Use of laboratory assays to predict subsequent growth and survival of Pacific oyster (Crassostrea gigas) families planted in coastal waters

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A B S T R A C T
Selective breeding programs for improving Pacific oyster (Crassostrea gigas) stocks are expensive, labor-intensive, and typically rely on lengthy field trials in which selection for survival is compromised by inherent stochasticity of outbreaks of "summer mortality syndrome." Reliable laboratory assays that identify and eliminate poor-performing families prior to planting could improve selection efficiency. We tested the hypotheses that juvenile survival after heat shock and/or gene transcription before or after heat shock predicts adult weight and survival at harvest for full-sib families of the Pacific oyster. We heat-shocked (41 °C for 1 h) 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 also deployed replicated groups of unstressed siblings from all 46 families at one subtidal and one intertidal site in Yaquina Bay, Newport, OR for two years. At harvest, we estimated family-specific average survival (%) and average individual weight (g). We found no significant family-level correlations between juvenile survival after heat shock and subsequent survival or average individual weight at the subtidal site or the intertidal site (P>0.05). In a separate experiment, we heat-shocked (39 °C, 1 h) another 25 juveniles from four low-surviving and four high-surviving families from 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 candidate ESTs relative to a housekeeping gene (elongation factor 1) using real-time quantitative PCR. The mRNA concentration of galectin was greater in low-surviving families before heat shock, whereas that of cystatin B at all sampling times 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 both constitutive differences in transcription between family types as well as induced responses to heat shock. The concentrations of heat shock protein 27, catalase, prostaglandin E receptor, gpox, and superoxide dismutase mRNA in juveniles were significantly correlated with adult survival, final individual weight or both. Additionally, mRNA concentrations of galectin and a gene with no match in Genbank, BQ426658, were correlated with average weight at either the intertidal or subtidal site. 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.

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1. Introduction

Summer mortality syndrome is a serious problem for Pacific oyster (Crassostrea gigas) growers. During warm summer months, episodes of high mortality coincide with periods of elevated 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 appear to result from a poorly-understood interaction between reproductive effort, glycogen metabolism, and opportunistic infection by microorganisms (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 using field trials at sites where summer mortality is predictable and severe (Dégremond et al., 2007). However, in the Pacific Northwest region of the United States, outbreaks of summer mortality are sporadic and unpredictable, making selective breeding based upon survival alone problematic because environmental conditions and the impact of summer mortality vary from year to year. It is therefore difficult to make progress in selection based solely upon field data. This difficulty could be circumvented and efficiency increased by subjecting spat to
controlled conditions that are expected to contribute to summer mortality in the hatchery, and selectively culling poor-performing families prior to lengthy and costly field trials, assuming that hatchery assays are correlated with adult weight and survival at harvest.

We conducted two experiments to test the hypotheses that adult family performance at harvest can be predicted using laboratory assays that quantify juvenile survival or whole-body transcript levels of candidate genes after immersion in heated water (heat shock). We chose heat shock as our stressor because heat stress is thought to be an important component of summer mortality (Cheney et al., 2000; Li et al., 2007; Samain et al., 2007), and because it does not involve the use of pathogens or hazardous chemicals. Heat shock damages cells and proteins (Feder and Hofmann, 1999), increases production of reactive oxygen species (Flanagan et al., 1998; Bruskov et al., 2002; Arnaud et al., 2002), results in oxidative stress (Kaur et al., 2005; Heise et al., 2006; Bagnyukova et al., 2007; Abele et al., 2002; Verlecar et al., 2007), and 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). Furthermore, mounting a heat shock response is metabolically expensive, and during summer months when energy reserves are heavily allocated to reproduction, heat shock may facilitate mortality caused by opportunistic infections or metabolic exhaustion (Li et al., 2007).

This paper reports the results of two experiments, in which we 1) tested for correlations between average juvenile survival (%) of heat shock and both adult survival (%) and average individual weight (meat and shell, g) at harvest in families reared in intertidal or subtidal locations, and 2) measured transcript concentrations of selected candidate ESTs before and after heat shock in a subset of families characterized by high or low juvenile survival after heat shock, in order to a) compare transcription between high- and low-surviving families, and b) test for correlations between those transcript concentrations and survival and weight of adults of the selected families at harvest.

2. Materials and methods

2.1. Experiment 1: correlation between survival of heat shock and adult weight and survival

We produced a cohort [Molluscan Broodstock Program (MBP) Cohort #18] of 60 full-sib oyster families during Summer 2005 using single-pair matings; of these, 46 families were available for use in this study. Detailed information on this cohort is available via the MBP internet website (http://hmsc.oregonstate.edu/projects/mbp/index.htm), including pedigrees. MBP selects for increased growth and survival (Langdon et al., 2003), in contrast to intentional production of highly divergent families (e.g. Degremont et al., 2007), and these families represent the standing genetic variation available in the MBP breeding population available for continued selection rather than a comparison between selected families and unselected wild or control populations.

During Fall 2005, we transferred juveniles from all families to troughs supplied with ambient temperature (9 °C–12 °C), sand-filtered water in a flow-through system that also received a continuous mixture of cultured algal food (Isochrysis galbana and Chaetoceros calcitrans) at a concentration of approximately 50,000–80,000 cells ml⁻¹. We heat-shocked three replicate groups of 50 juveniles (1–3 cm shell height) from each of the families by exposing them for 1 h to water heated 41 °C, and thereafter returned the juveniles immediately to ambient seawater (9 °C–12 °C) without addition of algal ration. The heat shock temperature and duration were based on conditions described by Clegg et al. (1998) and from the results of preliminary experiments. Troughs were cleaned daily, and the juveniles were monitored for mortality every other day for 6 d. Dead or dying animals were removed as discovered, and after 6 d the surviving animals were counted.

We planted non-stressed, full-sibling oysters from all families in the Yaquina estuary, Newport, Oregon, at subtidal and intertidal sites located approximately 15 km from the mouth of the estuary (44.6°N, 124.1°W) within two weeks of the heat shock trials. 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 × 0.81 m, 7-mm mesh) at a density of 50 animals per bag. We harvested the animals during Fall 2007, and estimated average survival (%) and average final individual weight (meat and shell, g) of surviving individuals for each of the 46 families. The raw field data is included as supplemental material (Appendix A).

We tested for significant correlations between the family mean of survival of the heat-shocked juveniles with the family mean of survival or weight as adults using the PROC CORR procedure in the SAS software package (PROC CORR; SAS/STAT Software, SAS Institute Inc., 1999), and considered correlations to be significant when P<0.05. We did not directly compare performance between the two sites in a single analysis because the animals were grown using different methods (e.g. mesh bags vs. suspended nets) and planted at different times, and thus experienced different rearing conditions.

2.2. Experiment 2: comparison of relative transcript abundance between high- and low-surviving families

During the cohort-level heat shock trials (Experiment 1), we set aside unstressed individuals from the four lowest-surviving (<31%) and four highest-surviving (>69%) families and used them in assays that measured whole-body transcript concentration of selected candidate ESTs before and after heat shock. The 14 candidate ESTs (Table 1) were chosen based on recent studies conducted in our laboratory (Lang et al., 2009; Taris et al., 2009) that used a complementary DNA (cDNA) microarray (Jenny et al., 2007) and cDNA amplified fragment length transcriptome profiling to identify ESTs whose transcription in gill or hemocytes differed between oyster families with high or low survival after heat shock, in response to chronic heat stress, and/or exposure to bacterial pathogens. In those studies, the candidate ESTs differed in transcription between high- and low-surviving families. We also selected ESTs for evaluation based on recent published studies on oyster stress responses.

These candidate ESTs putatively encode proteins whose function can be classified, based upon literature searches, as 1) antioxidant or detoxification enzymes; 2) heat shock proteins; 3) potential participants in cellular immunity; 4) extracellular matrix constituents, and 5) proteins with unknown functions. The ESTs, their accession numbers and species matches, and E-values are listed in Table 1 according to their predicted function. We acknowledge the uncertainty of these assignments, particularly those ESTs whose E-values are less than 1.0E−18 (heat shock protein 27-like, cystatin B-like, collagen-like, prostaglandin E receptor 4-like); further characterization of these ESTs will be required to confirm their identities and putative functions.

Immediately after deploying the full-cohort field (Experiment 1), we heat-shocked 25 unstressed juvenile animals from each of four low- and four high-surviving families by immersing them in water heated to 39 °C for 1 h, and returning them to sand-filtered ambient (~9 °C) water in a flow-through system without supplemental algae. In contrast to the lethal heat shock used to classify families as low- or high-surviving, this exposure temperature was intended to stress animals without killing them. We collected whole bodies (meat without shell) before heat shock and at 6 h and 24 h after heat shock and repeated the entire experiment three times to produce three independent temporal and biological replicates. The three replicates were all conducted during a single week. We chose to sample at 0, 6 and 24 h because they are proxies for non-stressed conditions, an acute stress response, and recovery from stress. Additionally, these
sampling times were consistent with those used in our microarray study (Lang et al., 2009) and thus served to further explore the value of the selected candidate ESTs.

For each replicate, three individuals from each family at each sampling time were pooled and 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 (not processed for RNA) for 6 d after heat shock to ensure that the heat shock treatment was not lethal and monitored five unستressed animals from each family to ensure that handling did not cause mortality. We isolated total RNA using RNAeasy® 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 used the included random primers.

We ran real-time quantitative PCR (RT-QPCR) reactions using an ABI7500 quantitative PCR thermocycler (Applied Biosystems, Foster City, CA) for the ESTs 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 to produce a total volume 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 s 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. During these tests, we also ran reactions using total RNA as template to ensure that genomic DNA contamination was minimized, and evaluated this by observing lack of PCR product.

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 = Ef1 χ target / Ef1 χ target, where Ef1 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; Lang et al., 2009; Taris et al., 2009), and in the present study did not differ among Sampling Times, between Family Types or for the interaction of Family Type and Sampling Time (P>0.05, 2-way ANOVA; PROC GLM, SAS Institute Inc., 1999).

We tested for differences in relative transcript concentration over time and between high- and low-surviving families using a repeated measures analysis of variance (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 (high and low-surviving) 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 patterns of gene transcription between 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) [see Ott (1999) for detailed explanation of and SAS code for profile analysis]. We log2-transformed the data when necessary 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 intended only to identify genes for further study, and we acknowledge the possibility of spurious significant P-values.

2.3. Experiment 2: correlations between relative transcript concentration and adult weight and survival

We tested for correlations of relative transcript concentration (RC) with average survival and average individual weight using data from
the four low-surviving and four high-surviving families collected during Experiment (PROC CORR, SAS Institute Inc., 1999) after log-transformation of the RC data to achieve normality when necessary. We considered differences to be significant when \( P < 0.05 \). We did not apply Bonferroni correction to control the experiment-wise error rate because this experiment was exploratory in nature and was used only to identify genes for further study in large-scale trials and we 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 as supplementary material (Appendix A).

3. Results

3.1. Experiment 1: correlation between survival of heat shock and adult weight and survival

There were no significant correlations between the family means of juvenile survival after heat shock and the family means of survival or final weight in either the intertidal or subtidal sites (Table 3). There were no significant differences (\( P > 0.05 \); data not shown) in survival or final weight at either site between the four high- and four low-surviving families subsequently studied in Experiment 2. The raw field data are available as supplemental material (Appendix A).

3.2. Experiment 2: comparison of relative transcript concentration between high- and low-surviving families

The ESTs that we studied in high and low-surviving families have been grouped into categories based on literature searches and there are results reported in the order that they appeared in Table 1.

3.2.1. Antioxidant or detoxification enzymes

The relative transcript concentrations (RCs) of superoxide dismutase (sod; Table 4, Fig. 1A) and glutathione peroxidase (gpx; Table 4, Fig. 1B) did not differ significantly over time or between family types. However, the RC of catalase increased significantly between the 6 h and 24 h sampling times in both family types (Table 4, Fig. 1C). The RC of glutathione S-transferase omega (gst) did not differ significantly over time (Table 4, Fig. 1D), but was significantly greater in high-surviving families at 24 h after heat shock.

3.2.2. Heat shock proteins

The RC of both heat shock protein 68 (hsp68; Fig. 1E) and heat shock protein 27-like (hsp27; Fig. 1F) increased significantly between 0 and 6 h and between 6 and 24 h after heat shock, but did not differ significantly between the family types (Table 4).

3.2.3. Potential participants in cellular immunity

The RC of cystatin B-like was significantly different overall between the family types (Table 4, Fig. 2A), greater in high-surviving families than in low-surviving families at each of the three sampling times, but not different among sampling times. We found a significant interaction between sampling time and family type for the transcription of galectin (Table 4, Fig. 2B). RC of galectin mRNA was greater before heat shock

### Table 2

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<tr>
<th>Accession</th>
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<th>C</th>
<th>Efficiency</th>
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<td>AB122062</td>
<td>Heat shock protein 68</td>
<td>TGGACCTTGAGAAGACCATATT</td>
<td>60</td>
<td>40</td>
<td>1.91 ± 0.003</td>
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<td>AB122066</td>
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<td></td>
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<td>AB179775</td>
<td>Lysozyme precursor</td>
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<td>60</td>
<td>40</td>
<td>2.01 ± 0.003</td>
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<td>AF321279</td>
<td>Tissue inhibitor of metalloprotease</td>
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<td>58</td>
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<td>2.00 ± 0.002</td>
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<td>AJ557141</td>
<td>Glutathione S-transferase</td>
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<td>45</td>
<td>1.94 ± 0.002</td>
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<td>AJ496219</td>
<td>Superoxide dismutase</td>
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<td>45</td>
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<td>Catalase</td>
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<td>1.98 ± 0.003</td>
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<tr>
<td>BQ426658</td>
<td>Heat shock protein 27-like</td>
<td>GCAGCAGCCGAGGAGAGAGA</td>
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<td>45</td>
<td>2.00 ± 0.003</td>
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<tr>
<td>None</td>
<td>None</td>
<td>GTATCTCTTTTCTGCTGCTGAC</td>
<td>58</td>
<td>45</td>
<td>1.98 ± 0.003</td>
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<td>CX069133</td>
<td>Cystatin B-like</td>
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<td>45</td>
<td>2.02 ± 0.002</td>
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<td>CX069163</td>
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<td>45</td>
<td>2.08 ± 0.004</td>
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<td>EF687775</td>
<td>Catalase</td>
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<td>2.03 ± 0.003</td>
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<tr>
<td>EF692639</td>
<td>Glutathione peroxidase</td>
<td>TTGGAACCTTGAGAAGACCATATT</td>
<td>60</td>
<td>40</td>
<td>1.91 ± 0.003</td>
</tr>
<tr>
<td>EX956398</td>
<td>Prostaglandin E receptor 4-like</td>
<td>AGATCGCTGGCATATACATGCA</td>
<td>58</td>
<td>45</td>
<td>1.95 ± 0.004</td>
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</tbody>
</table>

### Table 3

<table>
<thead>
<tr>
<th></th>
<th>Intertidal</th>
<th>Subtidal</th>
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<tbody>
<tr>
<td>r²</td>
<td>p</td>
<td>r²</td>
</tr>
<tr>
<td>Average survival (%)</td>
<td>−0.0226</td>
<td>0.3183</td>
</tr>
<tr>
<td>Average weight (g)</td>
<td>0.0007</td>
<td>0.4931</td>
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</table>
and decreased between 0 h and 6 h in low-surviving families but increased over the same period in high-surviving families. The RC of lysozyme precursor did not vary significantly over time or differ between family types (Table 4; Fig. 2C). The RC of prostaglandin E receptor 4-like (pe4) did not vary over time or differ between family types despite the visual trend of higher transcription in the low-surviving families between 6 h and 24 h after heat shock (Table 4; Fig. 2D). The RC of tissue inhibitor of metalloprotease (TIMP) increased significantly between 6 h and 24 h in both family types but did not differ between them (Table 4; Fig. 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). This trend of suppressed transcription is similar to what was observed for galectin.

3.2.4. Structural constituents of the extracellular matrix

The RC of collagen-like (Table 4; Fig. 2F) did not differ significantly over time or between family types.

3.2.5. No known function

The RCs of BQ426658 (Fig. 2G) and BQ426884 (Fig. 2H) did not differ significantly over time or between family types (Table 4).

### 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-surviving after heat shock). Abbreviations refer to: FT, Family Type (high- or low-surviving); T, sampling time. Differences were considered significant when P < 0.05.

<table>
<thead>
<tr>
<th>EST Identity</th>
<th>Between FT</th>
<th>Within FT</th>
<th>FT*T</th>
</tr>
</thead>
<tbody>
<tr>
<td>AJ496219 Superoxide dismutase</td>
<td>0.7264</td>
<td>0.1826</td>
<td>0.5137</td>
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<tr>
<td>EF692639 Glutathione peroxidase</td>
<td>0.6615</td>
<td>0.2218</td>
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<tr>
<td>EF687775 Catalase</td>
<td>0.6504</td>
<td>0.0066</td>
<td>0.4437</td>
</tr>
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<td>AJ557141 Glutathione 5-transferase</td>
<td>0.2151</td>
<td>0.0514</td>
<td>0.7018</td>
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<tr>
<td>AB122062 Heat shock protein 68</td>
<td>0.1127</td>
<td>&lt;0.001</td>
<td>0.0861</td>
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<tr>
<td>BQ426550 Heat shock protein 27-like</td>
<td>0.3928</td>
<td>&lt;0.001</td>
<td>0.0018</td>
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<tr>
<td>CX069133 Cystatin B-like</td>
<td>0.0373</td>
<td>0.0639</td>
<td>0.9688</td>
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<tr>
<td>AM237796 Galectin</td>
<td>0.2134</td>
<td>0.3023</td>
<td>0.0158</td>
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<td>AB179775 Lysozyme precursor</td>
<td>0.2238</td>
<td>0.4152</td>
<td>0.4694</td>
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<td>EX956398 Prostaglandin E receptor 4-like</td>
<td>0.1969</td>
<td>0.3222</td>
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<tr>
<td>AF321279 Tissue inhibitor of metalloprotease</td>
<td>0.9585</td>
<td>&lt;0.001</td>
<td>0.1348</td>
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<td>CX069163 Collagen-like</td>
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<td>BQ426658</td>
<td>0.2142</td>
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<td>BQ426884</td>
<td>0.4315</td>
<td>0.5257</td>
<td>0.3059</td>
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</table>

Differences were considered significant when P < 0.05, and are presented in bold font.

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Fig. 1. Average relative concentration (RC) or log2 relative concentration (Log2RC) (+ SEM) before and at 6 h and 24 after heat shock (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 HS (41 °C, 1 h). Asterisks above solid lines indicate significant differences between family types, and asterisks between sampling times indicate significant differences between sampling times. Differences were considered significant when (P < 0.05).
3.3. Experiment 2: correlations between relative transcript concentration and adult weight and survival

There were 13 instances of significant family means correlations between relative transcript concentrations (RC) and survival or weight of the eight families (Table 5; Figs. 3, 4). Of the 13 significant correlations, eight of them (∼62%) were negative. In all cases where an EST's RC was significantly correlated with performance at both the intertidal and the subtidal site, the correlation was consistent in direction (e.g. positive or negative). In only one case (pe4) was RC significantly correlated with both survival and weight but in opposite directions. Beyond the fact that significant correlations were observed, we observed two important trends: nearly half (46%) of the significant correlations occurred in unstressed animals, and the direction of most of the correlations was counterintuitive.

3.3.1. Negative correlations

Each of the four significant correlations between RC and survival were negative (Table 5, Fig. 3); these included hsp27 at 6 h after heat shock (Fig. 3A) and of catalase before heat shock (Fig. 3B) at the intertidal site, and pe4 at 6 h after heat shock (Fig. 3C) and sod at 24 h after heat shock (Fig. 3D) at the subtidal site. Four of the nine significant correlations between RC and weight were negative; these included hsp27 before heat shock at the intertidal site (Fig. 3E), gpx at 6 h after heat shock at the subtidal site (Fig. 3F), and BQ426658 before heat shock at both sites (Fig. 3G).
and are presented in bold font.

4.2. Experiment 2: comparison of relative transcript concentration between high- and low-surviving families

The ESTs that we studied in high and low-surviving families have been grouped into categories based on literature searches, and they are discussed below in the order that they appeared in Table 1 and that they were reported in Section 3.2.

4.2.1. Antioxidant or detoxification enzymes

Free oxygen radicals are converted by superoxide dismutase into hydrogen peroxide (H$_2$O$_2$) which is then detoxified by catalase and glutathione or thioredoxin peroxidases (Storey, 1996; Young and Woodside, 2001). It is reasonable to expect that heat shock would result in increased concentrations of reactive oxygen species (ROS) based on previous studies (Flanagan et al., 1998; Arnaud et al., 2002; Bruskov et al., 2002), and our observation of increased catalase transcription supports this contention. Interestingly, Samain et al. (2007) found that gill of summer mortality-resistant C. gigas families had higher catalase activity during normoxia and hypoxia than did susceptible families.

Reactive oxygen species may react with other substances to form toxic compounds that in turn cause lipid peroxidation and DNA damage (Storey, 1996). Glutathione S-transferase omega detoxifies these compounds by conjugating them to glutathione (Hayes and Pulford, 1995; Martinez-Lara et al., 2002; Bouet et al., 2004a,b). Our observation of lower post-stress GST transcription in low-surviving families adds to a growing body of evidence indicating that stress-sensitive oyster families may be more susceptible to tissue damage through oxidative stress. Huvet et al. (2004) found that 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, and Lambert et al. (2007) reported that production of ROS by hemocytes was higher in oyster families susceptible to summer mortality than in resistant families. Taris et al. (2009) studied gene transcription in the same oyster families used in the present study, and found that transcription of the detoxification enzyme cytochrome P450 was higher in high-surviving families than in low-surviving families that were acclimated to 25 °C (a temperature thought to be stressful to oysters) but not heat-shocked.

4.2.2. Heat shock proteins

The absence of significant differences between hsp27 RC in low- and high-surviving families contrasts with our previous finding that its transcription was higher in gills of low-surviving families than in high-surviving families (Lang et al., 2009). Samain et al., 2007 found that 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 resistant families. We speculate that gills may be more sensitive to the effects of heat shock than other tissues, and in the present study this sensitivity could have been masked by the contribution of transcripts from multiple tissues. For example, according to Meistertzheim et al. (2007), transcription of the hsp68 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.

4.2.3. Potential participants in cellular immunity

Under most 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), and therefore it 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, mortality could have been 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 remove bacterial pathogens. We stress that our suggested links between transcription and infection in the following discussion of this section are speculative and require further study.

The observation of greater overall transcription of cystatin B-like in high-surviving families at each of the three sampling times is compatible with our previous finding that its transcription in gill was greater in high- than in low-surviving families at 24 after heat shock (Lang et al., 2009). Cystatins inhibit proteolytic cathepsins.
that are secreted by pathogens to release nutrients from host cells (Brady et al., 1999; Vergote et al., 2005), and have been explicitly linked with response to infection in mollusks including a gastropod (*Biomphalaria glabrata*; Guillou et al., 2007) and bivalve (*Ruditapes philippinarum*; Kang et al., 2006).

Vergote et al. (2005) found that *B. glabrata* resistant to infection by blood flukes had higher protein levels of a type-2 cystatin after infection; therefore, cystatin B, and perhaps other protease inhibitors, could potentially be used as a marker for use in marker-aided selection programs, especially because differences are evident at the whole-body level and in the absence of stress.

In vertebrates, galectins promote cell adhesion and regulate both innate and adaptive immune responses (Perillo et al., 1998; Rabinovich et al., 2002; Levrony et al., 2005). In *Crassostrea* sp., galectins adhere to microbes, phytoplankton, and protozoans thereby serving both defensive and digestive roles by promoting phagocytosis of particles by hemocytes (Tasumi and Vasta, 2007). Greater pre-stress transcription of galectin in whole bodies of low-surviving families is compatible with our previous findings of higher overall galectin transcription in gill of low-surviving families (Lang et al., 2009). It is possible that relatively higher pre-stress levels of galectin in low-surviving families reflected an overall sensitivity to infection because many bivalve pathogens reduce hemocyte binding capacity (Choquet et al., 2003; Labreuche et al., 2006), and enhanced transcription could have resulted from an exaggerated need for protein product to compensate for less-efficient binding to pathogens.

The biological significance of the suppressed transcription of galectin after heat shock in low-surviving families is less clear. Yamaura et al. (2008) reported that transcription of galectin by *C. gigas* hemocytes was transiently suppressed by exposure to *Vibrio tubiashi*, and it is tempting to suggest that our observation of suppressed transcription could be linked to interaction with pathogens. However, after heat shock, increased transcription of heat shock proteins is usually accompanied by suppressed transcription and translation of non-heat shock proteins (Cuesta et al., 2000), and it is possible that suppressed galectin transcription was part of this larger phenomenon.

Given the possibility of opportunistic infection after heat shock and the need for energy after mounting a stress response, it was surprising that there were no differences in lysozyme transcription before or after heat shock. 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., 2006; Matsumoto et al., 2006; Itoh and Takahasi, 2007; Itoh et al., 2007). Lysozyme activity increased in tissue of mussel *Mytilus edulis* 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).

Our results for whole-body *pe4* transcription are not consistent with our previous finding that its transcription by hemocytes was greater in the same low-surviving families used for this study than in the high-surviving ones (Taris et al., 2009). 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 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).

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 mentioned earlier, it is possible that its suppressed transcription was a consequence of the general effects of heat shock. However, the consequence of heat shock induced suppression of immune-related
genes could be an increased susceptibility to infection. Samain et al. (2007) found that C. gigas families with high susceptibility to summer mortality syndrome had higher bacterial loads after heat shock than did families with low susceptibility. The possibility that sensitivity to infection in C. gigas results in part from suppressed transcription of immune-related genes merits further study.

4.2.4. Structural constituents of the extracellular matrix

Our results for collagen are inconsistent with our previous observation that its transcription was greater overall in gills of low-surviving C. gigas families (Lang et al., 2009). Altered collagen transcription has been reported 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). The physiological significance of these observations remains unclear.

4.2.5. No known function

We previously found that transcript concentrations for BQ426658 and BQ426884 were higher in gill of low- and high-surviving families, respectively (Lang et al., 2009), although these differences were not observed in the present study. 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.

4.3. Experiment 2: correlations between relative transcript concentration and adult weight and survival

The negative correlations between adult weight and survival and stress-induced transcription of genes thought to contribute to surviving stress seem counterintuitive; it is reasonable to expect that increased availability of a given protein that addresses injury or infection would be beneficial. We hypothesize that these negative correlations reflect genotype-specific differences in protein turnover that in turn had consequences for surviving stress and for growth. 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; Bayne and Hawkins, 1997). In general, when less energy is expended during maintenance metabolism, more energy is available to fuel growth and stress responses (Hawkins et al., 1987; Hawkins et al., 1989; Bayne, 1999, 2004). Thus, oyster families with a tendency to produce and turn over more proteins would be at an energetic disadvantage for surviving and growing, particularly during summer months when tissue glycogen reserves are expected to be low or exhausted (Berthelin et al., 2000). Heat shock proteins are generally assumed to enhance survival of stress (Parsell and Lindquist, 1993; Feder and Hofmann, 1999), although their production in oysters can be deleterious under certain conditions (Li et al., 2007). One potential example of deleterious protein turnover could be the negative correlations between survival and RC of hsp27 at the intertidal site where excessive investment of energy into heat shock protein synthesis following repeated periods of tidal emersion could become detrimental.

The positive correlations of both galectin and pe4 with weight before and after stress, however, do not support our proposed relationship between gene transcription rates and protein turnover or 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. Even so, galectin is thought to be used to acquire food (Tasumi and Vasta, 2007) and if an individual’s tendency was to produce more of that protein, particularly in the absence of stress, enhanced growth could result. It is difficult to speculate on the role of pe4 in weight or survival, but the contrasting correlations between its transcription and adult weight and survival make it an interesting candidate for further study.

5. Conclusions

Survival of immersion-based heat shock in the nursery was not predictive of adult weight and survival of C. gigas families with a history of artificial selection for fast growth and high survival in this study. Relative transcript concentrations of glutathione S-transferase, cystatin B, and galectin differed between families characterized by their tolerance to heat shock, and may be valuable molecular markers for use in selection programs. Gene transcript levels in juveniles were mostly negatively correlated with adult weight and survival and based on the trends observed here, families could be culled from the hatchery based upon higher transcription levels of certain genes.

An important outcome is that stress assays appeared to have been of no practical use in predicting adult weight and survival. We observed significant family-level correlations between transcription and adult weight and survival in the absence of stress, and also observed pre-stress differences in transcription of candidate ESTs between oyster families characterized by their tolerance of heat shock that were consistent with previous studies (Lang et al., 2009). Taris et al. (2009) also found that in the same families studied here, transcription by hemocytes of several putatively stress-related ESTs differed between the family types before experimental exposure to Vibrio tubiashii.

Our results suggest that the performance of selectively-bred oyster families, at least in the Pacific Northwest region of the United States, may be rooted more in overall physiological robustness, rather than in how they respond to stress. It is possible that being “prepared for stress” is just as important as the stress response itself, particularly in an animal that experiences routine intertidal emersion and associated variation in temperatures. This hypothesis raises the question of why after generations of selection, less-robust stocks have still not been eliminated from the breeding population. Dégremont et al. (2007) noted this phenomenon in French oyster populations and hypothesized that less-robust genotypes were maintained because environmental conditions vary greatly among growing regions and throughout time, thereby countering or precluding effective stabilizing selection.

Gene transcription assays in unstressed assays could simplify the identification and use of candidate ESTs to predict adult weight and survival and for use in marker-aided selection. However, links between gene transcription and maintenance metabolism require further clarification. Future studies should focus on differences in gene expression under non-stress conditions and take potential differences in maintenance metabolism and protein turnover into account when evaluating stocks.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.jaquaculture.2010.04.023.

References


