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# Intraspecific variation in thermal tolerance and heat shock protein gene expression in common killifish, *Fundulus heteroclitus*

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## Summary

**Populations of common killifish, *Fundulus heteroclitus*, are distributed along the Atlantic coast of North America through a steep latitudinal thermal gradient. We examined intraspecific variation in whole-animal thermal tolerance and its relationship to the heat shock response in killifish from the northern and southern extremes of the species range. Critical thermal maxima were significantly higher in southern than in northern fish by ~1.5°C at a wide range of acclimation temperatures (from 2–34°C), and critical thermal minima differed by ~1.5°C at acclimation temperatures above 22°C, converging on the freezing point of brackish water at lower acclimation temperatures. To determine whether these differences in whole-organism thermal tolerance were reflected in differences in either the sequence or regulation of the heat shock protein genes (*hsps*) we obtained complete cDNA sequences for *hsc70*, *hsp70-1* and *hsp70-2*, and partial sequences of *hsp90α* and *hsp90β*. There were no fixed differences in amino acid sequence between populations in either *hsp70-1* or *hsp70-2*, and only a single conservative substitution between populations in *hsc70*. By contrast, there were significant differences between populations in the expression of many, but not all, of these genes. Both northern and southern killifish significantly increased *hsp70-2* levels above control values ($T_{on}$) at a heat shock temperature of 33°C, but the magnitude of this induction was greater in northern fish, suggesting that northern fish may be more susceptible to thermal damage than are southern fish. In contrast, *hsp70-1* mRNA levels increased gradually and to the same extent in response to heat shock in both populations. *Hsc70* mRNA levels were significantly elevated by heat shock in southern fish, but not in northern fish. Similarly, the more thermotolerant southern killifish had a $T_{on}$ for *hsp90α* of 30°C, 2°C lower than that of northern fish. This observation combined with the ability of southern killifish to upregulate *hsc70* in response to heat shock suggests a possible role for these *hsps* in whole-organism differences in thermal tolerance. These data highlight the importance of considering the complexity of the heat shock response across multiple isoforms when attempting to make linkages to whole-organism traits such as thermal tolerance.**

Key words: killifish, thermal tolerance, acclimation, gene expression, heat shock proteins, Hsps, temperature, evolution, adaptation.

## Introduction

The pervasive effects of temperature on biochemical and physiological processes are thought to play a fundamental role in shaping the distribution and abundance of organisms. This is particularly true for ectotherms such as fish. Although studies comparing species that are widely divergent phylogenetically have revealed major patterns of thermal adaptation, understanding the role of temperature in establishing fine-scale patterns of thermal tolerance in closely related species or among populations within a species can provide additional insights into the nature of adaptive variation in thermal tolerance (Somero, 2002). The relationship between environmental conditions and variation in thermal tolerance, both intraspecifically and between closely related species, has been extensively studied in *Drosophila* (reviewed by Hoffmann et al., 2003). Comparisons of thermal tolerance of lab-bred flies derived from distinct geographic locations, however, have produced conflicting results. In some cases, geographic comparisons of thermal tolerance traits indicate that differences among populations are consistent with what is predicted by local environmental thermal regimes (Krebs and Loeschcke, 1995; Guerra et al., 1997; Sorensen et al., 2001). Other comparisons where latitudinal variation in thermal tolerance is expected have failed to reveal such differences (Davidson, 1990; Kimura et al., 1994). Similarly, in fish, evidence for intraspecific variation in thermal tolerance is mixed. Many studies have shown that populations of a species sampled from

differing thermal environments and acclimated to common temperatures have thermal tolerance limits such that fish from cooler latitudes exhibit lower tolerance limits than their warm-water counterparts (Hart, 1952; McCauley, 1958; Otto, 1973; Fields et al., 1987; Lohr et al., 1996; Strange et al., 2002). On the other hand, some studies have failed to show thermal tolerance differences in populations from thermally contrasting environments (Brown and Feldmeth, 1971; Elliott et al., 1994; Smale and Rabeni, 1995).

Common killifish (*Fundulus heteroclitus*), inhabit estuaries and salt marshes along the east coast of North America through a latitudinal temperature gradient, and thus have been studied extensively as a model to investigate mechanisms of thermal adaptation. It has been shown that substantial variation exists within the species in morphological, molecular, genetic and physiological traits (reviewed in Powers et al., 1993; Powers and Schulte, 1998; Schulte, 2001). This variation shows significant directional change with temperature/coastal latitude such that two distinct regional subspecies have been suggested – the northern form, *Fundulus heteroclitus macrolepidotus*, occurring from the Gulf of St Lawrence, Canada to New Jersey, USA, and the southern form, *Fundulus heteroclitus heteroclitus*, distributed from Virginia, USA to the North-eastern coast of Florida, USA (Morin and Able, 1983). At the extremes of the species' range, northern fish experience temperatures ranging from –1.4°C to 21°C, whereas southern fish encounter temperatures ranging from 7°C to 31°C, and monthly mean temperatures are on average, 13°C higher in the south than in the north at any given time of year [calculated from NOAA NERRS Data, Sapelo Island, GA and Wells Inlet, ME, USA (NOAA NERRS, 2004)]. Clearly, the ability to acclimate to seasonal and daily temperature fluctuations is critical to all killifish populations, but the temperature range over which they must make adjustments is very different between populations and seasons. Early work on the thermal tolerance limits of killifish confirmed that these fish do in fact possess the ability to acclimate and tolerate a wide range of temperatures (Bulger, 1984; Bulger and Tremaine, 1985). This work, however, was performed on a single killifish population from Virginia (southern subspecies) acclimatized to seasonal photoperiod and temperature combinations, and only upper thermal tolerance limits were quantified. These data, although useful in describing patterns of acclimation/acclimatization and tolerance for a single killifish population, provide no information about the nature of, or mechanisms involved in, intraspecific variation in thermal performance.

A number of physiological and biochemical traits that are influenced by temperature and may play important roles in thermal performance of organisms have been proposed (Hochachka and Somero, 2002). At the molecular level, many candidate genes have been identified as potential targets of adaptive evolution to temperature (reviewed in Hoffmann et al., 2003; Somero, 2005). In particular, heat shock proteins (Hsps) are thought to play an ecologically and evolutionarily important role in thermal adaptation (Parsell and Lindquist, 1993; Feder and Hofmann, 1999). As molecular chaperones, Hsps interact with proteins that are in their non-native conformation (stress denatured) in such a way that they prevent these proteins from interacting inappropriately with one another (for a review, see Lindquist, 1986; Hightower, 1991; Morimoto, 1998). It has been clearly shown that a species' threshold for Hsp expression is correlated with the levels of thermal stress they naturally experience, and that natural fluctuations in environmental temperatures are sufficient to elicit the heat shock response (Roberts et al., 1997; Tomanek and Somero, 1999; Buckley et al., 2001). Taken together, these findings suggest that Hsps are good candidates as ecologically relevant mechanisms used by animals to ameliorate thermal stress and likely have important roles in thermal adaptation.

It has long been known that heat shock proteins are encoded by multiple genes and are assigned to families based on sequence similarity and molecular mass. Two important Hsp families are Hsp90 and Hsp70, each containing several members, some of which are expressed constitutively under normal physiological conditions (i.e. Hscs) and some of which are induced in response to protein-denaturing stress (i.e. Hsps) (Gething, 1997). Members of both the Hsp90 and Hsp70 families are known to be important in folding of nascent polypeptides as well as renaturation of heat damaged proteins (Morimoto and Santoro, 1998). However, our understanding of the true biochemical diversity of the heat shock response in an ecologically relevant context remains limited, because much of the early work addressing this question was performed using one-dimensional gel electrophoresis, which often fails to discriminate among related heat shock proteins within a family. As a result of these technical limitations most of the work that has comprehensively addressed the relationship between the heat shock response, whole-organism thermal tolerance and population distribution and abundance has been performed on a few well-characterized model species (Feder and Hofmann, 1999; Sorensen et al., 1999; Sorensen et al., 2001; Sorensen et al., 2005; Michalak et al., 2001) (but see also White et al., 1994; Norris et al., 1995; Tomanek, 2005).

In this study, we quantified thermal tolerance and investigated the mRNA expression patterns of a variety of isoforms of heat shock proteins (Hsps) in killifish populations in order to address several questions. (1) Are there differences in thermal tolerance between killifish populations that correlate with latitudinal temperature ranges? (2) Is intraspecific variation in thermal tolerance related to differences in the sequence of *hsp* genes between populations? (3) Is intraspecific variation in thermal tolerance related to differences in the expression patterns of *hsp* genes? (4) Are differences in *hsp* expression patterns between populations consistent across multiple *hsp* genes? By addressing these questions we are able to provide valuable insight into how local adaptation can occur between fish populations from two different environments even when the local environments are themselves highly variable.

## Materials and methods

### *Experimental animals*

Adult killifish of the northern subspecies (*Fundulus heteroclitus macrolepidotus* Walbaum) were collected from three locations: Hampton, New Hampshire, USA (NH; 42° 54′ 46″ N), Salsbury Cove, Maine, USA (ME; 44° 25′ 54″ N) and Antigonish, Nova Scotia, Canada (NS; 45° 37′ 0″ N). Fish of the southern subspecies (*Fundulus heteroclitus heteroclitus* L.) were also collected from three sites: Brunswick, Georgia, USA (GA; 31° 7′ 31″ N), Whitney Island, Florida, USA (WI; 29° 39′ 34″ N) and Fernandina Beach, Florida, USA (FB; 30° 40′ 51″ N). All collections were made in late spring of 2002 [northern (NH) and southern (GA) thermal tolerance experiments] or 2004 [interpopulation thermal tolerance and heat shock experiments]. Fish were held in 75 l glass aquaria with biological filtration at 20 ppt salinity, 20±2°C, and 12 h:12 h L:D photoperiod for a minimum of 3 weeks before the experimental acclimations described below. Fish were fed TetraMin® fish flakes supplemented with commercial trout chow (PMI Nutrition International, Brentwood, MO, USA) daily to satiation, 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.

### *Upper and lower lethal limits*

Upper and lower thermal acclimation limits were quantified for northern (NH) and southern (GA) killifish populations using chronic thermal tolerance methodology. Following a two-week holding period at 20±0.5°C, 30 fish from each population were subjected to either increasing or decreasing water temperatures of 0.5°C per day. This rate is slow enough to allow the fish's thermal acclimation to keep pace with the temperature change but yet is ecologically realistic (Bennett et al., 1997). The respective chronic thermal maximum or minimum value is taken as the high or low temperature at which 50% morbidity is observed (Fields et al., 1987; Bennett and Beitinger, 1997).

### *Thermal tolerance methodology*

Temperature tolerance in killifish populations was determined using the critical thermal methodology (CTM). The critical thermal maximum (CTMax) and critical thermal minimum (CTMin) are typically defined as the upper and lower temperatures, respectively, at which fish lose the ability to escape conditions that will ultimately lead to death (Cox, 1974; Becker and Genoway, 1979; Beitinger et al., 2000). The CTM test chamber consisted of a plastic rectangular water bath (50×35×15 cm) containing 10 individual 1 l plastic test beakers. The water bath was filled with dilute ethylene glycol that could be heated or cooled with an immersion coil connected to a Lauda RM6 benchtop unit, and circulated with a Mag-Drive model 1.5 pump to ensure complete mixing. Each beaker was filled with seawater and individually aerated to maintain oxygen concentrations at saturation and prevent thermal stratification during the trials. Beaker temperatures were monitored with Fisherbrand® NIST certified mercury thermometers (Fisher Scientific, Nepean, ON, Canada) and heating/cooling rates were between 0.28 and 0.33°C $min^{-1}$ for all trials.

Loss of equilibrium (LOE) was chosen as our experimental endpoint, and critical thermal maxima and minima were calculated by taking the arithmetic mean of the LOE temperatures for each acclimation group (Cox, 1974; Beitinger et al., 2000). At the end of each trial, fish were weighed (wet mass ± 0.1 g), measured (total length ± 0.1 cm), and returned to their acclimation conditions for recovery. We achieved >95% post-trial survival in all acclimation groups.

### *Effects of acclimation*

We assessed the relationship between acclimation temperature and upper or lower thermal tolerance of northern (NH) and southern (GA) killifish by estimating CTMax and CTMin of fish acclimated to one of seven constant temperature treatments ranging from 2.3°C to 34.0°C. Acclimation temperatures were controlled with Fisherbrand® NIST traceable temperature controllers and Ebo Jager 250 watt submersible heaters. Killifish were acclimated for a minimum of 21 days to each treatment temperature under a 12 h:12 h (L:D) photoperiod and 20 ppt salinity. Three replicate 75 l acclimation tanks per temperature treatment were divided to house 10 northern fish on one side and 10 southern fish on the other. Five fish from each population per acclimation tank were randomly chosen to be in either a CTMax or CTMin trial. In total, 30 fish from each population ($N$=15, CTMax and $N$=15, CTMin) were tested from each acclimation group. All CTM trials were run between 10.00 am and 2.00 pm to minimize any effects of daily rhythms in thermal tolerance.

### *Intraspecific variation*

In a second experiment, we explored intraspecific variation in thermal tolerance between replicate northern and southern killifish populations. Three northern populations (NH, ME and NS) and three southern populations (GA, WI and FB) were acclimated to 22±0.25°C under identical experimental conditions to those described above. Thermal tolerance trials and calculations were performed as previously described.

### *Identification and sequencing of hsp genes*

#### *Isolation of genomic DNA, total RNA extraction, and reverse-transcriptase PCR amplification*

Several genes of interest were cloned from killifish tissues including *hsc70, hsp70-1* and *hsp70-2* (gill, liver and/or spleen), *hsp90α* (liver) and *hsp90β* (gill). The intronless *hsp70* genes were cloned from both cDNA and genomic DNA, whereas all other genes were cloned from cDNA only. Genomic DNA was isolated from killifish spleens either by proteinase K digestion followed by phenol:chloroform extraction essentially as described elsewhere (Sambrook et al., 1989), or by the salting out method (Medrano et al., 1990).

Total RNA was extracted from either heat shocked or control tissues using the guanidine isothiocyanate method

outlined elsewhere (Chomczynski and Sacchi, 1987) using TRIzol® Reagent (Invitrogen Life Technologies, Burlington, ON, Canada). Following isolation, RNA was quantified spectrophotometrically and electrophoresed on an agarose-formaldehyde gel (1% w/v agarose, 16% formaldehyde) to verify RNA integrity. RNA was stored at –80°C. First strand cDNA was synthesized from 5 μg total RNA using oligo($dT_{18}$) primer and RevertAid™ H Minus M-MuLV reverse transcriptase as per the manufacturer's instructions (MBI Fermentas Inc., Burlington, ON, Canada). cDNA was stored at –30°C for up to 1 month or at –80°C for longer-term storage.

Partial *hsp90* sequence was obtained using primers determined from conserved regions of European sea bass, *Dicentrarchus labrax* (accession no. AY395632), Atlantic salmon, *Salmo salar* (accession no. AF135117) and zebrafish, *Danio rerio* (accession no. AF042108). The forward primer was 5′-GGA CC(A/C) G(G/C/A)A ACC C(C/T)G A(C/T)G ACA T-3′ and the reverse primer was 5′-CCT G(G/T)G C(C/T)T TCA TGA TCC (T/G)CT CC-3′. All sequences were aligned with ClustalW and primers were designed with the assistance of GeneTool Lite software (www.biotool.com).

Complete sequences of two isoforms of the inducible *hsp70* and one isoform of the constitutive *hsc70* were obtained from northern killifish (NH). Degenerate primers were initially used to obtain a 1500 bp fragment in the central region of each gene. These primers were designed based on conserved regions of zebrafish *hsc70* (accession no. Y11413), *Ictalurus punctatus hsp70* (accession no. U22460), *Oncorhynchus tschawytscha hsp70* (accession no. U35064), *Xenopus laevis hsc70* (accession no. X01102) and *Gallus gallus hsp70* (accession no. J02579). The sequence of the forward primer (Hsp701F) was 5′ GGA CCA CAC C(C/A)A GCT TGG 3′ and the reverse primer (Hsp701R) was 5′ CGT TIG TGA TIG TGA TCT TGT TC 3′.

Polymerase chain reactions (PCRs) were carried out in a PTC-200 MJ Research thermocycler using *Taq* DNA polymerase (MBI Fermentas Inc., Burlington, ON, Canada) and either cDNA (for all genes) or genomic DNA (for *hsp70-1* and *hsp70-2*) isolated as described above. Each PCR consisted of 40 cycles of 30 s at 94°C, 30 s at the primer-specific annealing temperature, and 1 min for every 1,000 bp of expected product at 72°C.

PCR products were electrophoresed on 1.5% agarose gels containing ethidium bromide and bands of appropriate size were extracted from the gels using the QIAEX II gel extraction kit (Qiagen Inc., Mississauga, ON, Canada). Extracted PCR products were ligated into a T-vector (pGEM-T easy; Promega; Fisher Scientific, Nepean, ON, Canada), transformed into heat shock competent *Escherichia coli* (strain JM109; Promega; Fisher Scientific, Nepean, ON, Canada) and colonies were grown on ampicillin Luria-Bertani (LB) agar plates. Several colonies containing the ligated PCR product were selected and plasmids were isolated from liquid culture using GenElute Plasmid Miniprep kit (Sigma-Aldrich, Oakville, ON, Canada) and sequenced using an ABI 377 automated fluorescent sequencer at York University Molecular Biology core facility (Toronto, ON, Canada), or at the NAPS core facility at the University of British Columbia (Vancouver, BC, Canada). At least three clones of each fragment were sequenced bidirectionally. Consensus sequences for the *hsp90* fragments were submitted to GenBank (*hsp90α*, accession no. DQ202281; *hsp90β*, accession no. DQ202282).

To determine the complete cDNA sequences for *hsc70*, *hsp70-1* and *hsp70-2* genes, isoform-specific nested PCR primers were designed based on the central fragment sequences obtained above, and used for 5′ and 3′ rapid amplification of cDNA ends (Smart RACE cDNA amplification kit; BD Bioscience Clontech, Mississauga, ON, Canada). Primer sequences for 5′ RACE of *hsp70* were as follows: external primer (for both isoforms) 5′ TTC ACC TCA AA(C/T) ATG CCG TCC 3′; internal primer for *hsp70-1* 5′ CAT TGC GCT CTCCTC TTT TGC 3′; internal primer for *hsp70-2* 5′ TTT CTC TCT CCC GTC TTG CC 3′. Primer sequences for 3′ RACE of *hsp70* were as follows: external primer (for both isoforms) 5′ AGC CAT GAC CAA GGA CAA CAA 3′; internal primer for *hsp70-1* 5′ CCA GAG GAG TGC CAC AGA TAG AG 3′, internal primer for *hsp70-2* 5′ AGG TTT GAG CTG ACG GGA ATC 3′. Primer sequences for 5′ RACE of *hsc70* were as follows: external primer 5′ TTT GGC CGG GTG CTG TCA TTG A 3′, internal primer 5′ AAA CCG CCG GCC AAT CAA CC 3′. Primer sequences for 3′ RACE of *hsc70* were external primer 5′ CAC TGC TGG AGA TAC TCA TCT TGG TGG G 3′, internal primer 5′ GCG GTG TTC CAC AGA TTG AGG TGA CCT T 3′. Each PCR consisted of 3 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 60°C and 1 min at 72°C, followed by 7 min at 72°C. PCR products were then electrophoresed and cloned as described above.

At least three clones per fragment were sequenced in both directions at least twice, and a majority-rule consensus for the full-length cDNA transcript was developed for each isoform. Sequence assembly and analysis were performed using GeneTool Lite and DNAstar (Lasergene) software. Comparison with published sequences in GenBank was made with the BLAST algorithm (Altschul et al., 1997) and multiple alignments were produced using ClustalW (Thompson et al., 1994). Complete cDNA sequences have been deposited into GenBank (*hsc70*, accession no. DQ202278; *hsp70-1*, accession no. DQ202279; *hsp70-2*, accession no. DQ202280).

### *Sequence variation in hsc70 and hsp70 isoforms*

To determine whether there are any fixed differences in the sequences of *hsc70 or hsp70* isoforms between northern and southern populations of killifish that might affect the function of these proteins, a series of isoform-specific PCR primers were developed to amplify the complete coding region of each isoform: *hsp70-1* forward 5′ CTC AGA TCT TTT CCA CGT ACT CA 3′, *hsp70-1* reverse 5′CTC CAG TAG TGA AAT GAT GCA GT 3′; *hsp70-2* forward 5′ CTG AAA GGA AAG TGA GCC AAG ATG 3′, *hsp70-2* reverse 5′ TAA ACA GTC CAG GAG ATG AGA GT 3′; *hsc70* forward 5′ CCC GGA

GAG GTC TGC TGT GT 3′, *hsc70* reverse 5′ GGA GGT CTG AGG ATG GAA TGG T 3′. These primers were used to obtain the complete coding regions of these genes from at least three individuals from both NH and GA killifish populations. The sequences of a central fragment of the coding region of each gene (1409 bp) were also determined for an additional five individuals from each population using the following primers: for *hsp70-1* forward 5′ CAT GAA CCC CAC CAA CAC AAT C 3′, reverse 5′ CGA CAG CAG ACA CGT TTA GGA 3′; for *hsp70-2* forward 5′ CGC GTA CGG TCT GGA CAA AGG C 3′, reverse 5′ GCC CTT CAA GCT CTC GTC GTC CA 3′; for *hsc70* the original degenerate primers (HSP701F and HSP701R) were used on cDNA from control fish.

*Phylogenetic analysis*

Amino acid sequences were deduced from the nucleotide sequence of each isoform for both northern and southern fish using GeneTool Lite Software. Because there were few fixed differences between populations, only northern killifish sequences were used in the phylogenetic analysis. Protein sequences or deduced amino acid sequences were obtained from GenBank for all the available complete fish *hsp/hsc70* genes, and *Bos* and *Homo* sequences were used as representative mammalian species. Sequences were aligned using ClustalW and phylogenetic analysis was performed using the neighbor-joining method with pairwise deletion of gaps using MEGA2 software (Kumar et al., 2001). The support for each node was assessed using 1000 bootstrap replicates, and isoforms were named according to their position on the phylogenetic tree.

### *Relationship between thermal tolerance and heat shock proteins*

*Heat shock experiment*

To determine the threshold induction temperature of heat shock proteins in killifish, we acclimated northern (NH) and southern (GA) killifish to a common temperature of 20°C for 8 weeks as previously described. Groups of six fish per population were sampled directly from the acclimation tank (control) or transferred to one of several acute thermal challenge groups: 30, 31, 32, 33, 34, 35 and 36°C [GA only: preliminary experiments with NH killifish acutely transferred from 20°C to 36°C resulted in 100% mortality within 1 h] or to 20°C (handling control) for 2 h. Fish were then transferred back into 20°C recovery tanks for 1 h. We chose a 1-h recovery period based on literature values for eurythermal fish and several pilot experiments indicating that these exposure and recovery times were sufficient to induce changes in gene expression. Following the recovery period, fish were sacrificed by rapid decapitation and the gills were dissected and immediately frozen in liquid nitrogen. All tissues were stored at –80°C until analysis. We elected to examine expression in the gill because preliminary experiments indicated that inter-individual variation in *hsp* gene expression was lowest in this tissue, thus maximizing our ability to detect inter-population differences in gene expression.

Table 1. *Primers used for qRT-PCR of heat shock protein genes*

| Gene | Sequence (5′–3′) |
|---|---|
| *Hsc70* | F: ACA CCA CCA TCC CGA CAA A<br>R: CAC ACC AGG CTG GTT ATC AGA GT |
| *Hsp70-1* | F: CGG AAT AAA TGT CCT GCG GAT<br>R: CAA AAG TGC CTC CAC CAA GAT C |
| *Hsp70-2* | F: CTG ATC AAA CGC AAC ACC ACC<br>R: CTC CCC TTC GTA GAC CTG GAT |
| *Hsp90a* | F: CAG ATC TGC TGC GCT TCT ACA<br>R: CGA GAA ACA TAG TCT TTG AGG GAA AC |
| *Hsp90b* | F: TGA GCT GCT GCG CTA CCA<br>R: CAT ACG GGT GAG GTA CTC TGT CAA |
| *EF-1α* | F: GGG AAA GGG CTC CTT CAA GT<br>R: ACG CTC GGC CTT CAG CTT |

qRT-PCR, quantitative real-time PCR; F, forward; R, reverse.

*Quantitative real-time PCR analysis of hsc70, hsp70 and hsp90 gene expression*

Total RNA was extracted using TRIzol® Reagent, quantified spectrophotometrically, and cDNA was synthesized using 5 μg total RNA per sample (as previously described). Gene expression data were obtained using quantitative real-time PCR (qRT-PCR) on an ABI Prism 7000 sequence analysis system (Applied Biosystems Inc., Foster City, CA, USA). Gene-specific primers were designed using Primer Express software (version 2.0.0; Applied Biosystems Inc., Foster City, CA, USA) 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 Inc., Foster City, CA, USA) to a total volume of 22 μl under the following conditions: 1 cycle of 50°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. In addition, representative samples were sequenced to verify that the appropriate gene fragments were amplified.

Samples of RNA that had not been reverse transcribed were also subjected to qRT-PCR to detect the possible presence of genomic DNA contamination. For the constitutively expressed genes as well as the control gene elongation factor-1α, genomic DNA contamination was below 1:1024 starting cDNA copies for *hsc70*, 1:4096 for *hsp90β* and 1:2048 for *EF-1α*. For the inducible genes (*hsp70-1*, *hsp70-2* and *hsp90α*), we considered a sample to be induced when mRNA levels were at least 32-fold greater than background genomic contamination. 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).

*Statistical analyses*

Thermal tolerance data sets were analyzed by analysis of covariance (ANCOVA) with length or mass as covariates. Corrected CTM values differed by no more than 0.1°C from actual values; therefore, data sets from both the thermal tolerance and heat shock experiments were analyzed by multiple analysis of variance (ANOVA) with population, acclimation group, and/or heat shock temperature as factors without statistical adjustment for body size. Simple linear regression (SLR) and polynomial regressions were used to explore and model the statistical relationship between CTMax or CTMin of killifish and acclimation temperature. All data met the assumptions of normality, and data were log transformed where necessary to meet assumptions of homogeneity of variance. When interaction terms were not significant, *post-hoc* comparisons were performed among the groups with the Student–Newman–Keuls multiple range test (SNK MRT). If the interaction terms were significant, the data were separated and analyzed independently using one-way ANOVA. All statistical decisions were based on $P$=0.05.

## Results

*Thermal tolerance in killifish populations*

Using laboratory acclimation studies, we identified acclimation ranges and thermal tolerance scopes for killifish from northern (NH) and southern (GA) populations. Chronic thermal maximum and minimum experiments revealed pronounced differences in survival between northern and southern killifish populations (Fig. 1). Survival data were fit to a third order regression for each population, which predicted the chronic thermal maxima to be 36.4°C for northern fish and 38.2°C for southern fish. Chronic thermal minima experiments, however, revealed no difference between northern and southern populations with both populations surviving until the water ultimately froze at –1.1°C.

Acclimation temperature had a substantial effect on both CTMaxima and minima in northern (NH) and southern (GA) killifish populations such that CTM values increased with increasing acclimation temperature in both killifish populations (Fig. 2). Simple linear regressions of CTMax and CTMin on acclimation temperature for northern and southern killifish populations were highly significant (SLR, $P$<0.001, $r^2$=0.958 (CTMax) and 0.858 (CTMin), for northern fish and SLR, $P$<0.0001, $r^2$=0.935 (CTMax) and 0.910 (CTMin), for southern fish) (Table 2). Critical thermal maxima increased by 0.41°C (northern fish) and 0.36°C (southern fish) for every 1.0°C increase in acclimation temperature, whereas CTMin increased by 0.28°C (northern fish) and 0.35°C (southern fish) for every 1.0°C increase in acclimation temperature.

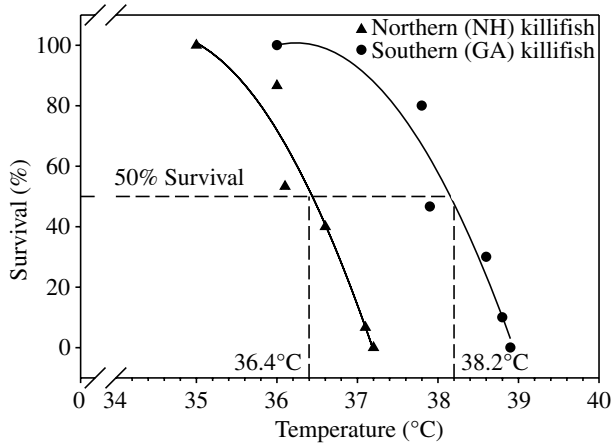


Fig. 1. Chronic thermal maxima for northern (NH, triangles) and southern (GA, circles) killifish. Chronic thermal minima (data not shown) were estimated to be –1.1°C for both populations. Data were fit with a third order regression for calculations of maxima and minima.

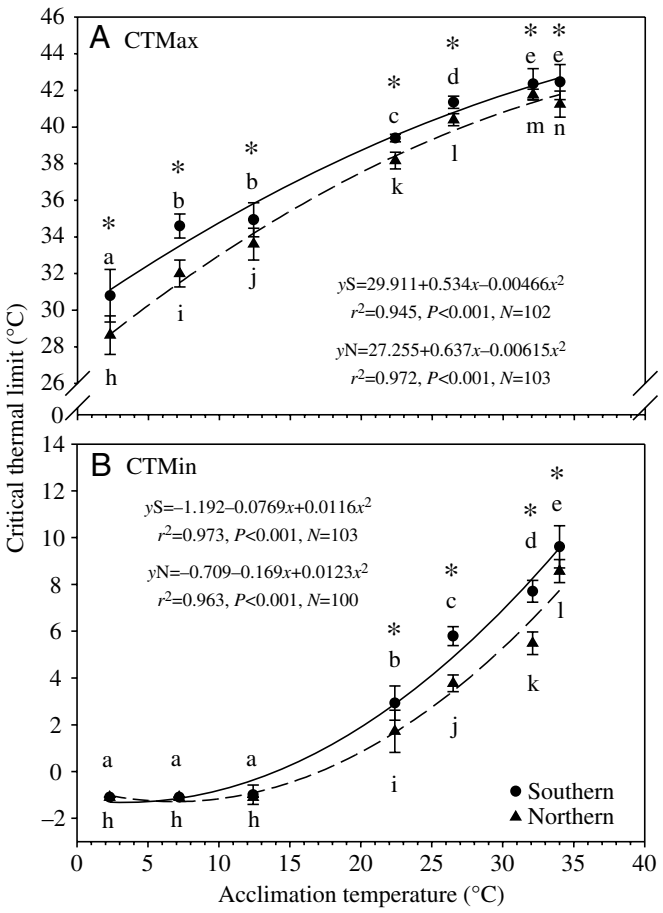


Fig. 2. Critical thermal maxima (CTMax; A) and minima (CTMin; B) for northern (NH, triangles) and southern (GA, circles) killifish acclimated to temperatures between 2.3°C and 34.0°C and a 12 h:12 h L:D photoperiod. Second order regression models of CTMax or CTMin within a population are shown. Significant differences in CTMax or CTMin within a population are indicated by different letters. An asterisk indicates a significant difference in critical thermal limit between populations at a given acclimation temperature. Values are mean ± s.d. ($N$=12–15, as indicated in Table 3); $P$<0.001 for all significant comparisons.

Table 2. *Simple linear regression equations and model comparisons of $r^2$*

| Population | Simple linear regression models | *N* | *P*-value | $r^2$ (SLR) | $r^2$ (2nd order) | $r^2$ difference (2nd order–SLR) |
|---|---|---|---|---|---|---|
| Northern (NH) | $CTMax_N$=28.525 + (0.410 * Acc. temp.) | 103 | <0.001 | 0.958 | 0.972 | 0.014 |
| Southern (GA) | $CTMax_S$=30.874 + (0.362 * Acc. temp.) | 102 | <0.001 | 0.935 | 0.945 | 0.010 |
| Northern (NH) | $CTMin_N$=–3.190 + (0.279 * Acc. temp.) | 100 | <0.001 | 0.858 | 0.963 | 0.105 |
| Southern (GA) | $CTMin_S$=–3.573 + (0.349 * Acc. temp.) | 103 | <0.001 | 0.910 | 0.973 | 0.063 |

Second order regressions are given in Fig. 2. Acc. temp., acclimation temperature.

Although simple linear regressions, as reported above, are traditionally used to model the relationship between acclimation temperature and thermal tolerance, we also used multiple regression techniques to determine the best-fit relationship for our data. Second order regression models (Fig. 2) increased the coefficients of determination ($r^2$) for each regression with the greatest improvements made in the CTMin models (Table 2). By using these second order regression models, we were able to explain 95% or more of the variation in critical thermal limit by variation in acclimation temperature in all four models.

### *Effects of body size on thermal tolerance*

There were significant differences within northern (NH) and southern (GA) killifish populations in mean length and mass among acclimation groups (Table 3). When CTM values were adjusted by analysis of covariance (ANCOVA) using either total length (cm) or wet mass (g) as covariates, the corrected CTM values differed by no more than 0.1°C from the actual values (Table 3). Therefore, no statistical adjustments of thermal tolerance values were necessary for either length or mass, and we used only the actual measured critical thermal tolerance values for all subsequent data interpretation and comparisons.

### *Intra- and interpopulation variation in thermal tolerance*

CTMax and CTMin were analyzed using two-way ANOVA with population and acclimation group as factors. Two-way ANOVA revealed a significant effect of population and acclimation group, as well as a significant interaction term for both the CTMax and CTMin data sets ($P$<0.001 for all comparisons). One-way ANOVA followed by *post-hoc* tests revealed that within a killifish population, CTMax were significantly different at each acclimation temperature with two exceptions: critical thermal maxima for southern fish acclimated to 7.2°C and 12.4°C, and critical thermal maxima for southern fish acclimated to 32.1°C and 34.0°C were not statistically significantly different from one another (SNK MRT, $P$=0.242 and $P$=0.709, respectively) (Fig. 2A). Critical thermal minima responses within a population of killifish were

Table 3. *Critical thermal maxima and minima, total length and mass for northern and southern killifish acclimated to a 12 h:12 h L:D photoperiod and constant temperatures for 21 days*

| Acc. temp. (°C) | Population | Total length (cm) | Mass (g) | CTMax (°C) (*N*) | CTMax (°C) (°C) | CTMin (°C) (*N*) | CTMin (°C) (°C) | CTMax (°C) corrected for: Length | CTMax (°C) corrected for: Mass | CTMin (°C) corrected for: Length | CTMin (°C) corrected for: Mass |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 2.3±0.47 | NH | 6.21±0.54 | 2.88±0.79 | 15 | 28.6±1.05 | 15 | –1.1±0 | 28.6 | 28.6 | –1.1 | –1.1 |
| | GA | 7.79±0.54 | 7.25±0.79 | 15 | 30.8±1.44 | 15 | –1.1±0 | 30.8 | 30.8 | –1.1 | –1.1 |
| 7.2±0.11 | NH | 6.18±0.54 | 2.76±0.77 | 14 | 32.0±0.73 | 15 | –1.1±0 | 32.0 | 32.0 | –1.1 | –1.1 |
| | GA | 7.38±0.95 | 5.13±1.92 | 14 | 34.6±0.65 | 15 | –1.1±0 | 34.6 | 34.6 | –1.1 | –1.1 |
| 12.4±0.24 | NH | 6.21±0.69 | 2.91±1.10 | 15 | 33.6±0.87 | 15 | –1.1±0 | 33.6 | 33.6 | –1.1 | –1.1 |
| | GA | 7.40±0.83 | 5.83±2.04 | 15 | 34.9±0.93 | 15 | –1.0±0.41 | 35.0 | 35.0 | –1.0 | –1.0 |
| 22.4±0.35 | NH | 6.44±0.73 | 3.24±1.29 | 15 | 38.2±0.45 | 14 | 1.7±0.90 | 38.2 | 38.2 | 1.8 | 1.8 |
| | GA | 7.76±0.92 | 7.08±2.73 | 15 | 39.4±0.20 | 15 | 2.9±0.73 | 39.4 | 39.4 | 2.9 | 2.9 |
| 26.5±0.28 | NH | 5.11±0.91 | 3.04±1.28 | 15 | 40.4±0.33 | 15 | 3.8±0.36 | 40.5 | 40.4 | 3.6 | 3.8 |
| | GA | 6.17±1.11 | 6.26±2.73 | 13 | 41.4±0.33 | 14 | 5.8±0.41 | 41.3 | 41.4 | 5.8 | 5.8 |
| 32.1±0.44 | NH | 6.57±0.75 | 3.33±1.33 | 14 | 41.8±0.29 | 14 | 5.5±0.49 | 41.7 | 41.8 | 5.5 | 5.5 |
| | GA | 7.82±0.87 | 7.13±2.23 | 15 | 42.4±0.84 | 15 | 7.7±0.47 | 42.4 | 42.4 | 7.7 | 7.7 |
| 34.0±0.32 | NH | 6.63±0.60 | 3.43±1.14 | 15 | 41.3±0.71 | 12 | 8.6±0.49 | 41.2 | 41.2 | 8.6 | 8.6 |
| | GA | 8.27±3.26 | 8.32±3.26 | 15 | 42.5±0.96 | 14 | 9.6±0.90 | 42.6 | 42.6 | 9.6 | 9.6 |

Values are mean ± s.d.; *N*, number of fish; Acc. temp., acclimation temperature; NH, northern population; GA, southern population.

also significantly different at each acclimation temperature with the exception of the three lowest acclimation temperature groups. There were no significant differences in the CTMin of northern fish acclimated to 2.3°C, 7.2°C and 12.4°C ($P$=1.000 for all comparisons). Similarly, no differences in CTMin within the southern population acclimated to 2.3°C, 7.2°C and 12.4°C were found ($P$-values between 0.544 and 1.000 for all comparisons; Fig. 2B).

Between-population comparisons revealed that the CTMax of southern fish within a temperature acclimation group was always significantly higher than the CTMax of northern fish ($P$<0.001 for all comparisons) (Fig. 2A). The CTMin of southern fish within an acclimation group were also significantly higher than that of northern fish, except at acclimation temperatures of 12.4°C or lower, where the CTMin converged at the freezing point of brackish water ($P$-values between 0.544 and 1.000 for all comparisons) (Fig. 2B).

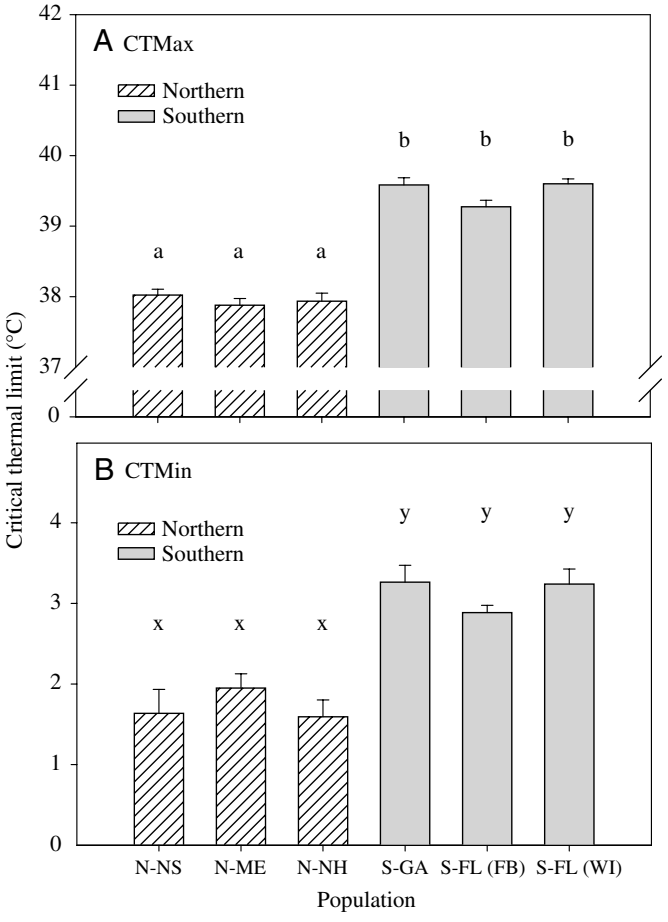


Fig. 3. Critical thermal maxima (CTMax; A) and minima (CTMin; B) for three northern [Nova Scotia (NS), Maine (ME) and New Hampshire (NH); hatched] and three southern [Georgia (GA), Fernandina Beach, FL (FB) and Whitney Island, FL (WI); grey] populations of killifish acclimated to 22°C and 12 h:12 h L:D photoperiod. Values are mean ± s.d., $N$=15; significant differences between populations within each panel are indicated by different letters ($P$<0.001 for all significant comparisons).

In order to determine if thermal tolerance differences between northern and southern killifish were consistent across multiple populations within a subspecies, we quantified CTMax and CTMin in samples of killifish from six different populations (three of the northern subspecies and three of the southern subspecies) acclimated to a common temperature of 22°C (Fig. 3). Within a subspecies, killifish populations did not differ in CTMax ($P$-values ranged from 0.060 to 0.928) or in CTMin ($P$-values ranged from 0.092 to 0.921). Between subspecies, however, southern killifish always had significantly higher CTMax ($P$<0.001 for all comparisons) and CTMin ($P$<0.001 for all comparisons) than the northern forms (Fig. 3A,B).

Northern (NH) and southern (GA) killifish were sampled from the same geographic location in two separate years and acclimated to 22°C (2002-thermal tolerance experiment, Fig. 2; 2004-intraspecific experiment, Fig. 3). There were no significant differences in CTM values within a population sampled in either year (compare Table 3 with Fig. 3).

### *Sequence variation in Fundulus hsps*

The degenerate primers used in this study allowed us to obtain three distinct heat shock protein 70-related transcripts from killifish gills. Phylogenetic analysis of the complete amino acid sequences of these transcripts (Fig. 4) revealed that one was highly similar to other fish *hsc70* sequences, whereas the remaining two transcripts were similar to other fish *hsp70* sequences. The putative *hsc70* from killifish was cloned from both control and heat shock samples, whereas the putative *hsp70* transcripts were found only in cDNA isolated from fish exposed to heat shock, confirming their identification as constitutive and inducible transcripts, respectively. The two *hsp70* transcripts did not group together phylogenetically, but instead each grouped with a distinct sequence from *Xiphophorus maculatus*. We tentatively named the *hsp70* transcripts *hsp70-1* and *hsp70-2*, following the convention established for *X. maculatus*.

In order to determine whether there were any fixed differences in *hsp* sequences between northern and southern killifish populations that might affect their function, we obtained the complete coding sequences of *hsp70-1*, *hsp70-2* and *hsc70* from a sample of individuals from each population (~10 individuals from each of the NH and GA populations). There were no fixed differences between populations in *hsp70-2*, and only a single fixed difference between populations in *hsp70-1*, which did not result in a change in the amino acid sequence. There were two fixed differences between populations in *hsc70*, one of which was silent, and one of which resulted in a change from serine in the southern population to threonine in the northern population at amino acid position 98.

Despite the high conservation of these sequences at the amino acid level, there was substantial silent polymorphism in these genes, most of which was found in the southern population, consistent with the pattern observed for other genes (e.g. Bernardi et al., 1993). There were 14 polymorphic sites

in *hsp70-1*, all of which were found in the southern population, whereas the sample from the northern populations exhibited no variation among individuals. Similarly, *hsp70-2* had five polymorphic sites, only one of which was found in the northern population. by contrast, there were three silent polymorphic sites in *hsc70* (in addition to the two fixed differences), two of which were present in both populations, and one of which was found only in the northern population.

### *Variation in hsp expression*

Prior to heat shock treatment, there were no differences in control and handling control mRNA levels in the gill between northern (NH) and southern (GA) killifish for any of the three *hsp70* genes measured. The mRNA levels for *hsc70* (constitutive isoform) in northern fish did not change with heat shock (Fig. 5A). Southern fish, however, had elevated *hsc70* mRNA levels in all heat shock groups, and this increase was significant in the 32°C group. As a result, southern fish had significantly higher *hsc70* mRNA at all heat shock temperatures when compared to northern fish ($P$-values $\leqslant$0.019 for all comparisons).

The patterns of mRNA expression differed substantially between *hsp70-1* and *hsp70-2* (inducible isoforms). Levels of *hsp70-1* increased gradually with increasing heat shock temperatures in both populations, and there was no difference between populations in the magnitude of this progressive induction (Fig. 5B). The induction profile for *hsp70-2* was more typical of an inducible gene with a clear onset temperature of expression (Fig. 5C). Both northern and southern killifish demonstrated a significant elevation above control values in mRNA levels ($T_{on}$) for *hsp70-2* at 33°C. However, northern fish had significantly higher levels of *hsp70-2* at 33°C, 34°C and 35°C than southern fish ($P$<0.05 for all comparisons).

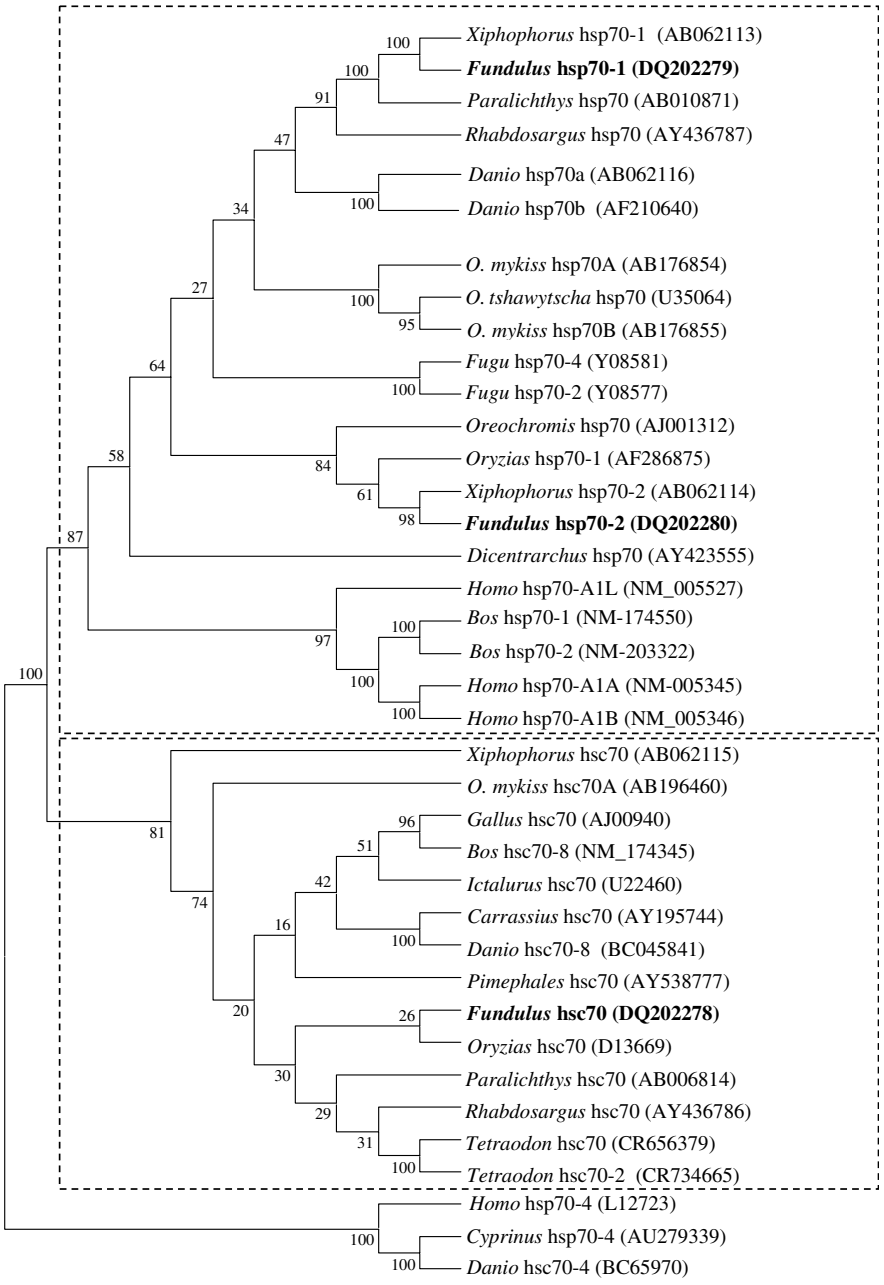


Fig. 4. Phylogenetic relationships among vertebrate *hsc/hsp70* amino acid sequences. The tree was constructed using the neighbor-joining method and bootstrap values (percentage of 1000 replicates) are shown at each branch point. Bold font indicates the sequences identified in this study. GenBank accession numbers of all sequences are given in brackets following the gene name.

Both northern and southern killifish populations showed a gradual increase in *hsp90α* expression (inducible isoform) with increasing heat shock temperature, and the overall magnitude of induction did not differ between populations (Fig. 6A). $T_{on}$ for *hsp90α*, however, did differ between populations, with significant induction occurring at 30°C in southern fish ($P$=0.021), and at 32°C in northern fish ($P$=0.012). Levels of *hsp90β* (constitutive isoform) did not change with heat shock in southern killifish. Northern killifish had slightly elevated *hsp90β* levels at 31–33°C. Two-way ANOVA for *hsp90β* mRNA levels, with population and heat shock temperature as factors, revealed a significant effect of heat shock temperature ($P$=0.001) and population ($P$=0.037) and no significant interaction ($P$=0.675) with southern individuals having overall higher *hsp90β* mRNA levels than northern fish. *Post-hoc* tests, however, revealed a significant difference in *hsp90β* mRNA levels between populations only in control fish.

## Discussion

### *Killifish thermal tolerance*

From the data presented here, it is clear that killifish are impressive eurytherms and can be acclimated to an exceedingly wide range of environmental temperatures. Both northern (NH) and southern (GA) killifish were able to survive temperatures approaching the freezing point of brackish water for several days and had chronic upper lethal limits of 36.4 and 38.2°C, respectively (Fig. 1). As a result, fish from a southern population (GA) had a chronic scope (chronic maximum – minimum) of 39.3°C (Fig. 1), which is larger than any previously published value for fishes (reviewed by Beitinger et al., 2000). Furthermore, both northern and southern killifish had CTMax that approached or exceeded 42°C and CTMin of approximately –1.1°C (Table 3), which are among the highest and lowest values, respectively, measured in fishes (reviewed by Beitinger et al., 2000). Of the available thermal tolerance scopes (CTMax – CTMin), only three of the most eurythermal fish have scopes that meet or exceed those of the killifish: the Amargosa pupfish, *Cyprinodon navadensis* (Feldmeth, 1981), the sheepshead minnow, *C. variegates* (Bennett and Beitinger, 1997), and the Atlantic stingray, *Dasyatis sabina* (Fangue and Bennett, 2003). The combined thermal acclimation and tolerance attributes of both northern and southern killifish

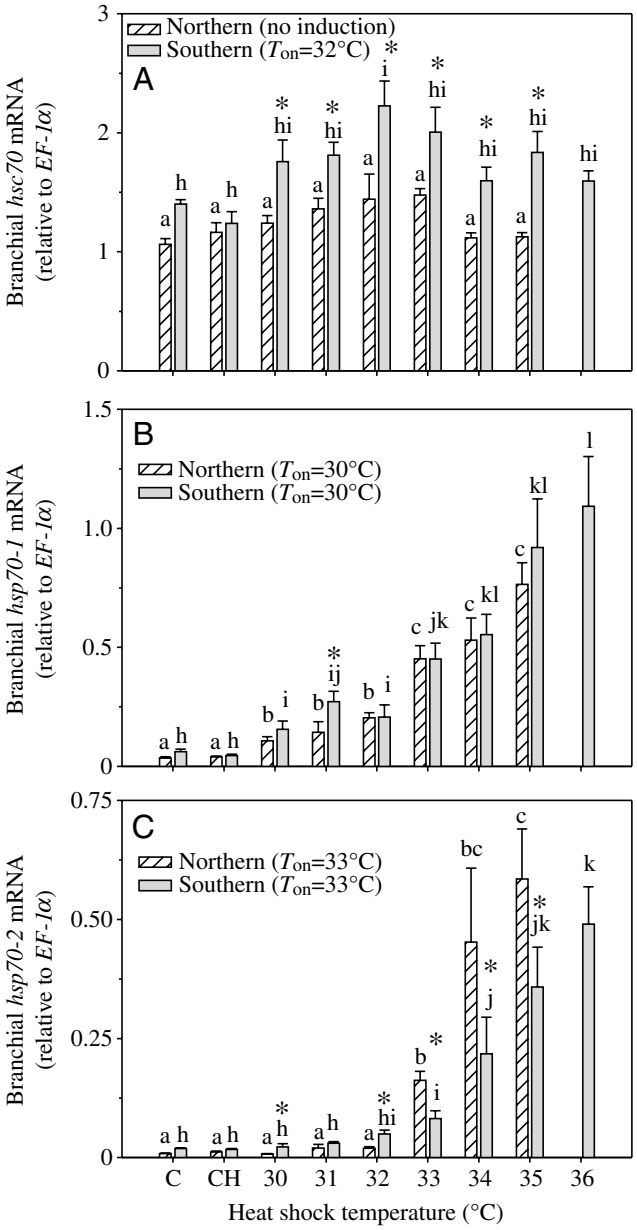


Fig. 5. Branchial *hsc70* (A), *hsp70-1* (B) and *hsp70-2* (C) mRNA levels in northern (NH, hatched bars) and southern (GA, grey bars) killifish in response to heat shock. Control (C) samples were taken prior to experimentation, and handling controls (CH) were treated the same as the experimental temperature groups but were transferred back to the acclimation temperature of 20°C. All mRNA data is normalized to the control gene *EF-1α* (values are mean ± s.e.m.; *N*=6). Different letters indicate significant differences between treatments but within a population, and an asterisk indicates a significant difference between northern and southern fish within a treatment.

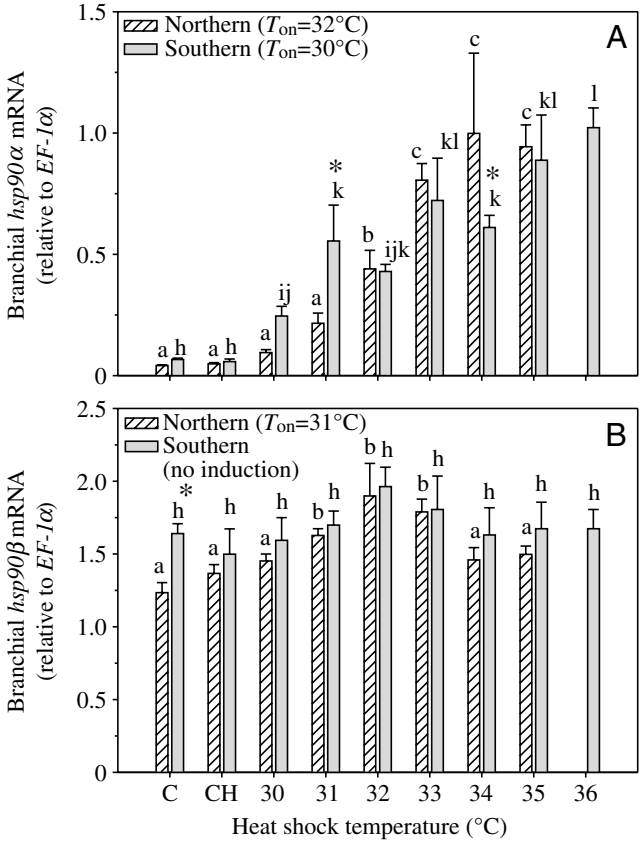


Fig. 6. Branchial *hsp90α* (A) and *hsp90β* (B) mRNA levels for northern (NH, hatched bars) and southern (GA, grey bars) killifish exposed to heat shock. Control (C) samples were taken prior to experimentation, and handling controls (CH) were treated identically to the heat shock groups but exposed only to the acclimation temperature of 20°C. All mRNA expression data is normalized to the control gene *EF-1α* (values are mean ± s.e.m.; *N*=6). Different letters indicates significant differences between treatments but within a population, and an asterisk indicates a significant difference between northern and southern fish within a treatment.

populations encompass the entire range of daily and seasonal temperatures they naturally experience and may allow killifish to move freely between dynamic thermal microhabitats.

Consistent with previous suggestions of local thermal adaptation, there were differences in thermal tolerance between killifish populations from different geographic locations. On average, southern (GA) killifish had critical thermal maxima that were 1.5°C higher than northern (NH) fish across all acclimation temperatures (Fig. 2A). This 1.5°C difference between populations was also maintained for critical thermal minima except at the three lowest acclimation temperatures (Fig. 2B). These differences in thermal tolerance between killifish populations are small relative to their large thermal acclimation ability, but comparisons within species for many physiological traits often show small overall intraspecific variation compared to the variation seem among species or higher taxa (Feder et al., 2000).

The difference in CTM values between northern and southern subspecies was consistent across multiple sampling sites: southern (GA, WI and FB) killifish populations had critical thermal tolerance values that were significantly higher than northern (NH, ME and NS) populations (Fig. 3). These differences in thermal tolerance were also maintained across samples obtained from the same locations in NH and GA in different years (2002 and 2004). Taken together, these data suggest that the critical thermal limits for each killifish subspecies are an intrinsic property of that subspecies and are constant from year to year. However, additional work on laboratory-reared killifish will be necessary to confirm this suggestion and to rule out the possibility of maternal or developmental effects. Killifish thermal tolerance limits vary in a direction consistent with that predicted for fish that have undergone localized adaptation to habitat temperatures. These functional differences between killifish subspecies are in agreement with a variety of work by others suggesting that the physiological specializations and genetic variation between subspecies are likely to be adaptive responses to temperature or some other factor correlated with latitude (reviewed by Powers and Schulte, 1998; Schulte, 2001).

### *Hsps and thermal tolerance*

The heat shock response is thought to be important for adaptation of organisms to their thermal environment (Feder and Hofmann, 1999; Hoffmann et al., 2003; Somero, 2005). A number of characteristics of the heat shock response could, in principle, respond to thermal selection, including: (1) the functional efficiency of the heat shock proteins themselves, (2) the onset temperature ($T_{on}$) at which *hsp* expression is induced, and (3) the magnitude of *hsp* expression under either basal or induced conditions. Although there is some evidence for each of these mechanisms in natural populations or following laboratory selection (Tomanek and Somero, 1999; Michalak et al., 2001; Feder et al., 2002; Sorensen et al., 2005) little is known about whether similar patterns are observed across multiple isoforms within a species, and few studies have assessed the relative roles and generality of these mechanisms.

We found no differences in the amino acid sequences of *hsp70-1* or *hsp70-2* within or between populations of killifish, and there was only a single highly conservative substitution between populations in *hsc70* (ser to thr at amino acid 98). These data strongly suggest that changes in the functional efficiency of the 70 kDa heat shock proteins have not been involved in the evolution of differences in thermal tolerance between northern and southern killifish populations.

The onset temperature of *hsp* induction ($T_{on}$) and magnitude of *hsp* expression exhibited a variety of patterns among isoforms when compared between northern and southern killifish populations. Among the three inducible genes we examined (*hsp90α*, *hsp70-1* and *hsp70-2*), *hsp90α* had a lower $T_{on}$ in southern populations, but populations did not differ in the magnitude of induction, whereas *hsp70-2* did not differ in $T_{on}$ between populations, but was induced to a greater magnitude by heat shock in northern fish than in southern fish. By contrast, neither $T_{on}$ nor the magnitude of expression differed between populations for *hsp70-1*. The observation that each isoform exhibited a different pattern of expression between populations suggests that differences in expression are not due to a global factor that affects all *hsps*, such as differences between populations in the stability of the total protein pool or overall rates of protein or mRNA turnover. Instead, this complex pattern of expression suggests that differences in mRNA levels between populations result from gene-specific mechanisms such as differences in transcription as a result of promoter sequence variation, or differences in mRNA stability as a result of sequence variation in the 5′ or 3′ untranslated region in a particular *hsp* gene (McGarry and Lindquist, 1986; Petersen and Lindquist, 1988).

The lower $T_{on}$ for *hsp90α* we observed in southern killifish (Fig. 6A) is in marked contrast to the results of most other studies, which have generally found that organisms from warmer environments induce Hsps at a higher temperature than closely related organisms from colder environments (Huey and Bennett, 1990; Dietz and Somero, 1992; Fader et al., 1994; Gehring and Werner, 1995; Hofmann and Somero, 1995; Hofmann and Somero, 1996; Tomanek and Somero, 1999; Tomanek and Somero, 2000). This discrepancy might be explained by the fact that our study examined mRNA levels, while most previous studies have focused on protein levels (Feder and Hofmann, 1999; Tomanek and Somero, 1999; Tomanek and Somero, 2000; Buckley et al., 2001). However, if this were the case, we would have to postulate a decoupling of transcription and translation for *hsp90α*. Alternatively, the lower $T_{on}$ for *hsp90α* could reflect an anticipatory response in southern killifish. Southern fish frequently experience peak water temperatures greater than 30°C, whereas water temperatures exceeding 30°C are rare in northern habitats. There is some support from experiments in *Drosophila* for maximum rather than mean environmental temperature as the important environmental thermal feature structuring adaptive thermal responses (Davidson, 1988; Anderson et al., 2003). An anticipatory upregulation of *hsp90α* could allow southern fish

to protect critical components of their protein pool in the face of high environmental temperatures.

The greater magnitude of *hsp70-2* upregulation in northern killifish (Fig. 5C) is consistent with the hypothesis that these fish are more sensitive to thermal stress. The magnitude of the heat shock protein response is typically proportional to the severity of the heat shock and associated protein damage (e.g. see DiDomenico et al., 1982). Similar to the results presented here, lines of *Drosophila* selected for high-temperature resistance have decreased expression of Hsp70 in response to heat shock compared to control lines, suggesting that a sub-lethal heat exposure is less stressful for heat adapted populations, thus leading to less cell damage and an overall smaller magnitude of stress protein induction (Sorensen et al., 1999; Sorensen et al., 2001). However, the hypothesis of differential thermal sensitivity is not supported by the results with *hsp70-1* mRNA levels, which exhibited no differences between populations in either $T_{on}$ or the magnitude of induction (Fig. 5B). Possible explanations for the differences in response between the two isoforms include differential sensitivity of these two isoforms to the denatured protein pool or differences in specificity between the *hsp70-2* response and the *hsp70-1* response.

Southern killifish had generally higher basal levels of the two constitutive mRNAs (*hsc70* and *hsp90β*) than northern killifish. If these mRNA levels are indicative of differences in the standing protein pool, these differences could be protective and are thus consistent with the greater thermal tolerance of southern fish. Work in poeciliid fishes (diIorio et al., 1996) suggests that higher constitutive levels of Hsc70 protein may be as or more important for thermal tolerance than changes in the inducible genes. However, there is some evidence in *Drosophila* and *Arabidopsis* that enhanced levels of Hsps under basal conditions can be deleterious (Krebs and Feder, 1997; Krebs and Feder, 1998; Zatsepina et al., 2001; Sung and Guy, 2003), and thus increased levels of the constitutive Hsps in killifish in unstressed fish could have a negative effect.

Basal levels of heat shock proteins, particularly Hsp90, can also affect the induction of heat shock protein genes (for a review, see Voellmy, 2004). Heat shock protein induction occurs *via* the binding of a transcription factor, the heat shock factor (HSF). Under unstressed conditions, HSF is present as a protein complex that includes Hsp90, Hsp70 and other chaperone molecules. This complex does not bind DNA effectively, and thus inducible heat shock genes are transcribed almost undetectably under unstressed conditions. During thermal stress, the chaperone proteins dissociate from the HSF protein complex and bind to unfolded proteins within the cell, releasing HSF from inhibition (Wu, 1995). Based on this mechanism, we would predict that southern killifish populations, which have a higher level of *hsp90β* mRNA under unstressed conditions, would require a higher level of thermal stress to remove the repression of HSF by Hsp90, and thus would have a higher $T_{on}$ and lower magnitude of expression at a given temperature for all inducible heat shock proteins. This was not the case. In fact, the only difference we observed in $T_{on}$ between populations was a lower $T_{on}$ for *hsp90α* in southern fish, in direct contrast to the predictions of this model. However, northern fish had a greater magnitude of *hsp70-2* induction, which is consistent with model predictions.

Although *hsc70* and *hsp90β* are considered to be constitutively expressed, we observed statistically significant increases in the expression of both of these genes in response to heat shock. It has been shown that some fish have the ability to upregulate *hsc70* levels with increasing acclimation temperatures as well as with heat shock (Deane and Woo, 2005), whereas other research has shown a pattern more typical of a constitutive isoform with no change in *hsc70* levels with heat shock (Yamashita et al., 2004; Ojima et al., 2005). The pattern of *hsc70* and *hsp90β* induction differed between killifish populations. Southern killifish exhibited a substantial increase *hsc70* levels in response to heat shock whereas no change was observed in northern fish. The upregulation of *hsc70* expression by southern killifish in response to heat shock may suggest an important role for Hsc70 in handling protein damage associated with daily fluctuations in environmental temperatures, and is consistent with the greater thermal tolerance exhibited by killifish from southern populations. In contrast, there was a small but statistically significant elevation of *hsp90β* in northern fish but not southern fish following heat shock, which is not obviously consistent with a hypothesis of thermal adaptation.

The modulation of *hsp* mRNA expression patterns involving multiple isoforms from several Hsp families has been suggested as one mechanism ectotherms use to maintain flexibility in thermal phenotype in response to changing thermal environments (Hightower, 1991; Hochachka and Somero, 2002). The combinations of Hsps expressed, however, vary widely between organisms and the reason for this variation in protein expression is unknown. Often, this variation reflects both the evolutionary histories of the species and the recent thermal acclimation conditions encountered by that organism (White et al., 1994; Hofmann and Somero, 1995; Roberts et al., 1997; Tomanek and Somero, 2002; Tomanek, 2005) suggesting that substantial adaptive variation exists in the heat shock response. To our knowledge, only a single study has addressed the mRNA expression profiles of multiple *hsp* genes from several gene families in fish (Ojima et al., 2005). This work was performed in an immortalized rainbow trout gonadal fibroblast cell line and only a single acclimation temperature and heat shock temperature treatment was evaluated. Even with this simple experimental design, however, the authors demonstrate gene-specific variation in *hsp* mRNA levels. Results from microarray studies in fish exposed to constant or cycling environmental temperatures (Podrabsky and Somero, 2004) or during cold acclimation (Gracey et al., 2004) show complex gene expression signatures that involve many gene classes known to be associated with thermal tolerance. However, these studies, and the study reported here, have examined these processes at the mRNA level, and mRNA levels are not necessarily predictive of the behaviour of the protein pool. Recently, $^{35}$S-labelling of newly

translated proteins followed by two-dimensional gel electrophoresis in turban snails exposed to heat shock, showed the induction of over 30 proteins from several Hsp families with varying patterns among isoforms (Tomanek, 2005). Whereas it is not yet known whether these proteins are coded by different genes or represent post-transcriptional modifications, it is clear that these protein variants are important and could contribute to the phenotypic plasticity seen in eurythermal organisms. Although correlative studies such as those of Tomanek (Tomanek, 2005) and the current study cannot directly establish a causal link between the patterns of *hsp* isoform expression and whole organism thermal tolerance, these studies provide critical evidence of the under-appreciated diversity of the patterns of *hsp* expression in natural populations, their relationship to differences in whole-organism thermal tolerance, and their possible role in the establishment of biogeographical patterns.

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## References

**Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J.** (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389-3402.

**Anderson, A. R., Collinge, J. E., Hoffmann, A. A., Kellett, M. and McKechnie, S. W.** (2003). Thermal tolerance trade-offs associated with the right arm of chromosome 3 and marked by the *hsr-omega* gene in *Drosophila melanogaster*. *Heredity* **90**, 195-202.

**Becker, C. D. and Genoway, R. G.** (1979). Evaluation of the critical thermal maximum for determining thermal tolerance of freshwater fish. *Environ. Biol. Fishes* **4**, 245-256.

**Beitinger, T. L., Bennett, W. A. and McCauley, R. W.** (2000). Temperature tolerances of North American freshwater fishes exposed to dynamic changes in temperature. *Environ. Biol. Fishes* **58**, 237-275.

**Bennett, W. A. and Beitinger, T. L.** (1997). Temperature tolerance of the sheepshead minnow, *Cyprinodon variegates*. *Copeia* **1997**, 77-87.

**Bennett, W. A., Currie, R. J., Wagner, P. F. and Beitinger, T. L.** (1997). Cold tolerance and potential overwintering of the red-bellied piranha *Pygocentrus nattereri* in the United States. *Trans. Am. Fish. Soc.* **126**, 841-849.

**Bernardi, G., Sordino, P. and Powers, D. A.** (1993). Concordant mitochondrial and nuclear DNA phylogenies for populations of the teleost fish, *Fundulus heteroclitus*. *Proc. Natl. Acad. Sci. USA* **90**, 9271-9274.

**Brown, J. H. and Feldmeth, C. R.** (1971). Evolution in constant and fluctuating environments: thermal tolerances of desert pupfish (Cyprinodon). *Evolution* **25**, 390-398.

**Buckley, B. A., Owen, M.-E. and Hofmann, G. E.** (2001). Adjusting the thermostat: the threshold induction temperature for the heat shock response in intertidal mussels (genus *Mytilus*) changes as a function of thermal history. *J. Exp. Biol.* **204**, 3571-3579.

**Bulger, A. J.** (1984). A daily rhythm in heat tolerance in the salt marsh fish *Fundulus heteroclitus. J. Exp. Zool.* **230**, 11-16.

**Bulger, A. J. and Tremaine, S. C.** (1985). Magnitude of seasonal effects on heat tolerance in *Fundulus heteroclitus. Physiol. Zool.* **58**, 197-204.

**Chomczynski, P. and Sacchi, N.** (1987). Single-step method of RNA isolation by acid quanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159.

**Cox, D. K.** (1974). Effects of three heating rates on the critical thermal maximum of bluegill. In *Thermal Ecology* (ed. W. Gibbons and R. R. Sharitz), pp. 158-163. Springfield, IL: National Technical Information Service.

**Davidson, J. K.** (1988). Extremes of climate and genetic heterogeneity in Australian populations of the dipteran species *Drosophila melanogaster. J. Biogeogr.* **15**, 481-487.

**Davidson, J. K.** (1990). Non-parallel geographic patterns for tolerance to cold and desiccation in *Drosophila melanogaste*r and *D. simulans*. *Aust. J. Zool.* **38**, 155-161.

**Deane, E. E. and Woo, N. Y. S.** (2005). Cloning and characterization of the hsp70 multigene family from silver sea bream: modulated gene expression between warm and cold temperature acclimation. *Biochem. Biophys. Res. Commun.* **330**, 776-783.

**DiDomenico, B. J., Bugaisky, G. E. and Lindquist, S.** (1982). The heat shock response is self-regulated at both the transcriptional and posttranscriptional levels. *Cell* **31**, 593-603.

**Dietz, T. J. and Somero, G. N.** (1992). The threshold induction temperature of the 90kDa heat shock protein is subject to acclimatization in the eurythermal goby fishes (genus *Gillicthys). Proc. Natl. Acad. Sci. USA* **89**, 3389-3393.

**diIorio, P. J., Holsinger, K., Schultz, R. J. and Hightower, L. E.** (1996). Quantitative evidence that both Hsc70 and Hsp70 contribute to thermal adaptation in hybrids of the livebearing fishes *Poeciliopsis*. *Cell Stress Chaperones* **1**, 139-147.

**Elliott, J. M., Elliott, J. S. and Allonby, J. D.** (1994). The critical thermal limits for the stone loach, *Noemacheilus barbatulus*, from three populations in north-west England. *Freshw. Biol.* **32**, 593-601.

**Fader, S. C., Yu, Z. and Spotila, J. R.** (1994). Seasonal variation in heat shock proteins (hsp70) in stream fish under natural conditions. *J. Therm. Biol.* **19**, 335-341.

**Fangue, N. A. and Bennett, W. A.** (2003). Thermal tolerance responses of laboratory-acclimated and seasonally-acclimatized Atlantic stingray, *Dasyatis sabina. Copeia* **2003**, 315-325.

**Feder, M. E. and Hofmann, G. E.** (1999). Heat shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annu. Rev. Physiol.* **61**, 243-282.

**Feder, M. E., Bennett, A. F. and Huey, R. B.** (2000). Evolutionary physiology. *Annu. Rev. Ecol. Syst.* **31**, 315-341.

**Feder, M. E., Bedford, B. C., Albright, D. R. and Michalak, P.** (2002). Evolvability of Hsp70 expression under artificial selection for inducible thermotolerance in independent populations of *Drosophila melanogaster. Physiol. Biochem. Zool.* **31**, 315-341.

**Feldmeth, R. C.** (1981). The evolution of thermal tolerance in desert pupfish (genus *Cyprinodon*). In *Fishes of North American Deserts* (ed. J. Naiman and D. L. Soltz), pp. 357-384. New York: John Wiley and Sons.

**Fields, R., Lowe, S. S., Kaminski, C., Whitt, G. S. and Philipp, D. P.** (1987). Critical and chronic thermal maxima of northern and Florida largemouth bass and their reciprocal $F_1$ and $F_2$ hybrids. *Trans. Am. Fish. Soc.* **116**, 856-863.

**Gehring, W. J. and Werner, R.** (1995). Heat shock protein synthesis and thermotolerance in *Cataglyphis*, an ant from the Sahara desert. *Proc. Natl. Acad. Sci. USA* **92**, 2994-2998.

**Gething, M.-J.** (ed.) (1997). Mammalian BiP. In *Guidebook to Molecular Chaperones and Protein-folding Catalysts*, pp. 59-64. New York: Oxford University Press.

**Gracey, A. Y., Fraser, E. J., Weizhong, L., Yongxiang, F., Taylor, R. R., Rogers, J., Brass, A. and Cossins, A. R.** (2004). Coping with cold: an integrative, multitissue analysis of the trascriptome of a poikilothermic vertebrate. *Proc. Natl. Acad. Sci. USA* **101**, 16970-16975.

**Guerra, D., Cavicchi, S., Krebs, R. A. and Loeschcke, V.** (1997). Resistance to heat and cold stress in *Drosophila melanogaster*: intra and inter population variation in relation to climate. *Genet. Sel. Evol.* **29**, 497-510.

**Hart, J. S.** (1952). Geographic variations in some physiological and morphological characters of certain freshwater fish. *Publ. Ont. Fish. Res. Lab.* **72**, 1-79.

**Hightower, L. E.** (1991). Heat shock, stress proteins, chaperones and proteotoxicity. *Cell* **66**, 191-197.

**Hochachka, P. W. and Somero, G. N.** (2002). *Biochemical Adaptation: Mechanism and Process in Physiological Evolution*. New York: Oxford University Press.

**Hoffmann, A. A., Sorensen, J. G. and Loeschcke, V.** (2003). Adaptation of *Drosophila* to temperature extremes: bringing together quantitative and molecular approaches. *J. Therm. Biol.* **28**, 175-216.

**Hofmann, G. E. and Somero, G. N.** (1995). Evidence for protein damage at environmental temperatures: seasonal changes in levels of ubiquitin conjugates and hsp70 in the intertidal mussel *Mytilus trossulus*. *J. Exp. Biol.* **198**, 1509-1518.

**Hofmann, G. E. and Somero, G. N.** (1996). Interspecific variation in thermal denaturation of proteins in the congeneric mussels *Mytilus trossulus* and *M. galloprovincialis:* evidence from the heat-shock response and protein ubiquitination. *Mar. Biol.* **126**, 65-75.

**Huey, R. B. and Bennett, A. F.** (1990). Physiological adjustments to fluctuating thermal environments: an ecological and evolutionary perspective. In *Stress Proteins in Biology and Medicine* (ed. R. I. Morimoto, A. Tissieres and C. Georgopoulos), pp. 37-59. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

**Kimura, M. T., Ohtsu, T., Yoshida, T., Awasaki, T. and Lin, F. J.** (1994). Climatic adaptations and distributions in the *Drosophila takahashii* species subgroup (Diptera: Drosophilidae). *J. Nat. Hist.* **28**, 401-409.

**Krebs, R. A. and Feder, M. E.** (1997). Deleterious consequences of Hsp70 overexpression in *Drosophila melanogaster* larvae. *Cell Stress Chaperones* **2**, 60-71.

**Krebs, R. A. and Feder, M. E.** (1998). Hsp70 and larval thermotolerance in *Drosophila melanogaster*: how much is enough and when is more too much? *J. Insect Physiol.* **44**, 1091-1101.

**Krebs, R. A. and Loeschcke, V.** (1995). Resistance to thermal stress in adult *Drosophila buzzatii*: acclimation and variation among populations. *Biol. J. Linn. Soc. Lond.* **56**, 505-515.

**Kumar, S., Tamura, K., Jakobsen, I. B. and Nei, M.** (2001). MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics* **17**, 1244-1245.

**Lindquist, S.** (1986). The heat shock response. *Annu. Rev. Biochem.* **55**, 1151-1191.

**Lohr, S. C., Byorth, P. A., Kaya, C. M. and Dwyer, W. P.** (1996). High-temperature tolerances of fluvial Arctic grayling and comparisons with summer river temperatures of the Big Hole River, Montana. *Trans. Am. Fish. Soc.* **125**, 933-939.

**McCauley, R. W.** (1958). Thermal relations of geographic races of *Salvelinus*. *Can. J. Zool*. **36**, 655-662.

**McGarry, T. and Lindquist, S.** (1986). The preferential translation of hsp70 mRNA requires sequences in the untranslated leader. *Cell* **42**, 903-911.

**Medrano, J. F., Aasen, E. and Sharrow, L.** (1990). DNA extraction from nucleated red blood cells. *Biotechniques* **8**, 43.

**Michalak, P., Minkov, I., Helin, A., Lerman, D. N., Bettencourt, B. R., Feder, M. E., Korol, A. B. and Nevo, E.** (2001). Genetic evidence for adaptation-driven incipient speciation of *Drosophila melanogaster* along the microclimate contrast in "Evolutionary Canyon", Israel. *Proc. Natl. Acad. Sci. USA* **98**, 13195-13200.

**Morimoto, R. I.** (1998). Regulation of the heat shock transcriptional response: crosstalk between a family of heat shock factors, molecular chaperones, and negative regulators. *Genes Dev.* **12**, 3788-3796.

**Morimoto, R. I. and Santoro, M. G.** (1998). Stress-inducible responses and heat shock proteins: new pharmacologic targets for cytoprotection. *Nat. Biotechnol.* **16**, 833-838.

**Morin, R. P. and Able, K. W.** (1983). Patterns of geographic variation in the egg morphology of the fundulid fish, *F. heteroclitus*. *Copeia* **1983**, 726-740.

**NOAA NERRS** (2004). Centralized data management office, Baruch Marine field lab, University of South Carolina http://cdmo.baruch.sc.edu.

**Norris, C. E., diIorio, P. J., Schultz, R. J. and Hightower, L. E.** (1995). Variation in heat shock proteins within tropical and desert species of poeciliid fishes. *Mol. Biol. Evol.* **12**, 1048-1062.

**Ojima, N., Yamashita, M. and Watabe, S.** (2005). Quantitative mRNA expression profiling of heat shock protein families in rainbow trout cells. *Biochem. Biophys. Res. Commun.* **329**, 51-57.

**Otto, R. G.** (1973). Temperature tolerance of the mosquitofish, *Gambusia affinis* (Baird and Girard). *J. Fish Biol.* **5**, 575-585.

**Parsell, D. A. and Lindquist, S.** (1993). The function of heat shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Annu. Rev. Genet.* **27**, 437-496.

**Petersen, R. and Lindquist, S.** (1988). The *Drosophila hsp70* message is rapidly degraded at normal temperatures and stabilized by heat shock. *Gene* **72**, 161-168.

**Podrabsky, J. E. and Somero, G. N.** (2004). Changes in gene expression associated with acclimation to constant temperatures and fluctuating daily temperatures in an annual killifish, *Austrofundulus limnaeus. J. Exp. Biol.* **207**, 2237-2254.

**Powers, D. A. and Schulte, P. M.** (1998). Evolutionary adaptations of gene structure and expression in natural populations in relation to a changing environment: a multidisciplinary approach to address the million-year saga of a small fish. *J. Exp. Zool.* **282**, 71-94.

**Powers, D. A., Smith, M., Gonzalez-Villasenor, I., DiMichele, L., Crawford, D., Bernardi, G. and Lauerman, T.** (1993). A multidisciplinary approach to the selectionist/neutralist controversy using the model teleost, *F. heteroclitus*. In *Oxford Survey of Evolutionary Biology* (ed. D. Futuyuma and J. Antonovics), pp. 43-107. Oxford: Oxford University Press.

**Roberts, D. A., Hofmann, G. E. and Somero, G. N.** (1997). Heat shock protein expression in *Mytilus californianus*: acclimatization (seasonal and tidal-height comparisons) and acclimation effects. *Biol. Bull.* **192**, 309-320.

**Sambrook, J., Fritsch, E. F. and Maniatis, T.** (1989). *Molecular Cloning: A Laboratory Manual*. New York: Cold Spring Harbor Laboratory Press.

**Schulte, P. M.** (2001). Environmental adaptations as windows on molecular evolution. *Comp. Biochem. Physiol.* **128**, 597-611.

**Smale, M. A. and Rabeni, C. F.** (1995). Hypoxia and hyperthermia tolerances of headwater stream fishes. *Trans. Am. Fish. Soc.* **124**, 698-710.

**Somero, G. N.** (2002). Thermal physiology and vertical zonation of intertidal animals: optima, limits, and costs of living. *Integr. Comp. Biol.* **42**, 780-789.

**Somero, G. N.** (2005). Linking biogeography to physiology: evolutionary and acclimatory adjustments of thermal limits. *Front. Zool.* **2**, 1-9.

**Sorensen, J. G., Michalak, P., Justesen, J. and Loeschcke, V.** (1999). Expression of the heat shock protein HSP70 in *Drosophila buzzattii* lines selected for thermal resistance. *Hereditas* **131**, 155-164.

**Sorensen, J. G., Dahlgaard, J. and Loeschcke, V.** (2001). Genetic variation in thermal tolerance among natural populations of *Drosophila buzzatii*: down regulation of Hsp70 expression and variation in heat stress resistance traits. *Funct. Ecol.* **15**, 289-296.

**Sorensen, J. G., Norry, F. M., Scannapieco, A. C. and Loeschcke, V.** (2005). Altitudinal variation for stress resistant traits and thermal adaptation in adult *Drosophila buzzatii* from the New World. *J. Evol. Biol.* **18**, 829-837.

**Strange, K. T., Vokoun, J. C. and Noltie, D. B.** (2002). Thermal tolerance and growth differences in orangethroat darter (*Etheostoma spectabile*) from thermally contrasting adjoing streams. *Am. Midl. Nat.* **148**, 120-128.

**Sung, D. Y. and Guy, C. L.** (2003). Physiological and molecular assessment of altered expression of Hsc70-1 in *Arabidopsis*: evidence for pleiotropic consequences. *Plant Physiol.* **132**, 979-987.

**Thompson, J. D., Higgins, D. G. and Gibson, T. J.** (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**, 4673-4680.

**Tomanek, L.** (2005). Two-dimensional gel analysis of the heat shock response in marine snails (genus *Tegula*): interspecific variation in protein expression and acclimation ability. *J. Exp. Biol.* **208**, 3133-3143.

**Tomanek, L. and Somero, G. N.** (1999). Evolutionary and acclimation-induced variation in the heat shock responses of congeneric marine snails (genus *Tegula*) from different thermal habitats: implications for limits of thermotolerance and biogeography. *J. Exp. Biol.* **202**, 2925-2936.

**Tomanek, L. and Somero, G. N.** (2000). Time course and magnitude of synthesis of heat shock proteins in congeneric marine snails (genus *Tegula*) from different tidal heights. *Physiol. Biochem. Zool.* **73**, 249-256.

**Tomanek, L. and Somero, G. N.** (2002). Interspecific and acclimation-induced variation in levels of heat shock proteins 70 (hsp70) and 90 (hsp90) and heat shock transcription factor-1 (HSF1) in congeneric marine snails (genus *Tegula*): implications for regulation of *hsp* gene expression. *J. Exp. Biol.* **205**, 677-685.

**Voellmy, R.** (2004). On mechanisms that control heat shock transcription factor activity in metazoan cells. *Cell Stress Chaperones* **9**, 122-133.

**White, C. N., Hightower, L. E. and Schultz, R. J.** (1994). Variation in heat shock proteins among species of desert fishes (Poeciliidae, Poeciliopsis). *Mol. Biol. Evol.* **11**, 106-119.

**Wu, C.** (1995). Heat shock transcription factors: structure and regulation. *Annu. Rev. Cell Dev. Biol.* **11**, 441-469.

**Yamashita, M., Hirayoshi, K. and Nagata, K.** (2004). Characterization of multiple members of the HSP70 family in platyfish culture cells: molecular evolution of stress protein HSP70 in vertebrates. *Gene* **336**, 207-218.

**Zatsepina, O. G., Velikodvorskaia, V. V., Molodtsov, V. B., Garbuz, D., Lerman, D. N., Bettencourt, B. R., Feder, M. E. and Evgenev, M. B.** (2001). A *Drosophila melanogaster* strain from sub-equatorial Africa has exceptional thermotolerance but decreased Hsp70 expression. *J. Exp. Biol.* **204**, 1869-1881.