Heat shock response of killifish (Fundulus heteroclitus): candidate gene and heterologous microarray approaches

Timothy M. Healy, Wendy E. Tymchuk, Edward J. Osborne, and Patricia M. Schulte

Department of Zoology, The University of British Columbia, Vancouver, British Columbia, Canada

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Healy TM, Tymchuk WE, Osborne EJ, Schulte PM. Heat shock response of killifish (Fundulus heteroclitus): candidate gene and heterologous microarray approaches. Physiol Genomics 41: 171–184, 2010. First published January 26, 2010. doi:10.1152/physiolgenomics.00209.2009.—Northern and southern subspecies of the Atlantic killifish, Fundulus heteroclitus, differ in maximal thermal tolerance. To determine whether these subspecies also differ in their heat shock response (HSR), we exposed 20°C acclimated killifish to a 2 h heat shock at 34°C and examined gene expression in fish from both subspecies during heat shock and recovery using real-time quantitative PCR and a heterologous cDNA microarray designed for salmonid fishes. The heat shock proteins Hsp70-1, hsp27, and hsp30 were upregulated to a greater extent in the high temperature-tolerant southern subspecies than in the less tolerant northern subspecies, whereas hsp70-2 (which showed the largest upregulation of all the heat shock proteins) in both gill and muscle and hsp90α in muscle was upregulated to a greater extent in northern than in southern fish. These data demonstrate that differences in the HSR between subspecies cannot be due to changes in a single global regulator but must occur via gene-specific mechanisms. They also suggest that the role, if any, of hsp90α in establishing thermal tolerance is complex and varies from gene to gene. Heterologous microarray hybridization provided interpretable gene expression signatures, detecting differential regulation of genes known to be involved in the heat shock response in other species. Under control conditions, a variety of genes were differentially expressed in muscle between subspecies that suggest differences in muscle fiber type and could relate to previously observed differences between subspecies in the thermal sensitivity of swimming performance and metabolism.

heat shock proteins; fish; temperature; gene expression; cGRASP

salmon microarray

THE MAXIMUM TEMPERATURE THAT an ectotherm can tolerate is typically correlated with the organism’s maximum habitat temperature (64). Therefore, understanding the physiological mechanisms underlying thermal tolerance could provide insights into the factors influencing species distributions and may provide the power to predict changes in these distributions in the face of increasing global temperatures (32, 50, 51, 53, 75). One possible cellular mechanism that could influence the upper thermal tolerance of an organism is the heat shock response (HSR) (66). The HSR is a highly conserved cellular response in which the transcription and translation of heat shock proteins (hsp hsps) are induced by a variety of protein-denaturing stressors (21). A causal relationship between the HSR and whole organism upper thermal tolerance has been most clearly demonstrated in genetically engineered Drosophila; flies with differing numbers of gene copies of the 70 kDa class of heat shock protein (hsp70) differ in survival following a severe heat shock (9). Similarly, RNA interference (RNAi) knockdown of hsp70 has been shown to prevent recovery from heat stress in the firebug, Pyrrhocoris apterus (36). While no studies have established a causal relationship between the HSR and thermal tolerance in fish, many studies have found a positive correlation between these two factors (7). In addition a QTL (quantitative trait locus) that is associated with differences in critical thermal limits in rainbow trout (13) has been shown to be closely linked to a heat shock cognate 71 gene (hsc71) (59).

Fundulus heteroclitus, the Atlantic killifish, represents an excellent model system in which to study the significance of the HSR, as the species is distributed through a latitudinal thermal gradient along the east coast of North America from Canada to northern Florida. There are two regional subspecies: F. heteroclitus macrolepidotus, the northern form, which is found from the Gulf of the St. Lawrence to approximately New Jersey, and F. heteroclitus heteroclitus, the southern form, which is found in the remainder of the species range (43). Populations in the north of the species range experience temperatures that are, on average, ~13°C lower than those experienced by populations in the south of the species range (18 and references therein). Associated with these differences in environmental temperature, the northern and southern subspecies have been shown to differ in a variety of aspects of their thermal biology (17–20, 62). Of specific relevance to the current study, both the maximum and minimum temperature at which fish can maintain their equilibrium (CTMax/CTMin) are ~2°C higher in the southern subspecies compared with the northern subspecies at any common acclimation temperature in the laboratory (18).

In F. heteroclitus, it has been previously shown that hsp70 sequence variation, and changes in the temperature of onset of the HSR did not correlate with thermal tolerance and although the extent of induction varied between subspecies for several hsps, the direction of this difference varied among genes (18). Because Fangue et al. (18) examined only a single time point during recovery from heat shock at various temperatures, the possibility remains that the observed differences actually represent differences in the timing of responses. Consequently, the current study addresses three main objectives: first, to examine if previously observed differences in hsp expression between subspecies can be accounted for by differences in the kinetics of induction of these genes; second, to extend this analysis by examining the expression patterns of additional subfamilies of hsps; and third, to utilize a heterologous microarray approach to examine whether there are other genes that differ between the subspecies in their response to heat shock or that differ in expression between the subspecies under control conditions potentially contributing to differences in thermal tolerance.
MATERIALS AND METHODS

Experimental animals. Adult killifish of the northern subspecies (Fundulus heteroclitus macrolepidotes) were collected from Hampton, NH (42° 54′ 46″ N), by Aquatic Research Organizations. Fish of the southern subspecies (Fundulus heteroclitus heteroclitus) were collected from Brunswick, GA (31° 7′ 31″ N). All fish were transported to the University of British Columbia where they were held in 12 replicate 75 l glass aquaria that were divided in half with water-permeable dividers. Each aquarium contained five northern and five southern fish separated by the divider. Holding conditions of 20 ppt salinity, 20 ± 2°C temperature and 12 h:12 h (light:dark) photoperiod were maintained for a minimum of 4 wk prior to experimentation. Fish were fed TetraMin fish flakes supplemented with commercial trout chow (PMI Nutrition International, Brentwood, MO) daily to satiety but were not fed for 24 h prior to experimental trials. Treatment of all experimental animals was in accordance with the University of British Columbia animal care protocol #A01-0180.

Heat shock experiment. We exposed 20°C-acclimated northern (NH) and southern (GA) killifish to a heat shock temperature of 34°C for 2 h, followed by 2 h of recovery at 20°C. Groups of six fish per population were sampled directly from the acclimation tank (time 0), during heat shock (times = 15, 30, 60, 90 and 120 min), and during recovery (times = 180 and 240 min following onset of heat shock). Following rapid decapitation of the fish, gills and lateral body musculature (directly ventral to the dorsal fin) were dissected and immediately frozen in liquid nitrogen. All tissues were stored at −80°C until analysis.

Quantitative real-time PCR analysis of hsp gene expression. The guanidine isothiocyanate method (12) was used to extract total RNA with TRIzol Reagent (Invitrogen Life Technologies, Burlington, ON, Canada). After isolation, integrity confirmation by gel electrophoresis and quantification by spectrophotometry, 5 μg total RNA per sample was used for cDNA synthesis (18). In brief, RevertAid H Minus M-MuLV reverse transcriptase (MBI Fermentas, Burlington, ON, Canada) and an oligo(dT18) primer were used to synthesize cDNA following the manufacturer’s instructions in a total volume of 20 μl. Gene expression was then obtained by quantitative real-time PCR (qRT-PCR) with an ABI Prism 7000 sequence analysis system (Applied Biosystems, Foster City, CA). Gene-specific primers for all hsp70 and hsp90 isoforms were taken from Fangue et al. (18), whereas primer pairs for hsp27 and hsp30 were designed from F. heteroclitus sequences in the GenBank expressed sequence tag database (hsp27: accession no. CN984618.1; hsp30: accession no. CN971867.1). Partial sequence of hsp60 was obtained using degenerate primers designed from conserved regions of sequences of this gene from Danio rerio (accession no. BC0684151), Carassius auratus (accession no. DQ872653.2), and Paralichthys olivaceus (accession no. DQ250130.1). Cloning primers were designed using GeneTool Lite cloning software. Cloning primer sequences were 5′ C(T/G) TCA CCA TGG G(A/G/T)C CAA AGG 3′ and 5′ C(T/G) CGT CTC TCC ATC ACG ATC ATG C(T/G) GC C(T/G) GC CCA 3′ for the forward and reverse primers respectively. PCR products were cloned and sequenced using standard techniques (18), and the resulting sequence information was deposited into GenBank (accession no. GQ903704) and used to generate F. heteroclitus-specific qRT-PCR primers. All qRT-PCR primers were designed using Primer Express software (version 2.0.0, Applied Biosystems) and are reported in Table 1. qRT-PCR reactions were performed using 2 μl cDNA, 4 pmol of each primer, and 2× SYBR Green Master Mix (Applied Biosystems) to a total volume of 22 μl under the following conditions: 1 cycle of 94°C for 2 min, 1 cycle of 94°C for 10 min, 40 cycles of 95°C for 15 s, 60°C for 1 min. At the end of each qRT-PCR reaction, PCR products were subjected to a melt curve analysis to confirm the presence of a single amplicon.

One highly induced sample was used to develop a standard curve relating threshold cycle to cDNA amount for each primer set. Results were then normalized using elongation factor-1α (EF-1α; accession no. AY430091) as mRNA levels of this gene do not change with heat shock in killifish gills (data not shown). However, in muscle, EF-1α levels varied such that southern fish had greater expression early in heat shock relative to constitutive levels and higher levels than northern fish throughout the heat shock (data not shown). Therefore we used the geometric mean (69) of EF-1α, lactate dehydrogenase subunit A (LDH-A), and cytochrome c oxidase subunit 2 (COXII) mRNA levels as the control in muscle because this quantity did not vary significantly with sampling time or subspecies (data not shown). Primer sequences for control genes were as previously published [18 (EF-1α), 57 (LDH-A), and 20 (COXII)] and are listed in Table 1. Several samples of RNA that had not been reverse transcribed were randomly chosen and subjected to qRT-PCR with each primer pair to detect the possible presence of genomic DNA contamination. In general, genomic contamination was detected to be in the range of 1:1,000 and was thus a negligible source of error.

All data are displayed relative to the northern control value to allow simpler comparison of the fold induction between genes. However, as these data are relative (both to the northern control and normalized to a control gene), we also estimated the absolute amount of mRNA expression for each gene (independent of normalization to a control gene or to the northern control) to directly compare total amounts of RNA for each gene. The absolute amount was estimated as in Ref. 63, using the following formula: Total amount of mRNA expression = Efficiency−Ct, where efficiency is determined from the slope of the standard curve (and theoretically has a value of 2) and Ct corresponds to the threshold cycle number. The threshold intensity was maintained constant across genes so that threshold cycle could be directly compared. This calculation was performed using data from control fish and from fish sampled at the time point that had the highest expression for each gene. Note that because the same amount of cDNA was loaded in each well, this quantity is effectively normalized to total RNA.

### Table 1. Quantitative real-time PCR primer sets for Fundulus heteroclitus

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence 5′ → 3′</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsp70-1</td>
<td>F: CGG AAT AAA TGT CCT GCG GAT</td>
</tr>
<tr>
<td>hsp70-2</td>
<td>R: CAA AGG TGC TCT CAC CAA GAT C</td>
</tr>
<tr>
<td>hsp90α</td>
<td>F: CAG TGC TCT GCG GTA CAT G</td>
</tr>
<tr>
<td>hsp90β</td>
<td>R: CAT AGG GAT GAA GTC CTA TCA</td>
</tr>
<tr>
<td>hsc70</td>
<td>F: ACA CCA CCA TCC CGA GAA A</td>
</tr>
<tr>
<td>hsp27</td>
<td>R: GAC TGG ATC TCG TCT TGT T</td>
</tr>
<tr>
<td>hsp30</td>
<td>F: GCA TGG AGG ATG AGT ATG C</td>
</tr>
<tr>
<td>hsp60</td>
<td>R: TGT CAT CAC GAG ACT TGG C</td>
</tr>
<tr>
<td>EF-1α</td>
<td>F: GGG AAA GGC CTT CTT CAA G</td>
</tr>
<tr>
<td>COXII</td>
<td>R: ACC TCT GGC CTT CAG GTT</td>
</tr>
<tr>
<td>Parvalbumin-β</td>
<td>TGA TGC TGA CAT TAG TGC AGC C</td>
</tr>
<tr>
<td>SERCA2</td>
<td>R: TGC GAT GCT GCT GTA CAT G</td>
</tr>
<tr>
<td>COXI</td>
<td>R: AGC TCG TGC AGG ATG AC</td>
</tr>
<tr>
<td>ATP synthase α-chain</td>
<td>R: GAA ACT TGG AGG CTC ACA AAG</td>
</tr>
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F, forward; R, reverse.
**HEAT SHOCK RESPONSE OF Fundulus heteroclitus**

Reverse transcription of RNA, labeling, and microarray hybridizations. Four muscle RNA samples were randomly selected from each subspecies under control (time = 0) conditions and at 60 min of the heat shock exposure (a total of 16 samples) for microarray analysis. This experiment was designed to comply with Minimum Information About a Microarray Experiment (MIAME) guidelines (10), and all scanned images and quantified raw data files have been deposited in the gene expression omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) with GEO platform accession number GSE18792. We chose to take a heterologous microarray approach in which *F. heteroclitus* cDNA was hybridized against a 16K cDNA array developed for salmonids (consortium for Genomics Research on All Salmon Project, cGRASP; 58, 71). Hybridizations were performed utilizing a reference-design (33) with a pooled RNA reference composed of an equal mixture (by RNA quantity) of all the samples used in the microarray experiment.

Microarray hybridizations were performed with a 3DNA Array 50 Expression Array Detection Kit (Genisphere, Hatfield, PA) according to the manufacturer’s instructions. Reverse transcription of 15 μg RNA per reaction was performed using SuperScript II reverse transcriptase (Invitrogen) and oligo (dT) primers designed with a unique 5′ sequence complementary to a “capture sequence” on a Cy3 or Cy5 fluorescent 3DNA capture reagent (Genisphere). Dyes were balanced across samples such that two individuals from each of the four treatment groups were labeled with each dye. Microarrays were prepared for hybridization by washing 2 × 5 min in 0.2% SDS, 5 × 1 min in MilliQ water, and drying by centrifugation (5 min at 500 g in 50 ml conical tubes). The arrays were prehybridized at 49°C for 45 min in 5× SSC, 0.1% SDS, 0.2% BSA (fraction V), washed 2 × 1 min in MilliQ water, and dried by centrifugation. Then, the cDNA was hybridized to the array in a formamide-based buffer for 16 h at 49°C. The arrays were washed 1 × 10 min at 49°C (2× SSC, 0.1% SDS), 2 × 5 min at room temperature (RT) 2× SSC, 0.1% SDS, 2 × 5 min in RT 1× SSC, and 2 × 5 min in RT 0.1× SSC, and again dried by centrifugation. The Cy3 and Cy5 3DNA capture reagents (Genisphere) were then hybridized to the bound cDNA on the microarray for 2 h at 49°C and washed and dried as before.

Fluorescent images of hybridized arrays were acquired immediately at 10 μm resolution using ScanArray Express (PerkinElmer, Waltham, MA). The same laser power (95%/e) and PMT settings were used for all slides.

TM4 Microarray Software Suite, a set of open-source freeware programs developed at the Institute for Genomics Research (TIGR), was used for quantification, normalization and analysis of microarray data. The OTSU quantification method within TIGR Spotfinder was used to quantify the scanned microarray files. Integrated intensities were corrected for background and any spots with expression levels <2 standard deviations above background for at least 50% of the samples were removed. TIGR Microarray Data Analysis System (MIDAS) was used to apply a locally weighted linear regression (LOWESS) normalization module. The resulting normalized and filtered expression files were analyzed using TIGR Multieperiment Viewer (MeV).

**Confirmation of microarray results.** By reanalyzing the qRT-PCR data for the heat shock proteins, we independently validated the microarray results. To parallel the analytical approach for the microarrays, absolute amounts of mRNA from qRT-PCR were calculated and expressed relative to the mean of the sample. Additionally, four genes of interest were selected from the microarray results to be investigated further with qRT-PCR. Genes chosen were parvalbumin-β, sarcolendoplasmic reticulum Ca²⁺-ATPase 2 (SERCA2), cytochrome c oxidase subunit 1 (COX1), and ATP synthase α-chain. For these genes the *F. heteroclitus* expressed sequence tag (EST) database was queried using the BLASTn algorithm using salmon sequences from the microarray annotation file. We chose one of the resulting EST sequences for each gene (parvalbumin-β: accession no. EV412830.2; SERCA2: accession no. CN958843.1; COX1: accession no. AY735174.1; ATP synthase α-chain: accession no. CN958293.1) to design Fundulus-specific qRT-PCR primers as previously described. Primer sequences are listed in Table 1.

**Statistical analyses.** Real-time PCR data were log transformed, if necessary, to meet assumptions of normality and homogeneity of variance, and were analyzed by multway analysis of variance (ANOVA) with subspecies and time point (during and following heat shock) as factors. When interaction terms were significant, the data were separated and reanalyzed using one-way ANOVA followed by a Student-Newman-Keuls post hoc test. Alpha was set at 0.05.

Statistical analysis of microarray data was performed in TIGR MeV using two-way ANOVA with subspecies and heat shock as factors. Alpha was set at 0.01. Because there is substantial replication on the 16K cGRASP salmon microarray, spots that were detected in MeV as differing significantly by subspecies, heat shock, or having a significant interaction were examined further. The statistically significant spots were grouped into clusters of putative genes based on their annotated gene descriptions. Data for these potentially replicate spots were averaged, log transformed, and reanalyzed by two-way ANOVA using SPSS statistics software, with subspecies and heat shock as factors. When interaction terms were significant, data were separated and analyzed independently by one-way ANOVA. Because of the exploratory nature of experiments utilizing a heterologous microarray, we did not perform any additional analyses on the microarray data set.

**RESULTS**

Effects of heat shock on 70 kDa hsps. Online Supplemental Table S1 provides a summary of the differences in gene expression for all hsps as a result of subspecies of origin or heat shock.1 In gill, hsp70-1 increased significantly with heat shock (*P* < 1 × 10⁻¹⁵), and there were significant differences between subspecies (*P* = 0.0001), with no significant interaction between factors (*P* = 0.120, Fig. 1A). Post hoc analysis indicated that hsp70-1 mRNA levels were greater in southern fish gills than in northern fish gills following 15 and 60 min of heat shock (*P* = 0.003 and 0.023 respectively) with a similar trend at 30 min (*P* = 0.051), whereas levels converged later in the time course (*P* = 0.388, 0.530, 0.159, and 0.081 for 90–240 min). Similar results were obtained in muscle, as hsp70-1 increased significantly with heat shock (*P* < 1 × 10⁻¹⁵), and there were significant differences between subspecies (*P* = 0.002) and a significant interaction between factors (*P* = 0.008, Fig. 1B), but neither subspecies nor interaction effects were detected in post hoc tests. However, consistent with the observations in gill tissue, there was a trend to increased mRNA levels in southern fish muscle early in the time course (*P* = 0.05 and 0.052 at 15 and 30 min of heat shock).

In gill tissue, hsp70-2 increased significantly with heat shock (*P* < 1 × 10⁻¹⁵) and differed significantly between subspecies (*P* = 0.018) with no significant interaction between the factors (*P* = 0.502, Fig. 1C). Although hsp70-2 mRNA levels tended to be lower in southern fish gills throughout the time course, post hoc tests did not detect significant differences between the subspecies (*P* > 0.1) except at 1 h into recovery (180 min *P* = 0.013). Again, in muscle the results were similar; there was a significant effect of heat shock (*P* < 1 × 10⁻¹⁵) and subspecies (*P* = 0.002) on hsp70-2 mRNA levels with no significant interaction of effects (*P* = 0.782, Fig. 1D). Hsp70-2 mRNA levels in muscle did not differ between the subspecies early in the time course (*P* > 0.3) but were significantly greater in

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1 The online version of this article contains supplemental material.
northern fish at 90 and 120 min of heat shock (P = 0.021 and 0.009, respectively) converging later during recovery (P = 0.072 and 0.931 at 180 and 240 min).

In gill tissue, two-way ANOVA detected a small (1.5-fold) but statistically significant induction of hsc70 by heat shock (P = 0.012), with no significant differences between subspecies (P = 0.584, Fig. 1E) or interaction between the factors (P = 0.637). Post hoc analyses, however, did not detect a significant effect of heat shock in either subspecies. In muscle, two-way ANOVA detected a significant effect of heat shock on hsc70 (P = 0.0009), but no significant effect of subspecies (P = 0.055) or an interaction (P = 0.377, Fig. 1F). As was the case in gill, post hoc analysis failed to reveal the effect of heat shock in either subspecies.

Effects of heat shock on 90 kDa hsps. Hsp90 was induced to a similar extent in both gill and muscle, and there were few differences in mRNA levels between fish of the northern and southern subspecies. In gill, two-way ANOVA revealed a significant effect of heat shock (P = 4 × 10^{-12}), but no significant effect of subspecies (P = 0.504) or an interaction between the factors (P = 0.762, Fig. 2A). In muscle, two-way ANOVA revealed a significant effect of heat shock (P = 4 × 10^{-12}) and subspecies (P = 0.032), and no significant interaction (P = 0.802, Fig. 2B). However, post hoc tests revealed only one significant difference between the subspecies (P = 0.038 at 90 min), with southern fish having lower expression than northern fish in muscle.

Hsp90β, the constitutive isoform, was not significantly induced by heat shock (P = 0.087 in gill, P = 0.094 in muscle) and did not differ between the subspecies in either tissue (P > 0.1), and there were no significant interactions (P > 0.7; Fig. 2, C and D).

Effects of heat shock on other hsps. We also examined the mRNA levels of three additional hsps, hsp27, hsp30, and hsp60 following heat shock. In gill, hsp27 was significantly affected by heat shock (P = 0.009) and subspecies (P = 0.00003), and there was no significant interaction between these factors (P = 0.534, Fig. 3A). In general, hsp27 levels were greater in the gill in southern fish than in northern fish, although this effect was detected as significant in post hoc tests at only 120 min (P =
0.045; \( P > 0.1 \) at all other time points except at 60 min where \( P = 0.058 \). In muscle, hsp27 was significantly affected by heat shock \( (P = 9 \times 10^{-15}) \), but there was no significant effect of subspecies \( (P = 0.080) \) or an interaction \( (P = 0.111, \text{Fig. 3B}) \). However, post hoc tests detected a significant difference between northern and southern fish in muscle at 15 min \( (P = 0.015) \) and 30 min of heat shock \( (P = 0.004) \), suggesting that, in muscle, southern fish induce hsp27 earlier and to a somewhat greater extent than do northern fish, although mRNA levels converge later in the time course.

In gill tissue, Hsp30 was not significantly induced by heat shock \( (P = 0.653) \), but there was a significant difference between the subspecies \( (P = 9 \times 10^{-7}) \), and no significant interaction between the factors \( (P = 0.731, \text{Fig. 3C}) \). Levels tended to be greater in southern fish than in northern fish, but post hoc tests detected significant differences between subspecies at only 15 min of heat shock \( (P = 0.019) \) and 2 h of recovery \( (240 \text{ min } P = 0.044, \ P > 0.1 \) for all other time points). The levels of hsp30 mRNA in muscle were significantly affected by both heat shock \( (P = 0.0002) \) and subspecies \( (P = 0.0001) \), and there was no significant interaction between the factors \( (P = 0.435, \text{Fig. 3D}) \). In general, hsp30 mRNA levels were greater in southern fish than in northern fish, although significant differences were only detected between subspecies at 15 min \( (P = 0.008) \) and 30 min \( (P = 0.01) \) in post hoc tests. The effect of heat shock was detected as significant by post hoc tests only in northern fish at 180 and 240 min \( (P = 0.029 \text{ and } 0.024 \text{ respectively}) \).

In gill, hsp60 was induced by heat shock \( (P < 1 \times 10^{-15}) \), but there was no significant effect of subspecies \( (P = 0.407) \) or an interaction between the factors \( (P = 0.064, \text{Fig. 3E}) \), and similar results were observed in muscle \( (P < 1 \times 10^{-15}, = 0.071, = 0.762 \text{ for heat shock, subspecies, and interaction, respectively; Fig. 3F}) \).

**Absolute levels of hsp mRNAs.** Figure 4 presents the absolute mRNA amounts for each gene in tissues under control conditions (Fig. 4, A and C) and at the time point of maximal induction for that mRNA in each tissue (Fig. 4, B and D) to examine the data as absolute rather than fold changes in mRNA. Since all real-time PCRs were performed using the same amount of input RNA these values are effectively normalized to total RNA within the tissue.

Under control conditions in gill only hsc70, hsp90\( \beta \), and EF-\( 1 \alpha \) were expressed at levels substantially above the detection limit of the technique (Fig. 4A), and the expression of these genes did not increase markedly with heat shock. In contrast, hsp27 and hsp90\( \alpha \) increased detectably, and hsp70-2 expression increased such that its absolute expression following heat shock was similar to that of the constitutively expressed genes (Fig. 4B). Although mRNA levels were different between tissues (note difference in scales, Fig. 4), similar overall patterns of induction were seen for these genes in both gill and muscle.

**Heterologous microarray analysis.** Heterologous hybridization of *F. heteroclitus* muscle cDNA to the cGRASP salmonid array resulted in binding to 1,708 of the 16,006 spots. Of the 1,708 spots that could be detected using hybridization of *F. heteroclitus* cDNA to this microarray, there were 161 that differed significantly either between subspecies (125 spots), with heat shock (34 spots) or showed a significant interaction between these two factors (2 spots) in two-way ANOVA. Replicated spots on the array were grouped into clusters of putative genes or gene families, based on their annotated gene descriptions, resulting in 50 identified genes or gene families,

![Fig. 2. Relative mRNA levels for hsp90\( \alpha \) (gill, \( A \); muscle, \( B \)) and hsp90\( \beta \) (gill \( C \); muscle, \( D \)) for northern (solid circles and lines) and southern (open circles and dashed lines) killifish over a 2 h 34°C heat shock and 2 h 20°C recovery. *Significant difference between subspecies at the time point. Points sharing the same letters do not differ significantly within subspecies for the expression of a given gene. Data are means ± SE, calculated relative to the expression of EF-1\( \alpha \) for gill and to the geometric mean of LDH-A, COXII, and EF-1\( \alpha \) for muscle. For clarity data are expressed relative to the mean of the northern fish at time zero for each gene and tissue. \( P < 0.05 \) for all significant comparisons.](http://physiolgenomics.physiology.org/)

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that differed by subspecies, nine that differed in response to heat shock, and two that showed a significant interaction between subspecies and heat shock (for a total of 61 identified genes/gene families). In addition, 19 differentially expressed spots had no known function (14 with a subspecies effect and 5 with a heat shock effect).

For 12 of the 61 identified genes with significant effects of subspecies, heat shock or an interaction, there were additional spots on the array with the same gene name that did not show any significant effect. This lack of technical consistency across the array suggested the possibility that some of the spots detected as significant might represent false positives or might represent members of a differentially regulated gene family. We reasoned that, since hybridization on the array occurs as a nucleotide to nucleotide binding reaction, using a nucleotide BLAST strategy to assign gene identity clusters (Ref. 3; BLASTn) might be more relevant to our heterologous experiment than the approach that had been used to annotate the array [using translated clone sequences to query either a protein sequence or translated nucleotide database (71)]. Thus, we used nucleotide BLAST to interrogate the nonredundant RefSeq database (55) with the sequences of all clones from the 12 gene identity clusters that had returned inconsistent results on the microarray. For example, 27 spots on the array were annotated as ubiquitin, and only a single spot met our cutoff of \( P = 0.01 \) as a gene significantly affected by heat shock. However, an additional six spots had \( P \) values close to 0.05, while 14 of the 27 spots had \( P \) values ranging from 0.13 to 0.99, and two spots gave no detectable hybridization. The 27 clones sequences corresponding to these spots retrieved three distinct accessions: one similar to ubiquitin-like protein (AF510711.1), one similar to ubiquitin (AB036060.1), and one similar to poly-ubiquitin (AF361365.1). Clones most similar to ubiquitin-like protein had \( P \) values for the effect of heat shock ranging from 0.38 or no detectable hybridization to \( F. \) heteroclitus cDNA, whereas those most similar to ubiquitin had \( P \) values for the effect of heat shock ranging from 0.13 to 0.99. In contrast, clones that shared greatest sequence identity with poly-ubiquitin had \( P \) values ranging from 0.005 to 0.060. Therefore, we divided the annotated “ubiquitin” spots into three groups, and computed the average signal from spots that had sequences most similar...
Fig. 4. Estimated absolute mRNA amounts for northern (black bars) and southern (grey bars) killifish under constitutive (gill; A; muscle, C) and maximal (gill; B; muscle, D) conditions. Bars representing each gene within a single panel that share the same letter do not differ in mRNA amount within a subspecies. Daggers indicate significant differences in mRNA amounts between gill and muscle for a given gene under either constitutive or maximally induced conditions. All data are expressed as means ± SE with P < 0.05 for all significant comparisons.

to “poly-ubiquitin” for each fish. When reanalyzed using two-way ANOVA, “poly-ubiquitin” was significantly affected by heat shock (P = 0.0098). We used this analytical approach for all 61 of the identified genes on the array. This more stringent analysis resulted in only 44 genes that differed in expression between subspecies or heat shock. One of the two genes that had initially shown a significant interaction between heat shock and subspecies, glutathione peroxidase, was detected as significantly expressed by only two of the >20 clones annotated as glutathione peroxidase on the salmon array, and only one of these clones showed a significant interaction between heat shock and subspecies, whereas the other clone did not show a significant effect of either factor or the interaction. However, BLAST analysis suggested that these two clones represented different isoforms, opening the possibility that at least one isoform of glutathione peroxidase may be differentially affected by heat shock between the two subspecies of F. heteroclitus, although this possibility remains to be confirmed. In contrast, the other gene that initially showed a significant interaction between heat shock and subspecies, 60S ribosomal protein P2, was detected as significantly expressed by three of the 12 clones with this annotation on the array, and only the originally identified clone showed any significant effect of subspecies or heat shock or an interaction between the main effects. BLAST analysis suggested that these two clones where highly similar in sequence, suggesting that the originally detected significant interaction effect was likely a false positive. Five additional genes could not be identified based on sequence similarity at the nucleotide level or retrieved entries that did not match those annotated for the array. We consider the identity of the F. heteroclitus cDNAs hybridizing to these latter spots as uncertain, and have classified these spots as “unknown.”

Table 2. Genes whose expression varied significantly with heat shock treatment

<table>
<thead>
<tr>
<th>Putative Identification</th>
<th>North Control</th>
<th>North Heat Shock</th>
<th>South Control</th>
<th>South Heat Shock</th>
<th>P Value</th>
<th>Fold Induction (Heat Shock/Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMGBl</td>
<td>1.20 ± 0.06</td>
<td>0.83 ± 0.07</td>
<td>1.00 ± 0.05</td>
<td>0.88 ± 0.19</td>
<td>0.00437</td>
<td>0.78</td>
</tr>
<tr>
<td>Hsc70a</td>
<td>0.47 ± 0.04</td>
<td>1.55 ± 0.20</td>
<td>0.44 ± 0.08</td>
<td>1.38 ± 0.14</td>
<td>0.00016</td>
<td>3.2</td>
</tr>
<tr>
<td>Hsp90a</td>
<td>0.52 ± 0.08</td>
<td>1.47 ± 0.21</td>
<td>0.40 ± 0.06</td>
<td>1.53 ± 0.27</td>
<td>0.00002</td>
<td>2.8</td>
</tr>
<tr>
<td>Hsp90B</td>
<td>0.58 ± 0.08</td>
<td>1.41 ± 0.15</td>
<td>0.54 ± 0.11</td>
<td>1.68 ± 0.26</td>
<td>0.00058</td>
<td>3.3</td>
</tr>
<tr>
<td>Hsp30</td>
<td>0.09 ± 0.06</td>
<td>1.94 ± 0.34</td>
<td>0.09 ± 0.07</td>
<td>1.93 ± 0.52</td>
<td>0.00023</td>
<td>22</td>
</tr>
<tr>
<td>Poly-ubiquitin</td>
<td>0.71 ± 0.04</td>
<td>1.15 ± 0.21</td>
<td>0.95 ± 0.08</td>
<td>1.27 ± 0.19</td>
<td>0.00980</td>
<td>1.5</td>
</tr>
<tr>
<td>Torb1</td>
<td>0.36 ± 0.03</td>
<td>0.96 ± 0.11</td>
<td>0.86 ± 0.13</td>
<td>1.42 ± 0.06</td>
<td>0.00192</td>
<td>1.7</td>
</tr>
<tr>
<td>Upstream stimulatory factor 1</td>
<td>0.41 ± 0.07</td>
<td>1.68 ± 0.12</td>
<td>0.39 ± 0.11</td>
<td>1.36 ± 0.19</td>
<td>0.00001</td>
<td>3.8</td>
</tr>
</tbody>
</table>

Means ± SE, n = 4. *Likely hybridizes to all members of the hsp70 gene family in F. heteroclitus.
on their probable functions. These subgroups fall into two major classes, one associated with contractile activity (subgroups 1–4) and another associated with muscle structure, growth, and development (subgroups 5–7). There was no consistent pattern either within or between these two major groups with respect to the direction of the difference in mRNA levels between populations. Seven of 20 (or 35%) of the mRNAs within the “contractile activity” group were upregulated in northern fish compared with southern fish, whereas four of eight (or 50%) of the mRNAs within the “structure, growth, and development” group were upregulated in northern fish compared with southern fish.

**Validation of microarray analysis.** Because the data presented here result from heterologous hybridization of *F. heteroclitus* cDNAs to a salmonid microarray, it is critical that the patterns of gene expression be validated with an alternative technique such as real-time PCR analysis. Interestingly, our initial real-time PCR analysis (Fig. 1) detected *hsp70-1* and *hsp70-2* as being significantly upregulated by heat shock, and *hsc70* as having no significant induction. In contrast, the...
microarray analysis detected no hybridization of F. heteroclitus cDNA to the many spots annotated as hsp70 and suggested that hsc70 was strongly upregulated by heat shock (Table 2). Examination of the sequences revealed that the arrayed clones of hsp70 contained largely 3’-untranslated region (UTR) sequences from which BLASTn analysis detected no significant sequence similarity with F. heteroclitus hsp70-1, hsp70-2, or hsc70, accounting for the lack of detectable hybridization. However, BLASTn analysis of the hsc70 clones present on the array indicated that the salmonid hsc70 clones had significant sequence identity with all of F. heteroclitus hsp70-1, hsp70-2, and hsc70. This suggests that detectable hybridization may occur between the salmonid hsc70 spots on the array and all of the F. heteroclitus hsp70 gene family members. Consequently, we summed the absolute expression levels obtained using real-time PCR across all of the hsp70 family members (hsp70-1, hsp70-2, and hsc70) to provide a rough estimate of the behavior of this gene family as a whole. These results, and results of the same technique applied to the hsp90 gene family (hsp90α and hsp90β) are presented in Fig. 5 for comparison to the microarray values. This analysis indicates that the microarray provides a good representation of the heat shock-mediated increase in expression of both the hsp70 and hsp90 gene families.

The results of real-time PCR validation studies for a selected set of genes are also summarized in Fig. 5. In all but one case, differences in expression that were detected as significant on the microarray were also detected as significant in data obtained by qRT-PCR, and the magnitude and direction of these differences were also similar across techniques. The one exception, hsp30, is a gene with very low expression levels under both control and heat shock conditions (Fig. 4). Its levels were close to the detection limits of the real-time PCR technique, and thus highly variable, which may have impeded the detection of a statistically significant increase in expression.

Further examination of genes with potential interactions. Although no genes were identified as having statistically significant interactions between heat shock and subspecies in the microarray analysis when we applied conservative criteria based on replication of spots across the array, the interaction P values of 15 genes fell close to our cutoff of α = 0.01. As heterologous microarray approaches are known to underestimate differences in expression (56), potentially inflating the probability of a type II statistical error, we chose to examine a subset of these genes further. In particular, several of these genes were involved in mitochondrial functions (including ATP synthase α-chain, ATP synthase β-chain, ATP synthase lipid-binding protein, and cytochrome oxidase subunit 1). Given that the F. heteroclitus subspecies differ in the acute response of mitochondrial respiration to thermal stress (20), we were interested in determining whether these genes varied in their responses to heat shock between the subspecies. Therefore, we used qRT-PCR to validate possible interactions for the ATP synthase α-chain (ATPSyna), and cytochrome oxidase subunit 1 (COXI) genes (Fig. 6). For ATPSyna, significant effects of population (P = 0.27), heat shock treatment (P = 0.23), and interaction (P = 0.14) were not detected using real-time PCR. For COXI, significant effects of population (P = 0.006) and heat shock treatment (P = 0.001) were detected using real-time PCR, but there was no significant interaction (P = 0.46). Yet, post hoc tests comparing levels within each population detected a significant effect of heat shock in southern (P = 0.026), but not northern (P = 0.47), fish.

**DISCUSSION**

F. heteroclitus subspecies have different maximal thermal tolerance (CTMax) and different HSRs; however, the pattern of variation in the HSR between subspecies was complex. Some inducible hsp50s showed greater induction in the high temperature-tolerant southern fish (hsp70-1, hsp70-2, hsp30), and others showed greater induction in less tolerant northern fish (hsp70-2, hsp90α); see online Supplemental Table S1 for a summary of these patterns. These results are largely consistent with, and extend, the results of Fangue et al. (18), who examined the expression of hsc70, hsp70-1, hsp70-2, and hsp90α in F. heteroclitus gill at a single time point during recovery from acute thermal stress. Because we have examined expression across a time course, the current data demonstrate
that differences in gene expression between the subspecies are not the result of differences in the kinetics of the HSR, that complex patterns are observed in both gill and muscle tissue, and that this complexity extends to additional classes of hsp5s. Although some of these hsp5s (90, 70, and 30 kDa classes) were detected as significantly upregulated by heat shock on the heterologous microarrays, these assays did not detect significant differences between the subspecies. This discrepancy between real-time PCR and heterologous microarray is most likely attributable to cross hybridization to the array among the members of a gene family, leading to a homogenized signal between constitutive and inducible isoforms. Despite these limitations of the heterologous hybridizations, we were able to detect differences in expression of a variety of genes between the subspecies and with heat shock that were subsequently confirmed using qRT-PCR.

**Differences in patterns of hsp expression between subspecies.** Our real-time PCR data indicate that the small hsp5s (hsp27 and hsp30) were induced by heat shock to a greater extent in both the gill and muscle of southern fish, whereas hsp90α was induced by heat shock earlier and to a greater extent in the muscle (but not the gill) of the northern subspecies. Additionally, there were also opposite patterns of expression within a single hsp gene family. In the 70 kDa class hsp70-1 showed earlier and greater induction in the gill (and to a certain extent in the muscle) of southern fish, whereas the closely related hsp70-2 was induced to a greater extent in both gill and muscle of northern fish.

These conflicting results highlight an ongoing lack of consensus within the literature on the expected relationship between the HSR and whole organism thermal tolerance. For example, it has often been suggested that because of the protective effect of the HSR, organisms with greater thermal tolerance should show greater induction of hsp5s (21), a contention that has been supported by the results of many studies (5, 23, 28, 38, 60, 68). However, it can also be argued (with equal plausibility) that organisms having greater thermal tolerance could be expected to have a reduced HSR relative to those with lower tolerance. For example, organisms with greater thermal tolerance could simply have a protein pool that is less susceptible to thermal damage, and thus a standardized thermal stress would be expected to cause less protein denaturation. Since protein denaturation is one of the primary signals that induces the HSR (70), less extensive denaturation would likely trigger a smaller HSR. Similarly, some organisms adapted to higher temperatures have high levels of constitutive HSPs (15, 44, 68), which may help to stabilize the protein pool during heat shock, resulting in less protein denaturation in response to a standardized thermal stress, thus triggering a smaller HSR. Increased levels of constitutively expressed HSPs are thought to act as negative regulators of the HSR (76), which would be expected to blunt the HSR of southern fish. A variety of studies provide support for a pattern in which organisms from warmer habitats exhibit a reduced HSR compared with organisms from cooler habitats (4, 5, 25, 34, 37, 44, 65, 78). In addition, neural (54) and hormonal (1, 6, 14, 31, 61) influences are also known to regulate the extent of the HSR, but the relative impact of each of these mechanisms on different hsp families is unknown.

The observation that the extent of induction of hsp5s can differ between two closely related subspecies in opposite directions for different genes implies that no single model can capture the complexities of the regulation of all classes of hsp5s (and hints at possible differences in their functional significance for whole organism thermal tolerance). It is possible that these differences relate to the distinct roles that different families of HSPs play within the cell. For example, the small heat shock proteins, shsps, typically act in an ATP independent manner to prevent the cytotoxic aggregation of unfolded proteins but do not appear to be involved in protein refolding following heat stress (26, 42), whereas the hsp70s and hsp90s are ATP-dependent chaperones that are involved in ensuring appropriate protein folding during synthesis, refolding denatured proteins, and targeting damaged proteins for degradation (48). It is only recently, with the advent of techniques such as RNAi, that it has become possible to begin to disentangle the relative functional contributions of these proteins. Using siRNA, a recent study in tomatoes (67) demonstrated that certain shsps and hsp70 were critical for the protection of cellular proteins from unfolding. Similar studies in which
multiple hsps are knocked down singly and in combination to assess effects on thermal tolerance have not yet been performed in animals. Our data suggest that such studies will be necessary to effectively distinguish the functions and regulation of the various hsp family members.

Efficiency of a heterologous microarray approach. Although the family Salmonidae and the family Fundulidae are separated by 200–300 million yr of evolution (30, 35, 45, 77), it was possible to obtain detectable and interpretable signals of gene expression in F. heteroclitus using a distant heterologous salmonid cDNA microarray. As expected because of phylogenetic distance (56), the total number of spots that could be detected in these cross-species hybridizations (11% of the spots) was lower than that typically observed with homologous hybridization for muscle tissue on this array (56% of the spots; 71). However, the decrease that we observed was also somewhat greater than has been estimated in previous work with heterologous hybridization in fish (56). Renn et al. (56) tested a small (4,500 feature) cDNA microarray for an African cichlid fish (Astatotilapia burtoni) against various other fish species. When testing the array against Atlantic salmon brain cDNA, they detected hybridization to 66% of the features on the array compared with detection of >94% of the features in homologous hybridizations. The slightly greater detection efficiency of heterologous hybridization by Renn et al. (56) compared with our study cannot be explained by differences in phylogenetic distance, since salmon are approximately equally distant from F. heteroclitus and A. burtoni. Instead, the differences in the efficiency of hybridization are most likely to be a product of the characteristics of the arrays used in these studies. The A. burtoni array was constructed using full-length cDNAs (56), whereas the cGRASP Atlantic salmon array (cGRASP and Refs. 58, 71) was biased toward clones containing the 3′ regions of genes to provide discrimination among closely related paralogs. The bias toward 3′-UTRs would tend to reduce the probability of sequence conservation between F. heteroclitus and salmonids, because of the high evolutionary substitution rate of these sequences compared with those in coding regions (39), which could account for our somewhat decreased detection efficiency in cross-species hybridizations. In addition, it is likely that these two arrays differ in the subset of genes that they contain. Although a direct comparison is not possible, because the A. burtoni array has not yet been annotated, differences in the degree of conservation of the genes represented on the A. burtoni array compared with the cGRASP array could also result in differences in the relative efficiency of cross-species hybridization.

In general, when real-time PCR was used to confirm the results of the microarray, both the direction and magnitude of detected changes were supported (Fig. 5). One important exception to this pattern, however, was the hsp70 gene family. Because of the high sequence similarity in the protein-coding regions of the members of this gene family, it is likely that cross-hybridization occurs and the observed signal on the microarray represents the summed expression of all family members. The important message from this observation is that heterologous microarray experiments may be unable to detect differences in expression among closely related members of a single gene family and that this homogenization of expression could obscure substantial, and potentially functionally important, differences in expression among isoforms. However, this concern may not be generally applicable since we were clearly able to distinguish among three members of the ubiquitin gene family. The relative degree of sequence conservation among the members of these families is likely to have played a role in the difference between these two observations.

Other genes affected by heat shock. More than half of the genes detected as upregulated in response to heat shock on the heterologous microarray were hsps, suggesting either that these proteins play the largest role in mitigating the heat damage to the cell, or that the other proteins involved in the HSP are poorly conserved and thus not detectable in cross-species hybridizations. Other microarray experiments have shown that hsps are the most responsive genes to heat shock in fish (11), which is typical of a classic HSR (40). The lack of other proteins upregulated in our experiment may be an indicator of the severity of the stress, as the heat shock temperature used in this experiment is within a few degrees of the critical thermal maximum for F. heteroclitus (18). The other three genes that were identified as upregulated also have known functions consistent with a response to heat shock. Ubiquitin is a protein involved in targeting other proteins for proteosomal degradation (73). TOB1, or Transducer of ERBB2, is an antiproliferative protein that suppresses cell growth, and upstream stimulatory factor (USF) is a transcription factor that has been suggested to interact with the heat shock factor at the promoters of some genes (24). The single mRNA that was downregulated, high mobility group protein-1 (HMGB1), has been shown to be negatively associated with high temperature exposure in Austrofundulus limnaeus (49). HMGB1 regulates the global accessibility of DNA to transcription factors such that higher levels increase the accessibility of the DNA (2, 46). It is possible that this gene is, in part, responsible for the global decrease in transcription that is typical of the HSR (40).

Constitutive differences between subspecies. We observed extensive differences in gene expression in body musculature between subspecies and among individuals under control conditions. However, there were limited similarities between the gene expression differences between populations detected here for white muscle and those previously detected for this species in cardiac muscle (47, 72). In fact, in the two previous studies on cardiac muscle, only two genes were detected as differentially regulated between subspecies in both studies: cytochrome c oxidase and glutathione peroxidase. Interestingly, both of these genes were detected as differentially regulated in the current study in body muscle. Glutathione peroxidase was detected as having a significant interaction between heat shock and subspecies in body muscle, at least for one of the two cross-hybridizing spots on the array. Although we did not detect a significant difference between subspecies (P = 0.097), the direction of the trend was consistent with the previously detected significant differences in cardiac muscle in one study (72), but not another (47). In contrast, cytochrome c oxidase was detected as significantly different between subspecies in all studies (current study and Refs. 47, 72), although the effects varied across subunits of this multiprotein complex (current study and Ref. 72). The small number of genes whose expression differed consistently between studies can, in part, be attributed to differences in tissue-specific gene regulation and in part to differences in gene representation across the arrays used. For example, only 8 of the 13 genes that Whitehead and Crawford (72) detected as having a significant phylogenetically independent association with habitat temperature/latitude were
represented on the cGRASP salmon array, and only two of these (glutathione peroxidase and cytochrome oxidase) showed significant cross-hybridization with *F. heteroclitus* cDNA.

In body muscle, the genes that differed in expression between populations were associated with excitation-contraction coupling and contraction and metabolism and could represent differences in muscle fiber type between the subspecies. These differences in gene expression could underlie previously observed differences in swimming performance between these subspecies (16, 19). For example, we have shown that the critical swimming speed of northern killifish plateaus as temperature is acutely increased >20°C, whereas the performance of southern killifish increases (19). Our results suggest that differences in muscle phenotype may underlie these differences in the thermal sensitivity of performance.

Compared to southern fish, northern fish had higher levels of parvalbumin, which is known to decrease relaxation time and increase contraction speed (8, 27, 29, 74) and lower levels of both creatine kinase and SERCA2. Interestingly, there is evidence of a connection between parvalbumin and the creatine-kinase shuttle; Gallo et al. (22) found that a supplemented creatine-phosphate shuttle in rats reduces levels of parvalbumin. These authors suggested that higher capacity of high-energy phosphate shuttling reduces the need for intracellular calcium buffering by parvalbumin. This observation is consistent with the differences in expression of parvalbumin and creatine kinase between northern and southern killifish. Additionally, lower parvalbumin levels should increase the efficiency of SERCA2 and sarcoplasmic calcium uptake (22), which is again consistent with our observed expression differences between northern and southern fish. The subspecies also differed in the expression of cytochrome c oxidase, although the direction of this difference was variable among several of the subunits of this multisubunit enzyme. These data are consistent with the data of Whitehead and Crawford (72), who detected evidence of significant directional selection on the expression of two subunits of cytochrome c oxidase in *F. heteroclitus* cardiac muscle but found that the direction of this difference was opposite across the subunits. Levels of cytochrome c oxidase have been correlated with swimming performance in cod, *Gadus morhua* (41), suggesting a possible functional role for this enzyme, although the exact mechanistic impacts of this variation are unclear given the differing results among subunits.

**Potential effects on mitochondrial genes.** Our microarray data indicate the possibility of a consistent effect of heat shock on the expression of mitochondrial genes. When comparing the mean expression values from the microarray for four of the genes with mitochondrial function (*ATPsynthase-α, ATPsynthase-β, COXI, ATP synthase lipid binding protein*), all four decreased with heat shock in northern fish (0.7- to 0.8-fold) and increased in southern fish (1.2- to 1.9-fold). Although this potential interaction between subspecies and the response to heat shock was not detected as statistically significant in either the microarray array data (*P* < 0.01) or in validations with real-time PCR (*P* < 0.05), these observations suggest the potential for a unified, although minor, difference in the response of the mitochondria to acute thermal stress between subspecies. This hypothesis is consistent with the observations of Fangue et al. (20), who demonstrated that mitochondrial respiration rates respond differently to increased temperature between the subspecies with southern fish increasing oxygen use at a faster rate. Additionally, Fangue et al. (20) found that this trend was exaggerated at low acclimation temperatures. Therefore, it is possible that the trend we detected in gene expression experiments in fish acclimated at 20°C could become more evident in fish acclimated at low temperatures.

**Summary**

We have demonstrated that the HSR differs between the northern and southern subspecies of *F. heteroclitus* and that the differences in extent of *hsp* expression between subspecies cannot simply be accounted for by differences in the kinetics of induction of these genes. Several *hsp*s, including *hsp*70-1 and the small *hsp*s, *hsp*27, and *hsp*30, were upregulated to a greater extent in southern fish than in northern fish. In contrast, *hsp*70-2 (which was induced at by far the highest level of all of the *hsp*s) was upregulated to a greater extent in northern fish than in southern fish. These data indicate that a simple global model for the regulation of the HSR in *F. heteroclitus* (such as overall differences between subspecies in the sensitivity of the protein pool to denaturation) is unlikely to fully account for the differences in the HSR between subspecies. Heterologous microarray analyses revealed few genes that differed between subspecies in their response to heat shock but suggested that differences in muscle structure or function could be involved in whole organism phenotypes such as differences in swimming performance in response to thermal stress.

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**DISCLOSURES**

No conflicts of interest are declared by the authors.

**REFERENCES**

HEAT SHOCK RESPONSE OF Fundulus heteroclitus


