually do not result in disease outbreaks
72 until other disease-favoring factors are prevalent (Gast et al., 2008; Liu et al., 2009; Lyons et
73 al., 2005). Such factors may include the presence of highly virulent parasite variants,
74 susceptible host strains and shifts of environmental conditions that either add to the pathogen
75 abundance or infectivity, or lower the resistance of the host (Dahl et al., 2011; Perrigault et
76 al., 2011).

77 Previous studies of QPX disease have demonstrated a significant association between
78 temperature and *M. mercenaria* genetic background and disease development. For instance,
79 QPX infection is considered as a "cold-water disease" since the infection has never been
80 detected in clams further south of Virginia where the water temperature is comparatively
81 warmer. Laboratory infection trials also reported higher disease prevalence in clams held at
82 13 °C as compared to temperatures of 21 °C and higher, where parasite establishment was
83 largely inhibited and signs of host healing were detected (Dahl and Allam, 2008; Dahl et al.,
84 2011a; Perrigault et al., 2011). In fact, environmental factors, such as temperature, salinity
85 and dissolved oxygen, can substantially affect the physiology of aquatic animals.
86 Temperature, of special importance, has been demonstrated to significantly modulate host
87 immune performance and pathogen virulence, thus changing the pattern of host-pathogen
88 interactions and affecting the development of disease (Chu and LaPeyre, 1993; Paillard et al.,
89 2004; Perrigault et al., 2012; Perrigault et al., 2011). On the other hand, marked differences in
90 QPX susceptibility has been observed between clams from different genetic backgrounds.
91 Generally, clams originating from the warm southern states (e.g. Florida and South Carolina)
92 appear to be more sensitive to QPX infection than those from relatively cold northern states
93 (e.g. Massachusetts, New York, New Jersey), suggesting the existence of genetically
94 controlled factors that regulate *M. mercenaria* resistance against this parasite (Calvo et al.,
95 2007; Dahl et al., 2008; Dahl et al., 2010; Ford et al., 2002).

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

varied considerably between resistant and susceptible clam stocks (Perrigault and Allam, 2012a).

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 (Anderson et al., 2003; Smolowitz et al., 1998). 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 (Calvo et al., 1998; Dahl and Allam, 2007; Dahl et al., 2010; Dove et al., 2004). Interestingly, the presence of factors inhibiting QPX growth has been reported in clam plasma (Perrigault et al., 2008), and preliminary molecular investigations also demonstrated the modulation of several stress- and defense-related genes during QPX disease development (Perrigault et al., 2009d). 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 (Buchmann, 2014; Degnan, 2015; Soderhall, 2010). 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 (Allam et al., 2014; Leite et al., 2013; Milan et al., 2011; Moreira et al., 2014; Nunez-Acuna et al., 2013; Rubin et al., 2014; Wang et al., 2010). 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 (Farm Raised Clams, St James City, FL) and MA clams presumably infected by QPX were collected from an enzootic clamming area in Wellfleet Harbor. Disease

155 status of subsets from each batch was checked by a specific quantitative real-time PCR assay
156 (Liu et al., 2009) and QPX prevalence was equal to 63% in the MA clams and 0% in the
157 Florida stock (n = 30 clams/batch). Clams were acclimated at 21 ± 1 °C in 150-L tanks with
158 re-circulating water (30 ppt) upon arrival. After 1-week acclimation, half of the FL clams
159 were challenged with QPX as described previously (Dahl et al., 2011b) by injecting *in vitro*
160 cultured parasite cells into the pericardial cavity (5×10^4 cells/clam), and the other half
161 received injection of sterile culture media as controls. Following injection, both control (FLc)
162 and QPX challenged (FLq) FL clams, as well as the naturally infected MA clams were
163 separately transferred to 40-L tanks and maintained at 13 and 21 °C. Temperature adjustment
164 for the 13 °C treatment was performed within 8 days by decreasing the temperature by 1 °C
165 per day as previously described in (Dahl et al., 2011b) and (Perrigault et al., 2011). For each
166 clam group (MA, FLc, FLq), a total of 60 clams were randomly assigned into 3 replicate
167 tanks (20 clams per tank) held at each temperature condition (13 and 21 °C). All tanks were
168 individually equipped with re-circulating filtration systems and aerated continuously. Water
169 quality, temperature, salinity (30 ppt) and ammonia level were monitored and adjusted
170 weekly. Clams were fed daily with commercial algae (DT's Live Phytoplankton, Sycamore,
171 IL) and monitored twice a day for mortality.

172 *2.2 RNA samples*

173 After 2-month incubation at targeted temperatures, three clams were randomly sampled
174 from each replicate tank, totaling 9 clams per clam type and per temperature condition. This
175 timeframe was chosen because it was shown to be sufficient for QPX to initiate infection in
176 susceptible clams or for clams to prompt healing under optimal conditions (Dahl and Allam,
177 2007; Dahl et al., 2011b). Clams were individually processed to collect hemolymph and
178 mantle tissues. Hemolymph (generally 1.2–1.8 ml) was withdrawn from the adductor muscle
179 sinus with a 1-ml syringe and centrifuged (700g, 10 min, 4 °C) to pellet the hemocytes from
180 the acellular fraction. Mantle biopsies and hemocyte pellets were flash-frozen in liquid
181 nitrogen and immediately stored at -80 °C until processed for RNA extraction. Trizol reagent
182 (MRC, Inc., Cincinnai, OH, USA) was used to isolate RNA from hemocytes and mantle
183 tissues according to the manufacturer's protocol. RNA quality and quantity were assessed
184 using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, WI, USA).
185 RNA samples were pooled using the same RNA quantity from each individual to generate 3
186 pools per condition (tissue type, clam strain, temperature). A total of 30 RNA pools (only
187 mantle RNA samples were assessed for MA clams) were generated and used for the
188 downstream reactions and oligoarray hybridization (Table. 1).

189 *2.3 Oligoarray design, construction and hybridization*

190 Our *in situ* synthesized oligoarrays were produced based on sets of expressed sequence
191 tags (ESTs) obtained from a previous SSH libraries (Perrigault et al., 2009d) as well as a
192 transcriptome library generated from 454 sequencing of RNA obtained from *M. mercenaria*
193 mantle tissues, gills, digestive gland and hemocytes (*unpublished data*). Sequences were
194 annotated using the Blast2GO software (http://www.blast2go.com/b2ghome) with blast
195 search against NCBI non-redundant (nr) database (blastx, E-value cut off of 10 E^{-5}). These
196 included 3,092 curated annotated sequences and 11,166 non-annotated sequences with a focus
197 on sequences generated from hemocytes (7,839 sequences) to emphasize the discovery of
198 novel immune-related transcripts. All sequences were submitted to Agilent eArray application
199 (https://earray.chem.agilent.com/earray/) for probe production, with 1 probe (60-mer)
200 produced for each single submitted sequence. In parallel, a set of 595 probes designed based
201 on annotated QPX ESTs (Rubin et al., 2011) were also incorporated into this array in an
202 attempt to target potential molecular evidence of parasite response to the host in different

203 clam broodstocks and temperature conditions. Probes were synthesized *in situ* (14,853) along
204 with positive and negative controls (891) using 8×15K-feature Agilent format slides and a
205 total of 15,744 probes were included on the oligoarray.

206 Cyanine dyes (Cy3 or Cy5) labeled complementary RNA (cRNA) was synthesized from
207 150 ng of RNA purified from mantles and hemocytes of FLc, FLq and MA clams (as
208 described in 2.2, Table 1) using the Two-Color Microarray-Based Gene Expression Analysis
209 Protocol (Quick Amp Labeling) according to the manufacturer's manual. Labeled cRNA was
210 purified using Illustra CyScribe GFX Purification Kit (GE Healthcare). cRNA quantity and
211 quality (including dye incorporation) were determined by spectrophotometry (Nanodrop 1000
212 ND-1000 spectrophotometer, Thermo Scientific, Wilmington, WI, USA). Samples were
213 considered satisfactory if cRNA concentration and incorporation efficiency exceeded 300
214 ng/ul and 8 pmol Cy/ug cRNA, respectively. All arrays were hybridized following a balanced
215 block design with the same amount of cRNA (300 ng of each Cy3- and Cy5-labeled cRNA).
216 Arrays hybridization and washes were conducted according to the kit protocol and the arrays
217 were scanned with a GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA, USA)
218 using the suggested Agilent scan settings.

219 *2.4 Oligoarray data analysis*

220 Fluorescence intensities of each spot were extracted using GenePix software and the
221 generated intensity data were normalized using the LIMMA package in R software to remove
222 within-array (method: global lowess) and between-array (method: quantile) non-biological
223 variation (Smyth and Speed, 2003). After normalization, probes with intensities less than two-
224 fold of background intensities were eliminated from further analysis (applied to all biological
225 replicates for the probes). The filtered data were then submitted to statistical analysis using
226 the Multi Experiment Viewer (MeV) program (Saeed et al., 2003). Gene expressions in
227 hemocytes were compared between healthy (FLc) and QPX challenged (FLq) Florida clams
228 at 13 °C and 21 °C (n=3 for each treatment) to examine the molecular response induced by
229 QPX infection and temperature. Mantle samples were examined within and between MA and
230 FL infected clams at temperatures of 13 and 21 °C, in an attempt to identify immune genes
231 and pathways potentially associated with *M. mercenaria* resistance against QPX and their
232 regulation patterns at different temperature. The data analysis was based on the relative gene
233 expression levels among compared samples, which was calculated as the ratio of intensity for
234 a transcript in each treatment against the mean intensity of that transcript in all treatments.
235 The criteria for final determination of differentially expressed genes were the significance by
236 statistical testing (p-value < 0.01, t-test or ANOVA) together with one and half fold increase
237 or decrease from the mean (up- or down-regulation). K-means clustering and hierarchical
238 clustering were then used to cluster those significantly differentially expressed genes with
239 similar expression profiles (Soukas et al., 2000; Yeung et al., 2001). The complete dataset for
240 *M. mercenaria* oligoarray can be found at the Gene Expression Omnibus public database
241 (GEO) (http://www.ncbi.nlm.nih.gov/geo/) under the accession number XXXXX.

242 **3. Results**

243 The QPX infection status in clams after 2 months was described in (Dahl et al., 2011b).
244 Briefly, temperature significantly impacted disease development. Among, FL clams
245 experimentally-injected with QPX (FLq), disease development was maximal in clams
246 maintained at 13 °C (73 % QPX disease prevalence) as opposed to those held at 21 °C (10%
247 prevalence). Disease prevalence in naturally-infected (MA) clams was 33% and 20% in clams
248 held at 13 and 21ºC, respectively. Furthermore, moderate to heavy infections constituted most
249 of the positive clams maintained at 13 °C, while none of the positive clams from the 21°C

250 treatment showed heavy infection, and signs of healing were only noticeable in clams held at
251 21°C (Dahl et al., 2011b).

252 *3.1 Differential gene expression in FL clams*

253 The modulatory effect of temperature and QPX infection on *M. mercenaria* gene
254 expression was investigated in hemocyte and mantle samples of FL clams maintained at 13
255 and 21 °C. Based on stringent criteria for the identification of significant differentially
256 expressed (DE) genes (one-way ANOVA $p<0.01$ in conjunction with relative fold change >
257 1.5), 887 and 311 DE genes were respectively identified in hemocyte and mantle samples in
258 response to QPX challenge and temperature modulation (Supplementary file 2 and 3). The
259 DE genes were then clustered by K-means clustering (KMC) into groups based on the
260 expression pattern similarities in order to gain further insight into their biological functions.
261 Within each KMC group, DE genes were further clustered using hierarchical clustering
262 (HCL) to explore the potential functional information of novel (unannotated) DE genes, since
263 genes clustered closely usually have linked or co-regulated expression performances thus
264 possibly share similar function or biological importance (Eisen et al., 1998).

265 The 887 DE genes in FL hemocyte samples were clustered into 8 groups based on the
266 results of K-means clustering (Fig 1A, Supplementary file 2). The cluster 1 (CL1) contained
267 135 DE genes that showed lower expression in QPX infected clams (FLq) held at 13 °C as
268 compared to those held at 21 °C. Several genes from this cluster are known to play a role in
269 mollusk immunity such as the angiopoietin-related protein, cathepsin L2 cysteine protease,
270 complement c1q-like protein (C1q), complement c1q tumor necrosis factor-related protein 2
271 (C1qTNF2) and vitelline membrane outer layer protein 1. Cluster 2 (CL2) contained 129 DE
272 genes that exhibit maximal expression in naïve FL (FLc) clams held at 21 °C. Some immune-
273 related genes featured in this cluster include beta-glucan recognition protein, complement
274 factor b-like protein and galectins. Cluster 3 (CL3) consisted of 26 DE genes that were found
275 to be over-expressed in FLq hemocytes at 13 °C but under-expressed at 21°C, with most of
276 these being unannotated genes, except the neurogenic locus notch homolog protein 1-like,
277 proteasome subunit beta type-6-like and ribosomal proteins. Cluster 4 (CL4) and cluster 7
278 (CL7) contained DE genes that were significantly up-regulated in QPX-injected clams held at
279 either 13 °C or 21°C, or at both temperatures (Fig 1). Out of the 69 DE genes clustered in
280 CL4, those encode molecules potentially involved in clam defense system were selected and
281 presented in Fig 1B, including immune effector proteins (cathepsin k, endo-1,3-β-glucanase,
282 ferritin and cytochrome peroxidase) and immune pattern recognition proteins (ARPs, C1qs,
283 galectins, tenascin and fibrinogen domain containing proteins). CL7 contains 133 DE genes
284 that presented the highest expression pattern in FLq clams held at 21 °C (Fig 1C). About 70%
285 (102) of the DE genes in CL7 are unannotated genes, while those annotated DE genes were
286 mostly pathogen pattern recognition proteins (PRRs) such as C1qs, thioester-containing
287 protein (TEP) and sialic acid-binding lectins (Fig 1C). Cluster 5 contains a list of 41 DE
288 genes that were significantly down-regulated in FL clams after QPX challenge for both
289 temperatures. This collection featured genes encoding structural proteins associated with
290 cytoskeleton and ribosome (e.g. ribosomal proteins, actins, tubulins and ATP synthases), and
291 one immune protein (lipopolysaccharide-induced tumor necrosis factor-alpha factor-like
292 protein). Cluster 6 (CL6) contains 209 DE genes that were highly expressed at 13 °C as
293 compared to 21 °C. Many DE genes from this cluster represent stress-related proteins, such as
294 heat-shock proteins, cytochrome oxidase, metallothionein, and omega glutathione s-
295 transferase. On the contrary, cluster 8 (CL8) was characterized by DE genes (147) that are
296 under-expressed in hemocytes from the low temperature treatment (13 °C) of both naïve and
297 infected FL clams. Genes clustered in this group were typically those associated with growth
298 and metabolism (eg. ribosomal proteins, latent-transforming growth factor beta-binding

299 protein 4, short-chain collagen partial), except a few proteins involved in defense (eg.
300 transmembrane serine 6, cytochrome p450 1a1, complement c1q subcomponent subunit b and
301 caprin-2).

302 As for the mantle tissues of FL clams, a total of 311 DE genes have shown significant
303 modulation in response to temperature and QPX infection and were clustered into 6
304 expression pattern groups by KMC (Fig. 2 and Supplementary file 3). Cluster 1 contained 62
305 DE genes that were down-regulated in mantle tissues from clams held at 13 °C as compared
306 to 21°C (Fig. 2). These included some structural and metabolism-related proteins (e.g.
307 ribosomal proteins, actins, tubulins, acyl-CoA-dehydrogenase and d-beta-hydroxybutyrate
308 mitochondrial), as well as several immune-related proteins, such as antioxidant cytochrome c,
309 the tumor suppressor protein inhibitor of growth protein 3-like, the immune signal transducer
310 serine threonine-protein kinase ctr1 and chitin deacetylase-like protein (Supplementary file 3,
311 Fig. S1). In contrast, Cluster 3 (Supplementary file 3, Fig. S1) was comprised of 84 genes and
312 highlighted DE genes that were highly expressed at 13 °C, including acyl-CoA desaturase
313 (lipid metabolism), mucin-associated protein (biomineralization), NADH dehydrogenase
314 subunit and heavy metal-binding protein hip (detoxification). Cluster 2 and Cluster 5
315 respectively included 56 and 22 DE genes that were highly expressed in FLq clams at 21 and
316 13 °C, respectively (Fig. 2 and 3). A considerable fraction of the annotated DE genes in
317 Cluster 2 (Fig. 3A) belonged to the immune-related protein category; such as the tandem
318 repeat galectin, lysozyme, polyubiquitin-c-like isoform1, C1q, and CD209 antigen-like
319 protein. Meanwhile, only genes of the PRR C1qDCs were found in Cluster 5, whereas two
320 QPX genes, the pyruvate dehydrogenase component x and 60s ribosomal protein, were amid
321 the DE genes over-expressed following QPX injection at 13 °C, which could be linked to
322 ongoing active infection in this batch. The remaining two clusters (Cluster 4 and Cluster 6,
323 Fig. 3) included DE genes that were either down-regulated (Cluster 4) or up-regulated
324 (Cluster 6) in infected FL clams as compared to control FL clams. Fifty-six DE genes were
325 present in Cluster 4, of which the annotated genes were mostly associated with cell structural
326 components (cytoskeleton, collagen and actin cytoskeleton), protein synthesis and processing
327 (nucleotide binding, translation elongation factor, mRNA splicing), as well as several genes
328 involved in apoptosis (Apoptosis 2 inhibitor, C1q-tumor necrosis factor 3) and detoxification
329 processes (cytochrome p450, small heat shock protein). Out of the 28 DE genes revealed by
330 Cluster 6, only 5 were functionally annotated, which interestingly included one QPX gene
331 involved in polysaccharide biosynthesis and one *M. mercenaria* tandem repeat galectin gene.

332 *3.2 Differential gene expression in MA clams*

333 The modulation of gene expression in mantle tissues from infected MA clams in response
334 to temperature was evaluated. A total of 563 genes were differentially expressed between 13
335 and 21 °C (t-test $p < 0.01$ with at least 1.5 fold change, Supplementary file 4). Among these,
336 217 DE genes exhibited higher expression at 13 °C, and the remaining 346 were
337 comparatively higher in the 21 °C samples. The DE genes from this comparison were
338 categorized by their putative functions and are summarized in Fig. 4 and Supplementary file
339 4. DE genes that are highly expressed at 13 °C are dominated by those related to stress
340 response and immune recognition (Fig. 4), which included HSP70, universal stress protein,
341 metallothionein, angiopoietin-related proteins and fibrinogen domain-containing proteins
342 (FREP). In addition, several structural proteins were also overexpressed in mantle tissues
343 from MA clams held at 13 °C (Fig. 4, Supplementary file 4), particularly the tubulins which
344 are known to associated with macrophage activity (Robinson and Vandre, 1995).

345 On the other hand, structural proteins overexpressed at 21 °C were mostly ribosomal
346 proteins, with functions pertaining to mitochondria or ribosome biogenesis processes

347 (Supplementary file 4). In parallel, genes associated with metabolic processes, signal
348 transduction and protein synthesis were also overexpressed at 21 °C (Fig. 4), reflecting a
349 higher metabolic rate in these clams as compared to those held at 13 °C. Interestingly, DE
350 genes with functions related to apoptotic processes were exclusively overexpressed at 21 °C,
351 implying a general underexpression of these genes at 13 °C (Fig. 4, Supplementary file 4).

352 *3.3 Comparison of gene expression profiles between FL and MA clams*

353 Gene regulation associated with QPX infection was also compared between the relatively
354 susceptible FL and resistant MA clams (Dahl et al., 2008) in order to get further insights into
355 genes potentially related to the *M. mercenaria* resistance to QPX. Comparisons were made
356 among mantle samples of MA and FLq clams held at either 13 or 21 °C, and DE genes were
357 identified based on the same criteria as above (p <0.01 by ANOVA in conjunction with over
358 1.5 fold change). A total of 1,569 DE genes were revealed from this comparison
359 (Supplementary file 5). K-means and hierarchical clustering were applied to categorize
360 expression patterns among annotated DE genes (Supplementary file 5). A total of 227
361 annotated DE genes were assigned into 6 K-means clusters based on the similarity of the
362 expression patterns across samples (Supplementary file 5, Fig. 5). The first three clusters
363 (CL1 to 3, Fig. 5) featured DE genes that displayed higher expression in MA clams than in
364 FL clams. These 3 clusters were remarkably enriched with DE genes involved in immune and
365 stress response, particularly in pathogen recognition, such as the C1q domain containing
366 proteins, fibrinogen related proteins and angiopoietin-related proteins (both contain
367 fibrinogen related domains, FReD), which comprised the three main PRRs families that were
368 overexpressed in MA clams as compared to FL clams (Fig. 5 and Supplementary file 5).
369 Interestingly, the highest expression of these PRRs was found in MA clams held at 13 °C,
370 suggesting a link between these immune genes and active host-pathogen interactions.

371 On the other hand, the remaining 3 clusters (CL 4 to 6, Fig. 5) highlighted DE genes that
372 were overexpressed at 13 °C (Cluster 4) and 21 °C (Cluster 5) in both MA and FL clams, and
373 those only up-regulated in FL clams at both temperatures (Cluster 6). DE genes up-regulated
374 at 13 °C (Cluster 4) in both clam strains were enriched with functions related to immune and
375 stress response. For example, sialic acid binding lectin, which is a pathogen binding protein,
376 clathrins and tubulins, which are known to regulate macrophage activity, stress protein
377 HSP70, immune enzyme serine protease and kazal-type proteinase inhibitor, as well as
378 cytochrome b, which is involved in the ROS detoxification, were concomitantly up-regulated,
379 suggesting active host-pathogen interactions are taking place under this condition. Whereas
380 DE genes involved in other biological processes were largely under-expressed at 13 °C as
381 compared to 21 °C (Cluster 5), such as those associated with protein biosynthesis processes
382 (ribosome biogenesis protein, eukaryotic translation initiation factor 3, transcription factor
383 containing protein, translation elongation factor hbs1-like protein, protein folding chaperon
384 heat shock proteins) and metabolic processes (cytochrome oxidase, ATPase inhibitor, ATP-
385 binding protein, cytochrome c, beta-1,4-galactosyltransferase; Cluster 5, Supplementary file
386 5). These changes were very similar to what have been observed in FL hemocytes from clams
387 held at 13ºC (described above), reflecting a systemic modulatory effect of temperature and
388 QPX infection on clams regardless of strain and tissue difference. In addition, a large fraction
389 of DE genes that are overexpressed in FL clams as compared to MA clams were related to
390 metabolic and protein biosynthesis processes (Cluster 6, Supplementary file 5), such as
391 ribosomal proteins, histone ribonucleoproteins, and ATP synthase. Interestingly, in term of
392 immune-related genes, none of the PRRs that were induced upon QPX infection in MA clams
393 (e.g. FREPs, C1q and angiopoietin) was overexpressed in infected FL clams. In contrast,
394 expression levels of some other defense-related genes were higher in FL clams than in MA,
395 such as the LPS-induced TNF factor, peptidoglycan recognition protein, galactose-specific c-

396 type lectin, proteasome subunit and several hydrolases (cathepsin 1, lipase, lysosomal
397 cholesterol esterase), many of which were especially higher at 21 °C as compared to 13 °C.
398 Finally, higher expression of stress proteins (superoxide dismutase, universal stress protein,
399 omega glutathione s-transferase) was noticed in FL clams injected with QPX or exposed to
400 13ºC as compared to their MA counterparts suggesting that these conditions are more
401 stressful to the former clam stock.

402 4. Discussion

403 Successful management and control of QPX disease is of great importance for the hard
404 clam industry, since mortalities caused by this parasite have resulted in significant
405 economical losses (Calvo et al., 1998; Ford et al., 2002; Smolowitz et al., 1998). Thus,
406 understanding the pathobiology of QPX disease and host-pathogen interactions is urgently
407 needed to set forth disease mitigation strategies and develop resistant clam stocks. Currently,
408 our knowledge about QPX disease mostly focused on clam physiology and baseline immune
409 processes. Information on the molecular aspects of clam response to QPX is still very limited,
410 with a very narrow collection of common immune-related genes thought to be involved in
411 host-pathogen interactions (Perrigault and Allam, 2009; Perrigault and Allam, 2012a;
412 Perrigault et al., 2011; Perrigault et al., 2009c). These investigations have described different
413 clam defense strategies mounted against bacterial and QPX infections (Perrigault and Allam,
414 2012a), and the interwoven genetic and environmental determinants associated with clam
415 resistance (Calvo et al., 2007; Dahl et al., 2008; Ford et al., 2002). Our study used high-
416 throughput genomic tools to comprehensively assess clam response to QPX at molecular
417 levels. The comparisons between healthy and infected clams, and between relatively resistant
418 and susceptible stocks provided insights into mechanisms involved in disease development
419 and host-QPX interactions. At the same time, the evaluation of QPX-associated responses in
420 mantle and hemocytes allowed for the understanding of tissue-specific defense strategies as
421 mantle represents the main infection site (Smolowitz et al., 1998) while hemocytes reflect an
422 overall systemic clam response toward the invader (Perrigault et al., 2008).

423 *4.1 Modulatory effects of temperature on gene expression in hemocytes*

424 We have previously shown that temperature significantly regulates *M. mercenaria*
425 immunity and QPX disease dynamics (Dahl et al., 2011b; Perrigault et al., 2011). In particular,
426 cold temperatures (13 °C) were shown to dampen cellular immunity and significantly
427 promote the establishment of QPX disease, while warmer temperatures (21 and 27°C)
428 prohibit infection development and favor healing of pre-existing lesions (Dahl et al., 2011b;
429 Perrigault et al., 2011). Results from the current study show that temperature changes cause
430 alterations in both constitutive and QPX-induced gene expression in FL clams. In parallel,
431 this study shows that QPX challenge alters the expression profiles of several immune genes
432 (at either 13 °C or 21 °C, or both; clusters CL4 and CL7, Fig. 1 and Supplementary file 2.
433 Among these, several DE genes belonged to the fibrinogen-related protein families (FREPs)
434 and the complement C1q domain containing proteins (C1qDCs).

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

444 (cluster CL7, Fig.1C), suggesting a fundamental role of these proteins in *M. mercenaria*'s
445 resistance to QPX.

446 It should be stated, however, that the exact functions of FREPs and C1qDCs have not been
447 clearly characterized in bivalves. Accumulating evidence supports their important roles in
448 innate immunity as pattern recognition receptors that mediate non-self recognition (Gerdol et
449 al., 2011; Hertel et al., 2005; Zhang et al., 2008; Zhang et al., 2009). In fact, both FREPs and
450 C1qDCs are known to activate the complement pathway of the innate immune system in
451 vertebrates, however their modes of action are slightly different with C1qDCs triggering the
452 classical pathway, while FREPs activate the lectin pathway (Dodds and Matsushita, 2007;
453 Fujita et al., 2004). Up-regulation of both groups in hard clams upon QPX challenge may
454 indicate the activation and involvement of complement proteins in *M. mercenaria* defense
455 mechanisms. It can be further speculated that an ancestral complement system existed and
456 may be actively involved in the neutralization of QPX in *M. mercenaria*, possibly via parasite
457 recognition, immobilization, and tissue damage repair, which leads the way to ultimate
458 healing. Interestingly, another complement-related protein, the thioester-containing protein
459 (TEP), was also found overexpressed after QPX infection (Fig. 1Cure). TEP is a functional
460 homologue to vertebrate C3, which is the central component of the complement system, and
461 is widely recognized as an essential player in the anti-parasite defense mechanisms of insects
462 (Blandin et al., 2004; Blandin et al., 2008). The up-regulation of a TEP homologue in *M.*
463 *mercenaria* after QPX stimulation provides further support to the role of the complement
464 system in anti-QPX defense in *M. mercenaria*. Finally, other immune genes found in cluster
465 CL4 and CL7 suggest the mobilization of diverse immune mechanisms and pathways for the
466 purpose of parasite neutralization, with the active involvement of hydrolases (e.g. cathespsin
467 K, endo-1,3- β-glucanase, and hydrolase), lectins (e.g. tandem repeat galectin, sialic acid-
468 binding lectins), metal transporters (e.g. ferritin, heavy metal-binding protein hip) and
469 reduction/oxidation (redox) enzymes (e.g. cytochrome b peroxidase, cytochrome oxidase,
470 eosinophil peroxidase).

471 The constitutive expression of the above immune-related genes was higher in control
472 clams held at 21°C as compared to those maintained at 13 °C, potentially linking host
473 resistance at the former temperature to immune fitness of the host. In addition, other
474 biological pathways such as metabolic activities, protein synthesis and anti-oxidative
475 processes were also significantly influenced by temperature on the constitutive-level (without
476 QPX challenge). For example, generally higher expression of ribosomal proteins and fatty
477 acid-binding proteins were found in FLc clams at 21 °C (CL2 and CL8, Supplementary file 1),
478 indicating a more robust protein synthesis and growth at the higher temperature. On the other
479 hand, the production of several proteins involved in the oxidative stress response was
480 significantly induced at 13 °C, such as the omega-glutathione s-transferase, NADH-
481 dehydrogenase subunit 3 and metallothionein proteins (CL1 and CL6, Supplementary file 1).
482 This suggests the activation of oxidative defense mechanisms to protect against damaging
483 reactive oxygen species (ROS) produced in excess during exposure to sub-optimal
484 temperatures, which is known to frequently induce hypoxia stress (Heise et al., 2006). As
485 reported in many marine invertebrates, the reduction of blood circulation and oxygen
486 transport at temperatures below an animal optimal thermal range can significantly lower the
487 oxygen supply, sometimes below tissue demand, resulting in functional hypoxia and
488 oxidative stress (Heise et al., 2007; Pörtner, 2002; Pörtner et al., 2001). This phenomenon is
489 especially pronounced in ectothermal animals, which include all bivalve species. Thus, for *M.*
490 *mercenaria* held at 13 °C, higher levels of oxidative defense activities need to be maintained
491 in order to avoid oxidative damage, which may consume a considerable portion of energy that
492 otherwise could have been used for other purposes such as growth and immunity. This might

493 partially account for the overall lower host defense levels at 13 °C as compared to 21 °C, and
494 may also explain the dubbing of QPX disease as a "cold water disease" (Dahl et al., 2011b;
495 Perrigault et al., 2011), particularly since parasite growth *in vitro* is in fact optimal at 21ºC
496 (Perrigault et al., 2009b) and that disease onset is largely dependent upon clam immune
497 performances (Perrigault et al., 2011).

498 *4.2 Modulatory effects of temperature on gene expression in mantle tissue of FL clams*

499 QPX lesions are most often found inside clam mantle tissues, sometimes leading to the
500 development of nodules. The formation of nodules materializes intensive interactions
501 between host defense system and the invading parasite. In this context, mantle-related host
502 factors likely contribute to the inflammatory response and encapsulation of QPX cells at the
503 lesion sites (Anderson et al., 2003; Smolowitz et al., 1998). Interestingly, tissue extracts from
504 *M. mercenaria* mantle were shown to significantly inhibit QPX growth *in vitro*, even though
505 the nature of these antimicrobial factors remains unknown (Perrigault et al., 2009a). Gene
506 expression profiles of mantle tissues were determined in FL clams in response to temperature
507 and QPX challenge and allowed the identification of candidate anti-QPX factors (Fig. 2). For
508 example, the potent antimicrobial protein lysozyme was overexpressed in mantle from
509 challenged clams held at 21ºC as compared to all other treatments (Supplementary file 3)
510 suggesting a possible role of this enzyme in disease resistance. A similar role of lysozyme
511 was suggested in the resistance of the clam *Meretrix meretrix* to bacterial infections (Yue et
512 al., 2012). Several other immune-related genes were also identified in challenged clams held
513 at either or both temperatures. Similar to the observations in FL clam hemocytes, the
514 overexpressed DE genes induced by QPX challenge mainly included those complement C1q
515 like proteins, tandem repeat galectin and galectin-3, all of which are PRRs involved in
516 pathogen recognition and neutralization (Kim et al., 2008; Tasumi and Vasta, 2007; Wang et
517 al., 2012; Xu et al., 2012). This overrepresented induction of PRRs in mantle suggests that
518 parasite detection and targeting might be a key process for QPX neutralization. The induction
519 of complement C1qDCs in both mantle and hemocytes following QPX stimulation
520 corroborates the idea that the complement-like system may play a central role in bivalve
521 defense against protozoan parasites, despite the fact that the functionality of such pathway has
522 not been extensively characterized in most bivalve species (Leite et al., 2013; Venier et al.,
523 2011). On the other hand, the crucial role of galectins has been recently confirmed in bivalves
524 as these were shown to mediate hemocyte recognition and binding of the protozoan parasites
525 *Perkinsus marinus* in the oyster *Crassostrea virginica* and *Perkinsus olseni* in Manila clam
526 *Ruditapes philippinarum* (Kim et al., 2008; Tasumi and Vasta, 2007). In our study, the up-
527 regulation of multiple galectins and tandem-repeated glaectins upon QPX infection also
528 implies their contribution to the protection against protozoan infections, possibly facilitating
529 parasite recognition. Not surprisingly, higher induction levels of these galectins were also
530 noticed at 21°C as compared to 13°C (cluster 2), which again suggests a positive relationship
531 between suitable environmental conditions and better immune performances in *M.*
532 *mercenaria.* Interestingly, one QPX gene involved in polysaccharide biosynthesis showed up
533 as DE gene in infected FL clam mantle tissue (cluster 6), which could reflect the intensity of
534 the parasite proliferation at the temperature that favored disease establishment.

535 On the other hand, FL mantle samples constitutively exhibited a set of expression profiles
536 very similar to those observed in hemocytes (Fig. S1). For example, the DE genes up-
537 regulated at 21°C have annotated functions generally related to protein synthesis (e.g.
538 ribosomal proteins, small heat shock protein, alpha-B crystallin), cellular components (β-
539 actins, laminin receptor) and metabolism (chitin deacetylase-like, cytochrome, protein
540 kinases). Whereas at 13 °C, interestingly, the up-regulated DE genes were largely associated
541 with cell respiration (e.g. nadh dehydrogenase subunit 1 and 3) and fatty acid desaturation

542 (e.g. acyl-desaturase, such as, acyl delta desaturase), suggesting that these processes are
543 involved in the biological adjustments of *M. mercenaria* in response to cold environment.

544 The total number of DE genes regulated by temperature and QPX infection in FL clam
545 mantle is 311 (Fig. 2), which is a considerably smaller than that of hemocyte (877). This
546 directly reflects the fact that hemocytes are more sensible and responsive to stimuli, possibly
547 because they play a central role as sentinels that monitor and react immediately to changes
548 and danger signals. Hemocytes are the primary immune cells in invertebrates, and alterations
549 of host immune response are readily reflected by their gene expression profiles. Moreover,
550 bivalve hemocytes also function in many other processes besides immune protection, such as
551 tissue repair, shell production and nutrition (Donaghy et al., 2009), so temperature and QPX
552 impacts imposed on all other processes can also be recorded in hemocytes. On the other hand,
553 the makeup and functions of clam mantle tissues are more stable and homogeneous as
554 compared to hemocytes, and this property can also be reflected in gene expression data.
555 However, given the fact that bivalves have open circulatory system with hemocytes
556 wandering in all tissue, gene expression changes monitored in mantle could encompass the
557 response of circulating hemocytes in addition to constitutive mantle cells. Nevertheless,
558 considering the typical localization of QPX lesions in *M. mercenaria*, changes induced inside
559 mantle tissues provide insights into the molecular mechanisms at play during *in situ* host-
560 pathogen interactions, regardless of their contributors.

561 *4.3 Comparisons of gene expressions in FL and MA clams in response to temperature and*
562 *QPX infection*

563 Variation in resistance to QPX disease has been broadly reported in hard clams from
564 different geographic and genetic origins. In general, better QPX resistance and host survival
565 are more often associated with *M. mercenaria* stocks originating from northern states
566 (Massachusetts, New York and New Jersey), while higher susceptibility and mortality are
567 found in southern stocks (South Carolina, Florida) (Calvo et al., 2007; Dahl et al., 2010; Ford
568 et al., 2002; Smolowitz et al., 2008). The reasons leading to the above observations of
569 difference in resistance have been previously ascribed to 1) the poor adaptation of southern
570 strains to the enzootic northern cold waters and 2) variations in genetic background among
571 clam stocks that result in different abilities of the immune system to mount effective defense
572 against the invading parasite (Calvo et al., 2007; Ford et al., 2002). However, results from the
573 field and laboratory observations of Dahl et al. (2008; 2010) have played down the cause of
574 “poor acclimation” from being a main disease-aggravating factor in southern clam strains,
575 leaving the factor of genetic variations, likely driven by the selective mortality caused by
576 enzootic QPX in northern locales, to act as the most important determinant for hard clam
577 susceptibility to QPX disease. In our study, significant difference in molecular response to
578 QPX infection was observed between the susceptible (FL) and resistant (MA) clam strains,
579 providing additional support to the argument that clams ability to resist QPX infection is
580 closely associated with their genetic makeup. The comparison of expression profiles among
581 defense-related genes before and after QPX induction between the two strains give further
582 insights into gene candidates potentially regulating this trait.

583 As many as 1,569 DE genes were found between challenged clams from both stocks held
584 at both experimental temperatures (Supplementary file 5), highlighting a divergence in
585 molecular responses between FL and MA clams to QPX infection. This dissimilarity in gene
586 regulation might be related to myriad of biological processes, including pathways regulating
587 both immune response and metabolism, and the observed difference in QPX resistance
588 between the host strains might result from the collective effect of all processes involved.
589 Interestingly, a large portion of the DE genes were up-regulated in MA clams when compared

590 to FL clams (Fig. 5 and Supplementary file 5), especially those with putative functions related
591 to immune response (e.g. PRRs, C1q and FReDs containing proteins). This suggests that the
592 rather resistant MA clams can react to the parasite infection more strongly than susceptible
593 FL clams. Furthermore, higher expression levels of these DE genes were observed in MA
594 clams held at 13 °C as compared to those maintained at 21 °C, and also corresponded
595 positively with both infection severity and host resistance. Therefore, it seems that MA clams
596 can regulate the level of their immune proteins to tailor the severity of ongoing QPX
597 infections, with higher expression levels associated with heavy infections. On the other hand,
598 FL clams overexpressed DE genes that were related to metabolic and protein biosynthesis
599 (Cluster 6, Supplementary file 5), which may be related to fast growth typically observed in
600 southern aquacultured strains. Interestingly, the defense-related genes up-regulated in FL
601 clams upon infection did not overlap with those induced in MA clams by QPX, which may be
602 linked to the susceptibility of the former clams. This may also suggest the attempt of FL
603 clams to possibly compensate for their inability to produce effective anti-QPX factors by
604 triggering the up-regulations of universal defense-related proteins, and this compensatory
605 overexpression of immune molecules were more efficient and pronounced at their optimal
606 temperature (21 °C). Such strategy is energetically-savvy and might be considered as
607 evidence that resistant MA clams have gone through selection processes that endowed them
608 with better adaptations to survive in QPX enzootic areas (Gast et al., 2008; Lyons et al., 2005;
609 Ragone Calvo et al., 1998). The fact that QPX is widely distributed in northern cold waters
610 has led to speculations that certain levels of selective mortality have taken place in the local
611 clam stocks due to frequent QPX encounter, which may have increased the overall resistance
612 in surviving animals (Calvo et al., 1998; Gast et al., 2008). Whereas the southern clam stocks
613 may never have been exposed to the selection pressure imposed by this parasite since QPX
614 has never been reported in waters further south than Virginia (Gast et al., 2008).

615 The over-expressed immune genes associated with MA clams were mostly those
616 specifically induced by QPX as shown from the comparison between FLc and FLq clams
617 discussed above, such as the C1q and FReD domain containing proteins (Fig. 6). Efficient
618 targeting of foreign invaders is a key step for the activation of anti-parasite defense
619 mechanisms and often result in the prompt neutralization of the invader. Higher resistance to
620 QPX observed in MA clams could partly result from higher expression of these QPX-
621 responsive PRRs, which were speculated to be the primary recognition proteins for QPX-
622 specific molecular structures (see above). On the contrary, the broad under-expression of
623 these proteins in FL clams suggests apparent incompetency of susceptible clams to mount
624 sufficient defense response against QPX. In addition, expression level for these DE PRR
625 genes appears to be correlated with infection intensity. For instance, the highest over-
626 expression was generally measured in MA clams held at 13 °C, the temperature that causes
627 the most severe QPX infections; and the up-regulation was not uniform in MA samples at
628 21 °C (Fig.5). This last observation may come from the fact that MA clams held at 21ºC
629 displayed a range of infection severities with some clams displaying complete signs of
630 healing (Dahl et al., 2011a).

631 A great number of DE sequences containing C1q domain and FReD have been noticed
632 (Fig. 6), possibly reflecting the high sequence diversification in these two gene families. In
633 fact, beside *M. mercenaria*, members of these two gene families have been shown to display
634 extensive sequence diversity in many molluscan species (Ghai et al., 2007; Hanington et al.,
635 2012; Leite et al., 2013; Mone et al., 2010; Venier et al., 2011; Zhang et al., 2009; Zhang et
636 al., 2013). The broad sequence variability in these genes usually translates into immune
637 molecules with highly diversified pathogen recognition domain structures (Cerenius and
638 Soderhall, 2013; Mone et al., 2010; Zhang and Loker, 2004). Mechanisms leading to this

639 diversification may include recombinatorial diversification, alternative splicing and somatic
640 diversification through gene conversions and point mutations (Hanington et al., 2012; Zhang
641 and Loker, 2004). The somatic generation of polymorphism and diversification of these
642 putative immune receptor sequences is important for the host to maintain a dynamic and rich
643 repertoire of putative recognition molecules so that response against a variety of pathogen
644 epitopes could be promptly mounted. In this study, the differential expression of diverse
645 forms of C1qDCs and FREPs between MA and FL clams might suggest high responsiveness
646 of these molecules against QPX, particularly when taking into consideration that the
647 overexpression was mostly associated with the more resistant clam stocks held at the
648 condition favoring disease development (MA clams, 13 °C). The regulation of these genes
649 likely help MA clams fight QPX, but can also contribute to the elimination of putative
650 secondary pathogens.

651 It is widely recognized that invertebrates do not have acquired immunity, and their innate
652 immune system exhibits less diversity of receptor repertoire leading to reduced specificity.
653 Their ability to detect parasites exclusively relies on invariable germline-encoded immune
654 receptors and effectors that interact with universal microbial antigens (Medzhitov and
655 Janeway, 1997). However, increasing evidence suggests the presence of sophisticated
656 recognition systems in some invertebrate species, including echinoderms (sea urchin), insects
657 (*Drosophila melanogaster* and *Anopheles gambiae*), crustaceans and mollusks (*Biomphalaria*
658 *glabrata*) (Brites et al., 2008; Dong et al., 2006; Pancer, 2000; Watson et al., 2005; Zhang,
659 2004). In addition, remarkably different immune responses were observed in *M. mercenaria*
660 following QPX and bacterial challenge, suggesting the involvement of different immune
661 pathways for the discrimination and elimination of different pathogen types (Perrigault and
662 Allam, 2012b). Together, these observations imply the existence of a form of specific
663 immunity in invertebrates, which has been suggested to be linked to those highly diversified
664 immune molecules generated via various types of somatic diversification (Mone et al., 2010).
665 This broad reservoir of recognition molecules also serves as the source for the development of
666 host adaptation to parasite-driven selective pressures (Cerenius and Soderhall, 2013; Dheilly
667 et al., 2015; Zhang et al., 2013; Zhang and Loker, 2003). For example, the *B. glabrata* FREPs
668 are a group of highly variable receptors that precipitate variable antigens of trematode
669 parasites (Mone et al., 2010). Exceptional somatic diversifications exist among these
670 molecules, creating quite individualized FREP pools that vary from one snail to another.
671 Therefore, the recognition capacity has been dramatically enlarged in a random way, however
672 individuals possessing the receptor variants that are capable of recognizing specific antigens
673 would be favored in an environment where they are exposed to corresponding pathogens,
674 allowing these snails to survive the selective pressure (Mone et al., 2010). The higher
675 expression levels of C1qDCs and FREPs associated with QPX-resistant MA clams are very
676 likely the result of QPX-derived selection process via similar mechanisms.

677 Overall, more and more studies advocate that invertebrate innate immunity has
678 considerable specificity and is capable of discriminating between pathogens. Recent
679 identification of several components of the lectin-based complement pathway from ascidians
680 reveals that the primitive complement system is one of the most highly organized innate
681 immune systems in invertebrates (Fujita, 2002; Fujita et al., 2004). In fact, the complement
682 system plays a pivotal role in innate immunity before the evolution of an adaptive immune
683 system in vertebrates and is widely thought to act as an evolutionarily transitional mechanism
684 that links innate immunity to acquired immunity (Fujita, 2002; Sekine et al., 2001). Since
685 both the FREPs and C1q proteins are important components initiating the complement
686 system, a primitive complement-like system capable of providing tailored immune protection
687 against various pathogens is speculated to also exist in *M. mercenaria*.

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

697 **5. Conclusions**

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

719

720 **Acknowledgments**

721 This research was supported by projects R/XG-19 (to BA, EPE and AT) and R/FBM-34
722 (to BA and EPE), funded under award NA07OAR4170010 from the National Sea Grant
723 College Program of NOAA to the Research Foundation of State University of New York on
724 behalf of New York Sea Grant. The study was also partially supported by project IOS
725 1050596 (to BA and EPE) supported by the National Science Foundation. The statements,
726 findings, conclusions, views and recommendations are those of the authors and do not
727 necessarily reflect the views of any of those organizations.

728

References

Allam, B., Pales Espinosa, E., Tanguy, A., Jeffroy, F., Le Bris, C., Paillard, C., 2014. Transcriptional changes in Manila clam (Ruditapes philippinarum) in response to Brown Ring Disease. Fish Shellfish Immunol. 41, 2-11.

Anderson, R. S., Kraus, B. S., McGladdery, S. E., Reece, K. S., Stokes, N. A., 2003. A thraustochytrid protist isolated from Mercenaria mercenaria: molecular characterization and host defense responses. Fish & Shellfish Immunology. 15, 183-194.

Blandin, S., Shiao, S. H., Moita, L. F., Janse, C. J., Waters, A. P., Kafatos, F. C., Levashina, E. A., 2004. Complement-like protein TEP1 is a determinant of vectorial capacity in the malaria vector Anopheles gambiae. Cell. 116, 661-70.

Blandin, S. A., Marois, E., Levashina, E. A., 2008. Antimalarial responses in Anopheles gambiae: from a complement-like protein to a complement-like pathway. Cell Host Microbe. 3, 364-74.

Brites, D., McTaggart, S., Morris, K., Anderson, J., Thomas, K., Colson, I., Fabbro, T., Little, T. J., Ebert, D., Du Pasquier, L., 2008. The Dscam homologue of the crustacean Daphnia is diversified by alternative splicing like in insects. Mol Biol Evol. 25, 1429-39.

Buchmann, K., 2014. Evolution of Innate Immunity: Clues from Invertebrates via Fish to Mammals. Frontiers in Immunology. 5.

Calvo, L. M. R., Ford, S. E., Kraeuter, J. N., Leavitt, D. F., Smolowitz, R., Burreson, E. M., 2007. Influence of host genetic origin and geographic location on QPX disease in Northern quahogs (=hard clams), Mercenaria mercenaria. Journal of Shellfish Research. 26, 109-119.

Calvo, L. M. R., Walker, J. G., Burreson, E. M., 1998. Prevalence and distribution of QPX, Quahog Parasite Unknown, in hard clams Mercenaria mercenaria in Virginia, USA. Diseases of Aquatic Organisms. 33, 209-219.

Cerenius, L., Soderhall, K., 2013. Variable immune molecules in invertebrates. Journal of Experimental Biology. 216, 4313-4319.

Chu, F. L. E., LaPeyre, J. F., 1993. Development of Disease Caused by the Parasite, Perkinsus-Marinus and Defense-Related Hemolymph Factors in 3 Populations of Oysters from the Chesapeake Bay, USA. Journal of Shellfish Research. 12, 21-27.

Dahl, S. F., Allam, B., 2007. Laboratory transmission studies of QPX disease in the northern quahog (=hard clam): Development of an infection procedure. Journal of Shellfish Research. 26, 383-389.

Dahl, S. F., Allam, B., 2008. QPX disease progress in cultured and wild type hard clams in New York waters. Journal of Shellfish Research. 27, 1000-1000.

Dahl, S. F., Perrigault, M., Allam, B., 2008. Laboratory transmission studies of QPX disease in the hard clam: Interactions between different host strains and pathogen isolates. Aquaculture. 280, 64-70.

Dahl, S. F., Perrigault, M., Liu, Q., Collier, J. L., Barnes, D. A., Allam, B., 2011a. Effects of temperature on hard clam (Mercenaria mercenaria) immunity and QPX (Quahog Parasite Unknown) disease development: I. Dynamics of QPX disease. Journal of Invertebrate Pathology. 106, 314-21.

Dahl, S. F., Perrigault, M., Liu, Q. Q., Collier, J. L., Barnes, D. A., Allam, B., 2011b. Effects of temperature on hard clam (Mercenaria mercenaria) immunity

and QPX (Quahog Parasite Unknown) disease development: I. Dynamics of QPX disease. Journal of Invertebrate Pathology. 106, 314-321.
Dahl, S. F., Thiel, J., Allam, B., 2010. Field Performance and Qpx Disease Progress in Cultured and Wild-Type Strains of Mercenaria Mercenaria in New York Waters. Journal of Shellfish Research. 29, 83-90.
Degnan, S. M., 2015. The surprisingly complex immune gene repertoire of a simple sponge, exemplified by the NLR genes: A capacity for specificity? Developmental & Comparative Immunology. 48, 269-274.
Dheilly, N. M., Duval, D., Mouahid, G., Emans, R., Allienne, J.-F., Galinier, R., Genthon, C., Dubois, E., Du Pasquier, L., Adema, C. M., Grunau, C., Mitta, G., Gourbal, B., 2015. A family of variable immunoglobulin and lectin domain containing molecules in the snail Biomphalaria glabrata. Developmental & Comparative Immunology. 48, 234-243.
Dodds, A. W., Matsushita, M., 2007. The phylogeny of the complement system and the origins of the classical pathway. Immunobiology. 212, 233-243.
Donaghy, L., Lambert, C., Choi, K. S., Soudant, P., 2009. Hemocytes of the carpet shell clam (Ruditapes decussatus) and the Manila clam (Ruditapes philippinarum): Current knowledge and future prospects. Aquaculture. 297, 10-24.
Dong, Y., Taylor, H. E., Dimopoulos, G., 2006. AgDscam, a Hypervariable Immunoglobulin Domain-Containing Receptor of the Anopheles gambiae Innate Immune System. PLoS Biology. 4, e229.
Dove, A. D. M., Bowser, P. R., Cerrato, R. M., 2004. Histological analysis of an outbreak of QPX disease in wild hard clams Mercenaria mercenaria in New York. Journal of Aquatic Animal Health. 16, 246-250.
Eisen, M. B., Spellman, P. T., Brown, P. O., Botstein, D., 1998. Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci U S A. 95, 14863-8.
Ford, S. E., Kraeuter, J. N., Barber, R. D., Mathis, G., 2002. Aquaculture-associated factors in QPX disease of hard clams: density and seed source. Aquaculture. 208, 23-38.
Fujita, T., 2002. Evolution of the lectin–complement pathway and its role in innate immunity. Nature Reviews Immunology. 2, 346-353.
Fujita, T., Matsushita, M., Endo, Y., 2004. The lectin-complement pathway - its role in innate immunity and evolution. Immunological Reviews. 198, 185-202.
Gast, R. J., Moran, D. M., Audemard, C., Lyons, M. M., DeFavari, J., Reece, K. S., Leavitt, D., Smolowitz, R., 2008. Environmental distribution and persistence of Quahog Parasite Unknown (QPX). Diseases of Aquatic Organisms. 81, 219-229.
Gerdol, M., Manfrin, C., De Moro, G., Figueras, A., Novoa, B., Venier, P., Pallavicini, A., 2011. The C1q domain containing proteins of the Mediterranean mussel Mytilus galloprovincialis: a widespread and diverse family of immune-related molecules. Developmental and Comparative Immunology. 35, 635-43.
Ghai, R., Waters, P., Roumenina, L. T., Gadjeva, M., Kojouharova, M. S., Reid, K. B., Sim, R. B., Kishore, U., 2007. C1q and its growing family. Immunobiology. 212, 253-66.

Hanington, P. C., Forys, M. A., Loker, E. S., 2012. A somatically diversified defense factor, FREP3, is a determinant of snail resistance to schistosome infection. PLoS Negl Trop Dis. 6, e1591.
Heise, K., Estevez, M. S., Puntarulo, S., Galleano, M., Nikinmaa, M., Pörtner, H. O., Abele, D., 2007. Effects of seasonal and latitudinal cold on oxidative stress parameters and activation of hypoxia inducible factor (HIF-1) in zoarcid fish. Journal of Comparative Physiology B. 177, 765-777.
Heise, K., Puntarulo, S., Nikinmaa, M., Lucassen, M., Pörtner, H.-O., Abele, D., 2006. Oxidative stress and HIF-1 DNA binding during stressful cold exposure and recovery in the North Sea eelpout (Zoarces viviparus). Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology. 143, 494-503.
Hertel, L. A., Adema, C. M., Loker, E. S., 2005. Differential expression of FREP genes in two strains of Biomphalaria glabrata following exposure to the digenetic trematodes Schistosoma mansoni and Echinostoma paraensei. Developmental & Comparative Immunology. 29, 295-303.
Kim, J. Y., Kim, Y. M., Cho, S. K., Choi, K. S., Cho, M., 2008. Noble tandem-repeat galectin of Manila clam Ruditapes philippinarum is induced upon infection with the protozoan parasite Perkinsus olseni. Developmental & Comparative Immunology. 32, 1131-1141.
Leite, R. B., Milan, M., Coppe, A., Bortoluzzi, S., dos Anjos, A., Reinhardt, R., Saavedra, C., Patarnello, T., Cancela, M., Bargelloni, L., 2013. mRNA-Seq and microarray development for the Grooved carpet shell clam, Ruditapes decussatus: a functional approach to unravel host -parasite interaction. BMC Genomics. 14, 741.
Liu, Q., Collier, J. L., Pawagi, S., Barnes, D., Allam, B., 2008. Quahog parasite unknown (QPX) dynamics in wild hard clams from Raritan Bay, New York. Journal of Shellfish Research. 27, 1024-1024.
Liu, Q. Q., Allam, B., Collier, J. L., 2009. Quantitative Real-Time PCR Assay for QPX (Thraustochytriidae), a Parasite of the Hard Clam (Mercenaria mercenaria). Applied and Environmental Microbiology. 75, 4913-4918.
Loker, E. S., Adema, C. M., Zhang, S. M., Kepler, T. B., 2004. Invertebrate immune systems--not homogeneous, not simple, not well understood. Immunol Rev. 198, 10-24.
Lyons, M. M., Smolowitz, R., Gomez-Chiarri, M., Ward, J. E., 2007. Epizootiology of Quahog Parasite unknown (QPX) disease in northern quahogs (=hard clams) Mercenaria mercenaria. Journal of Shellfish Research. 26, 371-381.
Lyons, M. M., Ward, J. E., Smolowitz, R., Uhlinger, K. R., Gast, R. J., 2005. Lethal marine snow: Pathogen of bivalve mollusc concealed in marine aggregates. Limnology and Oceanography. 50, 1983-1988.
Maas, P. A. Y., Kleinschuster, S. J., Dykstra, M. J., Smolowitz, R., Parent, J., 1999. Molecular characterization of QPX (Quahog Parasite Unknown), a pathogen of *Mercenaria mercenaria*. Journal of Shellfish Research. 18, 561-567.
MacCallum, G. S., McGladdery, S. E., 2000. 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. Journal of Shellfish Research. 19, 43-50.

Medzhitov, R., Janeway, C. A., Jr., 1997. Innate immunity: the virtues of a nonclonal system of recognition. Cell. 91, 295-8.
Milan, M., Coppe, A., Reinhardt, R., Cancela, L. M., Leite, R. B., Saavedra, C., Ciofi, C., Chelazzi, G., Patarnello, T., Bortoluzzi, S., Bargelloni, L., 2011. Transcriptome sequencing and microarray development for the Manila clam, Ruditapes philippinarum: genomic tools for environmental monitoring. BMC Genomics. 12, 234.
Mone, Y., Gourbal, B., Duval, D., Du Pasquier, L., Kieffer-Jaquinod, S., Mitta, G., 2010. 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.
Moreira, R., Milan, M., Balseiro, P., Romero, A., Babbucci, M., Figueras, A., Bargelloni, L., Novoa, B., 2014. Gene expression profile analysis of Manila clam (Ruditapes philippinarum) hemocytes after a Vibrio alginolyticus challenge using an immune-enriched oligo-microarray. Bmc Genomics. 15, 267.
Nunez-Acuna, G., Aballay, A. E., Hegaret, H., Astuya, A. P., Gallardo-Escarate, C., 2013. Transcriptional responses of Mytilus chilensis exposed in vivo to saxitoxin (STX). Journal of Molluscan Studies. 79, 323-331.
Paillard, C., Allam, B., Oubella, R., 2004. Effect of temperature on defense parameters in Manila clam *Ruditapes philippinarum* challenged with *Vibrio tapetis*. Diseases of Aquatic Organisms. 59, 249-262.
Pancer, Z., 2000. Dynamic expression of multiple scavenger receptor cysteine-rich genes in coelomocytes of the purple sea urchin. Proc Natl Acad Sci U S A. 97, 13156-61.
Perrigault, M., Allam, B., 2009. Cytotoxicity of quahog parasite unknown (QPX) toward hard clam (*Mercenaria mercenaria*) haemocytes and interactions between different pathogen isolates and host strains. Parasitology. 136, 1281-1289.
Perrigault, M., Allam, B., 2012a. Differential immune response in the hard clam (mercenaria mercenaria) against bacteria and the protistan pathogen QPX (quahog parasite unknown). Fish & Shellfish Immunology. 32, 1124-1134.
Perrigault, M., Allam, B., 2012b. Differential immune response in the hard clam (mercenaria mercenaria) against bacteria and the protistan pathogen QPX (quahog parasite unknown). Fish & Shellfish Immunology. 32, 1124-34.
Perrigault, M., Bugge, D. M., Hao, C. C., Allam, B., 2009a. Modulatory effects of hard clam (Mercenaria mercenaria) tissue extracts on the in vitro growth of its pathogen QPX. Journal of Invertebrate Pathology. 100, 1-8.
Perrigault, M., Chen, C. H., Bugge, D. M., Allam, B., 2008. Investigations of anti-QPX factors in plasma from susceptible and resistant hard clam stocks. Journal of Shellfish Research. 27, 1039-1040.
Perrigault, M., Dahl, S. F., Espinosa, E. P., Allam, B., 2012. Effects of salinity on hard clam (Mercenaria mercenaria) defense parameters and QPX disease dynamics. Journal of Invertebrate Pathology. 110, 73-82.
Perrigault, M., Dahl, S. F., Espinosa, E. P., Gambino, L., Allam, B., 2011. Effects of temperature on hard clam (*Mercenaria mercenaria*) immunity and QPX (Quahog Parasite Unknown) disease development: II. Defense parameters. Journal of Invertebrate Pathology. 106, 322-332.

Perrigault, M., Dahl, S. F., Liu, Q. Q., Collier, J. L., Barnes, D., Allam, B., 2009b. Effects of Environmental Factors on Mercenaria Mercenaria Immunity, Qpx Survival and Host-Pathogen Interactions. Journal of Shellfish Research. 28, 720-720.

Perrigault, M., Tanguy, A., Allam, B., 2009c. Identification and expression of differentially expressed genes in the hard clam, Mercenaria mercenaria, in response to quahog parasite unknown (QPX). Bmc Genomics. 10, 377.

Perrigault, M., Tanguy, A., Allam, B., 2009d. Identification and expression of differentially expressed genes in the hard clam, Mercenaria mercenaria, in response to quahog parasite unknown (QPX). Bmc Genomics. 10.

Pörtner, H. O., 2002. Climate variations and the physiological basis of temperature dependent biogeography: systemic to molecular hierarchy of thermal tolerance in animals. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology. 132, 739-761.

Pörtner, H. O., Berdal, B., Blust, R., Brix, O., Colosimo, A., De Wachter, B., Giuliani, A., Johansen, T., Fischer, T., Knust, R., Lannig, G., Naevdal, G., Nedenes, A., Nyhammer, G., Sartoris, F. J., Serendero, I., Sirabella, P., Thorkildsen, S., Zakhartsev, M., 2001. 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). Continental Shelf Research. 21, 1975-1997.

Ragan, M. A., MacCallum, G. S., Murphy, C. A., Cannone, J. J., Gutell, R. R., McGladdery, S. E., 2000. Protistan parasite QPX of hard-shell clam Mercenaria mercenaria is a member of Labyrinthulomycota. Diseases of Aquatic Organisms. 42, 185-190.

Ragone Calvo, L. M., Walker, J. G., Burreson, E. M., 1998. Prevalence and distribution of QPX, Quahog Parasite Unknown, in hard clams Mercenaria mercenaria in Virginia, USA. Diseases of Aquatic Organisms. 33, 209-19.

Robinson, J. M., Vandre, D. D., 1995. Stimulus-dependent alterations in macrophage microtubules: increased tubulin polymerization and detyrosination. J Cell Sci. 108 (Pt 2), 645-55.

Rubin, E., Tanguy, A., Perrigault, M., Allam, B., 2011. Virulence-Related Genes in Qpx, the Thraustochytrid Parasite of the Hard Clam Mercenaria Mercenaria. Journal of Shellfish Research. 30, 550-550.

Rubin, E., Tanguy, A., Perrigault, M., Pales Espinosa, E., Allam, B., 2014. Characterization of the transcriptome and temperature-induced differential gene expression in QPX, the thraustochytrid parasite of hard clams. BMC Genomics. 15, 245.

Saeed, A. I., Sharov, V., White, J., Li, J., Liang, W., Bhagabati, N., Braisted, J., Klapa, M., Currier, T., Thiagarajan, M., Sturn, A., Snuffin, M., Rezantsev, A., Popov, D., Ryltsov, A., Kostukovich, E., Borisovsky, I., Liu, Z., Vinsavich, A., Trush, V., Quackenbush, J., 2003. TM4: a free, open-source system for microarray data management and analysis. Biotechniques. 34, 374-8.

Sekine, H., Kenjo, A., Azumi, K., Ohi, G., Takahashi, M., Kasukawa, R., Ichikawa, N., Nakata, M., Mizuochi, T., Matsushita, M., Endo, Y., Fujita, T., 2001. An ancient lectin-dependent complement system in an ascidian: novel lectin isolated from the plasma of the solitary ascidian, Halocynthia roretzi. J Immunol. 167, 4504-10.

Smolowitz, R., Defaveri, J., Walton, W., Murphy, D., Leavitt, D., 2008. The interaction of temperature and hard clam (Mercenaria mercenaria) strain on the occurrence of QPX disease in the laboratory and in the field. Journal of Shellfish Research. 27, 1052-1053.
Smolowitz, R., Leavitt, D., Perkins, F., 1998. Observations of a protistan disease similar to QPX in Mercenaria mercenaria (hard clams) from the coast of Massachusetts. Journal of Invertebrate Pathology. 71, 9-25.
Smyth, G. K., Speed, T., 2003. Normalization of cDNA microarray data. Methods. 31, 265-73.
Soderhall, K., Invertebrate immunity. Advances in experimental medicine and biology,. Landes Bioscience and Springer Science+Business Media, LLC, Boston, MA, 2010, pp. xxiv, 316 p.
Soukas, A., Cohen, P., Socci, N. D., Friedman, J. M., 2000. Leptin-specific patterns of gene expression in white adipose tissue. Genes Dev. 14, 963-80.
Stokes, N. A., Calvo, L. M. R., Reece, K. S., Burreson, E. M., 2002. Molecular diagnostics, field validation, and phylogenetic analysis of Quahog Parasite Unknown (QPX), a pathogen of the hard clam Mercenaria mercenaria. Diseases of Aquatic Organisms. 52, 233-247.
Tasumi, S., Vasta, G. R., 2007. A Galectin of Unique Domain Organization from Hemocytes of the Eastern Oyster (Crassostrea virginica) Is a Receptor for the Protistan Parasite Perkinsus marinus. The Journal of Immunology. 179, 3086-3098.
Venier, P., Varotto, L., Rosani, U., Millino, C., Celegato, B., Bernante, F., Lanfranchi, G., Novoa, B., Roch, P., Figueras, A., Pallavicini, A., 2011. Insights into the innate immunity of the Mediterranean mussel Mytilus galloprovincialis. BMC Genomics. 12, 69.
Wang, L., Wang, L., Kong, P., Yang, J., Zhang, H., Wang, M., Zhou, Z., Qiu, L., Song, L., 2012. A novel C1qDC protein acting as pattern recognition receptor in scallop Argopecten irradians. Fish & Shellfish Immunology. 33, 427-435.
Wang, S., Peatman, E., Liu, H., Bushek, D., Ford, S. E., Kucuktas, H., Quilang, J., Li, P., Wallace, R., Wang, Y., 2010. 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 Immunology. 29, 921-929.
Watson, F. L., Puttmann-Holgado, R., Thomas, F., Lamar, D. L., Hughes, M., Kondo, M., Rebel, V. I., Schmucker, D., 2005. Extensive diversity of Ig-superfamily proteins in the immune system of insects. Science. 309, 1874-8.
Xu, S.-y., Wang, L., Wang, L., Zhang, H., Zhou, Z., Siva, V. S., Song, L., 2012. A C1q Domain Containing Protein from Scallop Chlamys farreri Serving as Pattern Recognition Receptor with Heat-Aggregated IgG Binding Activity. PLoS ONE. 7, e43289.
Yeung, K. Y., Haynor, D. R., Ruzzo, W. L., 2001. Validating clustering for gene expression data. Bioinformatics. 17, 309-318.
Yue, X., Wang, H., Huang, X., Wang, C., Chai, X., Wang, C., Liu, B., 2012. 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 Immunology. 33, 559-568.
Zhang, H., Song, L., Li, C., Zhao, J., Wang, H., Qiu, L., Ni, D., Zhang, Y., 2008. A novel C1q-domain-containing protein from Zhikong scallop Chlamys farreri

with lipopolysaccharide binding activity. Fish Shellfish Immunol. 25, 281-9.
Zhang, H., Wang, L., Song, L., Song, X., Wang, B., Mu, C., Zhang, Y., 2009. A fibrinogen-related protein from bay scallop Argopecten irradians involved in innate immunity as pattern recognition receptor. Fish & Shellfish Immunology. 26, 56-64.
Zhang, L., Li, L., Zhu, Y., Zhang, G., Guo, X., 2013. Transcriptome Analysis Reveals a Rich Gene Set Related to Innate Immunity in the Eastern Oyster (Crassostrea virginica). Marine Biotechnology. 16, 17-33.
Zhang, S.-M., Loker, E. S., 2003. The FREP gene family in the snail Biomphalaria glabrata: additional members, and evidence consistent with alternative splicing and FREP retrosequences. Developmental & Comparative Immunology. 27, 175-187.
Zhang, S. M., 2004. Diversification of Ig Superfamily Genes in an Invertebrate. Science. 305, 251-254.
Zhang, S. M., Loker, E. S., 2004. 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, 255-66.

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

Figure legends

Figure 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 21ºC), challenge status (C: control, Q: challenged with QPX), and replicate number (1 to 3).

Figure 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 ± S.D.). Sample names are similar to those in Figure 1.

Figure 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.

Figure 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).

Figure 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 ± S.D.).

Figure 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.

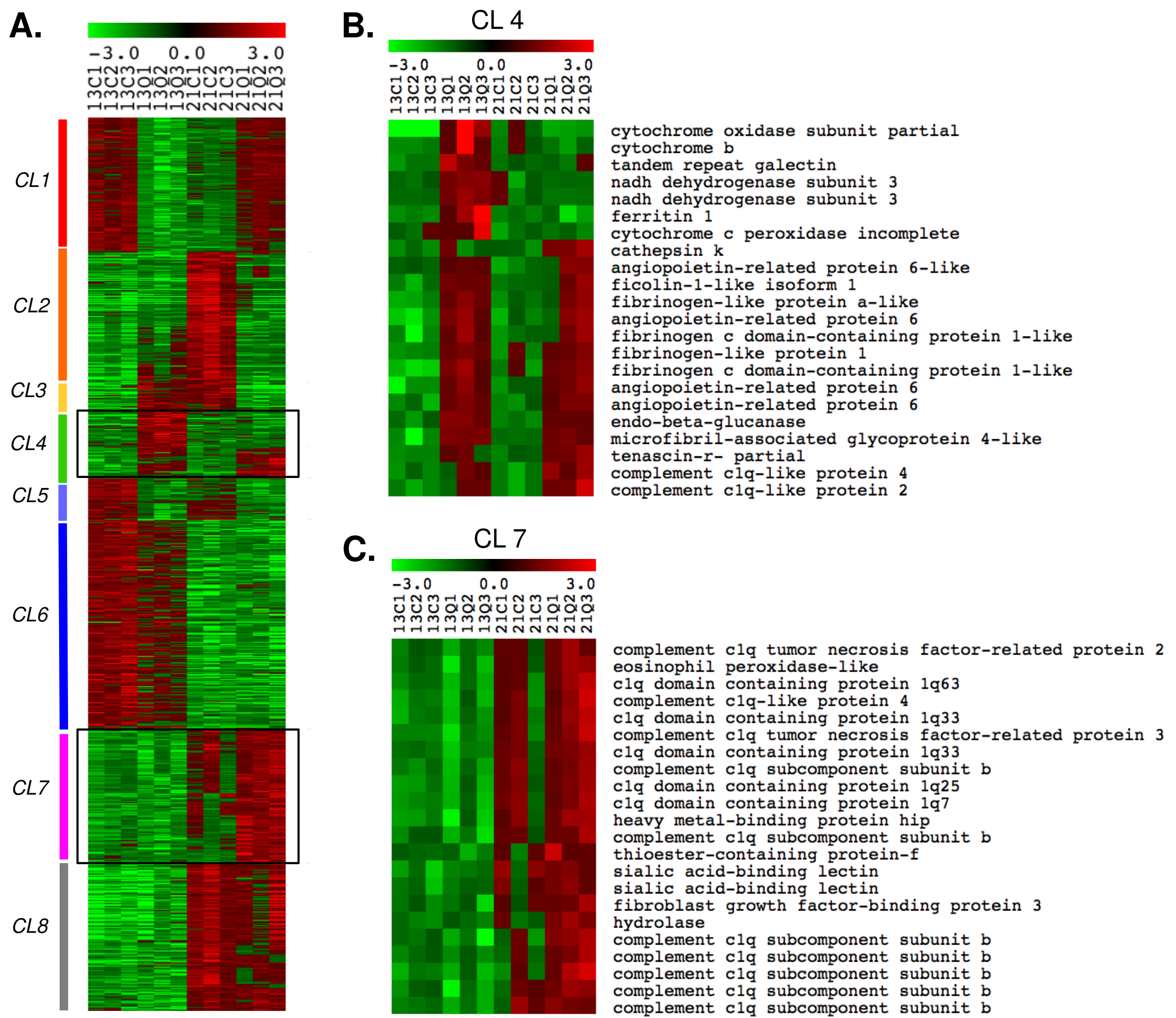

Figure 1

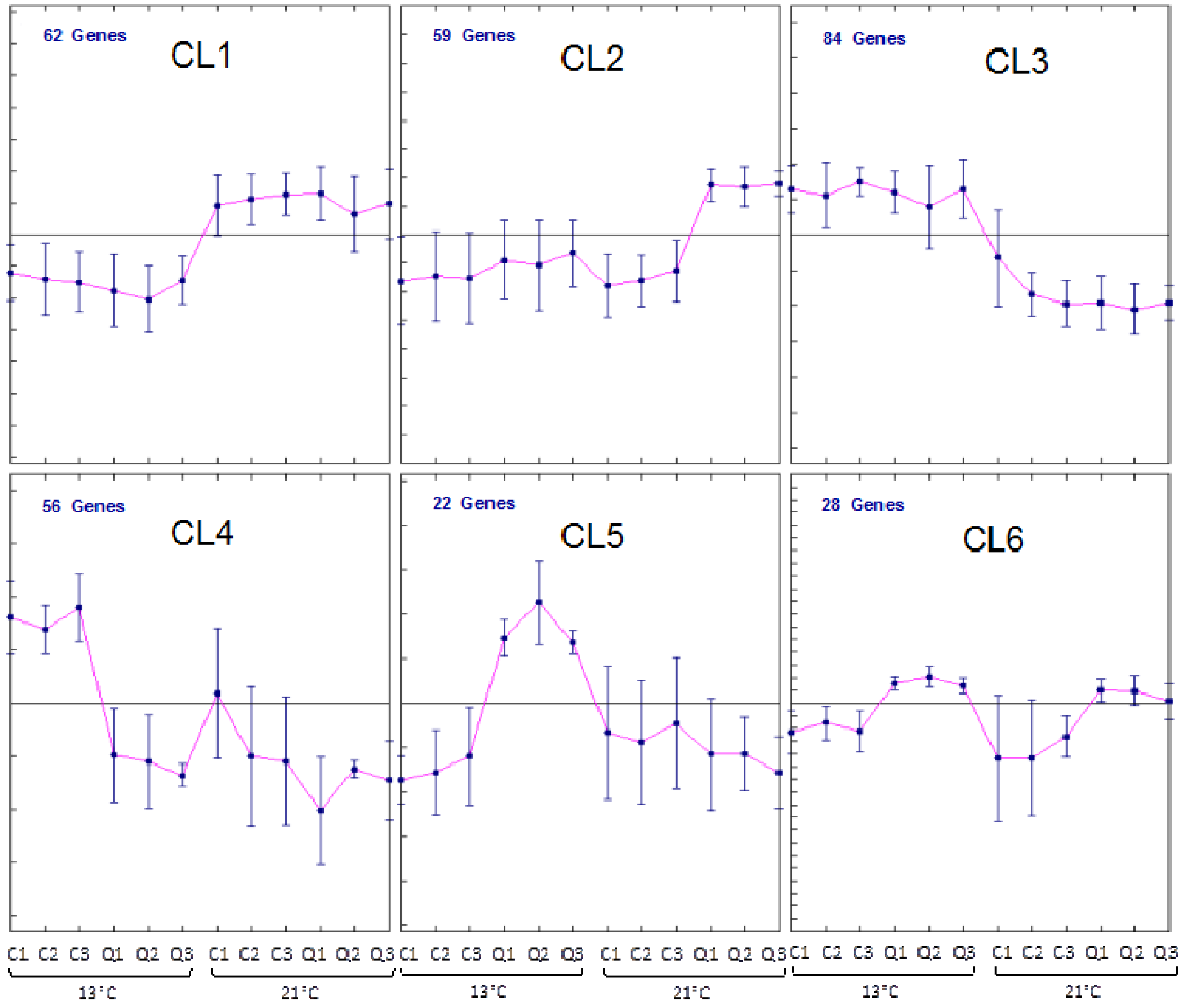

Figure 2

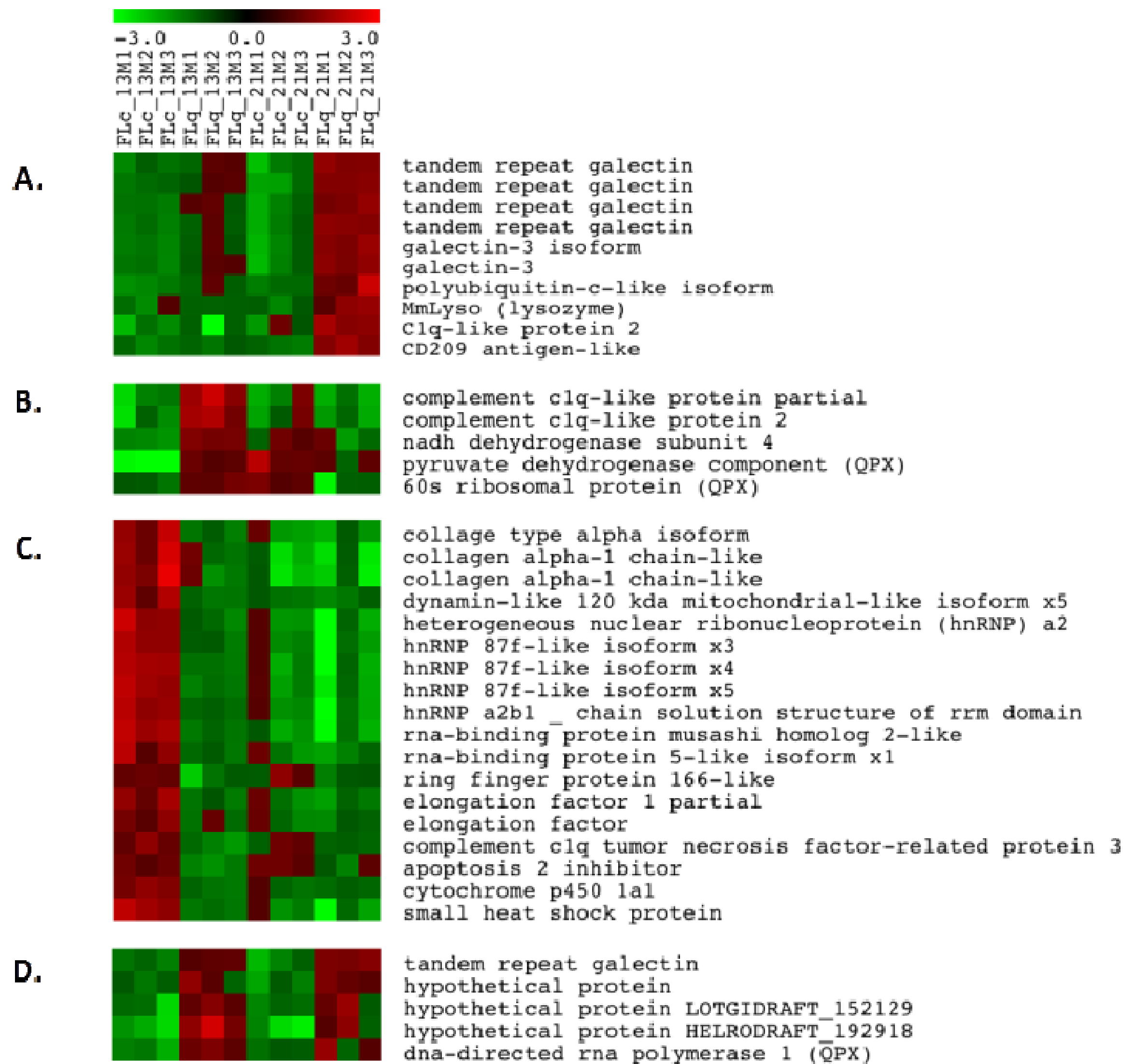

Figure 3

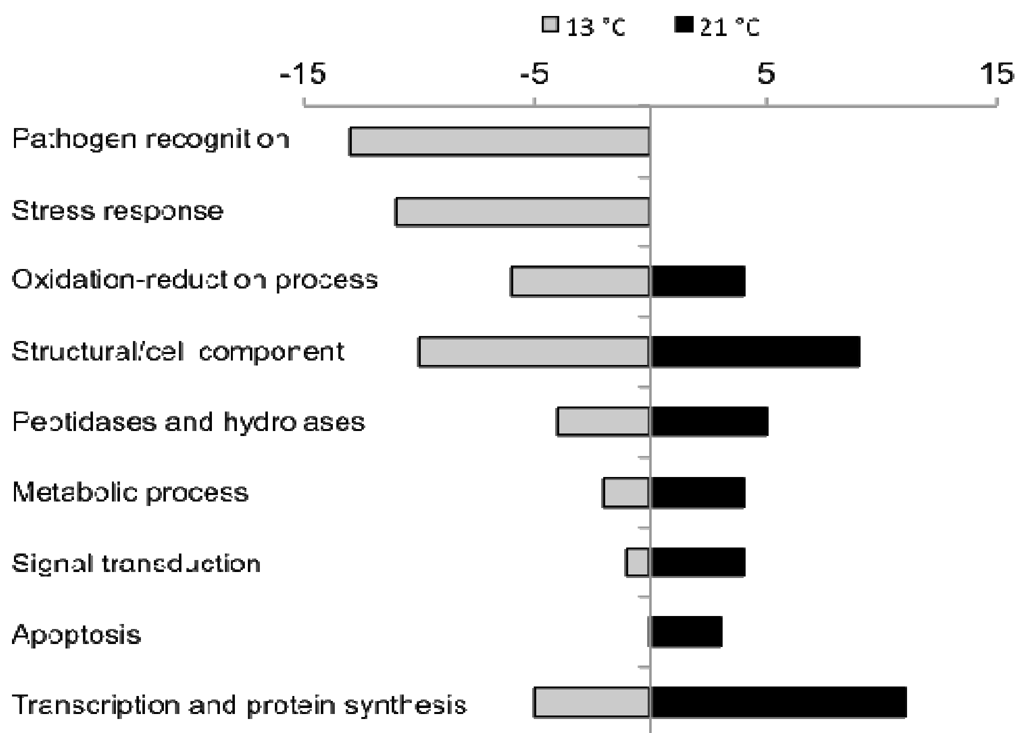

Figure 4

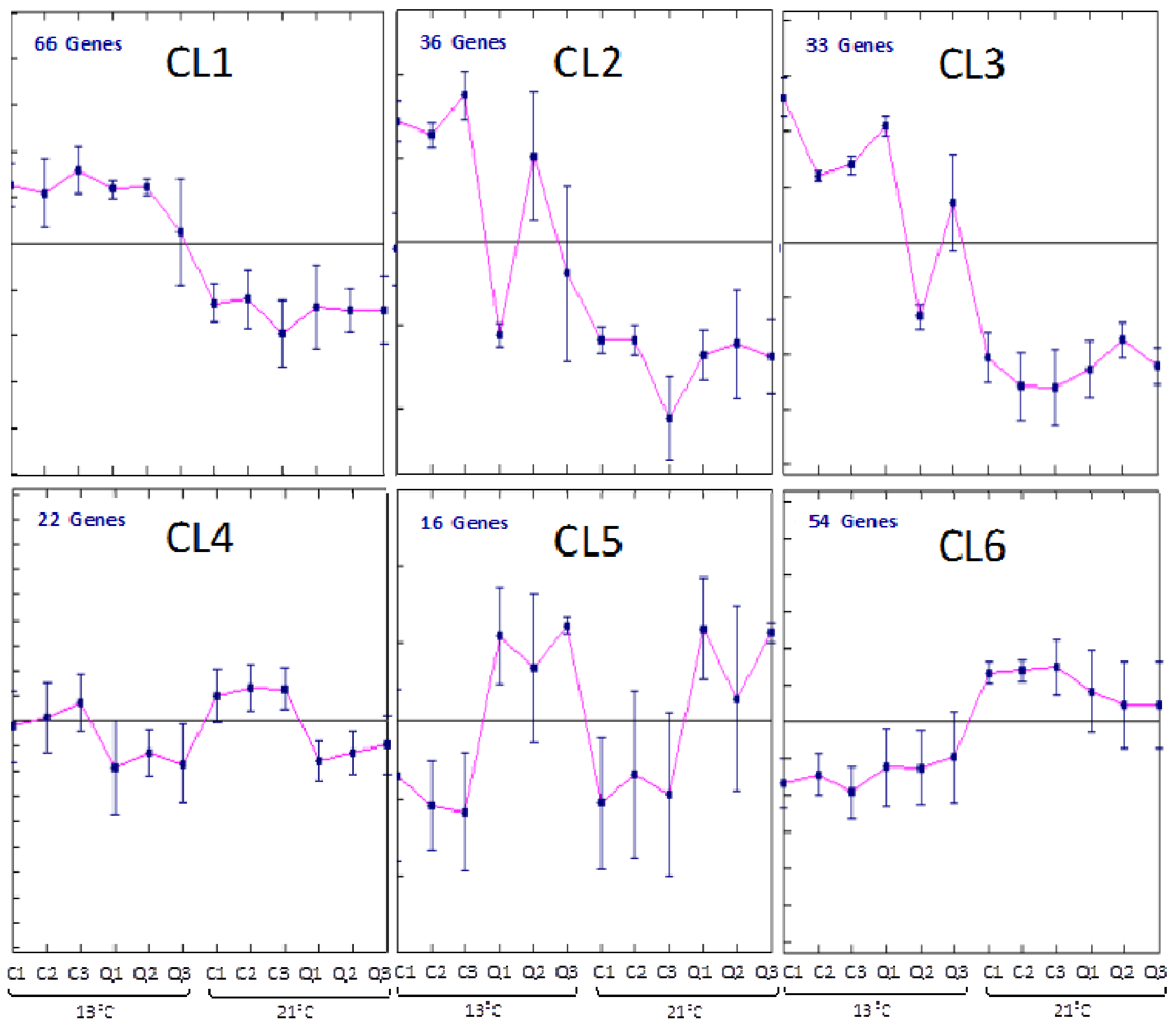

Figure 5

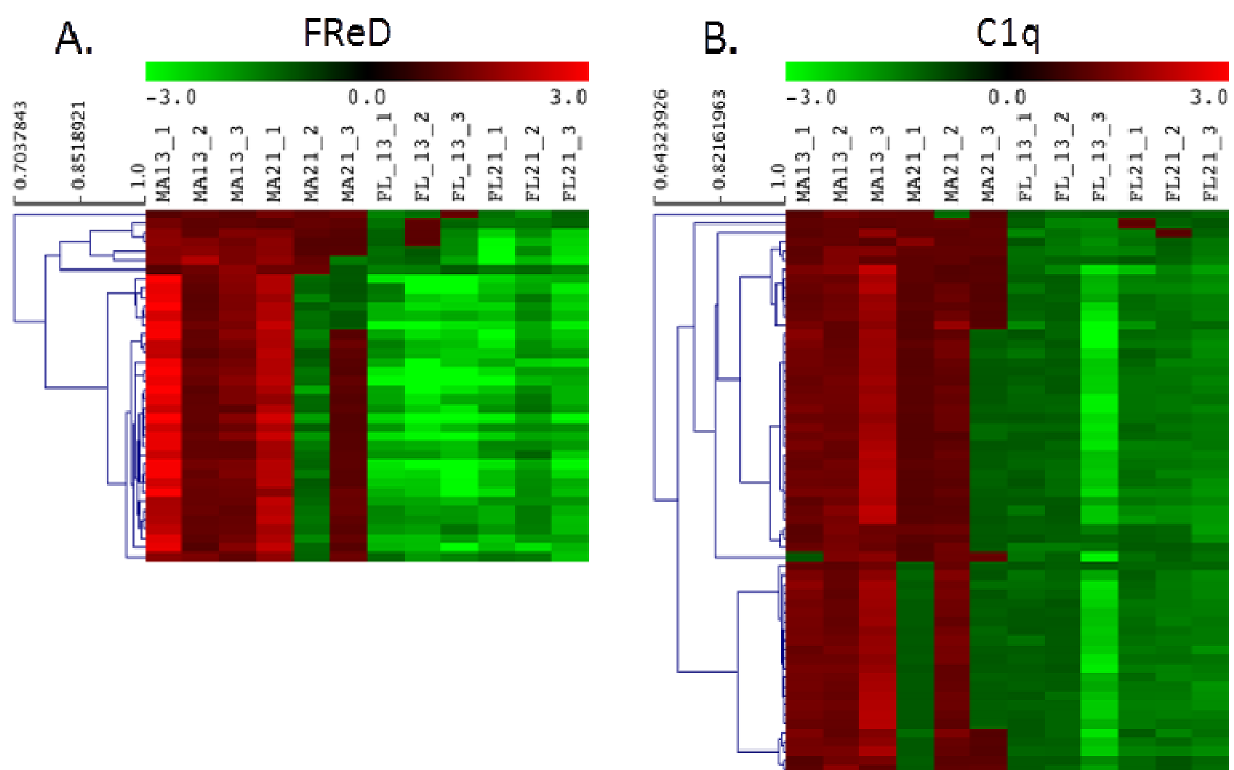

Figure 6

Differentially expressed (DE) transcripts in resistant and susceptible clams in response to QPX under different temperature conditions