AN ABSTRACT OF THE DISSERTATION OF

Robert Paul Lang for the degree of Doctor of Philosophy in Fisheries Science presented on May 29, 2008.

Title: Identification of Candidate Genes for Survival and Their Use in Predicting Field Performance of Pacific Oyster *Crassostrea gigas* Families in Coastal Waters

Abstract approved:

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Christopher J. Langdon

Summer mortality of farmed Pacific oysters causes financial losses for shellfish growers, and selective breeding to improve survival of offspring is a promising way to reduce losses. This dissertation has two objectives to assist the development of tools for use in selective breeding programs.

Objective 1: Identify candidate genes for selection and use in predictive assays by examining the transcriptome response to heat shock in gill of heat-shocked (40°C, 1 h) oysters and comparing gene transcription between families with high (>65%) or low (<30%) survival after heat shock (43°C, 1 h) by using a cDNA microarray and real-time quantitative polymerase chain reaction (RT-QPCR). The mRNA concentrations of *heat shock protein 27*, *peroxinectin*, *galectin*, *S-crystallin*, *collagen*, and of two ESTs with no match in Genbank, *BQ426658* and *BQ426884*, were greater overall or at individual time points in low-surviving families than in high-surviving families. The mRNA concentration of *cystatin-B* was greater in high-surviving families at 24 h after heat shock than in low-surviving families. These genes can be considered as candidate genes for use in selection programs and predictive assays.

Objective 2: Test for correlations between survival of heat shock as spat and field performance of adults at harvest at two growout sites, compare candidate gene transcription in whole bodies of four low-surviving and four high-surviving families, and test for correlations between candidate gene transcription in spat and field performance of adults at harvest. The correlations between survival of juveniles exposed to heat shock (41°C, 1 h) and survival, yield, or average body weight of adults at harvest were not significant. The mRNA concentration of *galectin* was significantly greater in low-surviving families before heat shock, and the mRNA concentrations of *cystatin B* at each sampling time and of *glutathione s-omega* at 24 h after heat shock were greater in high-surviving families. The correlations between mRNA concentrations of *heat shock protein 27, catalase, superoxide dismutase,* and *prostaglandin E receptor 4* in juveniles and survival of adults at harvest were significant. The correlations between mRNA concentrations of *galectin, BQ426658, heat shock protein 27, glutathione peroxidase,* and *prostaglandin E receptor 4* and weight of adults at harvest were significant. The correlations between mRNA concentrations of *galectin, BQ426658, BQ426884, heat shock protein 68, glutathione peroxidase,* and *prostaglandin E receptor 4* with yield of adults at harvest were significant. These results demonstrate that field performance can be predicted using gene transcription assays.

Identification of Candidate Genes for Survival and Their Use in Predicting Field Performance of Pacific Oyster *Crassostrea gigas* Families in Coastal Waters

by
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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Robert Paul Lang, Author

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Chimes restaurant in Baton Rouge; I'll raise a glass of Abita Purple Haze and leave a copy on the bar. My mother-in-law Julie passed away soon after my father, and I regret that she and her husband were not able to see me finish. Ms. Julie was a good friend who gave me selfless love and support, and I am grateful that she added wind into my sails.

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CONTRIBUTION OF AUTHORS

Dr. Christopher Langdon served as my departmental advisor, and Dr. Mark Camara served as an honorary co-advisor. They are included as authors on each of the manuscripts contained in this dissertation. They participated in design of experiments, analysis and interpretation of data, and preparation of manuscripts.

Drs. Matthew J. Jenny, Charles Cunningham, and Christopher J. Bayne are co-authors on the Chapter 2 manuscript entitled "Transcriptome profiling of selectively-bred Pacific oyster *Crassostrea gigas* families that differ in tolerance of heat shock". Dr. Matthew Jenny designed the cDNA microarray. Drs. Jenny, Cunningham, and Bayne each participated in interpretation of the dataset, and to preparation of the manuscript.

Dr. Nicolas Taris is a co-author on the Chapter 3 manuscript "Can laboratory assays using juveniles be used to predict the field performance of Pacific oyster (*Crassostrea gigas*) families planted in coastal waters". He contributed substantially towards developing analytical methods and interpretation of data.

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INTRODUCTION

Overview. Summer mortality syndrome in Pacific oysters *Crassostrea gigas* causes financial losses for shellfish growers in the United States, Japan, and France. Mortality episodes occur during periods of seasonal high temperatures, low dissolved oxygen, and high primary productivity (Soletchnick et al. 1999; Cheney et al. 2000; Soletchnick et al. 2005; Soletchnick et al. 2007). During these stressful periods, mortalities are thought to result from a poorly understood interaction of spawning-related glycogen and lipid metabolism (Perdue et al. 1981; Berthelin et al. 2000; Soletchnick et al. 2006), and infection by opportunistic pathogens (Lacoste et al. 2001; Friedman et al. 2005; Garnier et al. 2007). It is possible that the interaction of these factors causes mortality though metabolic exhaustion and opportunistic infection.

Survival of summer mortality is heritable in some oyster populations (Beattie et al. 1980; Dégremont et al. 2007). However, selecting broodstock based solely upon survival to produce resistant oyster strains is unreliable because environmental conditions vary from year to year and unexpected blooms of opportunistic pathogens may occur. This problem can be circumvented by selecting broodstock based upon genetic markers associated with candidate genes for surviving deleterious environmental conditions (Cnaani 2006; Rothschild and Ruvinsky 2007). Resistant strains could be reliably produced despite annual variability in biological and environmental parameters, but there is currently a need to identify genes associated with survival in the field, and to develop molecular markers for their use in selection programs for *C. gigas*.

Studies that address the transcription of stress-related genes in livestock can be used to better understand the physiological consequences of stress and to thereby identify candidate genes for use in selection programs. However, studying single genes in isolation based upon speculation about their potential importance for surviving summer mortality is time-consuming, expensive, and prone to producing null results. Complementary DNA (cDNA) microarrays overcome these obstacles by querying thousands of genes in parallel. Microarrays consist of bacterially-cloned cDNA gene fragments covalently-bound to glass slides to which fluorescently-labeled nucleic acid from samples of interest are applied. When a transcript is present in both the sample and on the microarray, the two hybridize through complementary base-pairing, and fluorescence can be detected using scanners equipped with lasers. In general, the intensity of the fluorescence corresponds to the quantity of transcript contained in the sample. Genes of interest based upon sample comparisons can then studied in isolation using real-time quantitative PCR to verify the microarray data.

Over the past five years, the amount of genetic information for oysters *C. gigas* and *Crassostrea virginica* has increased dramatically through the production of expressed sequence tag (EST) libraries. The "Oyster Microarray Consortium" was founded through a collaboration of North American and French scientists, and the outcome of this collaboration was a cDNA microarray that comprised most of the EST libraries published prior to 2007 and contained sequences from both *C. gigas* and *C. virginica* (Jenny et al. 2007). This tool shows promise for use in identifying candidate

genes for selection in *Crassostrea* sp., and will increase our understanding of the physiological consequences of stress that lead to summer mortality syndrome.

The process of rearing and evaluating oyster families separately in the nursery and field is expensive and labor-intensive. The ability to apply selection during the hatchery phase would alleviate some costs associated with evaluating families in the field. Brooks (2000) found that weight of juvenile *C. gigas* at plant-out was correlated with harvest weight, suggesting that poor and average-performing families could be identified prior to field trials. In a similar vein, the tolerance of oyster spat to conditions encountered during summer mortality might be used to predict performance of adults at commercial grow-out sites.

Research Approach. The goals of my dissertation research were to identify candidate genes for use in selection programs designed to improve survival of summer mortality, and to determine whether survival of heat shock or expression of candidate genes in juveniles would be predictive of subsequent growth and survival of adults in the field. I chose heat shock (39 – 43C, 1 h) as a proxy for estimating the tolerance of oyster families to summer mortality because repeated exposure to heat stress is a common feature of summer mortality sites in the Pacific Northwest region of the United States.

During tidal emersion, ambient air temperature frequently exceeds 40°C and can be as high as 53°C during low tide exposure on the Pacific coast, USA (Cheney et al. 2000; Hamdoun et al. 2003). Heat shock damages cells and proteins (Feder and

Hoffman 1999), causes increased production of reactive oxygen species (Flanagan et al. 1998; Arnaud et al. 2002; Bruskov et al. 2002;), increases oxidative stress in fish and marine bivalves (Abele et al. 2002; Kaur et al. 2005; Heise et al. 2006; Bagnyukova et al. 2007; Verlecar et al. 2007), and compromises bivalve cellular immunity (Hégaret et al. 2003; Hégaret et al. 2004; Gagnaire et al. 2006; Chen et al. 2007). The energetic cost of mounting heat shock responses in animals that are reproductively active can lead to opportunistic infection and death, especially when energetic reserves have been allocated towards reproduction (Li et al. 2007; Samain et al. 2007). Tolerance of heat shock has low heritability in bivalves (Elderkins and Klerks 1995), and marker-assisted selection could be useful for improving this trait.

To first identify candidate genes for survival of heat shock, I used a cDNA microarray to test the hypothesis that adult oysters from families with low survival (< 29% survival after heat shock) or high survival (> 65% survival after heat shock) differ in their transcriptional responses to stress. Heat shock perturbs different biological systems, and is likely to influence growth and survival under estuarine conditions. Therefore, survival of heat shock or the transcription in juveniles of genes that are thought to be relevant to surviving heat shock could be useful in predicting the growth and survival of animals in the field. To determine the value of assays using juveniles to increase the efficiency of selection programs, I tested the hypothesis that survival of heat shock and expression of candidate genes in juveniles was predictive of subsequent survival and growth of adults in the field.

Thesis Chapters. I report the use of the oyster cDNA microarray to identify candidate genes for increased thermal tolerance in the manuscript titled "Transcriptome profiling of selectively-bred Pacific oyster *Crassostrea gigas* families that differ in tolerance of heat shock" (Chapter 2). I report the use of measurements of survival of heat shock and candidate gene expression in juveniles in prediction of adult field performance in the manuscript titled "Can laboratory assays using juveniles be used to predict the field performance of Pacific oyster (*Crassostrea gigas*) families planted in coastal waters" (Chapter 3). In the final chapter, "Conclusions", I present a synthesis of the overall findings of this research project.

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TRANSCRIPTOME PROFILING OF SELECTIVELY-BRED PACIFIC OYSTER *CRASSOSTREA GIGAS* FAMILIES THAT DIFFER IN TOLERANCE OF HEAT SHOCK

R. Paul Lang, Mark D. Camara, Matthew J. Jenny, Charles Cunningham, Christopher J. Bayne, and Christopher J. Langdon

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ABSTRACT

Sessile inhabitants of marine intertidal environments commonly face heat stress, an important component of summer mortality syndrome in the Pacific oyster *Crassostrea gigas*. Marker-aided selection programs would be useful for developing oyster strains that resist summer mortality, but there is currently a need to identify candidate genes for surviving stress and to develop molecular markers associated with those genes. To identify candidate genes for further study, we used cDNA microarrays to test the hypothesis that oyster families that had high (>65%) or low (<30%) survival of heat shock (43°C, 1 h) differ in their transcriptional responses to stress. We extracted total RNAs from gills of two high- and low-surviving families before and at 1, 3, 6, and 24 h after oysters experienced a non-lethal heat shock (40°C, 1 h). The isolated mRNAs from each family, sampling time, and biological replicate were hybridized to single microarrays. We used an analysis of variance to detect significant differences in transcript concentrations among sampling times, between low- and high-surviving families, and significant interactions between these factors. The concentrations of 110 ESTs varied significantly among sampling times. We verified differential transcription of 15 ESTs using real-time quantitative PCR. Transcription increased after heat shock for genes encoding heat shock proteins and genes for proteins that synthesize lipids, protect against bacterial infection, and regulate spawning, whereas transcription decreased for genes that produce proteins that mobilize lipids and detoxify reactive oxygen species. RNAs encoding *heat shock protein 27*, *collagen*, putative *peroxinectin*, putative *S-crystallin*, and two genes with no match in Genbank

had higher transcript concentrations in low-surviving families than in high-surviving families, whereas concentration of *cystatin B* mRNA was greater in high-surviving families. These ESTs should be studied further for use in marker-aided selection programs. Low survival of heat shock could result from a complex interaction of cell damage, opportunistic infection, and metabolic exhaustion.

INTRODUCTION

Summer mortality syndrome causes mass mortalities of Pacific oysters *Crassostrea gigas* during warm summer months and has been problematic for oyster growers in the United States, Japan, and France since the 1960's (reviewed by Cheney et al. 2000 and Samain et al. 2007). Summer mortality kills both juvenile and adult oysters, and results from complex interactions between environmental factors such as temperature, dissolved oxygen, primary productivity and sediment quality (Soletchnik et al. 1999; Cheney et al. 2000; Soletchnik et al. 2005 Soletchnik et al. 2007), biological factors including reproductive condition and glycogen metabolism (Soletchnik et al. 1999; Berthelin et al. 2000), and pathogens (Lacoste et al. 2001; Friedman et al. 2005; Garnier et al. 2007). Resistance to summer mortality has a genetic basis in some populations (Dégremont et al. 2007), and selective breeding for oyster stocks that resist summer mortality is a promising way to reduce stock losses. However, the complex and unpredictable nature of summer mortality in U.S. waters complicates selection of broodstock solely upon the basis of phenotype. Marker-aided selection could be used to reliably produce stocks that have increased resistance to stress (Cnaani 2006; Rothschild and Ruvinsky 2007), but there is presently a need to identify genes and markers, as well as to understand the physiological basis of stress resistance in oysters.

Heat stress is an inevitable consequence of life in the marine intertidal zone, and during periods coinciding with summer mortality in the Pacific Northwest region of the United States, ambient temperature during tidal emersion can exceed 40°C

(Cheney et al. 2000; Hamdoun et al. 2003). In marine bivalves, heat shock results in oxidative stress (Abele et al. 2002; Verlecar et al. 2007), impairs immune defenses through reduced hemocyte phagocytosis and killing capacity (Hégaret et al. 2003; Hégaret et al. 2004; Chen et al. 2007), and may lead to metabolic exhaustion and susceptibility to opportunistic infection when coupled with energy investment in reproduction (Li et al. 2007). Each of these factors may contribute to summer mortality syndrome, and heat shock under laboratory conditions is a suitable means to elicit the transcription of genes that could be used as markers for selecting broodstock that produce offspring with improved survival under summer mortality-inducing conditions.

Transcriptome profiling is a useful first step towards identifying ways in which oysters differ in their responses to heat stress. Recently, an oyster cDNA microarray was made available that contains expressed sequence tag (EST) libraries from several different tissues of *C. gigas* and the Atlantic oyster *Crassostrea virginica* exposed to hypoxia, pesticides, bacterial and protozoan infection, hydrocarbons, hyperthermia, and summer mortality conditions (Jenny et al. 2007). We used this tool to test the hypothesis that oyster families that differ in survival after heat shock also differ in their transcriptional responses to this stress. The resulting data have identified several candidate genes that are potentially useful for marker-aided selection to enhance the resistance of Pacific oysters to heat stress and summer mortality.

METHODS

Screening of Families. To identify oyster families that differed in their tolerance of heat shock, we first heat-shocked one hundred juvenile oysters (1 – 2 cm) from each of 44 families from a selectively-bred cohort of oyster families produced according to Langdon et al. (2003). The heat-shock treatment consisted of exposure to UV-sterilized water heated to 43°C for 1 h (Clegg et al. 1998; Hamdoun et al. 2003) and recovery in troughs supplied with sand-filtered water at ambient temperature (~14°C) without the addition of algal ration, and was based upon preliminary experiments showing that this treatment killed approximately 50% of animals from pools of spat from all families (data not shown). We tentatively classified the families as low-surviving or high-surviving after heat shock when their survival after heat shock as juveniles was < 30% and > 65%, respectively.

One hundred and fifty unstressed juveniles from each of four low- and four high-surviving families were grown for two years in Yaquina Bay estuary, Newport, Oregon, USA at an intertidal site located approximately 15 km from the mouth of the Bay (44.6°N, 124.1 °W). Spat were deployed within individual rectangular mesh bags (0.53-m x 0.81m, 7-mm mesh) at a density of 50 animals per bag. During Summer 2005, we recovered the families, and allowed 1 month for acclimation in troughs supplied with sand-filtered water at ambient temperature (~14°C). Animals were fed a mixture of *Isochrysis galbana* and *Cheatoceros calcitrans* at a concentration of approximately 50,000 – 80,000 cells ml^{-1} during acclimation. We then heat-shocked (43°C, 1 h) 20 individuals from each of the eight families and monitored them as

before. Four of the eight families retained their classification as low- or high-surviving (two families of each type), and were used for the microarray experiment.

Tissue Collection. To elicit a heat shock response without killing the animals, we heat-shocked a separate group of 20 reproductively mature adult oysters (none were among the 20 oysters stressed as adults to assess survival) from the low- and high-surviving families in flow-through tanks supplied with heated (40°C) UV-sterilized water for 1 h. Animals were allowed to recover in troughs supplied with sand-filtered water at ambient temperature (~14°C). Five unstressed animals from each family were monitored alongside their heat-shocked siblings to confirm that no mortality was caused by handling, and 5 heat-shocked animals of the 20 were monitored for 6 d to confirm the non-lethality of the heat-shock treatment. We collected and pooled one piece of gill (~50 mg from the second lamellibranch) from each of three animals per family before heat shock and from separate individuals at 1, 3, 6, and 24 h afterwards, and homogenized the gill samples in buffer RLT (Qiagen, Inc., Valencia, CA) according to the manufacturer's directions. The entire experiment was conducted three times (from the step at which 20 animals were exposed at 40°C) to yield three biologically replicated tissue pools for each combination of family and sampling time. We chose to study gill tissue because of its ease of collection and because it is a large organ with high surface area that is perturbed by thermal stress (Meistertzheim et al. 2007).

RNA preparation and amplification. Total RNA was extracted from gill suspensions using Qiagen® RNeasy kits (Qiagen Inc., Valencia, CA) including the on-column DNAse treatment according to the manufacturer's instructions. We quantified total RNA using a SPECTRAmax PLUS spectrophotometer (Molecular Devices, Sunnyvale, CA), and produced amplified amino allyl RNA (aRNA) for hybridization to microarrays from 1 µg of total oyster RNA template using the Amino Allyl MessageAmp™ Kit (Ambion, Inc., Austin, TX), according to the manufacturer's instructions. In each reaction, we included a mixture of RNA standards produced from plasmid DNA stocks of *Karenia brevis* genes according to Jenny et al. (2007). The resulting RNA mixture contained 100 ng chlorophyll A/B binding protein (Genbank #CO059871) RNA, 10 ng photosystem core protein (Genbank #CO062297) RNA, 1 ng flavodoxin (Genbank #CO065421) RNA, and 0.1 ng photolyase (Genbank #CO064781) RNA.

Microarray Hybridization. We employed a single-dye approach, in which RNA pools representing one oyster family, sampling time, and biological replicate were prepared in 90-µl mixtures and hybridized to single arrays. Each 90-µl mixture contained 20 µg of Cy3-coupled aRNA added to a non-commercial hybridization buffer [50% formamide, 2.4% sodium dodecyl sulfate (SDS), 4x standard saline phosphate EDTA (SSPE), 2.5x Denhardt's solution, plus 1 µg Cot-DNA and 1 µg polydATP; Jenny et al. 2007]. We boiled the mixtures for 1 min and incubated them for 1 h in a covered thermocycler heated to 50°C, during which time we prepared the

microarrays for hybridization by soaking in 0.2% SDS solution for 2 min and boiling water for 2 min, and drying by brief pulsed centrifugation. To reduce non-specific background, dried slides were pre-hybridized with a non-commercial blocking solution (1.6x Denhardt's solution, 33.3% formamide, 1.6% SDS, 2.6x SSPE and 0.1 µM salmon sperm DNA; Jenny et al. 2007) for 1 h at 50°C in a humidified hybridization oven (Boekel Inslide-Out^{tm}, Boekel, Festerville, PA). We added the aRNA mixtures to the slides, covered the slides with coverslips (Lifterslips, Erie Scientific, Portsmouth, NH) and stored the slides overnight (12 h) at 50°C in the hybridization oven. We then washed away unbound aRNAs by quickly dipping the slides in 2x saline sodium citrate (SSC), 0.1% SDS to remove cover-slips and soaking them for 15 min each in 0.2x SSC, 0.1% SDS, followed by 0.2x SSC, and 0.1x SSC. Slides were dried by brief pulsed centrifugation, and scanned within 15 min.

Microarray Data Acquisition and Analysis. We used a ScanArray Express (Perkin Elmer, Boston, MA) microarray scanner to acquire images of each slide, using the included SpotArray software package. We scanned each slide using 70% PMT gain and 90% laser power. The QuantArray software package (Perkin Elmer, Boston, MA) was used to acquire raw fluorescence data, background, and spot quality information from the scanned images using the included histogram spot segmentation method. Each spot image was visually inspected for overall quality, and damaged spots were excluded prior to statistical analysis. The hybridization data and the MIAME protocols are currently available at the Marine Genomics website

(www.marinegenomics.org), and will be deposited into the Gene Transcription Omnibus website during July 2008.

We accounted for mean-versus-variance dependence of the raw data by transforming the entire dataset using a robust generalized log transformation, variance stabilized normalization (*vsn*) method (Huber et al. 2002) as implemented in the Bioconductor suite for the R software environment (Ihaka and Gentleman 1996; Gentleman et al. 2004). All spots were included in the transformation. To adopt a conservative approach towards data analysis, only spots with an average *vsn*-adjusted signal of 6 for at least one of 5 times points were included in our analysis; this intensity cut-off corresponds to the average signal generated by the *K. brevis* RNA gene for photolyase for all slides (average signal ± SEM = 5.8 ± 0.03).

Spots were printed in duplicate within each array (Jenny et al. 2007), and we averaged the two replicate spots after *vsn*-transformation but before statistical analysis. We used analysis of variance to compare the means of *vsn*-adjusted signals for ESTs among sampling times, between low- and high-surviving family types, between individual families within either family type, and for the interaction of time and family type using the SAS system (PROC GLM, SAS/STAT Software, SAS Institute Inc., 1999). The statistical model was: Signal = Time + Family Type + Family ID (Family Type) + Family Type * Time, where "Time" refers to sampling time, "Family Type" refers to low-surviving versus high-surviving families, "Family ID (Family Type)" refers to individual families within either family type, and "Family Type * Time" is the interaction of time and family type. To avoid issues with multiple hypothesis tests,

we calculated *Q*-values for each EST using the software *Q*-value (Storey 2002; Storey and Tibshirani 2003). The use of *Q*-values is based on false discovery rate approaches that estimate the proportion of false positives among large numbers of tests (Benjamini and Holchberg 1995). We adopted a false discovery rate of 5% and effects were considered to be significant when $Q < 0.05$.

The identities of the ESTs printed on the microarray were determined using the algorithms BLASTX and BLASTN which are available at the National Center for Biotechnology Information (NCBI). An EST was considered to have significant database match when the expected value (*E*) was less than 1.E-05. We aligned ESTs with matching identities using CLUSTALX, and excluded the shorter EST of a matching pair with identical nucleotide sequences.

To visually summarize temporal transcription for the purpose of discussing ESTs not studied by real-time quantitative PCR, we produced time-series clusters of differentially expressed ESTs using the software SSClust (Ma et al. 2006). This program plots each cluster as a mean times series with 95% confidence intervals for ESTs with similar profiles, and should be regarded as a guide to overall change over time rather than an absolute representation of the transcription profile of each EST contained within that cluster. Data for each EST were converted to a common scale by dividing mean *vsn*-adjusted signal for each sampling time by the average of all values for that EST. Raw clustering output is included in Appendix 1.

Real-Time Quantitative Polymerase Chain Reaction. We used real-time quantitative PCR (RT-QPCR) to verify the microarray data of 15 ESTs for the 0, 1, 6, and 24 h sampling times. We verified all ESTs that were significant for the terms Family Type and Family Type*Time, and for a subset of ESTs that were significantly different among sampling times that were considered to be relevant to heat shock based upon literature searches, and for which reliable primers could be designed. Complementary DNA (cDNA) was reverse-transcribed from the original total RNA samples using the ABI® High Capacity cDNA Synthesis kit (Applied Biosystems, Foster City, CA) using the included random primers, according to the manufacturer's instructions. All RT-QPCR reactions were conducted using an ABI7500 quantitative PCR thermocycler (Applied Biosystems, Foster City, CA), had concentrations of 50 nM forward and reverse primers, and contained 3 ng of cDNA,12.5 µl of 2X SYBR Green master mix (Applied Biosystems, Foster City, CA) and water resulting in a total reaction volume of 25 µl. We used the thermocycling protocol: 1) 50°C, 2 min, 2) 95°C, 10 min for transcription enzyme activation, and 3) 40 or 45 cycles of a) 95°C for 15 seconds and b) 58°C or 59°C for 1 min (Table 1).

We designed RT-QPCR assays using pools of RNA samples from each of the four families at each sampling time point. Taris et al. (2008) found that RT-QPCR analysis of pooled oyster RNA samples can lead to misleading inferences for gene transcription among oyster families due to polymorphisms among individuals within such pools. Therefore, we prepared pools of cDNA that each contained all replications of an individual family at a single sampling time (4 times * 4 families =

16 pools in total), and tested multiple primer pairs for each gene using each of the 16 pools. Assays were deemed suitable for use when multiple primer pairs produced similar amplification profiles among the pools and also produced a single PCR product, as determined by examining post-reaction dissociation plots. Preference for individual primer pairs that met these criteria was based on high amplification efficiencies which were estimated as described below.

Raw RT-PCR data were acquired using the SDS 1.4 software (Applied Biosystems, Foster City, CA) and expressed as the relative concentration of target to an internal endogenous control gene, elongation factor 1 (*ef1*; Genbank #AB122066). We used the formula [relative concentration = $(E_r^{cT\ (target)} / E_r^{cT(ef1)})$], where E_r is the reaction-specific amplification efficiency estimated using LinRegPCR software (Ramakers et al. 2003), cT is the fractional cycle at which amplification reached a detection threshold, target is the EST under consideration, and *ef1* is the endogenous control gene. We chose *ef1* because it was stably expressed in *C. gigas* exposed to a variety of conditions in other studies (Huvet et al. 2004; Taris et al. 2008). Additionally, analysis of variance revealed no significant difference in average cT of *ef1* among sampling times ($P = 0.3114$), between low- and high-surviving families ($P = 0.9209$), and the interaction of family type and time ($P = 0.9399$) in the samples used for this study.

We used analysis of variance to test for differences among RT-QPCR data using the same statistical model as used for the microarray data. Additionally, we

performed linear contrasts using the CONTRAST statement within the GLM procedure of SAS. These contrasts tested for differences in mean relative transcript concentrations between consecutive sampling times, and for differences in mean relative transcript concentrations between low- and high-surviving families at each time point. We did not include linear contrasts for the microarray data to avoid issues with multiple testing.

RESULTS AND DISCUSSION

Microarray. In total, 1,675 averaged spot pairs for individual ESTs were included in our analysis after quality control, of which 66% originated from *C. gigas* libraries, and 34% originated from *C. virginica* libraries (Table 2A). The mean *vsn*-adjusted signals of 110 ESTs (~7% of all ESTs included in the analysis) differed significantly among sampling times, of which the majority originated from *C. gigas* (Table 2A). Based on the microarray, mean *vsn*-adjusted signals of four ESTs differed significantly between family types, and one significant interaction of time and family type was detected (Table 2A). We classified the 110 ESTs that differed among sampling times into ten functional categories based on searches of published literature (Table 2B). Over half of the ESTs had no match in Genbank. The identities and accession numbers of these ESTs are reported in Tables 3A and 3B.

To visually summarize the temporal data, we grouped 73 of the 110 ESTs into six descriptive clusters (Figure 1). The cluster designation for each EST is listed alongside accession numbers in Table 3. Clusters A and F contained ESTs in which

transcript concentration increased and then decreased within the 24-h sampling period. Clusters D and E contain ESTs in which transcription increased monotonically. Cluster C contains ESTs in which transcription remained low until after the 6-h sampling period and then increased sharply. Cluster B contains ESTs in which transcription was suppressed after heat shock. Not all ESTs could be assigned to the six clusters because their expression pattern was unique and would have precluded unambiguous description of other ESTs. We identified these ESTs by visually inspecting the raw output and excluding ESTs whose expression profile was markedly dissimilar to each of the clusters.

RT-QPCR. We selected a subset of ESTs from six of the functional categories listed in Table 2B for verification using RT-QPCR and for discussion below based upon their potential relevance to heat shock and reports in other studies of summer mortality in bivalves. These data are reported in Figures 2, 3, and 4; in each figure, microarray data are presented on the left and RT-QPCR data on the right. The six categories we addressed were: molecular chaperones and co-chaperones (Figure 2), growth and reproduction (Figure 2), antioxidant and detoxification enzymes (Figure 3), lipid metabolism (Figure 3), cellular immunity (Figures 3 and 4), and ESTs with no match in Genbank (Figure 4). In general, patterns of change in transcript concentration over time were similar between the microarray and RT-QPCR methodologies (discussed below) and we therefore consider those microarray data to be reliable. The

transcription profiles for ESTs discussed below that were not verified using RT-QPCR are available in Appendix 1 of this dissertation

Microarray data and RT-QPCR data were not always similar for ESTs whose concentrations differed between family types. The outcomes of the statistical tests for both the microarray data and the RT-QPCR data are reported in Table 4A. The RT-QPCR data did not support one of the differences between family types (Genbank # *AM237729*) or the significant interaction indicated by the microarray data, (Genbank # *BQ426623*) - these are discussed below in the section "ESTs with no match in Genbank." In the RT-QPCR data only, we detected an overall effect of family type for mRNA concentrations of *galectin* and an EST with no macth in Genbank (*BQ426884*), a significant interaction of time and family type for *heat shock protein 27*. Linear contrasts revealed significant differences between the family types at individual sampling times for *S-crystallin* and *cystatin B.*

Discrepancies between the microarray data and the RT-QPCR data could have resulted from microarray EST probes that bound multiple transcripts from families of closely related gene families. In addition, gene polymorphisms specific to the oyster families may have influenced both microarray results and RT-QPCR results. Sequence polymorphisms in *C. gigas* coding regions are thought to occur on average every 60 base pairs (Sauvage et al. 2007), and sequence mismatches could have influenced transcript binding to microarray probes. Furthermore, studies that employ RT-QPCR to compare family-level gene transcription among pools of tissue from multiple *C. gigas* individuals and families may be influenced by polymorphisms

among individuals within the pools, and can lead to misleading inferences (Taris et al. 2008). We tested each EST using multiple primers to increase our confidence that inferences were not influenced by polymorphisms, but caution should still be excersized in interpreting these data.

Molecular Chaperones. Based on the microarray data, concentrations of mRNAs encoding *heat shock protein 70, heat shock protein 90,* and *small stress proteins* increased progressively following heat shock in both low- and high-surviving families (microarray data only; Table 3; Figure 1, Clusters A, D and E). This is not surprising because rapid synthesis of heat shock proteins is a nearly universal response to heat shock and other stressors (Lindquist 1986; Hochacka and Somero 2002). Heat shock proteins refold denatured proteins and fold newly synthesized proteins into functional conformations (Parsell and Lindquist 1993; Zhao and Houry 2007), and small stress proteins participate in protein folding, block apoptosis and protein translation during heat shock, and bind lipid membranes to ensure structural integrity (Bruey et al., 2000; Cuesta et al. 2000; Wang and Spector, 2000; Tsvetkova et al., 2002; Doerwald et al., 2006). The presence of increased levels of heat shock proteins after thermal stress confers the ability to withstand subsequent exposure to otherwise lethal temperatures in marine bivalves and other animals and is an important aspect of bivalve adaptation to their environment (Clegg et al. 1998; Hamdoun et al. 2003).

We verified the mRNA concentrations of *heat shock cognate 70* (Genbank #AJ305315) and *small heat shock protein 27* (Genbank #BQ426550) using RT-QPCR

(microarray and RT-QPCR data: Table 4; Figures 2A and 2B). The microarray probe for the *heat shock cognate 70* gene (Boutet et al. 2003) is a full-length mRNA sequence that contains six exons and encodes a highly conserved protein with homology to several other heat shock proteins. Therefore, we consider the microarray signal for this particular probe to be a measure of heat shock protein 70 family gene transcription rather than that of an individual gene. We designed primers to amplify only the single gene product of *heat shock cognate 70* and found that its relative mRNA concentration increased after heat shock and decreased after 6 h whereas transcription of *heat shock protein 70* family genes appeared to increase only after 1 h and remained elevated thereafter (Figure 2A). Although we detected no family-level differences in the transcription of this heat shock protein gene, heat shock protein 70 protein concentrations in gill before and during experimental hypoxia were greater in *C. gigas* families that were susceptible to summer mortality than in those of families that were resistant (Samain et al. 2007).

Based on the RT-QPCR data, there was a significant interaction of time and family type for the relative concentration of *heat shock protein 27* (Table 4). The significant interaction resulted from the significantly greater concentration of transcript in low-surviving families at 6 h after heat shock (Figure 2B). Taken together with the findings of Samain et al. (2007), it is possible that a consequence of stress sensitivity is an exaggerated requirement for heat shock protein transcription and production to repair damage caused by heat shock and environmental stress that may also impose considerable metabolic costs. Li et al. (2007) found that heat shock

and heat shock protein 70 production reduced tissue energy levels in reproductively mature *C. gigas*, and that the combination of reproductive activity and mounting a heat shock response lead to metabolic exhaustion. Therefore, we speculate that high production of heat shock protein gene transcripts and proteins could be deleterious under certain conditions due to trade-offs between the need to salvage damaged proteins and energetic costs this imposes on other biological functions.

Growth and Reproduction. The relative concentration of *suppressor of cytokine signaling-2* (Genbank #BQ426927; microarray and RT-QPCR data: Table 4; Figure 2C) mRNA increased between 1 and 6 h after heat shock and remained elevated thereafter. Suppressor of cytokine signaling proteins disrupt signaling cascades by targeting cytokines for proteasomal degradation (Alexander 2002; Yoshimura et al. 2007). In mammals, suppressor of cytokine signaling-2 negatively regulates growth by inhibiting signal transduction by insulin-like growth factor-1 and growth hormone (Greenhalgh and Alexander 2004; Rincón et al. 2007). In *C. gigas*, an insulin receptor related receptor has been identified, and recombinant human insulin-like growth factor-1 has been shown to stimulate tissue growth (Griscourt et al. 2003). Thus, it is possible that suppressor of cytokine signaling-2 expression after heat shock resulted in suppressed growth which would be advantageous for reallocating energy to meet the needs of mounting a stress response.

The mRNA concentration of *collagen* (Genbank #CX069163; microarray and RT-QPCR data: Table 4; Figure 2D) was greater overall in low-surviving than in

high-surviving families based on both the microarray data and RT-QPCR data, but in the RT-QPCR dataset the significant family effect may have resulted from the significantly greater concentration in low-surviving families at 1 h after heat shock (Figure 2D). Collagen is an abundant protein that is part of the extracellular matrix that is broken down and deposited as part of tissue growth (Woessner 1998; Ziegler et al. 2002; Montagnani et al. 2005). We speculate that its increased transcription may reflect differences in tissue growth and remodeling between the family types. Reduced growth in the high-surviving families could have consequences for stress tolerance because more energy would be available to fuel stress responses.

The concentrations of ***temptin*** (Genbank #CK172319; microarray data only: Table 3; Figure 1, Cluster C) and of ***neuropeptide Y*** (Genbank #BQ426456; microarray and RT-QPCR data: Table 4; Figure 2E) mRNAs increased after 6 h. Exposure to high temperature is a common method of inducing spawning in oysters in hatcheries (e.g. Tibile and Singh 2003), and the transcription data for ***temptin*** and ***neuropeptide Y*** suggest that spawning is influenced by simultaneous positive and negative regulation. Temptin is a component of the protein pheromone complex used by the mollusc ***Aplysia*** to attract mates and stimulate spawning behavior (Cummins et al. 2004) and may play a role in promoting ***C. gigas*** spawning. Neuropeptide Y secretion is associated with increased hunger and decreased sexual activity in mammals (Kalra and Kalra 2004).

Receptors for neuropeptide Y have been identified in molluscs (Tensen et al. 1998) and its expression could suppress spawning, which would be advantageous

under certain conditions. For example, in France oyster families that are susceptible to summer mortality invest more energy into gametogenesis and spawn repeatedly, whereas resistant families invest less energy into gametogenesis and fully spawn once per reproductive season (Samain et al. 2007). Tissue energy reserves in oysters are lowest during summer months (Berthelin et al. 2000) and the combination of reproductive activity and low energy reserves can lead to metabolic exhaustion and death by opportunistic infection (Li et al. 2007). Although we detected no differences at the family level in gill of heat-shocked oysters, neuropeptide Y may be involved in the differing patterns of spawning in summer mortality susceptible and resistant families and may be of interest for future research.

Antioxidant and Detoxification Enzymes. The concentration of *glutathione peroxidase* (Genbank #CX069146; microarray data only; Table 3, Figure 1, Cluster B) mRNA decreased after heat shock. Heat shock causes increased production of reactive oxygen radicals that react to form long-lived toxic byproducts, and these molecules damage cells and membranes (Flanagan et al. 1998; Arnaud et al. 2002; Bruskov et al. 2002). Antioxidant enzymes such as superoxide dismutase, catalse, and glutathione peroxidase reduce oxidative damage by detoxifying free oxygen radicals and their byproducts (Foreman and Fisher 1981; Storey 1996; Young and Woodside 2001). Although we detected no family-level differences in the microarray data, suppressed expression of glutathione peroxidase in gill after heat shock could be a potentially deleterious consequence of heat shock.

The concentration of *S-crystallin* (Genbank #AJ565456; microarray and RT-QPCR data: Table 4; Figure 3A) mRNA decreased after 6 h, and based on the RT-QPCR data was greater in low-surviving families at 6 h after stress. The protein S-crystallin is derived from glutathione S-transferase and is a major protein of vertebrate and invertebrate eye lenses that appears to lack enzymatic function (Chiou et al. 1995; Tomarev and Piatigorsky 1996; Chuang et al. 1999; Blanchette et al. 2007). As with most of the probes included on the microarray, it is possible that the probe for this *S-crystallin* EST hybridized with mRNA from multiple genes, leading to the discrepancy between the microarray data and the RT-PCR data. Although we have included the putative *S-crystallin* under the heading of "detoxification," further characterization of this EST is needed to determine whether it truly encodes S-crystallin, its function, and the significance of enhanced transcription in low-surviving families.

Lipid Metabolism. The transcription of *delta-9-desaturase* (Genbank #AJ565582; microarray and RT-QPCR data: Table 4; Figure 3B) and of *sterol regulatory element binding protein transcription factor 1* (Genbank #BQ426935; microarray data only; Table 3; Figure 1, Cluster C) increased after the 6 h sampling time. Heat shock damages lipid membranes through changes to the physical state of the membrane and through oxidative stress (Storey 1996; Hochacka and Somero 2002). Delta 9-desaturase is required for synthesis of unsaturated fatty acids and sterol regulatory element binding protein transcription factor 1 activates genes involved in cholesterol synthesis (Ntambi 1999; Martin et al. 2006; Bengoechea-Alsono and Ericcson 2007).

Therefore, the increased transcription of these genes could reflect repair and synthesis of lipid membranes that were damaged by heat shock. We verified the concentrations of *delta-9-desaturase* using RT-QPCR because in *C. gigas* its transcription is altered by exposure to chronic hypoxia (David et al. 2005), chronic elevated temperature (25°C, 3 d; Meistertzheim et al. 2007), and hydrocarbon exposure (Boutet et al. 2004), and because polymorphisms in this gene have been linked with summer mortality syndrome (David et al. 2007). However, we detected no family-level differences in its expression in heat-shocked gill.

The transcription of *patatin-like phospholipase* (#BQ426641; microarray data only; Table 3, Cluster B) and of *fatty acid binding protein* (Table 3; Genbank #CK172312; microarray data only; Table 3, Cluster B) decreased after heat shock. Patatin-like phospholipase metabolizes fatty tissue in mammals (Schweiger et al. 2006), and fatty acid binding protein binds and transport lipids (Storch and Thumser 2000). These data suggest that mobilization of lipid reserves was suppressed by heat shock, which would be deleterious to mounting stress responses and gametogenesis during seasonal periods when tissue reserves of glycogen are low (Berthelin et al. 2000). Disruption of lipid deposition into gonads during spring is thought to contribute to summer mortality in *C. gigas* reared in Marennes–Oleóron bay, France (Soletchnik et al. 2006), and *C. gigas* families that were susceptible to summer mortality syndrome had lower transcription levels of *fatty acid binding protein* than resistant families following injection with pathogenic *Vibrios* (Huvet et al. 2004).

Immune and Inflammatory Responses. The relative concentration of *nucleoredoxin* (Genbank #AJ565627; microarray and RT-QPCR data; Table 4; Figure 3C) mRNA increased after each of the sampling times which suggest a rapid and intense inflammatory response to heat shock. Nucleoredoxin resides in the nucleus and participates in the regulation of transcription factors including the rapid-acting nuclear factor κB (NF-κB) that enables cells to quickly react to harmful stimuli and to mount immune responses (Hirota et al. 2000).

Based on the RT-QPCR data, there was a significant interaction of time and family type for the relative mRNA concentration of *peroxinectin* (Genbank #AM237676; microarray and RT-QPCR data; Table 4; Figure 3D). In the low-surviving families, relative mRNA concentration was greater before and at 1 and 6 h after heat shock and then decreased after 6 h, whereas transcript concentration did not change in the high-surviving families (Figure 3D). Peroxinectin is a cell-adhesion protein with peroxidase activity identified in crustaceans including freshwater crayfish *Pacifastacus leniusculus* (Johansson et al. 1995), white shrimp *Litopenaeus vannamei* (Liu et al. 2005), and prawn *Machrobrachium rosenbergii* (Liu et al. 2007). It is synthesized and stored in hemocytes, and when released mediates hemocyte adhesion to foreign particles (Liu et al. 2007). *Peroxinectin* has not been previously studied in *C. gigas* but it may serve an adhesive / defensive function in oysters. The potential significance of the pattern of *peroxinectin* transcription in low-surviving families is discussed below.

The relative mRNA concentration of another cell-adhesion protein, *galectin* (Genbank #AM237796; microarray and RT-QPCR data; Table 4, Figure 3E), increased after 6 h and based on the RT-QPCR data was greater overall in low- than in high-surviving families. In vertebrates, galectins facilitate cell adhesion and participate in various aspects of cellular immunity (Perillo et al. 1998; Rabinovich et al. 2002; Levroney et al. 2005). In oysters, galectins serve a defensive and digestive role by binding to pathogens and algae and promoting phagocytosis of these particles by macrophages (Tasumi and Vasta 2007; Yamaura et al. 2008).

Oysters are constantly exposed to bacteria and pathogens during filtration of water. One interpretation of higher transcription of adhesive proteins in stress-sensitive oyster families is that they are more susceptible to opportunistic infection before and after heat shock owing to decreased ability of hemocytes to bind to and phagocytose bacteria; greater transcription of adhesive proteins may have been required to otherwise prevent opportunistic infection. Hemocyte adhesion in marine bivalves is reduced by some bacteria including *Vibrio aestuarianus* and *V. tapetis* (Choquet et al. 2006; Labreuche et al. 2006), and substrate adhesion by hemocytes of summer mortality-susceptible oysters reared at the rivière d' Auray, France, was compromised by *Vibrio* sp. strain S322 to a greater extent than in resistant families during summer months (Lambert et al. 2007). However, substrate adhesion was greater in hemocytes of the same susceptible families than in resistant families before the seasonal period of mortality (Samain et al. 2007), and therefore the linkage between expression of adhesion proteins and family-specific resistance to infection

requires further clarification. We note that we did not measure bacterial loads of dead and surviving oysters in this study and therefore do not know if infections lead to mortality. However, Samain et al. (2007) found that *C. gigas* families that were susceptible to summer mortality syndrome had higher quantities of *Vibrio aestuarianus* after heat shock than did resistant families, and it is not unreasonable to speculate that opportunistic infection contributed to morality in the low-surviving families used in the present study.

The relative concentration of *cystatin B* (Genbank #CX069133; microarray and RT-QPCR data; Table 4, Figure 4A) mRNA increased after 6 h and based upon the RT-QPCR data was greater in high-surviving than in low-surviving families at 24 h after heat shock (Figure 4A). Cystatins bind to and inhibit proteolytic cathepsins that are secreted by lysosomes as part of routine protein turnover and by pathogens as a means of acquiring nutrition from host cells (Ulrich 1995; Rinne et al. 2002; Vergote et al. 2004).

Protein turnover (the net result of synthesis and degradation) accounts for a large portion of maintenance metabolism energy expenditure in marine bivalves (Hawkins 1991; Hawkins and Day 1996), and lower rates of protein turnover in mussels *Mytilus edulis* afforded energy savings that in turn lead to enhanced survival under stressful conditions (Hawkins et al. 1987; Hawkins et al. 1989). One potential advantage of higher levels of cystatin B in the high-surviving oyster families could have been reduced energy loss due to protein turnover resulting in more energy for mounting stress responses.

In oysters, pathogens such as *P. marinus* secrete proteases that damage host cells, and during infection protease inhibitors such as cystatin B may serve to defeat these offensive processes (Romestead et al. 2002; Xue et al. 2006) or regulate host cathepsin activity. Protease inhibition in *C. virginica* families bred to resist *P. marinus* infection was negatively correlated with parasite loads (Oliver et al. 2000). Cystatin B has been explicitly associated with immune defense of arthropods (Kanost et al. 1998) snails (Guillou et al. 2007) and marine invertebrates (Guegan et al. 2003; Kang et al. 2006). Snail *Biomphalaria glabrata* strains that were resistant to infection by *Echinostoma caproni* had higher induced levels following infection of a type-2 cystatin protein that is thought to inhibit the parasite-derived cathepsins used to digest host cells (Vergote et al. 2004). Therefore, in addition to retarding protein turnover, higher levels of cystatin B in the oysters studied here could have augmented cellular defense following heat shock.

ESTs without Identity. In this study, transcript concentrations of dozens of unidentified ESTs were altered by heat shock, and some appeared to differ between family types. Transcription of Genbank EST #BQ426658 was greater overall in low-surviving families in the microarray dataset, but RT-QPCR data indicated a significant difference between low- and high-surviving families only at 1 h after stress (microarray and RT-QPCR data; Table 4; Figure 4B). Another unidentified EST (Genbank #BQ426884) was strongly upregulated after 6 h, and was more abundant in low-surviving families than in high-surviving families at this time (microarray and

RT-QPCR data; Table 4; Figure 4C). The significant differences in the microarray dataset for the interaction of time and family type observed in Genbank #BQ426623 and between family types for Genbank #AM237729 were not supported by RT-QPCR (microarray and RT-QPCR data; Table 4; Figures 4D, 4E). The field of genomics in marine bivalves is still in its infancy, and the results obtained from each of these unidentified ESTs are difficult to interpret. Recently, Tanguy et al. (2008) sequenced over 10,000 ESTs, and found that no more than 27% could be assigned to a functional category. As our knowledge of genes and the functions of their products expands in *C. gigas* and other molluscs, we expect that this dataset will continue to yield insights into the pathways that are perturbed by heat shock.

Conclusions. The response of oyster gills to heat shock included immediately increased transcription of genes that encode heat shock proteins, and increased transcription after 6 h of genes whose products synthesize lipids, participate in cellular immunity, and influence reproductive activity. Potentially deleterious consequences of heat shock included suppressed mobilization of stored lipid reserves and decreased transcription (relative to pre-stress levels) of antioxidant genes.

With the exception of one EST encoding *cystatin B*, transcript concentrations of ESTs that differed between family types was greater in low-surviving families than in high-surviving families. It seems counter-intuitive that low-surviving families expressed stress-relevant genes at higher levels than did high-surviving families. It is possible that low-surviving families required higher transcript levels to supply proteins

(e.g. heat shock proteins and lectins) that mitigate cellular damage and opportunistic infection. However, the increased production of both transcripts and proteins could come at an energetic cost above that of maintenance metabolism, and the resulting increase in protein turnover could have lead to metabolic exhaustion (Hawkins et al. 1987; Hawkins et al. 1989; Li et al. 2007). A complex interaction of cell damage, opportunistic infection, and exhaustion is a plausible explanation for mortality at 43°C when considering that tissue energy stores are low when oysters are in reproductive condition (Berthelin et al. 2000) and lipid mobilization may be suppressed by heat shock.

We identified a number of ESTs whose transcription differed between oyster families with high or low survival after heat shock. These ESTs will provide the basis for broodstock selection for increased thermal tolerance and potentially also for disease resistance.

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FIGURE LEGENDS

Figure1. Time-course clusters (95% confidence intervals) for ESTs in which transcription level differed among sampling times in gill of heat-shocked (40°C, 1 h) oysters before and at 1, 3, 6, and 24 h after heat shock. Data were clustered using the software SSClust (Ma et al. 2006). The *vsn*-transformed data for each sampling time are presented relative to the average for all sampling times for a given EST.

Figure 2. Average (±SEM) microarray-generated *vsn*-adjusted signal data and RT-QPCR-generated relative concentration data (target concentration relative to *elongation factor 1;* Log2RC) for heat shock protein genes and genes putatively related to growth and reproduction measured in gill before and at 1, 6, and 24 h after heat shock (40°C, 1 h). Each bar represents three replicates of two families (6 samples total) with either low survival (hatched bars) or high survival (white bars) after heat shock. Letters between sampling times indicate when significant changes between sampling times ("a", no change; "b", significant difference at $P < 0.05$). Asterisks ("*") above individual sampling times indicate significant ($P < 0.05$) differences between family types at that time.

Figure 3. Average (±SEM) microarray-generated *vsn*-adjusted signal and RT-QPCR-generated relative concentration data (target concentration relative to *elongation factor 1;* Log2RC) for genes putatively encoding antioxidant enzymes, genes involved in lipid synthesis, and genes involved in cellular immunity measured in gill before and at 1, 6, and 24 h after heat shock (40°C, 1 h). Each bar represents three replicates of two families (6 samples total) with either low survival (hatched bars) or high survival (white bars) after heat shock. Letters between sampling times indicate when significant changes between sampling times ("a", no change; "b", significant difference at $P < 0.05$). Asterisks ("*") above individual sampling times indicate significant ($P < 0.05$) differences between family types at that time.

Figure 4. Average (±SEM) microarray-generated *vsn*-adjusted signal data and RT-QPCR-generated relative concentration data (target concentration relative to *elongation factor 1;* RC or Log2RC) for genes involved in cellular immunity measured in gill before and at 1, 6, and 24 h after heat shock (40°C, 1 h). Each bar represents three replicates of two families (6 samples total) with either low survival (hatched bars) or high survival (white bars) after heat shock. Letters between sampling times indicate when significant changes between sampling times ("a", no change; "b", significant difference at $P < 0.05$). Asterisks ("*") above individual sampling times indicate significant ($P < 0.05$) differences between family types at that time.

Figure 1.

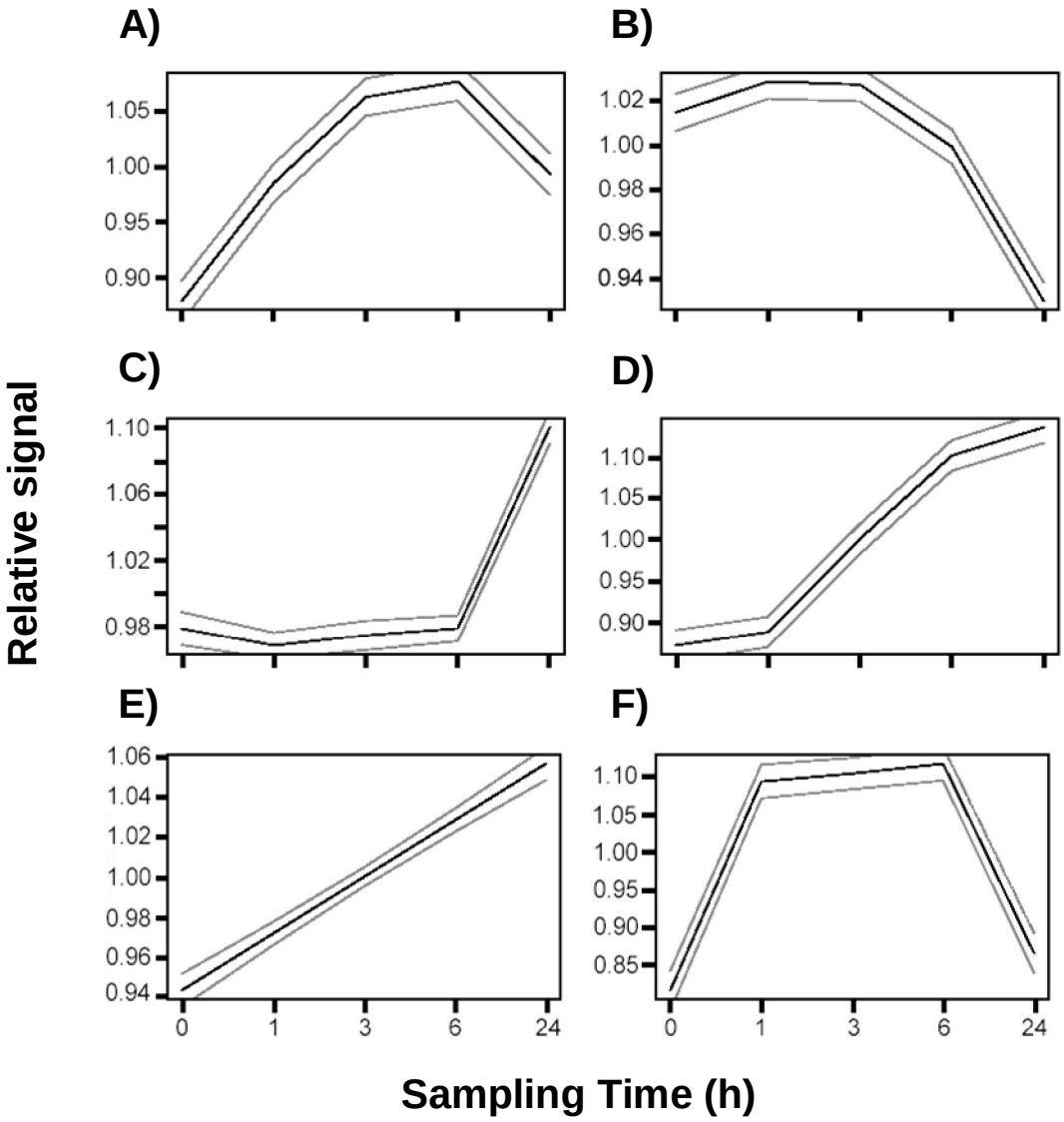

Figure 2.

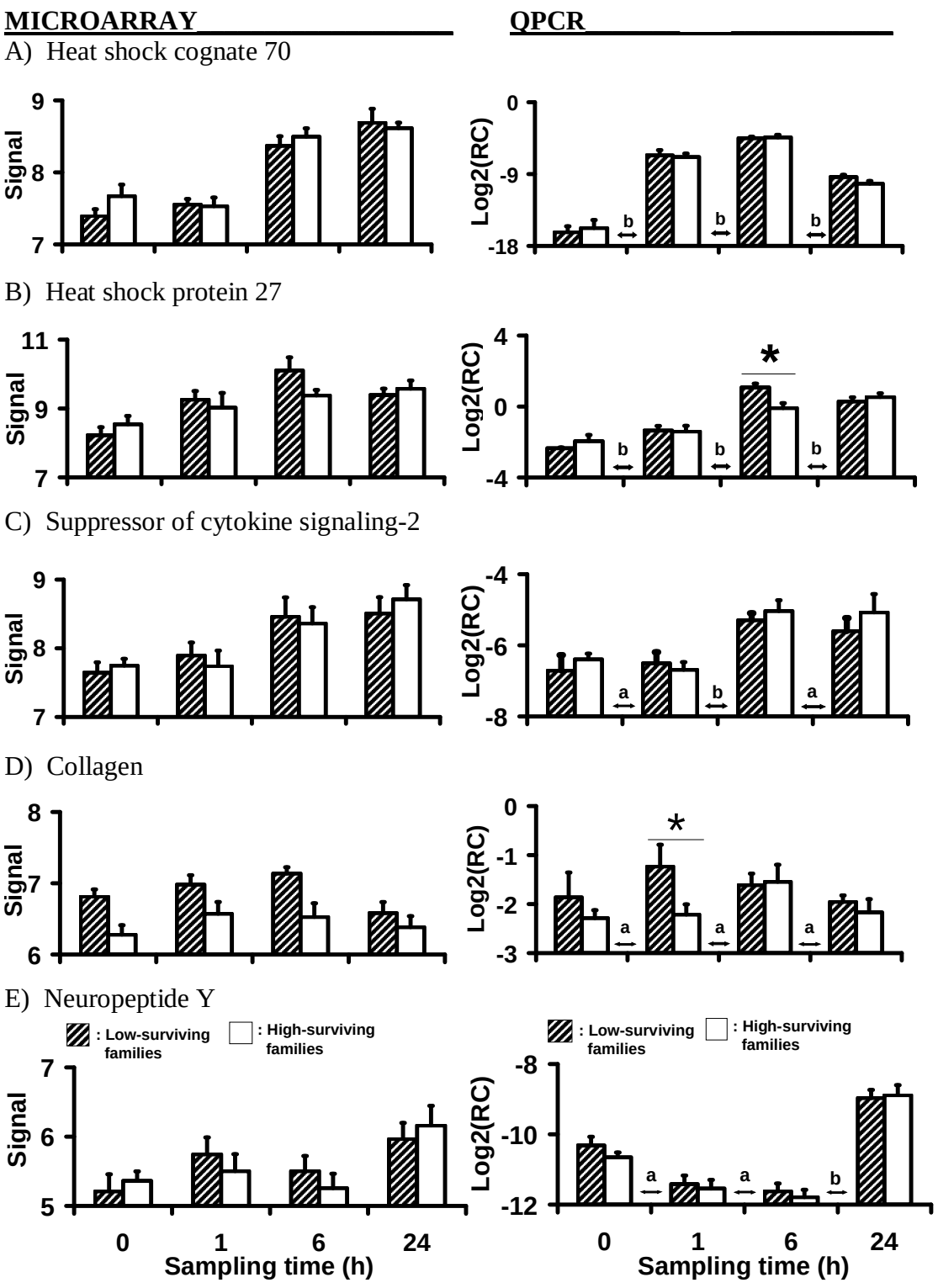
MICROARRAY
QPCR
A) Heat shock cognate 70
Signal
Log2(RC)
B) Heat shock protein 27
C) Suppressor of cytokine signaling-2
D) Collagen
E) Neuropeptide Y
: Low-surviving families
: High-surviving families
Sampling time (h)
0
1
6
24
a
b
*

Figure 3.

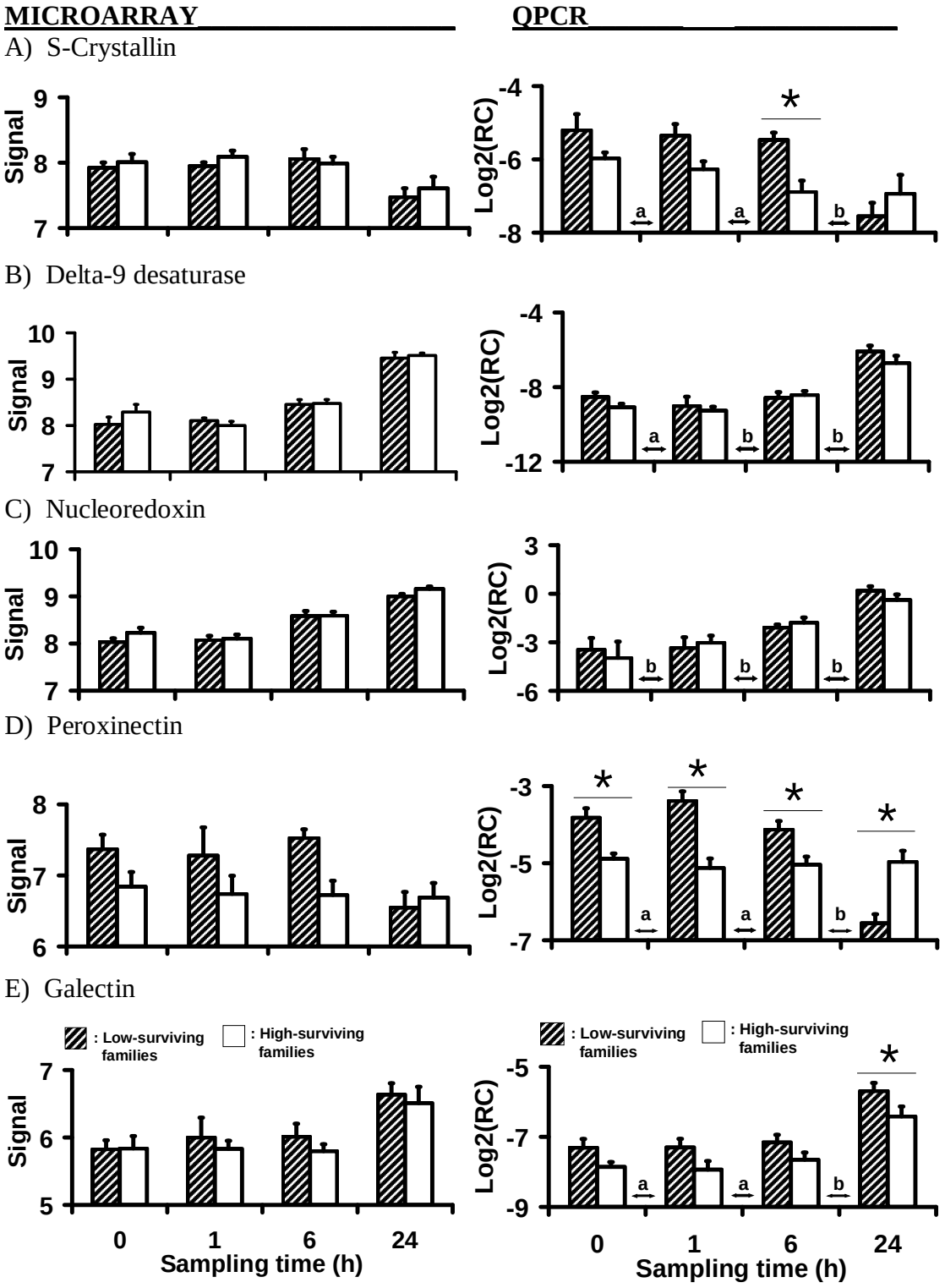
MICROARRAY
QPCR
A) S-Crystallin
B) Delta-9 desaturase
C) Nucleoredoxin
D) Peroxinectin
E) Galectin
Signal
Log2(RC)
: Low-surviving families
: High-surviving families
Sampling time (h)
0
1
6
24
a
b
*

Figure 4.

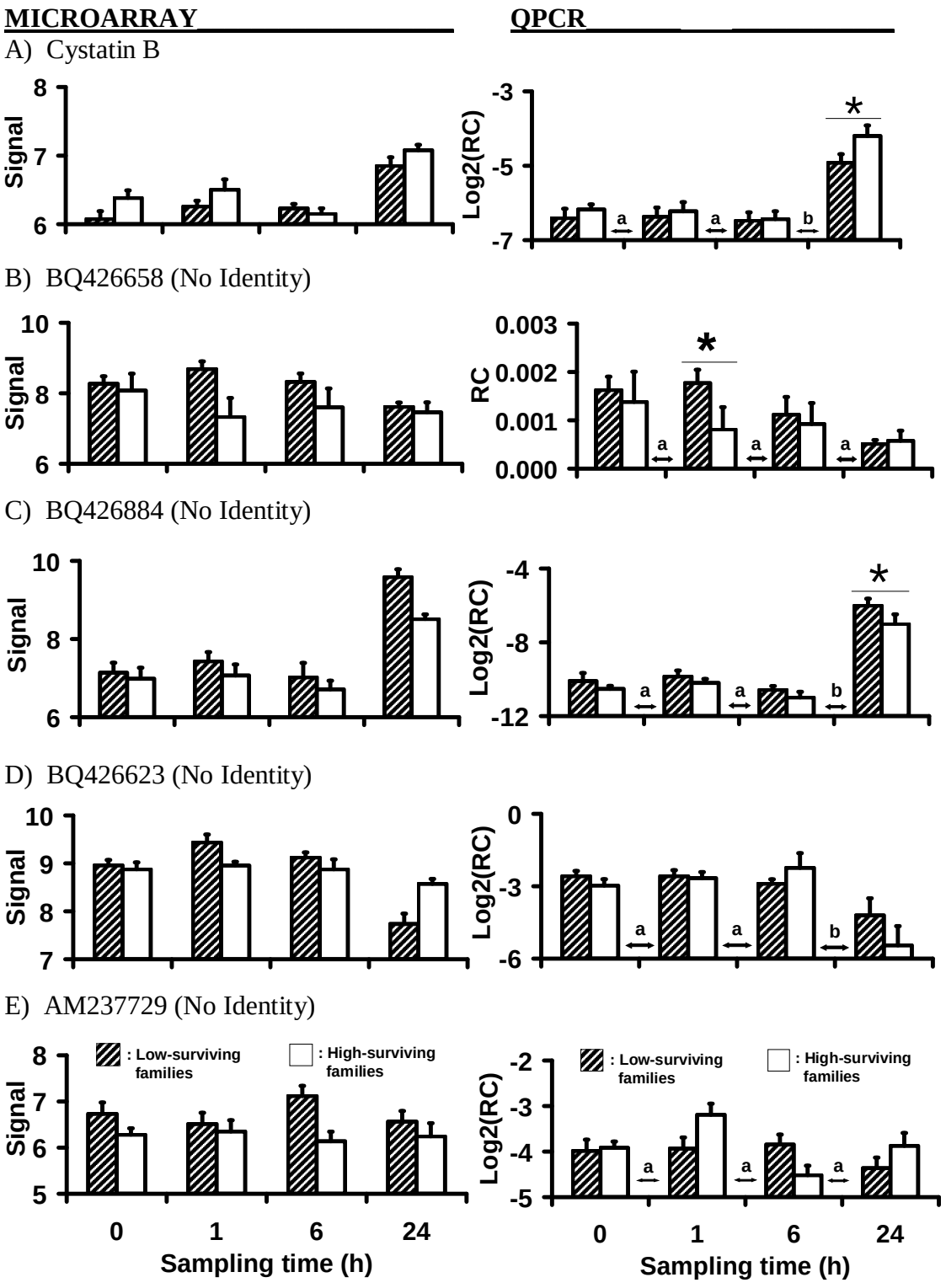
MICROARRAY
QPCR
A) Cystatin B
Signal
Log2(RC)
B) BQ426658 (No Identity)
Signal
RC
C) BQ426884 (No Identity)
Signal
Log2(RC)
D) BQ426623 (No Identity)
Signal
Log2(RC)
E) AM237729 (No Identity)
: Low-surviving families
: High-surviving families
Signal
Log2(RC)
Sampling time (h)
0
1
6
24
a
b
*

Table 1. Accession number, identity, forward primer sequence, reverse primer sequence, annealing temperature (T_a) and number of cycles (C) for quantitative RT-QPCR assays. Mean efficiency (E_r) ± SEM was calculated by using the software LinReg PCR (Ramakers et al. 2003). Target concentration was normalized to that of the endogenous control *ef1* (AB122066). "SOCS2" (BQ426927) refers to suppresssor of cytokine signaling 2.

EST Putative Identity	Forward Primer Reverse Primer	T_a	C	Efficiency
AB122066 Elongation factor 1α	F : GGAAGCTGCTGAGATGGGAA R : TCCAACACCCAGGCGTATTT	*	*	1.98 ± 0.004
AJ305315 Heat shock protein 70	F : AGGGCATTTCATGTCCGAAG R : TGCTCAGACATCCAAGGAAGG	58	40	2.01 ± 0.002
AJ565456 S-crystallin	F : ACCACAAAAGCAGTTGCCGT R : TGAGGGATAAGGCGACCATC	59	40	2.08 ± 0.01
AJ565627 Nucleoredoxin	F : GTACTTCAATGAGATGCCGTGG R : AGGTCACGTTCACTGAAGGGA	59	40	1.84 ± 0.153
AJ565582 Delta-9 desaturase	F : CCTTCGGATCGTCTGGAGAA R : CCGAAGTGTAAAAGAGCCATCAG	59	40	1.98 ± 0.004
AM237676 Peroxinectin	F : GCCAAACCTCGCCTACCTTC R : GTGGAGTTGACGCGTGACATA	58	40	2.00 ± 0.004
AM237729 None	F : GAGATCGTGGAGGACAAGAAAGA R : CTGCTCCGATCTCGTCAGC	58	45	1.97 ± 0.005
AM237796 Galectin	F : GCTGTGGAGTGGTTACCAGGA R : GAGAGTAACCCAGCTCCCCG	59	40	2.01 ± 0.004
BQ426456 Neuropeptide Y	F : AGGCCATTGAAGCCACAAAT R : TCCACAAAGGACTCTTGCCAT	58	40	2.00 ± 0.005
BQ426550 Heat shock protein 27	F : TGACGCAATGGATTTTCTGC R : GCCTGGTATCCAGGTCGGTAG	58	40	1.86 ± 0.005
BQ426623 No match	F : GCGCGACATTTTCGATTTTC R : CCATTGGTGATGACGTAACGC	59	40	1.88 ± 0.007
BQ426658 No match	F : AGAAGTGTGGAGAAATTTTGTCCAG R : TGGGATCAGATCCCATGCTC	59	40	1.92 ± 0.005
BQ426884 No match	F : ACGATGTCAGGGACAACTTTCTGT R : CGCAGAGGTAATCTTTTAAACGTCA	58	45	1.96 ± 0.002
BQ426927 SOCS2	F : GACATGGTCGTCCAGATCGG R : CCACCCTGATTGACTTAACGTGA	59	40	2.02 ± 0.005
CX069163 Collagen	F : TATGAAGTAGCAGCATGATGACTCC R : TCGTGGCTACCTAATGGCG	58	40	2.02 ± 0.004
CX069133 Cystatin B	F : GTTCCTGCCACGACCTGAGT R : GCTGGACACTTACACCCCAGTC	58	40	2.01 ± 0.004

Table 2. A) Summary of results for microarray experiment: Source of ESTs included in the analysis and the percentage that originated from either *Crassostrea gigas* or *C. virginica*; Number and percentage of ESTs included in the analysis from either species that differed among sampling times; Number and percentage of ESTs included in the analysis from either species that differed between family types or had significant interactions of time and family type. B) Number and percentage of ESTs that differed among sampling times from 10 functional categories, and the number and percentage of those that originated from *C. gigas* or *C. virginica* EST libraries.

A)

Source	Included in analysis	Different among times	Different between family types	Interaction of time and family type
C. gigas	1,102 (66%)	90 (8.1%)	4 (0.004%)	1 (> 0.001%)
C. virginica	573 (34%)	21 (3.6%)	0	0
Total	1,675 (100%)	111 (6.5%)	4 (0.002%)	1 (> 0.001%)

B)

Functional category	# Total	*# C. Gigas*	*# C. Virginica*
Molecular chaperones	10 (9.0%)	8 (80%)	2 (20%)
Protein degradation	4 (3.6%)	3 (75%)	1 (25%)
Growth / reproduction	3 (2.7%)	3 (100%)	0
Antioxidant / detoxification	2 (1.8%)	2 (100%)	0
Lipid metabolism	5 (4.5%)	5 (100%)	0
Cellular immunity	5 (4.5%)	5 (100%)	0
Transcription / Translation	5 (4.5%)	1 (20%)	4 (80%)
Cytoskeletal organization	7 (6.3%)	7 (100%)	0
Other processes	10 (9.0%)	8 (80%)	2 (20%)
No match in Genbank	59 (53.6%)	47 (80%)	12 (20%)
Total	110 (100.0%)	90 (81%)	21 (19%)

Table 3. Accession number (accn#), cluster (clst; Figure 1), *Q*-value, identity, species match and accession number, and *E*-scores of ESTs for which *vsn*-adjusted signals in gill of oysters differed significantly ($Q < 0.05$) A) among sampling times, and B) between family types or the interaction of time and family type.

A)

Accn#	Cluster	*Q*	Identity	Species match	Accn #	*E*
			Molecular Chaperones / Co-chaperones			
AJ305315	E	1.E-10	Heat shock cognate 70	*Crassostrea gigas*	AJ305315	0
AJ565518	E	1.E-03	EKN1	*Strongylocentrotus purpuratus*	XP_787717	4.E-47
AM237785	A	6.E-14	Heat shock protein 27	*Carassius auratus*	AAV97950	8.E-07
BQ426550	A	1.E-04	Heat shock protein 27	*Homo sapiens*	AAA62175	8.E-07
BQ426606	E	3.E-08	Heat shock protein 90	*Chlamys farreri*	AAR11781	6.E-112
BQ426928	E	3.E-09	Heat shock protein 70	*Crassostrea gigas*	AF144646	1.E-114
CB617443	D	1.E-09	Heat shock protein 90	*Crassostrea gigas*	ABS18268	2.E-110
CB617445	NA	4.E-02	Chaperonin subunit 6	*Nasonia vitripennis*	XP_001606486	3.E-27
CD648461	NA	1.E-05	Small heat shock protein	uncultured cnidarian	ABA42878	3.E-10
CV089199	D	6.E-06	Heat shock cognate 72	*Crassostrea gigas*	AAD31042	6.E-133
			Protein Degradation			
BQ427148	NA	4.E-02	Siah-interacting protein	*Xenopus laevis*	NP_000180214	8.E-18
CB617402	C	2.E-06	Ubiquitin activating enz. 1	*Xenopus laevis*	AAH47256	2.E-52
CB617456	C	3.E-06	Valosin-containing protein	*Homo sapiens*	AAH07562	4.E-84
CV132727	E	2.E-02	Ribosomal Protein L40a	*Lycosa singoriensis*	ABX75386	3.E-35
			Growth and Reproduction			
BQ426456	NA	2.E-03	Neuropeptide Y	*Haliotis discus hannai*	ABH10673	7.E-12
BQ426927	E	8.E-03	Suppressor of cyto. sig. 2	*Apis mellifera*	XP_394764	4.E-13
CK172319	C	4.E-07	Temptin	*Aplysia brasiliana*	AAS92605	6.E-27
			Antioxidant / Detoxification			
AJ565456	B	1.E-03	S-crystallin	*S. purpuratus*	XP_791441	9.E-33
CX069146	B	3.E-03	Glutathione peroxidase	*Hymeniacidon perlevis*	ABB91779	4.E-53
			Lipid Metabolism			
AJ565582	NA	8.E-14	Delta-9-desaturase	*Danio rerio*	NP_942110	8.E-08
BQ426641	B	9.E-03	Patatin-like phospholipase	*S. purpuratus*	XP_001185333	5.E-47
BQ426935	C	6.E-03	Sterol reg. element BP-1	*Gallus gallus*	NP_989457	2.E-41
CB617498	C	9.E-12	Delta-9-desaturase	*Ctenopharyngodon idella*	CAB53008	4.E-69
CK172312	B	4.E-03	Fatty acid binding protein	*Crassostrea gigas*	AAT44355	5.E-08

A), continued.

Accn#	Cluster	*Q*	Identity	Species match	Accn #	*E*
			Cellular Immunity			
AJ565627	E	6.E-14	Nucleoredoxin	*S. purpuratus*	XP_781174	3.E-30
AM237796	C	2.E-02	Galectin 4	*Crassostrea gigas*	CAD79473	4.E-88
BQ427041	B	2.E-03	5-lipoxygenase	*S. purpuratus*	XP_001198535	3.E-24
CB617516	B	3.E-05	Protein w/ COMM domain	*Xenopus laevis*	NP_001087091	8.E-44
CX069133	C	5.E-08	Cystatin B	*Gallus gallus*	XP_416492	3.E-18
			RNA Transcription / Protein Translation			
CD648403	D	2.E-08	FUSE binding protein 1	*Xenopus tropicalis*	NP_989293	6.E-08
CD649011	B	5.E-02	Y-box factor protein	*Lymnaea stagnalis*	AAT97092	6.E-31
CV087081	B	6.E-03	Ribosomal protein S27-1	*Crassostrea gigas*	CAD91436	0
CV087137	A	1.E-02	18S rRNA gene	*Crassostrea gigas*	AM182263	0
CX069191	NA	3.E-02	Ribosomal protein P1	*Biomphalaria glabrata*	AAZ39530	4.E-24
			Cytoskeletal Organization			
AJ565479	NA	2.E-03	Fascin homolog 2	*Xenopus tropicalis*	NP_001093724	1.E-60
BQ426318	C	5.E-02	Ankyrin w/ MYND	*Bos taurus*	NP_00168852	1.E-52
BQ426459	A	6.E-03	Ankyrin 2,3/unc44	*S. purpuratus*	XP_001179527	3.E-20
BQ426555	B	5.E-02	Arp2/3 16kd subunit	*Aedes aegypti*	EAT39104	2.E-10
BQ426750	E	9.E-04	Transgelin	*Bombyx mori*	NP_001040372	7.E-46
BQ426862	E	5.E-05	Profilin	*Entamoeba histolytica*	CAA62418	5.E-08
CB617442	NA	1.E-03	Tubulin β5	*Crassostrea gigas*	AAU93877	1.E-65
			Other processes			
AM237712	NA	6.E-12	Inhibitor of apoptosis	*Ochlerotatus triseriatus*	AAL46972	2.E-12
BQ426894	NA	9.E-03	hypothetical dehydrogenase	*Gallus gallus*	XP_426310	2.E-16
BQ426907	C	1.E-02	Arginase	*Equus caballus*	XP_001503335	8.E-58
BQ426918	B	3.E-02	Aldehyde dehydrogenase 1	*Mus musculis*	NP_082546	3.E-42
BQ426932	B	1.E-04	Diaphorase 1	*Xenopus laevis*	NP_001080477	2.E-84
BQ427069	NA	2.E-03	Alcohol dehydrogenase	*Lysiphlebus testaceipes*	AAY63990	6.E-104
CD526814	F	8.E-15	Senescence associated protein	*Brugia malayi*	EDP31077	2.E-55
CD526816	F	6.E-14	Tetraspanin 66E	*D. melanogaster*	NP_523985	2.E-07
CD647828	NA	3.E-02	Bm1_17870	*Brugia malayi*	EDP36122	6.E-22
CF369127	B	1.E-02	LOC496342 protein	*S. purpuratus*	XP_792838	3.E-46
			ESTs without significant identity			
AJ565488	E	4.E-02	Not known			
AJ565533	A	1.E-06	Not known			
AJ565662	E	7.E-03	Not known			
AJ565673	B	1.E-03	Not known			
AJ565697	A	5.E-24	HSP24	*Branchiostoma lan*	CAE83570	8.E-02
AJ565827	NA	2.E-20	Not known			

A), continued.

Accn#	Cluster	*Q*	Identity	Species match	Accn #	*E*
AJ565831	A	9.E-10	Not known			
AJ565846	NA	3.E-07	Not known			
AM237667	A	1.E-04	Not known			
AM237694	NA	2.E-09	Not known			
AM237730	A	5.E-10	Not known			
AM237767	A	5.E-14	Not known			
AM237771	NA	2.E-12	Not known			
BG624437	B	8.E-03	Not known			
BG624595	A	5.E-10	Not known			
BG624747	A	1.E-03	Not known			
BQ426330	C	4.E-02	Not known			
BQ426395	NA	3.E-04	tRNA transferase	*Nematostella vectensis*	XP_001626290	4.E-04
BQ426399	NA	2.E-05	Not known			
BQ426402	C	6.E-03	Not known			
BQ426405	C	7.E-04	Not known			
BQ426413	A	2.E-17	Not known			
BQ426454	NA	2.E-02	Not known			
BQ426620	A	1.E-07	Not known			
BQ426623	B	2.E-02	Not known			
BQ426654	C	9.E-08	Not known			
BQ426851	NA	2.E-14	Transcription factor	*Ciona intestinalis*	BAE06318	5.E-04
BQ426884	NA	5.E-09	Not known			
BQ426924	NA	9.E-04	Not known			
BQ426995	NA	1.E-06	Not known			
BQ427058	NA	3.E-18	Not known			
BQ427084	NA	1.E-02	Not known			
BQ427134	NA	2.E-03	Not known			
BQ427151	A	9.E-09	Uroplakin 1	*Heliocidaris tuberculata*	ABE27955	1.E-04
BQ427169	NA	9.E-06	Not known			
CB617327	NA	5.E-03	Not known			
CB617408	D	7.E-08	Not known			
CB617424	NA	3.E-04	Not known			
CB617426	D	5.E-14	Not known			
CB617465	C	2.E-04	Not known			
CB617473	NA	1.E-05	Not known			
CB617482	NA	1.E-06	Not known			
CB617484	NA	4.E-04	Not known			
CB617540	B	1.E-04	Not known			
CD526789	F	9.E-09	Not known			
CD526790	F	4.E-08	Not known			
CD526794	A	3.E-04	Not known			
CD526795	NA	8.E-06	Not known			
CD526862	F	9.E-14	Not known			
CD526863	F	3.E-20	Not known			
CD526872	F	1.E-06	Not known			
CD648405	E	3.E-02	Not known			
CD648964	NA	4.E-02	Not known			
CK172325	NA	9.E-15	Not known			
CK172347	NA	1.E-02	Not known			
CK240435	NA	4.E-03	Not known			
CV132419	A	2.E-05	Not known			
CV132796	NA	8.E-03	Not known			
CV132891	C	5.E-03	Not known			

B)

Accn#	Cluster	*Q*	Identity	Species match	Accn #	*E*
ESTs that differed for the main effect of family type						
AM237676	NA	3.E-02	Peroxinecin	*Pacifastacus leniusculus*	CAA62752	6.E-29
AM237729	NA	3.E-02	No identity			
BQ426658	NA	3.E-03	No identity			
CX069163	NA	6.E-03	Collagen protein	*Suberites domuncula*	CAC81019	7.E-09
ESTs with significant interaction of time and family type						
BQ426623	NA	2.E-02	No identity			

Table 4. Statistical results for ESTs studied in gill of families with high (>64%) or low (<29%) survival ("FT") after heat shock (43°C, 1 h) in which transcription was measured using a cDNA microarray and RT-QPCR. Differences were considered significant at $Q < 0.05$ or $P < 0.05$.

	Microarray data (*Q*)			RT-QPCR data (*P*)		
EST	Time	FT	Time*FT	Time	FT	Time*FT
Heat shock cognate 70	< **0.0001**	0.9935	0.9842	< **0.0001**	0.7884	0.6863
Heat shock protein 27	**0.0001**	0.9881	0.8032	< **0.0001**	0.4360	**0.0150**
Suppressor cytokine signaling-2	**0.0078**	0.9877	0.9881	< **0.0001**	0.3032	0.7171
Collagen	0.7152	**0.0065**	0.9823	0.1621	**0.0417**	0.2461
Neuropeptide Y	**0.0023**	0.9865	0.9619	< **0.0001**	0.2264	0.4574
S-Crystallin	**0.0013**	0.8785	0.9865	**0.0041**	0.0610	0.1578
Delta 9-desaturase	< **0.0001**	0.9596	0.9823	< **0.0001**	0.1580	0.5841
Nucleoredoxin	< **0.0001**	0.4558	0.9842	< **0.0001**	0.4798	0.0709
Peroxinectin	0.5497	**0.0254**	0.7992	**0.0228**	< **0.0001**	< **0.0001**
Galectin	**0.0207**	0.8660	0.9894	< **0.0001**	**0.0021**	0.9720
Cystatin B	< **0.0001**	0.4962	0.8309	< **0.0001**	0.0661	0.3960
BQ426658	0.4715	**0.0027**	0.5672	**0.0115**	0.1054	0.3353
BQ426884	< **0.0001**	0.1474	0.9164	< **0.0001**	**0.0041**	0.5580
BQ426623	< **0.0001**	0.9557	**0.0171**	< **0.0001**	0.4356	0.2682
AM237729	0.9842	**0.0329**	0.8127	0.1843	0.4669	0.1160

CAN LABORATORY ASSAYS USING JUVENILES BE USED TO PREDICT THE FIELD PERFORMANCE OF PACIFIC OYSTER (*CRASSOSTREA GIGAS*) FAMILIES PLANTED IN COASTAL WATERS?

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Unpublished (to be submitted)

ABSTRACT

We tested the hypotheses that survival after heat shock or gene transcription before or after heat shock in juveniles is predictive of the field performance of adult hatchery-produced full-sib families of the Pacific oyster *Crassostrea gigas*. We heat-shocked (41°C for 1h) juveniles from each of 46 families, monitored their survival for 6 d and classified families with > 69% survival as high-surviving and those with < 31% survival as low-surviving. We deployed unstressed siblings from all 46 families at one subtidal and one intertidal site in Yaquina Bay, Newport, OR. In a separate experiment, we heat-shocked (39 °C, 1 h) another 25 juveniles from four low-surviving and four high-surviving families of the same cohort, extracted mRNAs from whole bodies collected before and at 6 h and 24 h after heat shock, and measured the mRNA concentrations of 14 genes relative to a housekeeping gene (*elongation factor 1*) using real-time quantitative PCR. Oysters from the 46 families deployed were harvested after two years, and we estimated family-specific average survival (%), individual weight (g), and yield (kg per bag). There were no significant family-level correlations between juvenile survival after heat shock and subsequent survival, yield, or weight at the subtidal site or the intertidal site ($P > 0.05$). The mRNA concentration of *galectin* was greater in low-surviving families before heat shock, whereas those of *cystatin B* at each sampling time and of *glutathione S-transferase omega* at 24 h after heat shock were greater in high-surviving families. The pre-stress differences in transcription between the family types suggest that survival of stress may be related to constitutive differences in transcription between family types in

addition to induced responses to heat shock. The concentrations of *heat shock protein 27, catalase, prostaglandin E receptor*, and *superoxide dismutase* mRNA were significantly correlated with survival and average weight at the intertidal or subtidal sites. Additionally, the concentrations of *galectin* and an unidentified mRNA *BQ426658* were correlated with average weight at either the intertidal or subtidal site, and concentrations of *tissue inhibitor of metalloprotease* mRNA were correlated with yield at both sites. We conclude that assays measuring gene transcription in whole bodies of juveniles hold promise for predicting performance of *C. gigas* families planted in coastal waters.

INTRODUCTION

Summer mortality syndrome is a serious problem for Pacific oyster (*Crassostrea gigas*) aquaculture. During warm summer months, summer mortality episodes coincide with periods of high water temperature, low dissolved oxygen, and high primary productivity (Soletchnik et al. 1999; Cheney et al. 2000; Soletchnik et al. 2005; Soletchnik et al. 2007). Mortalities are most likely caused by a poorly understood interaction of reproductive effort, glycogen metabolism, and opportunistic pathogens that attack oysters weakened by stress (Perdue et al. 1981; Soletchnik et al. 1999; Berthelin et al. 2000; Lacoste et al. 2001; Friedman et al. 2005; Garnier et al. 2007).

Resistance to summer mortality is heritable in French hatchery-reared oyster populations, and phenotypic selection has been used to produce resistant strains (Dégremont et al. 2007). However, in the United States, outbreaks of summer mortality are sporadic and unpredictable making selection of broodstock based upon survival alone difficult because environmental conditions and the impact of summer mortality vary from year to year. As opposed to relying solely on field evaluation to select broodstock, the use of marker-assisted selection based upon either quantitative trait loci or candidate genes whose protein products are involved in tolerance of stressful conditions could increase efficiency and effectiveness of breeding programs (Cnaani 2006; Rothschild and Ruvinsky 2007). However, the physiological processes that result in summer mortality remain poorly understood, and markers and genes

associated with resistance to summer mortality have not been sufficiently characterized to be useful.

To reduce costs and increase the efficiency of oyster breeding programs, we tested the hypothesis that laboratory assays with juveniles at an early stage of rearing can predict a family's subsequent performance in the field, and thereby provide a basis for selective culling prior to lengthy and costly field trials. We measured 1) survival after heat shock and 2) the transcription levels of several candidate genes whose products are thought to be important for susceptibility to stress. We chose heat shock as our stressor because it is easy to implement under laboratory conditions, because sessile marine bivalves located in the intertidal zone are repeatedly exposed to heat stress during tidal emersion, and because heat stress is an important component of summer mortality (Cheney et al. 2000; Samain et al. 2007).

Heat shock damages cells and proteins (Feder and Hoffman 1999) and causes increased production of reactive oxygen species (Flanagan et al. 1998; Bruskov et al. 2002; Arnaud et al. 2002) resulting in oxidative stress in both fish (Kaur et al. 2005; Heise et al. 2006; Bagnyukova et al. 2007) and bivalves (Abele et al. 2002; Verlecar et al. 2007). Heat shock impairs bivalve immune defense through reduced activity and destruction of hemocytes (Hégaret et al. 2003; Hégaret et al. 2004; Gagnaire et al. 2007; Chen et al. 2007). Mounting a heat shock response is metabolically expensive, and during summer months when energy reserves have been diverted into reproduction, heat shock may cause mortality by opportunistic infection or metabolic exhaustion (Li et al. 2007).

Recently, we used the complementary DNA (cDNA) microarray produced by Jenny et al. (2007) (Chapter 2) and the cDNA amplified fragment length polymorphism technique (N. Taris, P. Lang, and M. Camara, unpublished) to identify genes whose transcription in gill or hemocytes differed between oyster families with high or low survival after heat shock. In the present study we measured whole-body transcription of some of these genes as potential predictors of field performance. The genes studied include antioxidant or detoxification enzymes, potential participants in cellular immunity, heat shock proteins, genes involved with the extracellular matrix, and genes with no known function (Table 1). This study comprised three experiments whose objectives were 1) to test for correlations between survival of heat shock as juveniles and survival, yield, and final weight of adults at harvest in families reared at two sites in Yaquina Bay estuary, Newport, OR; 2) to compare whole-body gene transcription in juveniles between oyster families that had high or low survival after heat shock, and 3) to test for correlations between whole-body gene transcription in juveniles and survival, yield, and weight of adults at harvest.

METHODS

Experiment 1.

Production and identification of families. We produced a cohort of 46 full-sib oyster families during Summer 2005 using standard techniques (Langdon et al. 2003). Prior to stress trials, juveniles from all families were held in troughs supplied with ambient (9°C) sand-filtered water in a flow-through system and fed a mixture of *Isochrysis*

galbana and *Cheatoceros calcitrans* at a concentration of approximately 50,000 – 80,000 cells ml^{-1}. During Fall 2005, we exposed three replicate groups of 50 juveniles (1 – 3 cm) from each family to heat shock (41°C - 43°C, for 1 h), returned them immediately to ambient seawater (9°C - 12°C) without addition of algal ration, and monitored survival every other day. Based upon average survival (% ± S.E.M.), we classified 4 families as low-surviving and 4 as high-surviving when their average survival at 6 d after heat shock as juveniles was < 31% or > 69%, respectively. We used different unstressed animals from each of these eight families for Experiment 2.

Harvest and field data collection. During Fall 2005, we planted the entire cohort in Yaquina Bay Estuary, Newport, Oregon, at subtidal and intertidal sites located approximately 15 km from the mouth of the Bay (44.6°N, 124.1 °W). At the subtidal site, we planted oysters in 10-tier lantern nets (0.51-m diameter, 5-mm mesh) at a density of 50 animals per tier, and at the intertidal site in individual rectangular mesh bags (0.53-m x 0.81m, 7-mm mesh) at a density of 50 animals per bag. We harvested the animals during Fall 2007, and estimated average survival (%), average yield (total weight per bag, kg), and average individual weight (meat and shell) of surviving individuals (g) for each of the 46 families (Langdon et al. 2003). We tested for significant correlations between average survival of the heat-shocked oyster families as juveniles with average survival, yield, or weight using the SAS software package (PROC CORR; SAS/STAT Software, SAS Institute Inc., 1999), and considered correlations to be significant when $P < 0.05$. We also used a one-way analysis of

variance (PROC GLM; SAS/STAT Software, SAS Institute Inc., 1999) to compare average survival, average yield, and average individual weight between high- and low-surviving families at the interidal site and subtidal sites.

Experiment 2.

Tissue collection, RNA extraction and cDNA synthesis. Within two weeks of planting the full cohort in the field, we heat-shocked 25 juvenile animals from each of the four low-surviving and four high-surviving families at 39°C for 1 h and then returned them to sand-filtered ambient (~9°C) water in a flow-through system without supplemental algae. We collected whole bodies (meat without shell) before and at 6 h and 24 h after heat shock in an experiment that was repeated three times to produce three indepedent biological replicates. The three replicates were all collected during a single week. For each replicate, three individuals from each family at each sampling time were pooled, ground in RLT Buffer (Qiagen, Valencia, CA) containing 0.05% (v/v) β-mercaptoethanol using a Teflon®-coated homogenizer and an electric drill. We monitored the remaining animals for 6 d after heat shock to ensure that the heat shock treatment was not lethal, and monitored five unstressed animals from each family to ensure that handling did not cause mortality. We isolated total RNA using RNeasy® Miniprep kits (Qiagen, Valencia, CA) including the on-column DNase treatment, and after isolation we reverse-transcribed total RNA into cDNA using the ABI® High Capacity cDNA Synthesis kit (Applied Biosystems Inc., Foster City, CA) in reactions that contained 2 µg of template and that used the included random primers.

Quantitative PCR. We ran real-time quantitative PCR (RT-QPCR) reactions using an ABI7500 quantitative PCR thermocycler (Applied Biosystems, Foster City, CA) for the genes listed in Table 1. Reactions had a concentration of 50 nM forward and reverse primers and contained 12.5 µl of 2X SYBR green master mix (Applied Biosystems, Foster City, CA), 3 ng of cDNA, and water for a total of 25 µl. We used the reaction conditions: 1) 50°C, 2 min, 2) 95°C, 10 min, and 3) 40 or 45 cycles of a) 95°C for 15 seconds and b) 58°C or 59°C for 1 min (Table 2). As suggested by Taris et al. (2008), we tested multiple primer pairs for each RT-QPCR target using pools of cDNA that represented all animals from each family at one sampling time to assess whether polymorphic primer binding sites would introduce error into our dataset, and confirmed that only one amplicon was produced by examining post-reaction dissociation plots. The full list of primers used in this research is provided in Appendix 3.

RT-QPCR data analysis. We analyzed raw fluorescence data by expressing it as the relative concentrations (RC) of target mRNAs to that of the housekeeping gene *elongation factor 1* (Genbank # AB122066; *ef1*) using the formula $RC = E_r^{cT\ (target)} / E_r^{cT(ef1)}$, where E_r is the reaction-specific amplification efficiency estimated using LinRegPCR software (Ramakers et al. 2003), cT is the fractional cycle where amplification reached a detection threshold, target is the EST under consideration, and *ef1* is the endogenous control gene. We selected *ef1* as the endogenous control

because its transcription in *C. gigas* was not influenced by heat shock, bacterial challenge, or genotype in other studies (Huvet et al. 2004; Taris et al. 2008).

We analyzed the RT-QPCR data using a repeated measures analysis of variance with the SAS software package (PROC GLM; SAS Institute Inc., 1999). The model used in this analysis contained the following sources of variation: the between subject effects of Family Type and the nested effect of Individual Families within Family Types, and the within-subject effects of Sampling Times and the interaction of Sampling Times with Family Type. The dependent variables were gene transcription before and at 6 h and 24 h after heat shock. We used profile analyses to compare the temporal gene transcription profiles of high- and low-surviving families using tests for co-incidence (representing an overall effect of family type), level (representing an effect of time), and parallelism (representing an interaction of time and family type). We $\log_2$-transformed the data when needed to achieve normality. We also conducted *a priori* linear contrasts that compared mean transcript concentrations between the family types at individual sampling times. We considered differences to be significant when $P < 0.05$. We did not apply Bonferroni correction because this experiment was exploratory in nature and was used only to identify genes for further study.

Experiment 3.

Correlations between gene transcription and field performance. We tested for correlations between gene transcription data collected during Experiment 2 with average survival, final weight, and yield data for the four low-surviving and four high-

surviving families collected during Experiment 1 using the SAS software (PROC COR; SAS Institute Inc., 1999). We $\log_2$-transformed the relative mRNA concentration data when needed to achieve normality. We considered differences to be significant when $P < 0.05$. We did not apply Bonferroni correction for family-wise error rate because this experiment was exploratory in nature and was used only to identify genes for further study, and acknowledge that some significant correlations maybe spurious. We report only the significant correlations in this manuscript, but the full table of all correlations is available in Appendix 2.

RESULTS

Experiment 1.

Correlation between survival of heat shock and field performance**.** All juveniles exposed to 42°C and 43°C heat shock died within 3 – 4 d. However, when the cohort was exposed to 41°C, four families could be classified as low-surviving or high-surviving. There were no significant correlations between juvenile survival of heat shock and average survival, individual final weight, or yield of adults for either the intertidal or subtidal sites (Table 3A). There were also no significant differences in average survival, individual final weight, or yield between the 4 high- and 4 low-surviving families ($P > 0.05$; data not shown). There was a significant positive correlation between yield and survival for the four low- and high-surviving families at the subtidal site, but we found no other significant correlations between field parameters at either site (Table 3B).

Experiment 2.

Summary of statistical analyses. The statistical results for the overall between-subject effect of family type, the overall within-subject effects of sampling time, and the interaction of family type and time for each of the genes are summarized in Table 4. The results of the profile analysis are indicated by letters in Figures 1 and 2, and significant differences in average relative mRNA concentration between high- and low-surviving families at individual sampling times are denoted by asterisks in Figures 1 and 2.

ESTs that encode antioxidant or detoxification enzymes**.** The relative concentration (RC) of s*uperoxide dismutase* mRNA did not differ significantly over time or between family types (Table 4; Figure 1A). The RC of *catalase* mRNA increased significantly between the 6 h and 24 h sampling times in both family types (Table 4, Figure 1B). The RC of *glutathione S-transferase omega* mRNA did not differ significantly over time (Table 4, Figure 1C), but was significantly greater in high-surviving families at 24 h after heat shock (Figure 1C). The RC of *glutathione peroxidase* mRNA did not differ significantly across sampling times or between family types (Table 4, Figure 1D), although the profile analysis test for level was suggestive of an increase between 6 h and 24 h after heat shock, ($P = 0.07$)

Heat shock proteins. The RC of both *heat shock protein 68* (Figure 1E) and *heat shock protein 27* mRNA (Figure 1F) increased significantly between 0 h to 6 h and 6 h

to 24 h after heat shock, but did not differ significantly between the family types (Table 4).

ESTs whose products are associated with cellular immunity. The RC of *cystatin B* mRNA was significantly different overall between the family types (Table 4; Figure 2A) and was greater in high-surviving families than in low-surviving families at each of the three sampling times (Figure 2A). No significant differences in RC across sampling times were detected (Table 4).

A significant interaction occurred for the effects of sampling time and family type for the concentration of *galectin* mRNA (Table 4, Figure 2B). The RC was greater in low-surviving families before heat shock, and between 0 h and 6 h, RC decreased in low-surviving families but increased in high-surviving families (Figure 2B).

The RC of *lysozyme* mRNA did not differ significantly over time or between family types (Table 4; Figure 2C). The RC of *prostaglandin E receptor 4* did not differ over time or between family types, (Table 4; Figure 2D), although visually it appears that transcription increased in the low-surviving families between 6 h and 24 h after heat shock. The RC of *tissue inhibitor of metalloprotease* (*TiMP*) mRNA increased significantly between 6 h and 24 h in both family types but did not differ between them (Table 4; Figure 2E). We note the contrast tests for within-subject interactions, although not statistically significant, were suggestive of an interaction of family type with sampling time; the RC in low-surviving families appeared to

decrease between the 0 h and 6 h sampling times whereas the RC in high-surviving families appeared not to change between those times ($P = 0.054$).

Collagen. The RC of *collagen* mRNA (Figure 2F) did not differ significantly over time or between family types (Table 4).

ESTs that have no match in Genbank. The RCs of *BQ426658* mRNA (Figure 2G) and *BQ426884* mRNA (Figure 2H) did not differ significantly over time or between family types (Table 4).

Experiment 3.

Correlations between gene transcription and field performance. Four correlations between relative mRNA concentration and survival at either site were significant (Table 5). The RC of *heat shock protein 27* mRNA (Figure 3A) at 6 h after heat shock and of *catalase* mRNA before heat shock (Figure 3B) were negatively correlated with survival at the intertidal site. The RC of *prostaglandin E receptor 4* mRNA (Figure 3C) at 6 h after heat shock and of *superoxide dismutase* mRNA at 24 h (Figure 3D) after heat shock were negatively correlated with survival at the subtidal site.

Seven correlations between relative mRNA concentration and average weight at either site were significant (Table 6). The RC of *heat shock protein 27* mRNA (Figure 4A) before heat shock was negatively correlated with average weight at the intertidal site. The RC of *galectin* mRNA at 24 h after heat shock (Figure 4B) was

positively correlated with average weight at the intertidal site. The RC of *prostaglandin E receptor 4* mRNA before heat shock (Figure 4C) was positively correlated with average weight at the subtidal site. The RC of *glutathione peroxidase* mRNA at 6 h after heat shock (Figure 4D) was negatively correlated with average weight at the subtidal site. The RC of *galectin* mRNA before heat shock (Figure 4E) was positively correlated with average weight at the subtidal site. The RC of *BQ426658* mRNA before (Figure 4F) and of *prostaglandin E receptor* mRNA at 24 h after heat shock (Figure 5) were positively correlated with average weight at both sites.

Eight correlations between relative mRNA concentration data and yield data at either site were significant (Table 7). The RC of *galectin* mRNA at 24 h after heat shock (Figure 6A) was positively correlated with yield at the intertidal site. The RCs of *BQ426658* mRNA before heat shock (Figure 6B) and at 6 h after heat shock (Figure 6C), and of *glutathione peroxidase* mRNA at 6 h after heat shock (Figure 6D) were negatively correlated with yield at the intertidal site. The RC of *BQ426884* at 24 h after heat shock (Figure 6E) was negatively correlated with yield at the intertidal site, and the RC at 6 h after heat shock (Figure 6F) was negatively correlated with yield at the subtidal site. The RC of *heat shock protein 68* mRNA before heat shock (Figure 7A) was positively correlated with yield at the subtidal site. Finally, RC of *tissue inhibitor of metalloprotease* at 6 h after heat shock (Figure 7B) was negatively correlated with yield at both sites.

DISCUSSION

Experiment 1.

Predictive value of heat shock assays on field performance. Juvenile survival of heat shock was not predictive of adult field performance in Yaquina Bay, Oregon. Survival and growth of *C. gigas* in the estuary are influenced not only by temperature, but by food availability and salinity (Mann et al. 1991; Soletchnik et al. 2002; Soletchink et al. 2007). Our immersion-based heat shock assay may not have adequately reproduced the selective biological pressures imposed by these factors, or juveniles may have different tolerances than adults.

Our results are in contrast to those of Boudry et al. (2008) who reported significant positive correlations between juvenile survival of heat shock (2 h at 40°C in a drying oven) and adult survival of summer mortality in oyster *C. gigas* families. A fundamental difference between our experiment and that study is that the families studied here represent the highest- and lowest-surviving families of a cohort produced for fast growth and high survival in the Pacific Northwest (Langdon et al. 2003), whereas the families used by Boudry et al. were produced by divergent selection for high or low survival during summer mortality (Degremont et al. 2007). Therefore, while survival of laboratory stress may be predictive in families that are expected to thrive or perish in the field, such assays may not be useful for cohorts produced strictly for high growth and survival.

There were also practical issues associated with thermal exposure to consider when developing a predictive assay for oyster performance in the field. The thermal

range over which oysters live or perish after heat shock has been found to span only ~2° - 3°C (Shamseldin et al. 1997; Hamdoun et al. 2003), and this is consistent with our data. Furthermore, tolerance of *C. gigas* to heat shock varies seasonally (Hamdoun et al. 2003). Therefore, assay temperature would have to be tightly controlled and adjusted for each assay as determined by season and thermal history of the test oysters.

Experiment 2.

ESTs that encode antioxidant or detoxification enzymes. The upregulation of *catalase* suggests that heat shock resulted in increased concentrations reactive oxygen species (ROS), as was expected (Flanagan et al. 1998; Arnaud et al. 2002; Bruskov et al. 2002). Free oxygen radicals are converted by superoxide dismutase into hydrogen peroxide (H_2O_2) which is then detoxified by catalase and glutathione or thioredoxin peroxidases (Storey 1996; Young and Woodside 2001). We found previously that glutathione peroxidase transcription in gill of adult oysters was suppressed by heat shock (40°C, 1 h) (Lang 2008); although there was a visual decrease between 0 h and 6 h in whole bodies in the present experiment, the effect was not significant.

The lower levels of *glutathione S-transferase* mRNA at 24 h after heat shock in the low-surviving oyster families suggests that they could face a greater risk of cell damage caused by ROS and their byproducts. Reactive oxygen species react with other substances to form toxic compounds that cause lipid peroxidation and DNA damage (Storey 1996), and glutathione S-transferase omega detoxifies these

compounds by conjugating them to glutathione (Hayes and Pulford 1995; Martinez-Lara et al. 2002; Boutet et al. 2004b). The difference between high- and low-surviving families for *glutathione S-transferase omega* transcription adds to a growing body of evidence indicating that stress-sensitive oyster families may be more susceptible to tissue damage through oxidative stress. Gene transcription of *cavortin*, a protein with superoxide dismutase activity, was greater in mantle and gonad of summer mortality-resistant *C. gigas* oyster families than in susceptible ones (Huvet et al. 2004). Production of ROS by hemocytes was higher in hemocytes of oyster families that were susceptible to summer mortality than in resistant ones (Lambert et al. 2007). Furthermore, gill of summer mortality-resistant *C. gigas* families had higher catalase activity during normoxia and hypoxia than did susceptible ones (Samain et al. 2007).

Heat shock proteins. We found previously that *heat shock protein 27* gene transcription was higher in gills of low-surviving families than in high-surviving families (Table 8), but this difference was not observed at the whole-body level in the present study. In other studies, heat shock protein 70 protein levels were greater before and during hypoxia in gill of oyster families that were susceptible to summer mortality syndrome than in those that were resistant (Samain et al. 2007). Gills may be more sensitive to the effects of heat shock than other tissues, but in the present study this sensitivity could have been masked by the contribution of transcripts by multiple tissues at different rates. For example, transcription of the *heat shock protein*

68 gene studied here (Genbank #AB122062) increased significantly in gill of *C. gigas* exposed to 25°C water after 3 d exposure, but did not increase significantly in mantle tissue until 7 d exposure (Meisterzhein et al. 2008).

ESTs whose products are potentially involved with cellular immunity.

Under normal conditions, oysters are in constant contact with bacteria and protists that pose a threat of opportunistic infection. Heat shock damages hemocytes and reduces both phagocytotic and cell-killing activities (Hégaret et al. 2003; Hégaret et al. 2004; Chen et al. 2007; Li et al. 2007), which increases the risk of opportunistic infection. We did not measure bacterial loads in this experiment and therefore do not know if opportunistic infection contributed to mortality after exposure to 41°C. However, given the overwhelming evidence that heat shock is detrimental to oyster cellular immunity, we speculate that mortality could have been somehow influenced by interactions with pathogens because the water that supplied the troughs used to hold animals after heat shock was not UV-sterilized nor filtered sufficiently to preclude opportunistic bacterial pathogens.

The results for *cystatin B* mRNA in the high-surviving families studied here are consistent with our previous findings (Table 8). Higher expression of cystatin B before and after stress could result in greater ability to stave off infection and thus facilitate survival of heat shock. Cystatins inhibit proteolytic cathepsins (Ulrich 1995; Rinne et al. 2002) that are secreted by pathogens to derive nutrient from host cells (Brady et al. 1999; Vergote et al. 2005). Cystatins have been explicitly linked with

response to infection in invertebrates including snails *Biomphalaria glabrata* (Guillou et al. 2007) and clams *Ruditapes philippinarum* (Kang et al. 2006). Snails *B. glabrata* that were resistant to infection to blood flukes had higher protein levels of a type-2 cystatin after infection (Vergote et al. 2005). Although cystatin has not been explicitly studied within the context of cellular immunity in *Crassostrea* sp., it is known that protease inhibition is crucial for resisting infection (Oliver et al. 2000 Romestead et al. 2002; Montagnani et al. 2007). It is possible that cystatin B could contribute to enhanced protease inhibition capacity in heat-shocked *C. gigas*.

Another benefit of cystatin B expression that could facilitate survival is conservation of energy reserves. Maintenence metabolism accounts for a large portion of energy expenditure and includes the energy spent synthesizing and degrading proteins (protein turnove; Hawkins and Day 1996). Mussels *Mytilus edulis* with lower rates of protein turnover had higher tolerance of otherwise lethal temperature and metals exposure (Hawkins et al. 1987; Hawkins et al. 1989), possibly because more energy was available to fuel stress responses. Therefore, it is possible that higher expression of cystatin B conferred an advantage for survival by reducing rates of protein turnover thereby providing more energy to fuel stress responses.

The greater concentration of *galectin* mRNA before and at 24 h after heat shock in low-surviving families is consistent with our previous findings (Table 8). In vertebrates, galectins promote cell adhesion and regulate both innate and adaptive immune responses (Perillo et al. 1997; Rabinovich et al. 2002; Levroney et al. 2005). In *Crassostrea* sp., galectins adhere to microbes, phytoplankton, and protozoans

thereby serving defensive and digestive roles by promoting phagocytosis of particles by hemocytes (Tasumi and Vasta 2007).

We speculate that in low-surviving families, sensitivity to infection through lowered capacity of hemocytes to bind particles could have lead to exaggerated transcription of adhesive proteins to effect cellular defense. Pathogens of marine bivalves reduce hemocyte adhesive capacity (Choquet et al. 2003; Labrueche et al. 2006), and hemocytes of *C. gigas* that were susceptible to summer mortality syndrome had lower rates of substrate adhesion than did resistant families when exposed to *Vibrio* sp. S322 (Lambert et al. 2007). The significance of galectin downregulation between 0 h and 6 h in low-surviving families is unclear. However, increased transcription of heat shock proteins is usually accompanied by decreased transcription and translation of non-heat shock proteins (Cuesta et al. 2000) and it is possible that galectin transcription was decreased as part of the overall heat shock response. Alternatively, transcription of galectin has been reported to be transiently suppressed by exposure to *Vibrio tubiashii* (Yamaura et al. 2008) and the decreased transcription observed here could reflect interaction with pathogens. However, whether this depressed transcription would increase susceptibility to opportunistic infection remains to be determined.

Multiple types of lysozymes may be secreted by hemocytes or tissues in marine bivalves and participate in self-defense and digestion of food (Xue et al. 2004; Matsumoto et al. 2006; Itoh and Takahashi 2007; Itoh et al. 2007). Lysoszyme activity increased in tissue of mussel *Mytilus eduilis* during summer mortality months

in association with the liberation of tissue energy reserves, and this effect was more pronounced in summer mortality-susceptible mussel populations (Tremblay et al. 1998). Given the possibility of opportunistic infection after heat shock and the need for energy after mounting a stress response, it was surprising to observe no differences in lysozyme transcription before or after heat shock.

Prostaglandins are oxygenated compounds derived from polyunsaturated fatty acids (PUFAs) that play various physiological roles including mounting immune and inflammatory responses (Rowley et al. 2005). In a previous study (N. Taris, P. Lang, and M. Camara, *unpublished data*) we found that transcription of this gene by hemocytes in *Vibrio tubiashii*-challenged oysters was greater in the same low-surviving families that were used in the present study. In molluscs, prostaglandin production has been linked with chemical defense (Di Marzo et al. 1991), maintaining ionic balance in gill (Saintsing et al. 1983; Freas and Grollman 1980), and inducing spawning (Osada et al. 1989). Without further characterization of this EST it is impossible to evaluate its function in this study.

The transcription profile of another protease inhibitor thought to be important to cellular defense, *tissue inhibitor of metalloprotease,* appeared similar to the pattern observed for *galectin* - transcription appeared to decrease after heat shock in low-surviving families only but this trend was not statistically significant. *Tissue inhibitors of metalloproteases* remodel the extracellular matrix and inhibit pathogen-derived proteases (Montagnani et al. 2001; Gagnaire et al. 2007; Montagnani et al. 2007). As with *galecitn,* it remains to be determined whether temporarily suppressed

transcription simply reflects downregulation of non-heat shock protein genes and whether it would enhance susceptiblity of low-surviving families to opportunistic infection.

Collagen. In a previous study, transcription of *collagen* was greater after heat shock in gills of low-surviving families (Table 8), but this difference was not evident at the whole-body level. The significance of higher or lower collagen transcription is unclear. It appears that altered collagen transcription can be expected in bivalves exposed to stress; it increased in clams *Ruditapes decussates* exposed to a mixture of *Vibrio* sp. and *Micrococcus lysodeikticus* (Gestal et al. 2007), and decreased in *C. gigas* exposed to experimental hypoxia (David et al. 2005). Because collagen is an extremely abundant protein that is a major constituent of tissue and the extracellular matrix (Voet and Voet 1995) we speculate that differences in transcription could reflect higher rates of protein turnover which in turn would decrease the available energy for mounting stress responses, thereby leading to mortality.

ESTs that have no match in Genbank. The lack of difference between the family types observed here for *BQ426658* and *BQ426884* contradicts our previous findings (Table 8). It is difficult to speculate as to the causes of or significance of these results without information on the full gene sequence or function of these genes. The field of oyster genomics is still in its infancy, and while the amount of EST and genomic resources for marine bivalves continues to grow, thousands of ESTs without

homology to known genes continue to be identified in *C. gigas*. Tanguy et al. (2008) found that no more than 27% of over 10,000 recently sequenced ESTs could be assigned a functional category.

Experiment 3.

Correlations between gene transcription and field performance. Most of the significant correlations between gene transcription in juveniles and field performance of adults were negative. If we assume that gene transcription is a reasonable proxy for protein production, it is possible that the negative correlations reflect genotype-specific differences in protein turnover that in turn had consequences for growth and surviving stress. Proteins are in a constant state of turnover (the net result of protein breakdown, synthesis, and deposition) and the energy required to drive these processes comprises a large portion of energy expended during maintenance metabolism (Hawkins 1991; Hawkins and Day 1996). In general, when less energy is expended during maintenance metabolism, more energy is available to fuel growth and stress responses (Hawkins et al. 1987; Bayne 1999; Bayne 2004).

Each of the correlations between gene transcription in juveniles and survival of adults were negative. In the field, higher protein turnover rates could have contributed to metabolic exhaustion and mortality, particularly when animals were ripe and tissue glycogen reserves were low (Berthelin et al. 2000). Many of the correlations between transcription in juveniles and final adult weight were also negative, such as for both

heat shock protein 27 and *BQ426658*, perhaps indicating higher protein turnover rates and lower growth efficiencies in families with lower final weights.

Relative concentrations of ESTs for *galectin*, *prostaglandin E receptor 4*, and *heat shock protein 68* in juveniles were positively correlated with adult growth or yield, which does not conform to the proposed positive relationship between gene transcription rates and protein turnover/metabolic rates. We acknowledge that caution is needed in interpreting the significance of individual genes transcribed under hatchery conditions and performance in the field because the two environments differ substantially, although some explanations are possible based upon the predicted functions of these three ESTs. *Galectin* is thought to be used to acquire food and a positive relationship between its transcription in juveniles and adult growth seems plausible. Heat shock proteins are generally assumed to confer survival of stress (Lindquist et al. 1981; Feder and Hoffmann 1999) and a positive relationship between expression of *heat shock protein 68* and yield (which was driven by survival) at the subtidal site is consistent with this assumption. Further characterization of *prostaglandin E receptor 4* is needed in order to propose explanations about its role in growth or survival. We advocate further study of this EST because among the ESTs studied here, it was the only one in which transcription was negatively correlated with survival but positively correlated with growth.

CONCLUSIONS

We conclude that survival of heat shock in the nursery is not predictive of field performance of *C. gigas* families produced by selecting parents based on fast growth and high survival. Differences in *gluathione S-transferase*, *cystatin B*, and *galectin* gene transcription in low-surviving families suggest that sensitivity to heat shock may have resulted from exaggerated damage by ROS and opportunistic infection. Gene transcript levels in juveniles were mostly negatively correlated with field performance and it is therefore reasonable that families could be culled from the hatchery based upon higher transcription levels of certain genes. A key finding is that many differences in gene transcription between low- and high-surviving families and correlations between gene transcription and field performance occurred in juvenile oysters sampled before heat shock. This implies that the relationship between gene transcription and stress tolerance or field performance may be rooted in constitutive genetic differences rather than a response to heat shock. To simplify identification of candidate genes and to further develop predictive assays of field performance, future studies should focus on differences in gene expression under non-stress conditions and should take potential differences in maintenance metabolism and protein turnover into account when evaluating stocks.

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FIGURE LEGENDS

Figure 1. Average relative concentration (RC) or $\log_2$ relative concentration (Log_2RC) (± SEM) before and at 6 h and 24 after HS (39°C, 1 h) of ESTs encoding antioxidant enzymes (A – D) and heat shock proteins (E – F) in pools of whole bodies of oyster families that had low survival (L; hatched bars) or high survival (H; white bars) after heat shock (HS) (41°C, 1 h). Letters indicate no significant ("a") or significant ("b") ($P < 0.05$) change in RC between sampling times. Asterisks indicate significant ($P < 0.05$) differences between the two family types at individual sampling times.

Figure 2. Average relative concentration (RC) or $\log_2$ relative concentration (Log_2RC) (± SEM) before and at 6 h and 24 after HS (39°C, 1 h) of ESTs in which products contribute towards cellular defense (A-E), encode collagen (F), or have no known function (G-H) in pools of whole bodies of oyster families that had low survival (L; hatched bars) or high survival (H; white bars) after heat shock (HS) (41°C, 1 h). Letters indicate no significant ("a") or significant ("b") ($P < 0.05$) change in RC between sampling times, or ("c") significant interactions of time and family type. Asterisks indicate significant ($P < 0.05$) differences between the two family types at individual sampling times.

Figure 3. Correlation of average survival (%) of adults at harvest at intertidal (points represented by triangles) or subtidal (points represented by circles) sites and average log2 relative concentration of gene transcript ($\log_2\text{RC}$) in heat-shocked (39°C, 1 h) spat of individual families, as measured using real-time quantitative PCR. Significant correlations ($P < 0.05$) are denoted by asterisks.

Figure 4. Correlation of average weight (g) of adults at harvest at intertidal (points represented by triangles) or subtidal (points represented by circles) sites and average log2 relative concentration of gene transcript ($\log_2\text{RC}$) in heat-shocked (39°C, 1 h) spat of individual families, as measured using real-time quantitative PCR. Significant correlations ($P < 0.05$) are denoted by asterisks.

Figure 5. Correlation of average weight (g) of adults at harvest at intertidal (points represented by triangles) or subtidal (points represented by circles) sites and average log2 relative concentration of gene transcript ($\log_2\text{RC}$) in heat-shocked (39°C, 1 h) spat of individual families, as measured using real-time quantitative PCR. Significant correlations ($P < 0.05$) are denoted by asterisks.

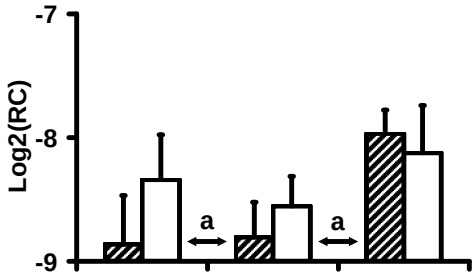
FIGURE LEGENDS, CONTINUED

Figure 6. Correlation of average yield (kg) of adults at harvest at intertidal (points represented by triangles) or subtidal (points represented by circles) sites and average log2 relative concentration of gene transcript (log_2RC) in heat-shocked (39°C, 1 h) spat of individual families, as measured using real-time quantitative PCR. Significant correlations ($P < 0.05$) are denoted by asterisks.

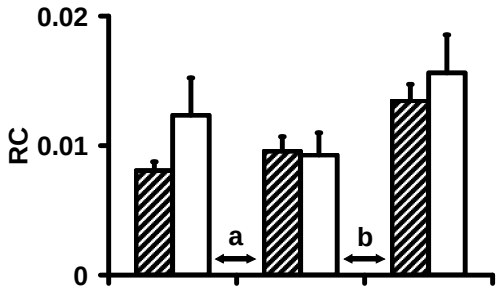
Figure 7. Correlation of average yield (kg) of adults at harvest at intertidal (points represented by triangles) or subtidal (points represented by circles) sites and average log2 relative concentration of gene transcript (log_2RC) in heat-shocked (39°C, 1 h) spat of individual families, as measured using real-time quantitative PCR. Significant correlations ($P < 0.05$) are denoted by asterisks.

Figure 1.

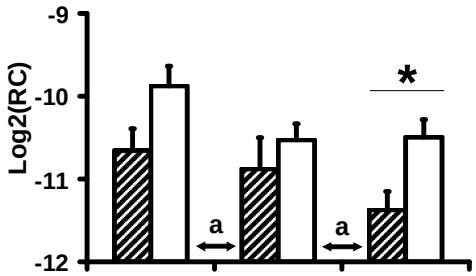
A) Superoxide dismutase:



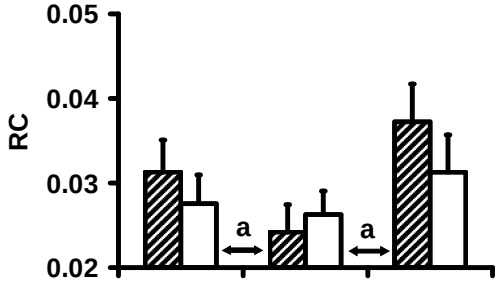
B) Catalase



C) GST-Omega



D) Glutathione peroxidase



E) HSP68

F) HSP27

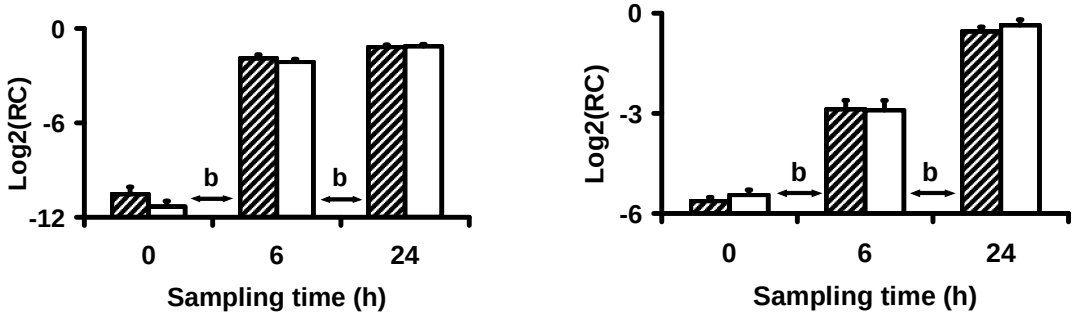
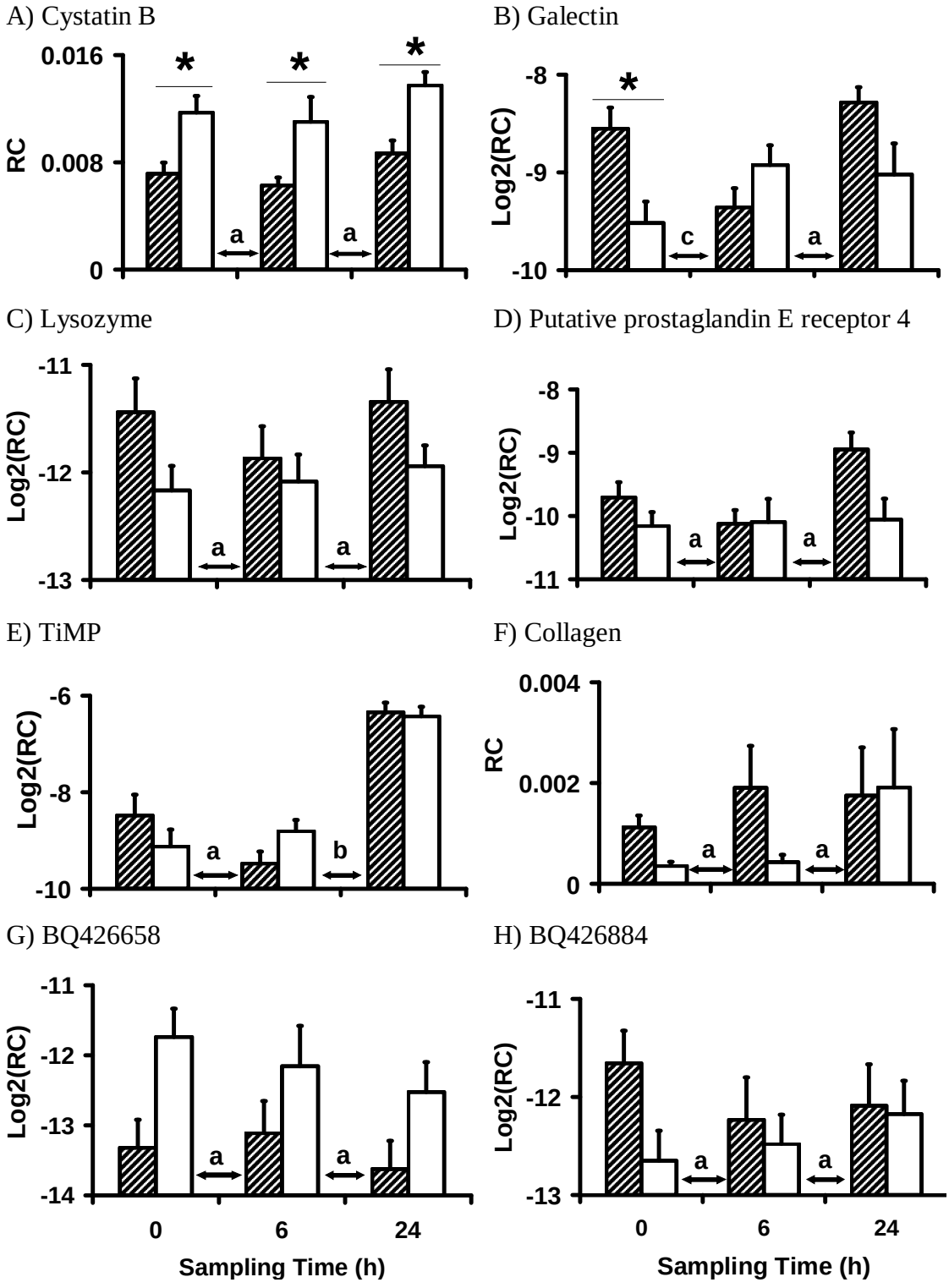


Figure 2.


A) Cystatin B
RC
0.016
0.008
0
a
a
B) Galectin
Log2(RC)
-8
-9
-10
c
a
C) Lysozyme
Log2(RC)
-11
-12
-13
a
a
D) Putative prostaglandin E receptor 4
Log2(RC)
-8
-9
-10
-11
a
a
E) TiMP
Log2(RC)
-6
-8
-10
a
b
F) Collagen
RC
0.004
0.002
0
a
a
G) BQ426658
Log2(RC)
-11
-12
-13
-14
a
a
0
6
24
Sampling Time (h)
H) BQ426884
Log2(RC)
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a
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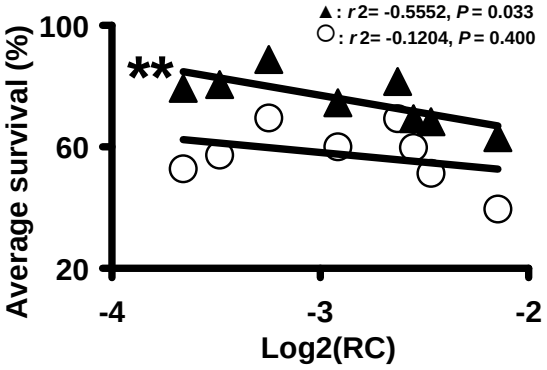
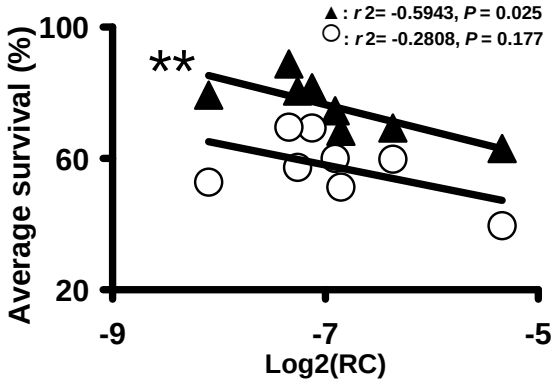
Sampling Time (h)

Figure 3.

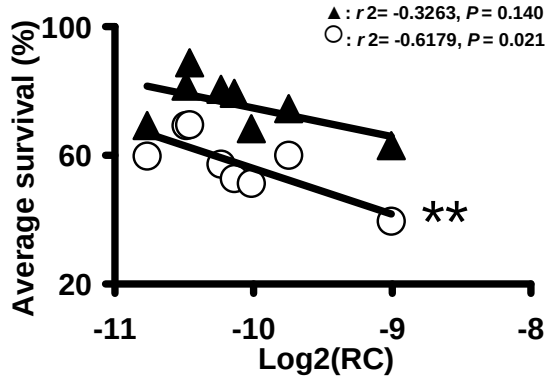
A) Heat shock protein 27 at 6 h after heat shock



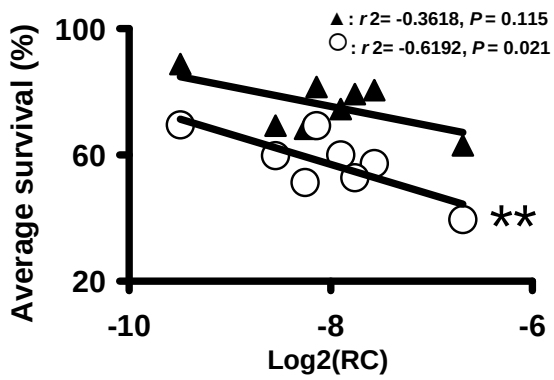
B) Catalase before heat shock



C) Prostaglandin E receptor 4 at 24 h after heat shock



D) Superoxide dismutase at 24 h after heat shock

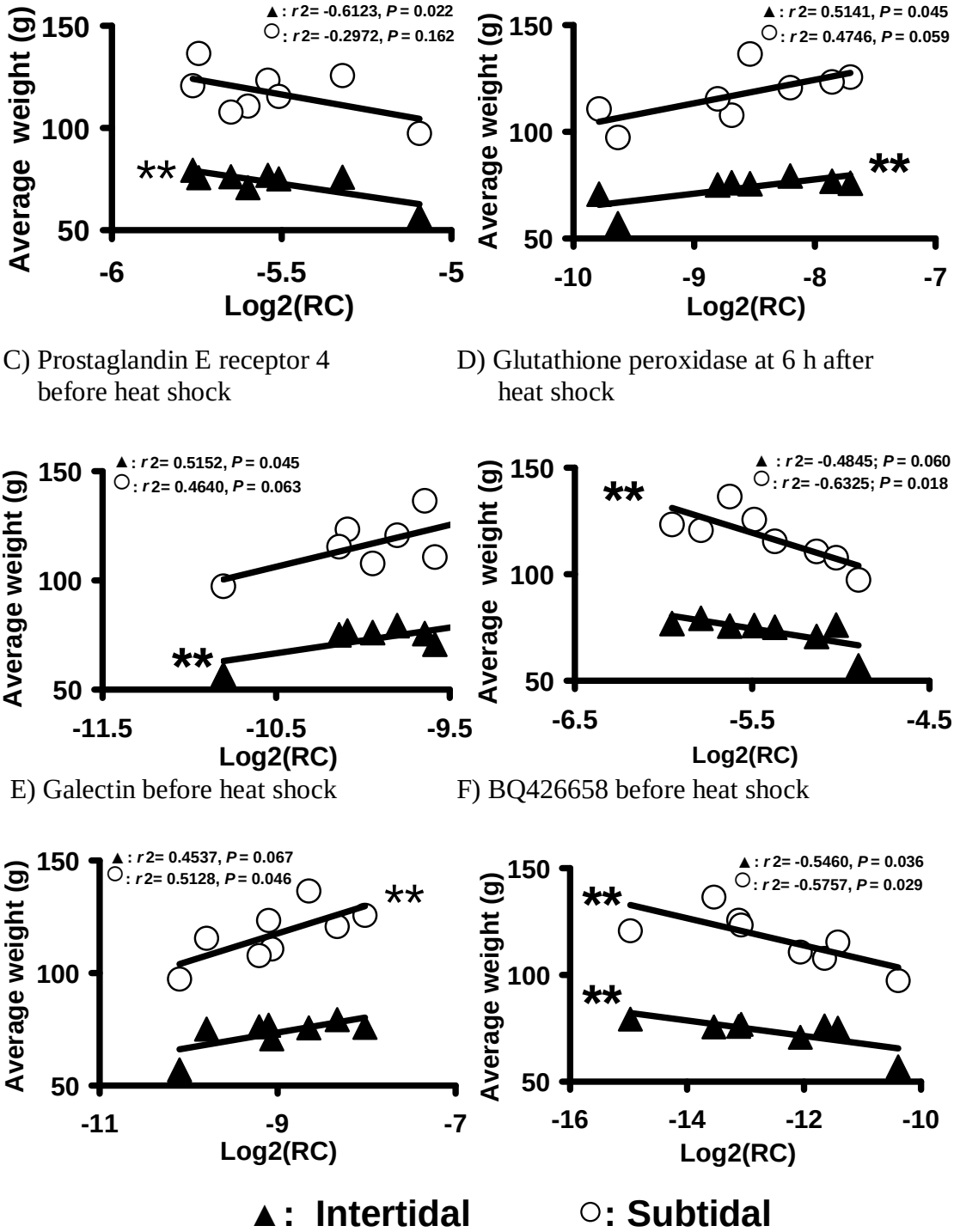


▲: Intertidal ○: Subtidal

Figure 4.

A) Heat shock protein 27 before heat shock

B) Galectin at 24 h after heat shock



C) Prostaglandin E receptor 4 before heat shock

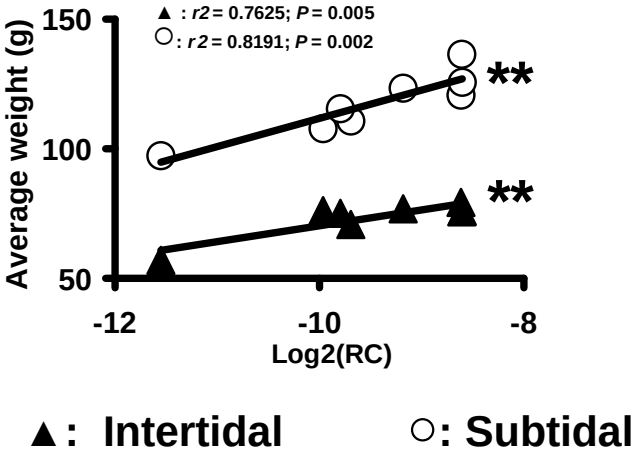
D) Glutathione peroxidase at 6 h after heat shock

E) Galectin before heat shock

F) BQ426658 before heat shock

Figure 5.

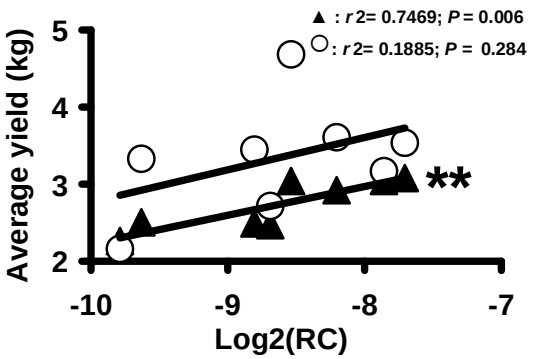
A) Prostaglandin E receptor 4 at 24 h after heat shock



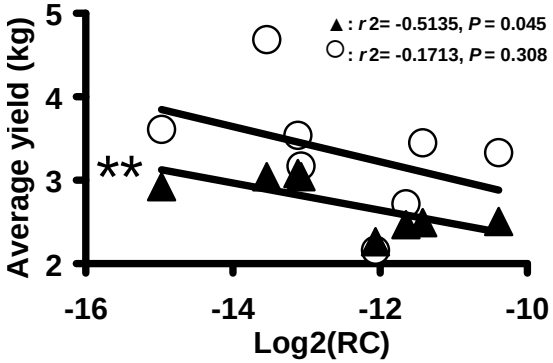
▲: Intertidal ○: Subtidal

Figure 6.

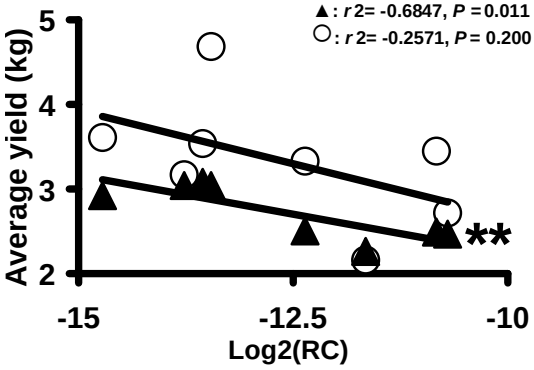
A) Galectin at 24 h after heat shock



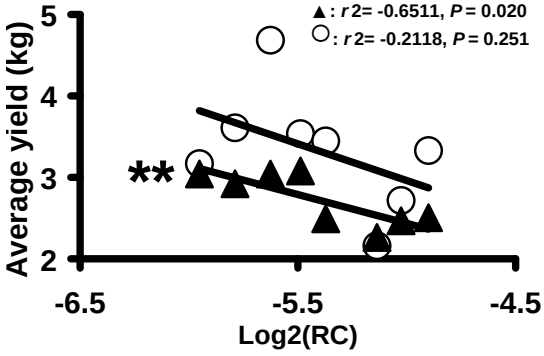
B) BQ426658 before heat shock



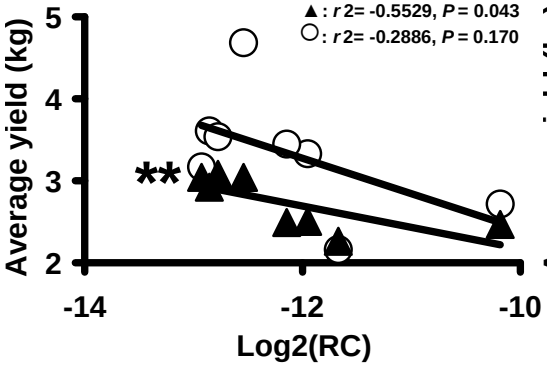
C) BQ426658 at 6 h after heat shock



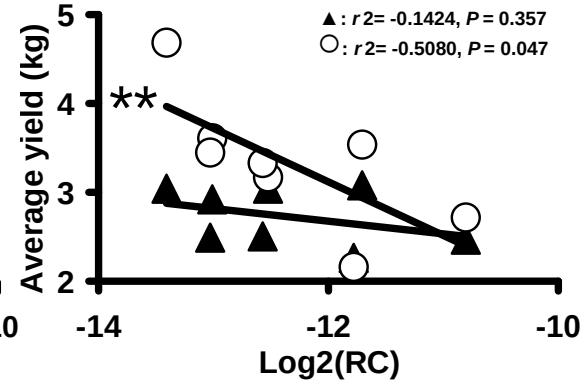
D) Glutathione peroxidase at 6 h after heat shock



E) BQ426884 at 24 h after heat shock



F) BQ426884 at 6 h after heat shock



▲: Intertidal ○: Subtidal

Figure 7.

A) Heat shock protein 68 before heat shock

B) Tissue inhibitor of metalloprotease at 6 h after heat shock

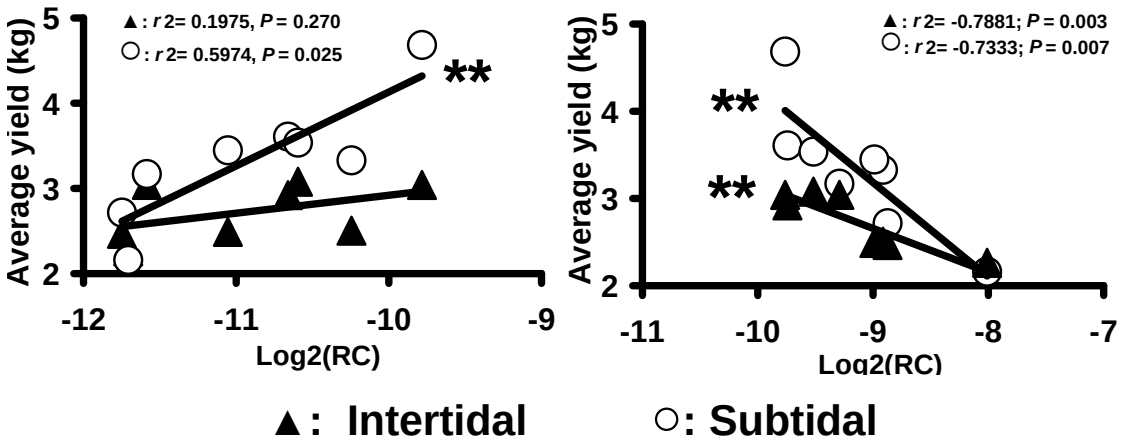


Table 1. Accession numbers, identity, species match with corresponding accession number and *E*-value, and source of ESTs whose transcription was studied in whole bodies of oyster juveniles in the present study.

Accn#	Identity	Species Match	Accession	E	Source
ESTs that encode antioxidant enzymes					
CX069146	Glutathione peroxidase	*Hymeniacidon perlevis*	ABB91779	4.E-53	David et al. (2005)
AJ558252	Glutathione S-transferase omega	*Crassostrea gigas*		0	Boutet et al. (2004a)
AJ496219	SOD	*Crassostrea gigas*		0	Boutet et al. (2004b)
EF687775	Catalase	*Crassostrea gigas*		0	Unpublished
Heat shock proteins					
AB122062	Heat shock protein 68	*Crassostrea gigas*		0	Unpublished
BQ426550	Heat shock protein 27	*Homo sapiens*	AAA62175	8.E-07	Guegan et al. (2003)
ESTs whose products are involved in cellular immunity					
CX069133	Cystatin B	*Gallus gallus*	XP_416492	3.E-18	David et al. (2004)
AM237796	Galectin 4	*Crassostrea gigas*	CAD79473	4.E-88	Unpublished
AB179775	Lysozyme	*Crassostrea gigas*			Matsumoto et al. (2006)
EX956398	Prostaglandin receptor E4	*Homo sapiens*	EAW55996	1.E-03	Unpublished
AF321279	Tissue inhibitor of metalloprotease	*Crassostrea gigas*		0	Montagnani et al. (2001)
ESTs whose products are associated with the extracellular matrix					
CX069163	Collagen	*Suberites domuncula*	CAC81019	7.E-09	David et al. (2004)
ESTs with no match in Genbank					
BQ426658	--				Guegan et al. (2003)
BQ426884	--				Guegan et al. (2003)

Table 2. Accession numbers, identity, forward and reverse primer sequences of ESTs studied using real-time quantitative PCR (RT-QPCR). Reactions were run using the annealing temperature (Ta) and number of cycles (C) listed. Efficiency was calculated using LinReg PCR (Ramakers et al. 2003). RT-QPCR data were normalized to levels of the gene *elongation factor 1* (AB121066).

EST Identity	Forward Primer Reverse Primer	C	T_a	Efficiency
AB122062 Heat shock protein 68	TGGAACCTGTGAAGAAAGCATTATT TTGATCCTCCTACCAAAACCACTT	60	40	1.91 ± 0.003
AB122066 Elongation factor 1	GGAAGCTGCTGAGATGGGAA TCCAACACCCAGGCGTATTT	*	*	2.01 ± 0.001
AB179775 Lysozyme	GCAGTGGTTGTCCAGCAAACT CACCATTGTGGATTCGTGCA	60	40	2.01 ± 0.003
AF321279 Tissue inhibitor metalloprot.	CATTGCCGTGGTGAGAACTG CTGGTCCGGATTCAATGACTG	58	45	2.00 ± 0.002
AJ557141 Glutathione S-transferase	TATTTGGACCAGGTGTATCCCGA AGAATACGTTCAAACCATGGCCA	60	40	1.94 ± 0.002
AJ496219 Superoxide dismutase	GAGGATCACGAGAGGCACGT TTCGCCAGCGGTGACATTA	60	40	2.04 ± 0.004
AM237796 Galectin	GCTGTGGAGTGGTTACCAGGA GAGAGTAACCCAGCTCCCCG	58	40	2.02 ± 0.003
BQ426550 Heat shock protein 27	GACGAGCTAGGCTTTCCCGT TCTCTTTCTGTGCTGCAAGGC	60	40	1.98 ± 0.003
BQ426658 None	CTCAAACCTACAGTCATCACCACAT GTGACTTTTGTTCTGTGTTGCCA	58	45	2.00 ± 0.003
BQ426884 None	ACGATGTCAGGGACAACTTTCTGT CGCAGAGGTAATCTTTTAAACGTCA	58	45	1.98 ± 0.003
CX069133 Cystatin B	GTTCCTGCCACGACCTGAGT GCTGGACACTTACACCCCAGTC	58	45	2.02 ± 0.002
CX069163 Collagen	TATGAAGTAGCAGCATGATGACTCC TCGTGGCTACCTAATGGCG	59	45	2.08 ± 0.004
EF687775 Catalase	TTCTCAGACCGAGGCACACC GCCATAGCCGTTCATTCTCC	60	40	2.03 ± 0.003
EF692639 Glutatuione peroxidase	TGACAATGCGACGGAGCTC CTACCGGGCCGAACGTACTT	60	40	2.04 ± 0.002
EX956398 Prostaglandin E receptor	ACCAATTCTACTGGTGTACCCAACA AAGTCCGTGGCATATCTAACCATAA	58	40	1.95 ± 0.004

Table 3. A) Correlation between survival of heat shock as juveniles and average survival (%), yield (kg), or average individual weight (g) in 46 oyster families reared at a subtidal and intertidal site in Yaquina Bay estuary, Newport, OR. No significant correlations were observed when $P < 0.05$. B). Correlation between field parameters at the intertidal or subtidal site in four families that had high (> 69%) survival and four that had low (< 29%) survival at 6 d after heat shock. Correlations were significant when $P < 0.05$.

A)

	Intertidal		Subtidal	
	r^2	P	r^2	P
Average survival (%)	-0.0226	0.3183	0.0041	0.6708
Average weight (g)	0.0007	0.4931	-0.0004	0.8829
Average yield (kg)	-0.0107	0.8611	0.0017	0.7842

B)

	Survival		Body weight		Yield	
Intertidal	r^2	P	r^2	P	r^2	P
Survival	--	--	0.1931	0.2760	0.3156	0.1473
Body weight	0.1931	0.2760	--	--	0.2219	0.2386
Yield	0.3156	0.1473	0.2219	0.2386	--	--
Subtidal	r^2	P	r^2	P	r^2	P
Survival	--	--	0.0027	0.6966	0.7105	**0.0086***
Body weight	0.0027	0.6966	--	--	0.4403	0.0728
Yield	0.7105	**0.0086***	0.4403	0.0728	--	--

Table 4. Repeated measures analysis of gene transcription before and at 6 h and 24 h after heat shock (39°C, 1 h) in whole bodies of two types of oyster families (high or low survival after heat shock). Abbreviations refer to: FT, Family Type (high- or low-surviving); T: sampling time. Differences were significant when $P < 0.05$.

		Between	Within	
EST	Identity	FT	T	FT * T
AJ496219	*Superoxide dismutase*	0.7264	0.1826	0.5137
EF687775	*Catalase*	0.6504	**0.0066**	0.4437
AJ557141	*Glutathione S-transferase*	0.2151	0.0514	0.7018
EF692639	*Glutathione peroxidase*	0.6615	0.2218	0.3964
AB122062	*Heat shock protein 68*	0.1127	**<.0001**	0.0861
BQ426550	*Heat shock protein 27*	0.3928	**<.0001**	0.9018
CX069133	*Cystatin B*	**0.0373**	0.0639	0.9688
AM237796	*Galectin*	0.2134	0.3023	**0.0158**
AB179775	*Lysozyme*	0.2238	0.4152	0.4694
EX956398	*Prostaglandin E receptor 4*	0.1969	0.3222	0.3818
AF321279	*Tissue inhibitor of metalloprotease*	0.9585	**<.0001**	0.1348
CX069163	*Collagen*	0.4887	0.3127	0.7361
BQ426658	--	0.2142	0.3758	0.6200
BQ426884	--	0.4315	0.5257	0.5059

Table 5. Correlation between average survival (%) at harvest of adult oyster families that had high (> 69%) and low (< 29%) survival at 6 d after heat shock as juveniles, with average log2 relative concentrations in juveniles of *heat shock protein 27, catalase, prostaglandin E receptor,* and *superoxide dismutase* in juveniles. Correlations were considered to be significant when $P < 0.05$.

	Intertidal		Subtidal	
Time 0	r^2	*P*	r^2	*P*
Heat shock protein 27	0.3041	0.1565	0.0727	0.5183
Catalase	-0.5943	**0.0251***	-0.2808	0.1767
Prostaglandin E receptor 4	-0.1886	0.2823	-0.1855	0.2867
Superoxide dismutase	-0.1395	0.3622	-0.1562	0.3325
Time 6	r^2	*P*	r^2	*P*
Heat shock protein 27	-0.5552	**0.0339***	-0.1204	0.3998
Catalase	-0.0383	0.6424	-0.0253	0.7068
Prostaglandin E receptor 4	-0.3263	0.1392	-0.6179	**0.0207***
Superoxide dismutase	-0.0367	0.6495	-0.0074	0.8394
Time 24	r^2	*P*	r^2	*P*
Heat shock protein 27	0.0435	0.6200	0.0592	0.5614
Catalase	-0.2965	0.1629	-0.1199	0.4008
Prostaglandin E receptor 4	-0.0252	0.7075	-0.0132	0.7863
Superoxide dismutase	-0.3618	0.1147	-0.6192	**0.0205***

Table 6. Correlation between average weight at harvest of adult oyster families that had high (> 69%) and low (< 29%) survival at 6 d after heat shock as juveniles with average log2 relative concentrations of various transcripts in juveniles measured before and at 6 h and 24 h after heat shock. Correlations were considered to be significant when $P < 0.05$, and are in bold and denoted by asterisks.

	Intertidal		Subtidal	
Before heat shock	r^2	P	r^2	P
Heat shock protein 27	-0.6123	**0.0217***	-0.2972	0.1623
Galectin	0.4537	0.0670	0.5128	**0.0457***
Prostaglandin E receptor 4	0.5152	**0.0450***	0.4640	0.0629
Glutathione peroxidase	-0.0063	0.8518	0.0099	0.8151
BQ426658	-0.5460	**0.0362***	-0.5757	**0.0291***
	Intertidal		Subtidal	
6 hours after heat shock	r^2	P	r^2	P
Heat shock protein 27	-0.0058	0.8582	-0.0249	0.7089
Galectin	0.0101	0.8124	-0.0009	0.9434
Prostaglandin E receptor	0.0119	0.7974	-0.0242	0.7132
Glutathione peroxidase	-0.4845	0.0552	-0.6325	**0.0183***
BQ426658	-0.0693	0.5288	-0.3254	0.1398
	Intertidal		Subtidal	
24 hours after heat shock	r^2	P	r^2	P
Heat shock protein 27	-0.0363	0.6514	0.0145	0.7765
Galectin	0.5141	**0.0453***	0.4746	0.0588
Prostaglandin E receptor 4	0.7625	**0.0046***	0.8191	**0.0020***
Glutathione peroxidase	-0.0245	0.7113	0.0575	0.5673
BQ426658	-0.1636	0.3202	-0.3624	0.1143

Table 7. Correlation between average yield (kg per bag) at harvest of adult oyster families that had high (> 69%) and low (< 29%) survival at 6 d after heat shock as juveniles with average log2 relative concentrations of various transcripts in juveniles measured before and at 6 h and 24 h after heat shock. Correlations were considered to be significant when $P < 0.05$, and are in bold and denoted by asterisks.

Before heat shock	Intertidal r^2	Intertidal P	Subtidal r^2	Subtidal P
Galectin	0.4472	0.0698	0.0767	0.5067
BQ426658	-0.5135	**0.0455***	-0.1713	0.3080
Glutathione peroxidase	-0.2265	0.2333	0.0279	0.6925
BQ426884	0.0311	0.6761	0.0074	0.8392
Heat shock protein 68	0.1975	0.2700	0.5974	**0.0245**
Tissue inhibitor of metalloprotease	-0.0087	0.8266	-0.0133	0.7860

6 hours after heat shock	Intertidal r^2	Intertidal P	Subtidal r^2	Subtidal P
Galectin	-0.0518	0.5879	-0.1398	0.3615
BQ426658	-0.6847	**0.0112***	-0.2571	0.1996
Glutathione peroxidase	-0.6511	**0.0155***	-0.2118	0.2512
BQ426884	-0.1424	0.3567	-0.5080	**0.0472**
Heat shock protein 68	0.0121	0.7952	0.0200	0.7358
Tissue inhibitor of metalloprotease	-0.7881,	**0.0032***	-0.7333,	**0.0066***

24 hours after heat shock	Intertidal r^2	Intertidal P	Subtidal r^2	Subtidal P
Galectin	0.7469	**0.0056***	0.1885	0.2825
BQ426658	-0.1987	0.2683	-0.0229	0.7207
Glutathione peroxidase	-0.0649	0.5426	0.0120	0.7960
BQ426884	-0.5229	**0.0426***	-0.2886	0.1697
Heat shock protein 68	0.0339	0.6625	0.0654	0.5335
Tissue inhibitor of metalloprotease	-0.0278	0.6933	-0.0211	0.7312

Table 8. Comparison of gene expression in adult gill (Chapter 2) and in whole bodies of juvenile oysters from families showing either high (H) or low (L) survival after heat shock (HS; 41°C, 1 h). Tissue samples were collected before and at 6 and 24 h after HS (40°C, 1 h for adults; 39°C, 1 h for juveniles). Incidences where transcript concentration was significantly greater ($P < 0.05$) in L families than in H families are denoted as L > H; greater in H families than in L families is denoted as L < H.

Gene	Gill (Chapter 2)	Whole Body
Heat shock protein 27	L > H at 6 h after HS	No differences
Cystatin B	L < H at 24 h after HS	L < H at all times
Galectin	L > H overall	L > H before HS
Collagen	L > H at 1 h after HS	No differences
BQ426658	L > H at 1 h after HS	No differences
BQ426884	L > H at 24 h after HS	No differences

GENERAL CONCLUSIONS

Summary of results. The objectives of this research were 1) to identify genes for which mRNA concentrations differed between selectively-bred oyster families characterized by high survival or low survival after heat shock, and 2) to determine whether laboratory assays of juvenile survival after heat shock and gene transcription before or after heat shock could be used to predict field performance of adults.

I first used cDNA microarrays to identify genes for which mRNA concentrations in gill were altered by heat shock in low- and high-surviving families (Chapter 2). The mRNA concentrations for eight ESTs (Table 1) differed significantly between high- and low-surviving families. This study of transcriptome profiling in a marine bivalve enabled us to identify candidate genes for subsequent use in predictive assays.

Based on these results, I used a RT-QPCR approach to determine whether the mRNA concentration of the candidate genes and other related genes would differ in expression at the whole-body level in a different group of low- or high-surviving families, and whether survival of heat shock or gene expression in juveniles was predictive of growth and survival of adults in the field (Chapter 3). I found that the differences in transcript concentrations between low- and high-surviving families for *galectin* and *cystatin B* in whole bodies were similar to those observed in gill of adult low- and high-surviving families (Table 1). I found that the concentrations of mRNAs for *glutathione S-transferase* were greater in high-surviving families at 24 h after heat shock. Finally, I found that survival of heat shock was not predictive of field

performance; however, mRNA concentrations of several genes were significantly correlated with survival, final weight, or yield of adults reared at intertidal and subtidal growing sites (Table 2). Most of these correlations were negative.

The transcription data, when considered in terms of current literature on summer mortality and energy metabolism in marine bivalves, suggest that mortality at high temperatures could have been driven by enhanced sensitivity to cellular damage, metabolic exhaustion, and opportunistic infection. The negative relationships between gene transcription and survival or final weight could have reflected whole-body protein turnover rates, whereas the positive correlations may relate to the longevity or function of their protein products.

Transcription and cellular damage in high- and low-surviving families.

Molecular chaperones. The transcriptional response of gill to heat shock in families that had low survival after heat shock included higher transcription of ***heat shock protein 27***. This result seems consistent with Samain et al. (2007)'s finding that heat shock protein 70 levels in gills before and during hypoxia were higher in summer mortality-susceptible *C. gigas* families than in resistant ones.

Small heat shock proteins perform a wide variety of constitutive and induced functions, and it is therefore difficult to speculate as to the effects of higher ***heat shock protein 27*** transcription in our study. For example, it is accepted that heat shock denatures proteins that can be refolded by heat shock proteins. Oysters are known to possess a highly polymorphic genome (Sauvage et al. 2007) and mutations could lead

to decreased stability of various proteins during heat shock and, in turn, could require a greater chaperone response to salvage them. Alternatively, small stress proteins stabilize lipid membranes that have been perturbed by high temperatures (Tsvetkova et al. 2002), and it is possible that lipid membranes of low-surviving families are more prone to thermal disorganization. Damage to lipid membranes in gills of all families after heat shock was suggested by increased transcription of *delta 9-desaturase* and *sterol regulatory element binding protein* genes after heat shock.

It seems counter-intuitive that elevated transcription of proteins that are essential to surviving life in the intertidal zone would correspond to lower tolerance of heat shock or summer mortality (Samain et al. 2007). The exaggerated transcriptional response could simply reflect an enhanced predisposition to cell damage by high temperatures. However, excessive heat shock protein transcription and translation could lead to metabolic exhaustion and opportunistic infection, thereby providing a basis for mortality. It has been established that heat shock in adult *C. gigas* reduces tissue energy levels (Li et al. 2007). In that study, energy expenditure following heat shock response and spawning rendered animals unable to synthesize heat shock proteins, and resulted in opportunistic infection.

Antioxidant and Detoxification Enzymes. Transcription of the antioxidant/ detoxification enzyme *glutathione S-transferase omega* (*GST*-Ω) at 24 h after heat shock was lower in whole bodies of low-surviving families. Lower *GST*-Ω protein availability could have led to greater damage by reactive oxygen species in low-

surviving families, which could, in turn, have led to greater levels of tissue damage. Reactive oxygen species (ROS), such as the superoxide radical, are produced by heat shock (Flanagan et al. 1998; Arnaud et al. 2002; Briskov et al. 2002), and *GST*-Ω detoxifies ROS directly and also their byproducts through conjugation to glutathione (Hayes and Pulford 1995; Martinez-Lara et al. 2002). It is thought that the source of ROS is respiring mitochondria and that increases result from enhanced metabolic rates as a function of temperature (Abele et al. 2002). Another source of reactive oxygen species is hemocytes; they produce ROS as a defensive reaction (Lambert et al. 2007), and *GST*-Ω may also directly detoxify ROS and their byproducts during opportunistic infection.

There is increasing evidence that summer mortality-susceptible *C. gigas* families are at greater risk of damage through ROS production. Catalase enzyme activity was higher in gills of summer mortality-resistant *C. gigas* families than in susceptible ones (Samain et al. 2007). In the same families, reactive oxygen species production by hemocytes was higher in the susceptible families (Lambert et al. 2007; Samain et al. 2007). Antioxidant enzyme activities are an important safeguard against self-induced tissue damage during infection or exposure to stress. Taken together with the findings of Lambert et al. (2007) and Samain et al. (2007), our transcriptional data raises the possibility that these systems are inadequate in oyster families that are sensitive to stress.

Transcription and opportunistic infection in high- and low-surviving families. Adhesive Proteins. The higher transcription of *galectin* and *peroxinectin* in gills of low-surviving families before and after heat shock, and of *galectin* in whole bodies before heat shock, suggests that those families may have been more susceptible to opportunistic infection overall. Oysters are in constant contact with bacteria in the marine environment, presenting a continuous threat of opportunistic infection. In bivalves, adhesive proteins bind to bacteria and encourage phagocytosis by macrophages (Tasumi and Vasta 2007), but *Vibrios* reduce the ability of hemocytes to adhere to particles (Choquet et al. 2006). I speculate that exaggerated transcription of adhesive proteins was a compensatory response to either enhanced sensitivity to infection or the suppressive effects of bacteria on hemocyte adhesion. I did not measure bacterial loads during my research, but it is plausible that opportunistic infection contributed to mortality in low-surviving families. Li et al. (2007) found that opportunistic infection after heat shock lead to mortality, and Samain et al. (2007) found that oyster families that were more susceptible to summer mortality had higher rates of infection by *Vibrio aestuarianus* after heat shock.

Interestingly, isolated unchallenged hemocytes from summer mortality-susceptible *C. gigas* families had higher hemocyte adhesion prior to the seasonal period of summer mortality (Samain et al. 2007) than those from resistant families, but suppression of hemocyte adhesion by *Vibrio* sp S322 was greater in the same susceptible oyster families reared at the riviére deAuray of France (Lambert et al. 2007). This suggests that potentially diminished adhesive capacity may be related to

how certain pathogens interact with hemocytes as opposed to some inherent defect in the hemocytes, such as mutations within sugar recognition sites. However, it should be noted that the differences between family types occurred before exposure to heat shock, which implies that the significance of exaggerated transcription is rooted in constituative genetic differences rather than an induced response to stress.

Protease Inhibitors. Concentrations of mRNAs encoding the protease inhibitor *cystatin B* were lower both in gill after heat shock and in whole bodies of low-surviving families before and after heat shock. Cystatins inhibit proteolytic lysosomal cathepsins (Ulrich 1995; Rinne et al. 2002), and serve a defensive role in invertebrates by deactivating cathepsins secreted by parasites. For example, the blood fluke *Schistosoma mansoni* obtains nutrients from host snail *Biomphalaria* sp. cells by digesting them using cathepsin L (Brady et al. 1999; El-Ansary 2003). Cystatins have not been explicitly addressed within the context of cellular immunity in *C. gigas*, but it is possible that insufficient levels of cystatin B could result in enhanced sensitivity of low-surviving families to opportunistic infection. As with blood flukes, oysters pathogens such as *Perkensus marinus* secrete proteases to defeat host defenses and possibly to derive nutrients (Romestead et al. 2002; Xue et al. 2006; Montagnani et al. 2007), and higher levels of protease inhibition in isolated plasma of *C. virginica* families have been linked with genotype-specific resistance to infection by *P. marinus* (Oliver et al. 2000). Cystatins have been implicated in genotype-specific differences in resistance to infection in other invertebrates; for example, snails *B. glabrata* that

resisted infection by blood flukes had higher protein levels of a type-2 cystatin after infection than did those that were susceptible (Vergote et al. 2004).

Transcription and energy metabolism in high- and low-surviving families. Transcript concentrations in gills of adults for genes that differed significantly at the family level were usually higher in low-surviving families before and after heat shock. Gills of marine invertebrates are highly active in regards to protein turnover (Lydon and Houlihan 1998), and additional turnover imposed by heat shock could have contributed to mortality in low-surviving families through metabolic exhaustion.

Energy requirements for maintenance metabolism must be met before energy can be allocated towards growth, reproduction, or stress responses. Maintenance metabolism accounts for a substantial proportion of energy expenditure in marine bivalves, and includes the energy expended during routine protein turnover (the net effect of synthesis and degradation) and RNA turnover (Hawkins 1991; Hawkins and Day 1996; Bayne and Hawkins 1997). In general, it appears that lower genotype-specific rates of protein turnover result in greater tolerance of stress through energy savings. It has been shown that the activities of cathepisns account for over 70% of genotype-specific variation in maintenance metabolism of mussels *Mytilus edulis* (Hawkins and Day 1996). Mussels *M. edulis* that had the greatest tolerance of lethal stress exposure had the lowest rates of whole-body protein turnover (Hawkins et al. 1987; Hawkins et al. 1989), and populations that were susceptible to summer mortality had greater tissue energy depletion during summer mortality than resistant populations

(Tremblay et al. 1998). It is possible that these relationships are also true for *C. gigas*, but to date studies of whole-body protein turnover and maintenance metabolism in *Crassostrea sp*. have focused on growth rates (Bayne 1999; Bayne 2004).

Higher *cystatin B* transcription in gill of high-surviving families and in whole bodies of high-surviving families before and after heat shock could reduce protein turnover through inhibition of lysosomal cathepsins and it is possible that genotypic differences in cystatin expression could underlie differences in overall rates of protein turnover, which would influence the amount of energy that was available for mounting stress responses. Importantly, the difference in *cystaitn B* transcription at the whole body level was present before heat shock, implying that possible regulation of protein turnover or disease resistance are due to constituative genetic differences among families.

Survival of heat shock, transcription in juveniles, and their relationship to field performance of adults.

Survival of heat shock is not predictive of field performance. Simple survival of heat shock was not useful in predicting field performance in the oysters used in my research. A number of environmental parameters such as salinity, food quality, and temperature may determine the performance of oyster families in the Pacific Northwest, and it is possible that heat shock did not adequately mimic the stress response that each of these factors would induce in combination. Furthermore, summer mortality results in part from an interaction of spawning-related glycogen

metabolism and reproductive investment (Perdue et al. 1981; Samain et al. 2007), and this is an important factor that any assays using juveniles would ignore.

Although we found that survival of immersion in heated water was not predictive of mortality in the field, Boudry et al. (2008) reported significant correlations between the survival of Pacific oyster *C. gigas* spat after heat shock (2 h at 40°C in a drying oven) and survival at two grow-out sites in France. Across several experiments, families that resisted summer mortality had low mortality both after heat shock and in the field, and families that were susceptible had high mortality after heat shock and in the field. It should be noted that the experiments conducted in France were fundamentally different from ours because they produced families by selecting parents based upon high or low survival during summer mortality (Degremont et al. 2007), whereas our oyster families were developed based upon high growth and survival of parental families in the Pacific Northwest (Langdon et al. 2003). It is possible that, in contrast to an immersion-based heat-shock assay, a desiccation-based heat shock assay could be useful for predicting field performance of families reared in the Pacific Northwest. This approach would circumvent the logistical problem of maintaining a consistent seawater temperature in assays that require large culture volumes.

Relationships between gene expression in juveniles and field performance. As has been demonstrated for survival in mussels, (Hawkins et al. 1987; Hawkins et al. 1989), lower genotype-specific rates of protein synthesis and protein degradation are thought

to facilitate faster growth in marine bivalves (Bayne 1999; Bayne 2004). Assuming that gene transcription is a reliable proxy for protein production, the negative correlations of final adult weight, survival, and yield with whole-body gene transcription described by this dissertation could be explained by differences in protein turnover rates. This explanation is compelling because many negative correlations were observed for transcription before exposure to heat shock. However, not all data support a relationship between gene transcription and protein turnover that ultimately influenced field performance.

If the families studied in Chapter 3 did truly differ in rates of protein turnover, it is surprising that there was no correlation between field performance and *cystatin B* transcription. One explanation is that protein turnover is controlled at other levels or by other protease inhibitors. Cystatins are represented by a large family of proteins, and it appears that each cystatin may inhibit multiple cathepsins (Rinne et al. 2002). To date only two cystatins, B and S are represented by expressed sequence tags in *Crassostrea sp*. In order to further explore the relationship between cystatins and field performance, more work is needed to characterize this gene family in oysters, to determine whether transcription of other cystatins are related to field performance, and whether protein levels reflect protein turnover.

The positive correlations between field performance and concentrations of mRNA encoding *heat shock protein 68, galectin,* and *prostaglandin E receptor 4* seem to refute the proposed relationship between protein turnover in juveniles and field performance of adults in which high rates of transcription reflect high rates of protein

breakdown and replacement. *Heat shock protein 68* transcription before heat shock was positively correlated with yield at the subtidal site, and at this site, yield was driven by survival. It is possible that higher levels of pre-existing heat shock protein transcripts conferred an advantage for surviving summer mortality. Galectins bind food particles that could account for the positive relationship of growth and transcription. The EST encoding *prostaglandin E receptor 4* is short and requires further characterization to determine its function, and the implications of its production for growth and survival.

Future Studies and Applications.

Candidate Genes. Based on the observed differences in transcription between high- and low-surviving families and their presumed functions, the candidate genes *cystatin B, galectin*, and *glutathione S-transferase* should be useful for marker-aided selection programs that seek to improve survival of farmed *C. gigas* stocks. The mRNA and gene encoding *cystatin B* remain to be fully characterized, but full-length mRNA sequences are already available for *galectin* (Yamaura et al. 2008) and *glutathione S-transferase omega* (Boutet et al. 2004). This will facilitate immediate development of single nucleotide polymorphism markers for these genes and determination of how their expression varies within breeding populations.

Galectin and glutathione S-transferase have been thoroughly studed in *C. gigas*, but the transcriptional regulation and physiological functions of cystatin B requires further study. These studies will be important to not only clarify how cystatin

B confers enhanced survival to oyster families, but to also clarify further the links between protein turnover and survival or field performance. Initial studies should characterize the full-length transcript; this information will facilitate its use in transcription assays. The transcript product of the cystatin B gene could then be used as a probe to screen genomic libraries, leading to the full characterization of its gene sequence and upstream regulatory regions. Knowledge of the full gene sequence would also facilitate the identification of single nucleotide polymorphism markers in the gene and its regulatory regions in oyster breeding populations.

Functional studies should be carried out to identify the tissues in which the cystatin B protein product is localized, and to identify potential cathepsin binding partners. To characterize tissue localization, anti-cystatin B antibodies could be produced and used to visualize the expressed proteins using histology, similar to Yamaura et al. (2008) for lectins in *C. gigas*. These data could be used to test the hypothesis that cystatin B deters protein turnover or opportunistic infection. To identify lysosomal cathepsin binding partners, an RNA interference approach could be used to reduce expression of the cystatin B gene in embryos. In this experiment, one could measure the enzymatic activities of cathepsins in both control and treated embryos, and based upon significant increases in cathepsin activity it could be inferred that the absence of an inhibitory binding partner lead to increased proteolytic activity. Hawkins and Day (1996), Gagnaire et al. (2003) and Donald et al. (2004) each described methods for measuring activities of cathepsins in various tissues of marine bivalves.

Predicting field performance. If taken at face value, the results obtained in this work suggest that transcription levels of several genes can be used to predict the field performance of farmed *C. gigas* families in the Pacific Northwest region. This contention partly relies on an as-of-yet untested assumption that genotype-specific differences in gene transcription correspond to differences in rates of protein degradation and replacement. Using the methodology described by Hawkins and Day (1996), families of a cohort could be characterized by their rates of maintenance energy expenditure and protein turnover under normal (non-stress) conditions, and these measurements could be coupled with whole-body gene transcription data. When significant correlations were detected between protein turnover and transcript concentrations, one could infer that transcription was a reliable estimator of protein turnover. It is generally accepted that faster growth results in part from lower rates of protein turnover (Bayne 2004); therefore, stocks could be selectively culled prior to plant-out to reduce costs and increase efficiency of selective breeding.

The hypothesis that transcription reflects protein turnover resulted from the synthesis of all data included in this dissertation, but in fact there are several other genes that could have been useful for predicting field performance. Ribosomal protein genes *L32* and *S3* are thought to contribute to non-additive differences in growth rate of *C. gigas* (Hedgecock et al. 2007), and could be useful descriptors of protein synthesis activities. Whole-body transcription of lysosomal cathepsins among families could complement the ribosomal protein transcription data, and presently, there are *Crassostrea sp.* EST sequences in Genbank for cathepsins B, L, Y, 1, and 8.

Two genes whose products have been functionally associated with protein turnover in *C. gigas*, aminopeptidase N and alanine aminotransferase could also be informative measures of protein turnover (Donald et al. 2001). Transcription of cystatin B was not predicitive of field performance, but other cystatins might be responsible for regulating protein turnover and warrant further study. Presently, an EST encoding cystatin S is present for *C. gigas* in Genbank.

Concluding Remarks. I identified candidate genes for use in marker-assisted selection programs designed to improve survival, growth and yields of oyster stocks. I also developed gene expression assays to predict field performance of adults. These results will be immediately useful for breeding programs to increase the efficiency of selection and reduce costs. The gene transcription data allowed me to hypothesize that a combination of cellular damage, energy metabolism, and opportunistic infection may drive mortality of low-surviving families after heat shock. A surprising and important finding was that differences in transcript concentrations between high- and low-surviving families, and correlations between gene transcription in juveniles and field performance of adults, occurred before stress. This implies that performance may be rooted in constiutative genetic expression rather than in induced responses to stress, and in turn has important implications for how future studies of marine bivalves could be conducted.

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Table 1. Summary of ESTs in which transcript concentrations differed between high- (H) and low-surviving (L) families before and after heat shock (HS).

Gene	Gill (Chapter 2)	Whole Body
Collagen	L > H at 1 h after HS	No differences
Cystatin B	L < H at 24 h after HS	L < H at all times
BQ426658	L > H at 1 h after HS	No differences
BQ426884	L > H at 24 h after HS	No differences
Galectin	L > H overall	L > H before HS
Glutathione S-transferase Ω	--	L < H at 24 h after HS
Heat shock protein 27	L > H at 6 h after HS	No differences
Peroxinectin	L > H before, 1 h, 6 h after HS L < H at 24 h after HS	--
S-Crystallin	L > H at 6 h after HS	--

Table 2. Summary of correlations between transcription of various ESTs and field performance of adults at an intertidal or subtidal site. Total RNA was collected before and at 6 h and 24 h after heat shock (39°C, 1 h).

	SURVIVAL	
Gene	Subtidal	Intertidal
Catalase	--	0 h (-)
Heat shock protein 27	--	6 h (-)
Prostaglandin E receptor 4	24 h (-)	--
Superoxide dismutase	24 h (-)	--

	WEIGHT	
Gene	Subtidal	Intertidal
BQ426658	0 h (-)	0 h (-)
Galectin	0 h (+)	24 h (+)
Glutathione peroxidase	6 h (-)	--
Heat shock protein 27	--	0 h (-)
Prostaglandin E receptor 4	24 h (+)	0 h (+), 24 h (+)

	YIELD	
Gene	Subtidal	Intertidal
BQ426658	--	0 h (-), 6 h (-)
BQ426884	6 h (-)	24 h (-)
Galectin	--	24 h (+)
Glutathione peroxidase	--	6 h (-)
Heat shock protein 68	0 h (+)	--
Tissue inhibitor of metalloprotease	6 h (-)	6 h (-)

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APPENDICES

APPENDIX 1

SUPPLEMENTARY MATERIAL FOR CHAPTER 2

FIGURE LEGENDS

Figure 1. Raw output for 95% confidence intervals for ESTs in which transcription level differed among sampling times in gill of heat-shocked (40°C, 1 h) oysters before and at 1, 3, 6, and 24 h after heat shock. Data were clustered using the program SSClust. The *vsn*-transformed data for each sampling time are presented relative to the average for all sampling times for a given EST.

Figure 2. Average microarray (*vsn*-transformed) signal (±SEM) for heat shock proteins that were discussed but not studied by real-time quantitative PCR in Chapter 2. Gill samples were collected before and at 1, 3, 6, and 24 h after heat shock (40°C, 1 h), and RNAs from those samples were hybridized to cDNA microarrays. In each plot, families with low survival (< 29%) after heat shock are represented by hatched bars, and those with high survival (>64%) are represented by white bars. Each bar represents 9 individuals from each of 2 individual families of the specified survival type.

Figure 3. Average microarray (*vsn*-transformed) signal (±SEM) for various ESTs that were discussed but not studied by real-time quantitative PCR in Chapter 2. Gill samples were collected before and at 1, 3, 6, and 24 h after heat shock (40°C, 1 h), and RNAs from those samples were hybridized to cDNA microarrays. In each plot, families with low survival (< 29%) after heat shock are represented by hatched bars, and those with high survival (>64%) are represented by white bars. Each bar represents 9 individuals from each of 2 individual families of the specified survival type.

Figure 4. Average microarray (*vsn*-transformed) signal (±SEM) for various ESTs that were discussed but not studied by real-time quantitative PCR in Chapter 2. Gill samples were collected before and at 1, 3, 6, and 24 h after heat shock (40°C, 1 h), and RNAs from those samples were hybridized to cDNA microarrays. In each plot, families with low survival (< 29%) after heat shock are represented by hatched bars, and those with high survival (>64%) are represented by white bars. Each bar represents 9 individuals from each of 2 individual families of the specified survival type.

Figure 1.

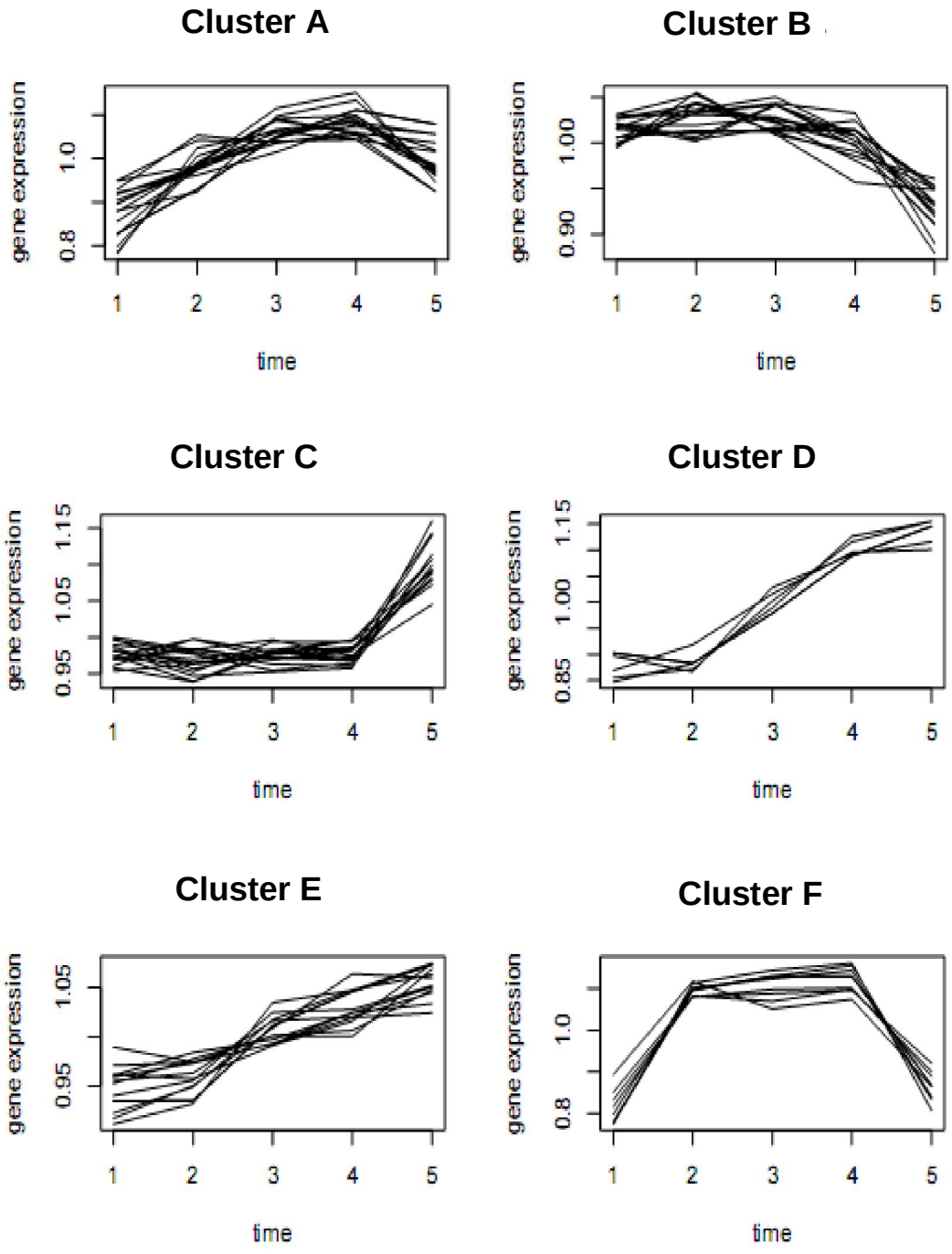
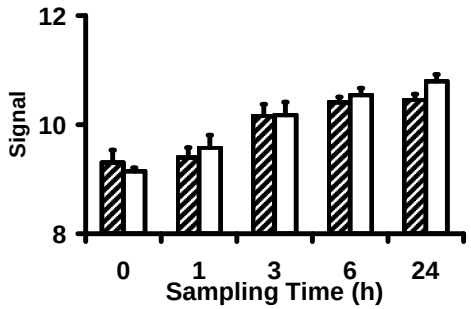

Cluster A
gene expression
0.8
1.0
1
2
3
4
5
time
Cluster B
gene expression
0.90
1.00
1
2
3
4
5
time
Cluster C
gene expression
0.95
1.05
1.15
1
2
3
4
5
time
Cluster D
gene expression
0.85
1.00
1.15
1
2
3
4
5
time
Cluster E
gene expression
0.95
1.05
1
2
3
4
5
time
Cluster F
gene expression
0.8
1.0
1
2
3
4
5
time

Figure 2.

BQ426928 (HSP70)



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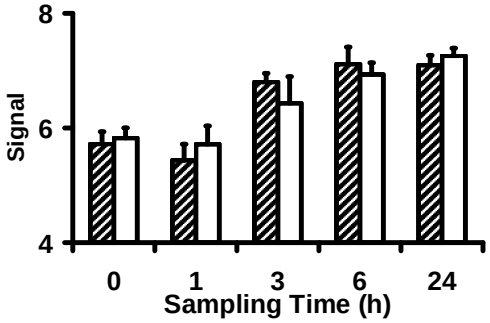

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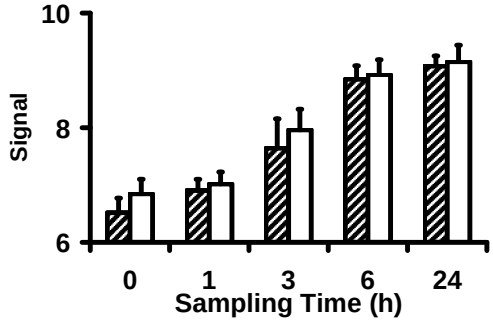

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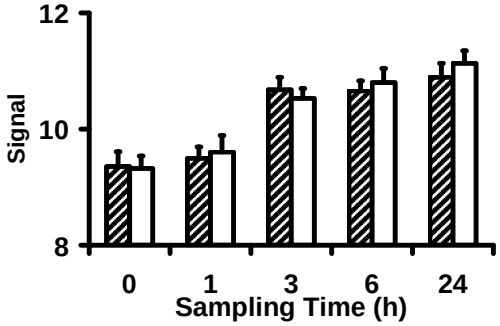

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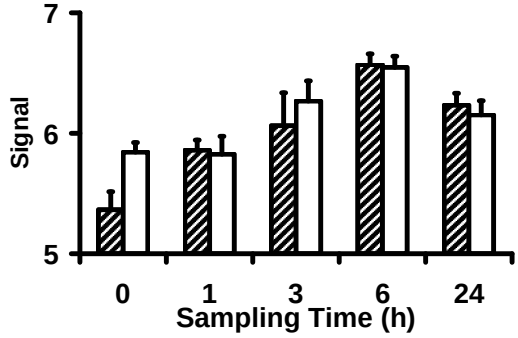


AM237785 (HSP27)

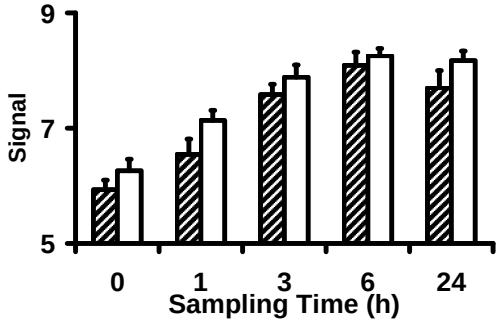
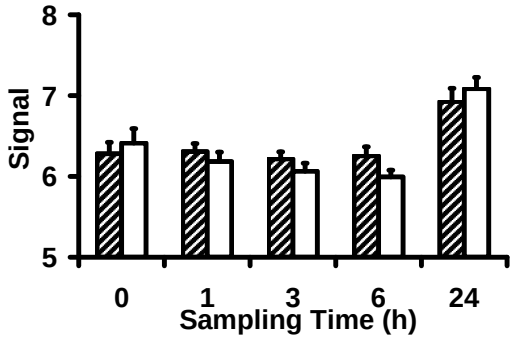


Figure 3.

CB617402 (Ubiquitin activating enzyme 1)



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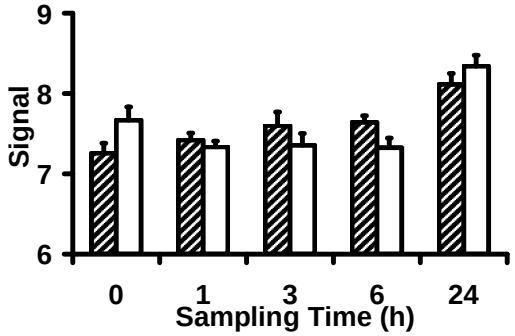


BQ426641 (Patatin phosholipase)

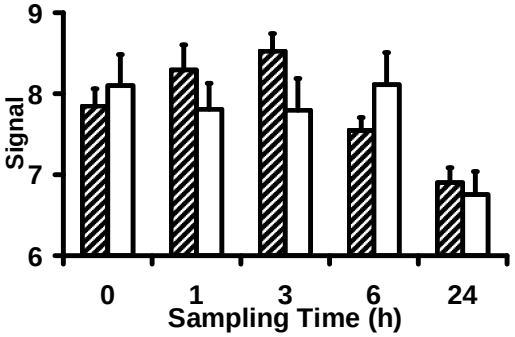
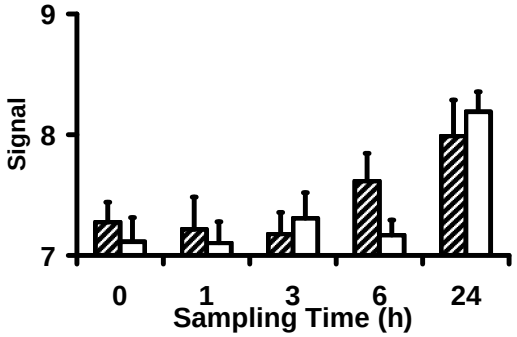
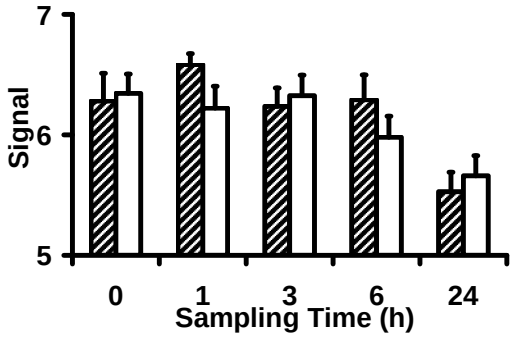


Figure 4.

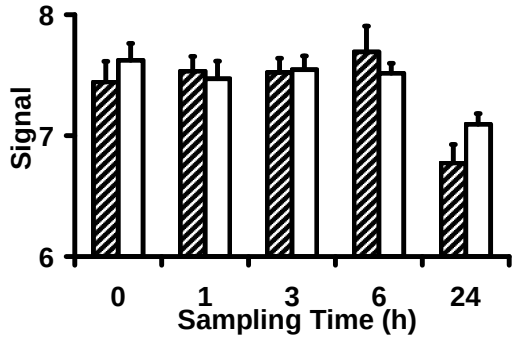
BQ426935 (SREBP1)



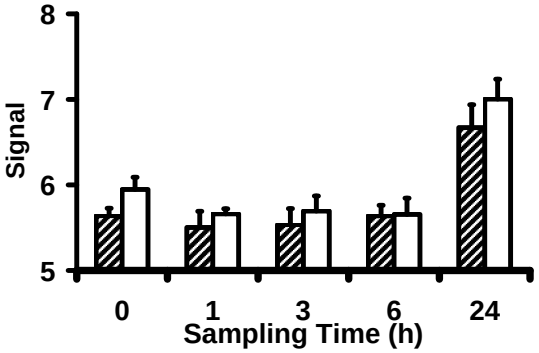
CK172312 (Fatty acid binding protein)



CX069146 (Glutathione peroxidase)



CK172319 (Temptin)



APPENDIX 2

SUPPLEMENTARY MATERIAL FOR CHAPTER 3

Table 1. Correlations between average survival (%) or average weight (g) of adults at intertidal or subtidal sites and log2-transformed mRNA concentration relative to elongation factor 1-alpha in juveniles measured before (0) and at 6 and 24 h after heat shock (39C, 1 h) for *BQ426658* (*BQ658*), *BQ426884* (*bq884*), *catalase* (*cat*), *collagen* (*col*), *cystatin B* (*cys*), *galectin* (*gal*), *glutathione peroxidase* (*gpox*), *glutathione S-transferase* omega (*gst*), and *heat shock protein 27* (*hsp27*). Significant correlations ($P < 0.05$) are denoted by bold font.

	Intertidal survival (%)		Intertidal weight (g)		Subtidal survival (%)		Subtidal weight (g)	
	r^2	P	r^2	P	r^2	P	r^2	P
BQ658-0	<0.0001	0.9867	**- 0.5460**	**0.0362**	- 0.0001	0.9817	**- 0.5757**	**0.0291**
BQ658-6	- 0.2744	0.1827	- 0.0693	0.5288	- 0.0970	0.4527	- 0.3254	0.1398
BQ658-24	0.0052	0.8651	- 0.1636	0.3202	0.0551	0.5759	- 0.3624	0.1143
BQ884-0	0.0013	0.9325	0.0027	0.9024	- 0.0535	0.5814	0.0287	0.6885
BQ884-6	- 0.1557	0.3333	<0.0001	0.9972	- 0.3971	0.0940	- 0.1803	0.2943
BQ884-24	- 0.2229	0.2375	- 0.0354	0.6557	- 0.1127	0.4162	- 0.3535	0.1201
cat-0	**- 0.5943**	**0.0251**	- 0.0011	0.9369	- 0.2808	0.1767	- 0.0663	0.5380
cat-6	- 0.0383	0.6424	0.0121	0.7955	- 0.0253	0.7068	0.1129	0.4157
cat-24	- 0.2965	0.1629	0.0020	0.9171	- 0.1199	0.4008	- 0.0024	0.9076
col-0	0.0019	0.9194	0.1149	0.4114	0.0754	0.5105	0.2306	0.2284
col-6	0.0060	0.855	0.0424	0.6247	0.1394	0.3624	0.2932	0.1657
col-24	- 0.2349	0.2236	0.0653	0.5412	- 0.0078	0.8357	- 0.0424	0.6246
cys-0	0.0043	0.8776	0.1887	0.2821	0.0636	0.5469	0.2689	0.1879
cys-6	- 0.1504	0.3424	0.1194	0.4019	0.0007	0.9498	0.1144	0.4126
cys-24	- 0.2230	0.2375	0.3062	0.1548	- 0.0025	0.906	0.0182	0.7504
gal-0	- 0.0010	0.9419	0.4537	0.0670	- 0.0183	0.7493	**0.5128**	**0.0457**
gal-6	- 0.2033	0.2622	0.0101	0.8124	- 0.2329	0.2258	- 0.0009	0.9434
gal-24	0.0539	0.5799	**0.5141**	**0.0453**	0.0182	0.7503	0.4746	0.0588
gpox-0	- 0.1128	0.4161	- 0.0063	0.8518	0.0361	0.6520	- 0.0099	0.8151
gpox-6	- 0.0188	0.7462	- 0.4845	0.0552	- 0.0061	0.8541	**- 0.6325**	**0.0183**
gpox-24	- 0.1461	0.3501	0.0245	0.7113	- 0.0096	0.8172	0.0575	0.5673
gst-0	0.0517	0.5882	- 0.1141	0.4132	- 0.0092	0.8209	- 0.0132	0.7862
gst-6	0.0848	0.4841	0.0244	0.7119	0.1007	0.4437	0.0825	0.4904
gst-24	- 0.0095	0.8188	- 0.0105	0.8089	- 0.1225	0.3953	- 0.0190	0.745
hsp27-0	0.3041	0.1565	**- 0.6123**	**0.0217**	0.0727	0.5183	- 0.2972	0.1623
hsp27-6	**- 0.5552**	**0.0339**	- 0.0058	0.8582	- 0.1204	0.3998	- 0.0249	0.7089
hsp27-24	0.0435	0.6200	- 0.0363	0.6514	0.0592	0.5614	0.0145	0.776

Table 1, continued. Abbreviations refer to: *heat shock protein 68* (*hsp68*), *lysozyme* (*lys*), *prostaglandin E receptor* (*per*), *superoxide dismutase* (*sod*), *and tissue inhibitor of metalloprotease* (*TiMP*).

	Intertidal survival (%)		Intertidal weight (g)		Subtidal survival (%)		Subtidal weight (g)	
	r^2	*P*	r^2	*P*	r^2	*P*	r^2	*P*
hsp68-0	0.0009	0.9423	0.2860	0.1720	0.2239	0.2363	0.4930	0.0522
hsp68-6	- 0.1403	0.3607	0.2502	0.2068	- 0.1719	0.3072	0.0818	0.4923
hsp68-24	- 0.0237	0.7160	0.0992	0.4474	0.0028	0.9005	0.1400	0.3611
lys-0	0.1096	0.4231	- 0.0905	0.4691	0.0590	0.5622	0.0018	0.9197
lys-6	- 0.0274	0.6955	- 0.0809	0.4947	- 0.0745	0.513	- 0.1817	0.2923
lys-24	- 0.0098	0.8156	0.1401	0.361	0.0399	0.6352	0.0424	0.6248
per-0	- 0.1886	0.2823	**0.5152**	**0.0450**	- 0.1855	0.2867	0.4640	0.0629
per-6	- 0.3263	0.1392	0.0119	0.7974	**- 0.6179**	**0.0207**	- 0.0242	0.7132
per-24	- 0.0252	0.7075	**0.7625**	**0.0046**	- 0.0132	0.7863	**0.8191**	**0.0020**
sod-0	- 0.1395	0.3622	0.1227	0.3949	- 0.1562	0.3325	0.1896	0.2809
sod-6	- 0.0367	0.6495	0.2892	0.1692	- 0.0074	0.8394	0.2887	0.1696
sod-24	- 0.3618	0.1147	0.2966	0.1628	**- 0.6192**	**0.0205**	0.1591	0.3277
TiMP-0	-0.2996	0.1603	0.3738	0.1073	- 0.0981	0.4501	0.3285	0.1375
TiMP-6	-0.3287	0.1373	- 0.1819	0.2919	- 0.4573	0.0656	- 0.4514	0.0680
TiMP-24	-0.2360	0.2223	0.0685	0.5311	- 0.1871	0.2844	- 0.0659	0.5394

Table 2. Correlations between yield at intertidal or subtidal sites and transcript concentration relative to elongation factor 1-alpha in juveniles measured before (0) and at 6 and 24 h after heat shock (39C, 1 h). Significant correlations ($P < 0.05$) are denoted by bold font.

EST	Intertidal		Subtidal	
	R^2	P	R^2	P
BQ426658-0	- 0.5135	0.0455	- 0.1713	0.3080
BQ426658-6	**- 0.6847**	**0.0112**	- 0.2571	0.1996
BQ426658-24	- 0.1987	0.2683	- 0.0229	0.7207
BQ426884-0	0.0311	0.6761	0.0074	0.8392
BQ426884-6	- 0.1424	0.3567	**- 0.5080**	**0.0472**
BQ426884-24	**- 0.5229**	**0.0426**	- 0.2886	0.1697
Catalase-1	**- 0.5308**	**0.0404**	- 0.2314	0.2276
Catalase-6	0.0031	0.8965	0.0070	0.8434
Catalase-24	- 0.1639	0.3198	- 0.0600	0.5587
Collagen-0	0.0565	0.5294	0.2481	0.2090
Collagen-6	0.0461	0.6094	0.3844	0.1010
Collagen-24	- 0.1598	0.3265	- 0.0215	0.7289
Cystatin B-0	0.1314	0.3774	0.2351	0.2226
Cystatin B-6	- 0.0191	0.7441	0.0712	0.5230
Cystatin B-24	- 0.0134	0.7853	0.0057	0.8670
Galectin-0	0.4472	0.0698	0.0767	0.5067
Galectin-6	- 0.0518	0.5879	- 0.1398	0.3615
Galectin-24	**0.7469**	**0.0056**	0.1885	0.2825
Glutatione perodisase-0	- 0.2265	0.2333	0.0279	0.6925
Glutatione perodisase-6	**- 0.6511**	**0.0155**	- 0.2118	0.2512
Glutatione perodisase-24	- 0.0649	0.5426	0.0120	0.7960
Glutathione S-transferase-0	0.003	0.8921	0.027	0.6943
Glutathione S-transferase-6	0.2047	0.2603	0.1366	0.3674
Glutathione S-transferase-24	- 0.0069	0.8445	0.1400	0.3611

Table 2, continued. *Tissue inhibitor of metalloprotease* is abbreviated as "*TiMP*".

	Intertidal		Subtidal	
EST	r^2	*P*	r^2	*P*
Heat shock protein 27-0	- 0.0247	0.7102	- 0.0178	0.7529
Heat shock protein 27-6	- 0.4631	0.0633	- 0.0738	0.5150
Heat shock protein 27-24	0.0001	0.9785	0.0654	0.5411
Heat shock protein 68-0	0.1975	0.2700	**0.5974**	**0.0245**
Heat shock protein 68-6	0.0121	0.7952	- 0.0200	0.7383
Heat shock protein 68-24	0.0339	0.6625	0.0678	0.5335
Lysozyme-0	0.0046	0.8729	- 0.0469	0.6065
Lysozyme-6	- 0.2004	0.266	- 0.1793	0.2958
Lysozyme-24	0.0081	0.8315	0.0832	0.4883
Prostaglandin E receptor-0	0.1090	0.4245	0.0036	0.8875
Prostaglandin E receptr-6	0.1056	0.4323	- 0.4443	0.0710
Prostaglandin E receptor-24	0.4891	0.0535	0.1624	0.3222
Superoxide dismutase-0	0.0108	0.8066	- 0.0036	0.8874
Superoxide dismutase-6	- 0.1273	0.3857	0.0481	0.6017
Superoxide dismutase-24	0.0042	0.8787	- 0.1410	0.3593
TiMP-0	0.0087	0.8266	0.0133	0.7860
TiMP-6	**- 0.7881**	**0.0032**	**- 0.7333**	**0.0066**
TiMP-24	- 0.0278	0.6933	- 0.0211	0.7312

APPENDIX 3

LIST OF PRIMERS

Table 1. Primers used in this dissertation: Accession number, identity, annealing temperature, and comments for each primer tested.

AB122062 (Heat shock protein 68)		T_A	Comments
P68	F: TGGACGTCACCCCTTATTCTTT R: TGGAATCTTGGTATTGCAGTTTATG	58	(C18) Used in Chapter 3
P68 853	F: GCCAGTCTTGATCCTTTGCACT R: AAGCCTTTGTGTACGATTTGCAC	57	(C18)1 product, matches 'P68'
P68 938	F: TCTGGAGTGTGTTACTGGATGTCA R: CATGAGGCGTAGAGTTTAAAAGGTC	57	not tested
P68 34	F: TTCATGGCTAGACCATAACAGTGG R: TTAAGTTCAAGTTCAAAAAGTGCAGC	56	(C18) 1 product, matches 'P68' very low efficiency when run at 60C
AB178775 (Lysozyme)		T_A	Comments
LYZ 198	F: TCGGCATGTCTGCGTTGTAT R: AATGGGTCTGCATCCCGAT	58	(C18) 1 product, matches '412'
LYZ 412	F: GCAGTGGTTGTCCAGCAAACT R: CACCATTGTGGATTCGTGCA	58	(C18) Used in Chapter 3
LYS 233	F: GGGATGCAGACCCATTGGT R: CCGCACGAGTATGAGTACACGT	60	(C18)1 product, matches '412' Efficiency is poor relative to '198' and '412'
LYS 476	F: TCGACATCCATCCACTCTCAGA R: CAACCTTGCTGATGCACCTTC	59	Very strange amplification – appears almost linear (C18)
AF321279 (Tissue inhibitor of metalloprotease)		T_A	Comments
TiMP 161	F: CCCAGGAGACGATGAAGCAG R: CGGTCAACACCACGAATAGAAG	59	Family 10 – two peaks (C18); Same profile as in '161' (C18)
TiMP 640	F: CATTGCCGTGGTGAGAACTG R: CTGGTCCGGATTCAATGACTG	61	(C18) Used in Chapter 3
TiMP Org	F:TAGCGTAGCAGTCGTTCGTG R: ATTGCCGTGGTGAGAACTG	60	Original primers published by Montagnagi Multiple family-specific peaks
AJ305315	(Heat shock cognate 72)	T_A	Comments
C70_A	F: TGCTCCTGTTGGATGTCACC R: CTCCGGCTGTTTCAATACCC	61	(C16) Profile unique among primer pairs 2 products in all families
C70_B	F: CTTCAGCTGTCCAGGCCG R: GTACCTCCCCGGATTTGTCTC	62	(C16) Profile similar to 'D' 2 products in families 6, 10 only
C70_C	F: GATGTCACCCCCCTGTCCT R: GGTCATCACTCCTCCGGCT	61	Not tested
C70_D	F: AGGGCATTTCATGTCCGAAG R: TGCTCAGACATCCAAGGAAGG	58	(C16) Used in Chapter 2
C70_E	F: CTCCATTCGCAGTTTCCACC R: TTAAGCCATCTTCGGACATGAA	58	(C16) Profile similar to 'D'

Table 1, continued.

ID	AJ496219 (Superoxide dismutase)	T_A	Comments
SOD 294	F: GAGGATCACGAGAGGCACGT R: TTCGCCAGCGGTGACATTA	62	(C18) Used in Chapter 3
SOD 264	F: AACCCCTTCAACAAAGAGCATG R: CACGTGCCTCTCGTGATCCT	61	(C18) Same profile as '294'
SOD 469	F: TCAGTAAGACGACCGGAAACG R: CGATCACTCCACAAGCCAATC	61	(C18) Family-specific multiple peaks Poor efficiency
SOD 192	F: GGACAGCATGGATTCCACG R: ACAGCCATTAGTATTGTCGCCG	59	not tested
SOD 392	F: CGGCCCTCAATCCATCATT R: TCAACATCGCCATGAATAACCA	58	(C18) Family-specific multiple peaks Poor efficiency
ID	AJ557141 (Glutathione S-transferase Omega)	T_A	Comments
GST-0	F: TATTTGGACCAGGTGTATCCCGA R: AGAATACGTTCAAACCATGGCCA	60	(C18) Used in Chapter 3 (C18) Original primers by Boutet et al. 2004
GST 497	F: CCTACTTTGGAGGGAACGCA R: ATGGCCACAGCAGGAAGTCT	60	(C18) Same profile as "GST-0" (C18) 1 product
GST 672	F: GGACCCTCAGCAGTTTTTGG R: GGTGCGCCAGCTTTTGTACT	57	(C18) Same profile as "GST-0" (C18) 1 product
GST 28	F: CCGACCCAACAATCATTTGC R: TGCCTCCAATTCCGGACA	60	(C18) Poor efficiency
ID	AJ565456 (S-Crystallin)	T_A	Comments
GSTS 176	F: ACCACAAAAGCAGTTGCCGT R: TGAGGGATAAGGCGACCATC	59	(C16) Used in Chapter 2
GSTS 520	F: ACATCACTGACCAGGTCGGC R: TTGGTGGGAAAGACGGAAGT	60	(C16) Sample-specific multiple products (C16) Same profile as in '176'
GSTS 424	F: TTTTCAGCCTGTTGGAGGACA R: AACCATCCCCGCCATTATTT	56	Not tested
GSTS 78	F: GAGGTCATCCGATTGGCCTT R: TTTATCCTCAAATGACTGTCCAGC	59	Not tested
GSTS 220	F: TCCCTCAGTCTGGAGCAATCA R: ACAACCCAAATTCCCTGGC	58	Not tested
GSTS 34	F: TGCCGGCATACAAGTTGATG R: TGACCTCGGCTCTCCCCT	59	(C16) Sample-specific multiple products (C16) Same profile as in '176'
ID	AJ565627 (Nucleoredoxin)	T_A	Comments
redox 422	F: CAAACTCCCGGACAAGTGGT R: ACACATTGACGACCGGCAC	60	(C16) Same profile as '251' Various sample-specific multiple peaks

Table 1, continued.

redox 251	F: GTACTTCAATGAGATGCCGTGG R: AGGTCACGTTCACTGAAGGGA	59	(C16) Used in Chapter 2 1 product
redox 1	F: TAAAGTCGAAGGCAAGGATGGA R: CTTGTACCAAATCGGAAACCT	58	(C16) Family-specific multiple peaks Same profile as '251'
redox 108	F: TTGTATTTCTCCGCACATTGGT R: TGGTGTATAGCTGGGCCAAGA	60	not tested
ID	AJ565582 (Delta 9 Desaturase)	T_A	Comments
CoA 95	F: CCTTCGGATCGTCTGGAGAA R: CCGAAGTGTAAAAGAGCCATCAG	59	(C16) Used in Chapter 2
CoA 10	F: CAAGAAATGCAATGGAGGCA R: CCTCTGTTGTTCCTCTAGATGGCT	58	(C16) Same profile as in '95' (C16) 1 product
CoA	F: TCGGAGCGATTTATGCTCTGA R: ACCATGCGCTTACACCATAGC	58	(C16) Same profile as in '95' (C16) 1 product
ID	AM237676 (Peroxinectin)	T_A	Comments
pero 164	F: GCCAAACCTCGCCTACCTTC R GTGGAGTTGACGCGTGACATA	58	(C16) Used in Chapter 2
pero 429	F: CCTCCGTATAATGCATGGCG R AGAGGTGGCTACCGTAAGTCCA	60	(C16) Same profile as in '164' 1 product
pero 328	F: AACTAGAAGGTGCGGTGCGA R: CTTTACCATGCGCGTTTTCA	58	(C16) Same profile as in '164' 1 product
ID	AM237729 (Oxioreductase)	T_A	Comments
Oxio 39	F: AACAGCTGAAGGAGATCGTGG R CGTCAGCGTAACCTGGGTCT	59	(C16) Family-specific multiple products
Oxio 300	F: AGGAGCCGGAAATTTTCGAG R: CCGACTGTTTTTCCTCCGTTT	60	(C16) Family-specific multiple products
Oxio 122	F: GTGGAGGAAAGGGTGCTCAA R: CTTCAGGCCCCGGATTTT	59	(C16) Family-specific difference in profile (F10) 1 product
Oxio 70	F: AGACCCAGGTTACGCTGACG R: TGGTATCTTTCACCTCCTCTGC	60	(C16) Family-specific multiple products
Oxio 14	F: TGGAACAGCAGGTGTTTCTTCT R: GTCCTCCACGATCTCCTTCA	59	(C16) Same profile as in '50' Family-specific multiple products
Oxio 50	F: AGATCGTGGAGGACAAGAAAGA R: TGCTCCGATCTCGTCAGC	60	(C16) Used in Chapter 2
ID	AM237796 (Galectin)	T_A	Comments
Gal 232	F: CGCAAAAGTGACCGTCAAGTC CCACACGCCATTTTGACATG	60	(C16, C18) Matches primer 81 in 1 product
Gal 81	F: GCTGTGGAGTGGTTACCAGGA GAGAGTAACCCAGCTCCCCG	58	(C16, C18) Used in Chapter 2, Chapter 3

Table 1, continued.

Gal 410	F: ATCGCCTGCCTTTGGAGAG TGCAGTCTCCATCCATGAACAG	58	(C16) Family 6 – profile differs from other primers (C16) Family 6 – multiple peaks (C18) Similar profile as in '81'
ID	BQ426456 (Neuropeptide Y)	T_A	Comments
XPV73	F: CTCACGACGGAACCAATTGA R: CATCGTTTCATTCCGTACCGA	59	(C16) Same profile as in 'XPV476' 1 product
XPV476	F: AGGCCATTGAAGCCACAAAT R: TCCACAAAGGACTCTTGCCAT	59	(C16) Used in Chapter 2
XPV509	F: GCAAGAGTCCTTTGTGGAGTGG R: CCATGAGAACAAAATGGCGAG	60	(C16) Same profile as in 'XPV476' (C16) 1 product
ID	BQ426623	T_A	Comments
623v5	F: GCGCGACATTTTCGATTTTC R: CCATTGGTGATGACGTAACGC	59	(C16) Used in Chapter 2
623v3	F: GACAGTCACAGTTAATGCCAAGATG R: GCCACACACGGGAATACATG	57	(C16) Same profile as in v5 (C16) 1 product
623v2	F: AATGGAACCGCGTGTAACGT R: GCTCTTTTGGAACGGCTCG	59	(C16) Same profile as in v5 (C16) 1 product
623v4	F: TGTGTCTCCATCCGCAGTTTT R: CGGTATTGCCTAGCACTGTTG	59	(C16) Same profile as in v5 (C16) 1 product
ID	BQ426658	T_A	Comments
658v5	F: AGCAGGAATGCCATCTTCGT R: TTTGAAGAATGTTTGGTATATGCCTTA	58	(C18) Same profile as 'v9', 1 product (C16) Same profile as 'v7', 1 product
658v6	F: TCATCACCACATGTTTGGCAA R: AAAGTGCTCGAAAGTGACTTTTGTT	56	(C18) Same profile as 'v9', 1 product (C16) Same profile as 'v7', 1 product
658v7	F: AGAAGTGTGGAGAAATTTTGTCCAG R: TGGGATCAGATCCCATGCTC	58	(C16) Used in Chapter 2
658v9	F: CTCAAACCTACAGTCATCACCACAT R: GTGACTTTTGTTCTGTGTTGCCA	57	(C16) Used in Chapter 3
658v12	F: AACGCGAGACGAAGCAGAAA R: AACTGGTTTAAGCAATCCTGTAGTGTT	56	(C18) Profile similar to v9 (C16) 1 product
ID	BQ426884	T_A	Comments
884_157	F: TCATTCTTTGTTACGAGTGTGGGA R: ATATTTGCCCCTGGTGGAATT	57	(C16, C18) Same profile as in '262' 1 product
884_262	F: ACGATGTCAGGGACAACTTTCTGT R: CAGAGGTAATCTTTTAAACGTCA	57	(C16, C18) Used in Chapter 2, Chapter 3

Table 1, continued.

ID	CX069133 (Cystatin B)	T_A	Comments
Cys_205	F: TGACTTTTTGCTTGACTGTGGC R: ATTGCAGAGATTCCCCCTCA	59	(C16, C18) Same profile as in '413' 1 product only
Cys_413	F: GTTCCTGCCACGACCTGAGT R: GCTGGACACTTACACCCCAGTC	60	(C16, C18) Used in Chapter 2, Chapter 3
ID	CX069163 (Collagen)	T_A	Comments
Col_1	F: ACAGCTTCTGGACGTGCGT R: TCATCATGCTGCTACTTCATACACTT	58	(C16, C18) Same profile as in '31/38' 1 product
Col_31/38	F: TATGAAGTAGCAGCATGATGACTCC R: TCGTGGCTACCTAATGGCG	58	(C16, C18) Used in Chapters 2, Chapter 3
Col_74	F: AGCCACGATACTCTAAGGTCCATC R: TTCCAGCGCGAAAAAACTG	59	(C16, C18) Same profile as in '31/38' 1 product
ID	BQ426550 (Heat shock protein 27)	T_A	Comments
550_110	F: TGACGCAATGGATTTTCTGC R: GCCTGGTATCCAGGTCGGTAG	58	(C16) Used for Chapter 2; Multiple products with C18
550_440	F: GGTACACTGCTTTATGGACCACG R: AAGGGCGCCTCTACAACGTA	59	(C16, C18) Same profile as other primers; 1 product
550_V3	F: ACGAGGTGAAGGTTTCGGTG R: GGCGGGCCTTGATCCTTA	61	(C16, C18) Same profile as other primers; 1 product
550_V4	F: GACGAGCTAGGCTTTCCCGT R: TCTCTTTCTGTGCTGCAAGGC	60	Used for Chapter 3 (C18, C16) Same profile as in '110'
ID	EF629639 (Glutathione Peroxidase)	T_A	Comments
gpox_185	F: ATTTGGATGGAAGCAATCGAA R: AAGTTACATTCCCGGCGAAA	58	(C18) Family-specific multipe peaks
gpox_366	F: TGACAATGCGACGGAGCTC R: CTACCGGGCCGAACGTACTT	60	(C18) Used in Chapter 2
gpox_34	F: GGCCGTGTGTTGTGGATAGAG R: CAGAGAGACAACGAGCGGACT	61	(C18) Family-specific multipe peaks (C18) Same profile as in '366'
gpox_324	F: GATGGGATTCCCCTGTAACCA R: GCATTGTCAGCCGGTTCCT	62	(C18) Multiple peaks in all families
gpox_415	F: AGTGACTTTGTTCCCACCTTCG R: GCCGTTGACATCGCCTATTC	59	(C18) Same profile as in '366' (C18) 1 product
gpox_751	F: AAATGACGTCCACCCTATCTGTG R: TTTCCCCGAAGAGGTTGTTG	58	(C18) Same profile as in '366' (C18) 1 product
ID	EF697775 (Catalase)	T_A	Comments
V1	F: TTCTCAGACCGAGGCACACC R: GCCATAGCCGTTCATTCTCC	60	(C18) Used in Chapter 3

Table 1, continued.

Cat_1326	F: AAGGAGGTGCCCCGAACTATT R: TGTCCATTGGGCCAGAGAAG	60	(C18) Same profile as V1 1 product
V4	F: GTGACCAAAGTGTGGTCCCAG R: CTTCCCGACCTCGATCAGAG	61	(C18) Same profile as V1 1 product
ID	EX956398 (Prostaglandin E receptor 4)	T_A	Comments
9 / 59	F: ACCAATTCTACTGGTGTACCCAACA R: AAGTCCGTGGCATATCTAACCATAA	57	(C18) Used in Chapter 3
1/51	F: GACTGCGTACCAATTCTACTGGTG R: GGCATATCTAACCATAACTGTTGGGT	57	(C18) Same profile as in '4-9/4-59'