Effects of thermal acclimation on transcriptional responses to acute heat stress in the eurythermal fish *Gillichthys mirabilis* (Cooper)

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Submitted 19 October 2010; accepted in final form 10 March 2011

Logan CA, Somero GN. Effects of thermal acclimation on transcriptional responses to acute heat stress in the eurythermal fish *Gillichthys mirabilis* (Cooper). *Am J Physiol Regul Integr Comp Physiol* 300: R1373–R1383, 2011. First published March 16, 2011; doi:10.1152/ajpregu.00689.2010.—The capacities of eurythermal ectotherms to withstand wide ranges of temperature are based, in part, on abilities to modulate gene expression as body temperature changes, notably genes encoding proteins of the cellular stress response. Here, using a complementary DNA microarray, we investigated the sequence in which cellular stress response-linked genes are expressed during acute heat stress, to elucidate how severity of stress affects the categories of genes changing expression. We also studied how prior acclimation history affected gene expression in response to acute heat stress. Eurythermal goby fish (*Gillichthys mirabilis*) were acclimated to 9 ± 0.5, 19 ± 0.5, and 28 ± 0.5°C for 1 mo. Then fish were given an acute heat ramp (4°C/h), and gill tissues were sampled every +4°C to monitor gene expression. The average onset temperature for a significant change in expression during acute stress increased by ~2°C for each ~10°C increase in acclimation temperature. For some genes, warm acclimation appeared to obviate the need for expression change until the most extreme temperatures were reached. Sequential expression of different categories of genes reflected severity of stress. Regardless of acclimation temperature, the gene encoding heat shock protein 70 (*HSP70*) was upregulated strongly during mild stress; the gene encoding the proteolytic protein ubiquitin (*UBQ*) was upregulated at slightly higher temperatures; and a gene encoding a protein involved in cell cycle arrest and apoptosis, cyclin-dependent kinase inhibitor 1B (*CDKN1B*), was upregulated only under extreme stress. The tiered, stress level-related expression patterns and the effects of acclimation on induction temperature yield new insights into the fundamental mechanisms of eurythermy.

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**THE CELLULAR STRESS RESPONSE (CSR) involves prevention and repair of macromolecular damage incurred as a consequence of environmental stress (23, 31, 40). A group of conserved stress-induced proteins, termed the “minimal stress proteome,” has been identified in eukaryotic and prokaryotic organisms and appears to be a universally conserved aspect of response to diverse physical and chemical stresses. The minimal stress proteome includes proteins controlling the cell cycle, protein folding and repair, DNA and chromatin stabilization and repair, removal of damaged proteins, and certain aspects of energy metabolism (31). Whereas the basic, common constituents of the CSR and minimal stress proteome have been most thoroughly characterized in a few model (genomically characterized) species (31), current efforts to characterize this conserved response in nonmodel organisms focus on several questions of ecological and evolutionary significance. For example, are certain species better suited physiologically than others to handle fluctuations in their environments, e.g., widely thermally tolerant (eurythermal) vs. narrowly thermally tolerant (stenothermal) ectotherms (26)? These varying abilities to employ the CSR in response to thermal stress may be key factors in determining how successfully an organism can respond to climate change (42).

One important facet of the CSR concerns the patterning and mechanisms by which increasing levels of stress sequentially induce different components of the response (23, 31, 40). For example, at mild levels of heat stress, molecular chaperones may be induced to refold proteins that have unfolded due to thermal perturbation of tertiary structure. At moderate levels of stress, proteolysis through the ubiquitin-proteasome pathway may be initiated to remove proteins that cannot be rescued through activities of chaperones. In addition, above a certain level of stress, basic activities like cell proliferation may cease due to cellular damage, notably to DNA, and to allow sufficient energy to be redirected from housekeeping functions toward the stress response. At severe levels of acute stress, whole cells may become sufficiently damaged to trigger induction of apoptotic pathways. Identifying gene or protein markers that define these different sublethal stress levels could be highly useful in assessing the health of individual organisms in their environment (27). In the context of the mechanisms underlying eurythermy, elucidation of the effects of acclimation or acclimatization on induction thresholds for the different components of the CSR might provide important insights into the fundamental mechanisms of acclimatization (phenotypic plasticity) to highly variable thermal environments.

One species that has proven to be an excellent study system for analyzing effects of abiotic factors on physiological systems, including gene expression, is the eurytolerant goby *Gillichthys mirabilis* (Cooper). The biogeographic range of *G. mirabilis* extends from Tomales Bay, CA (38.160°N, −122.8945°W) to both coasts of the Baja California peninsula and the northern coast of mainland Mexico bordering the Gulf of California (37). This intertidal and estuarine goby fish can survive drastic shifts in its environment, including changes in oxygen availability (25), salinity (22), and temperature (9). In its shallow estuarine habitats, this species can experience one of the widest temperature ranges of any fish, from −5 to 37°C (10). *Gillichthys*’s capacity for tolerating such wide ranges of abiotic conditions makes it an excellent model for investigating the molecular basis of physiological plasticity (24). Several studies have investigated the CSR of *G. mirabilis* in response to environmental stress to better elucidate its ability to cope with stress (9, 22, 25, 34).

The effects of temperature stress on marine fishes are of particular interest for predicting “winners” and “losers” in the context of climate change (42). Here, the ability of this eury-
transcriptional changes in response to acute heat stress. Thermal
acclimation effects on induction temperatures of gene expres-
sion are a largely unexplored facet of phenotypic plasticity.
Studies of specific, targeted proteins have, however, shown that
temperatures of acclimation or acclimatization can modify
induction temperatures for synthesis of the HSPs HSP70 and
HSP90 and heat shock factor 1 in *G. mirabilis* (10, 11, 19, 20,
35). Now, with the development of genomic methods, it is
possible to examine how patterns of heat stress-induced gene
expression vary with acclimation temperature in thousands of
possible genes in gill tissue that changed in expression during an acute shock exposure at
32°C. These genes were involved in protein homeostasis, cell
cycle control, cytoskeletal reorganization, metabolic regulation
and signal transduction, as well as several other categories (9).
Logan and Somero (34) examined responses to steady-state
thermal acclimation and found that the whole organism critical
temperature was 5.3°C higher in fish acclimated to 28 vs. 9°C for 3 wk. The transcriptomes of gill tissue from
differently acclimated populations were strikingly similar, rel-
tive to effects noted in studies of acute thermal stress on gene
expression. For example, no indication was found of higher
expression of genes encoding heat shock proteins (HSPs) in
28°C-acclimated fish, which may reflect an acclimation pro-
cess that has largely remedied the effects of acute thermal
stress and is perhaps a hallmark of eurythermy (34).

One issue that is only now receiving study is the effect of
prior thermal acclimation or acclimatization on patterns of
transcriptional change in response to acute heat stress. Thermal
acclimation effects on induction temperatures of gene expres-
sion are a largely unexplored facet of phenotypic plasticity.
Studies of specific, targeted proteins have, however, shown that
temperatures of acclimation or acclimatization can modify
induction temperatures for synthesis of the HSPs HSP70 and
HSP90 and heat shock factor 1 in *G. mirabilis* (10, 11, 19, 20,
35). Now, with the development of genomic methods, it is
possible to examine how patterns of heat stress-induced gene
expression vary with acclimation temperature in thousands of
genes simultaneously. Here, using a cDNA microarray devel-
opled for *G. mirabilis* (24), we investigated transcriptional
changes in gill tissue to address two main questions. First, how
is the onset temperature (T_onset) of increased or decreased ex-
pression of genes related to the CSR modified by acclimation
temperature? Second, is there a tiered stress response in gene
expression by which genes representing different components
of the CSR can define the severity of sublethal thermal stress?
Gill tissue was chosen for this study due to its high metabolic
activity and important physiological roles as the primary site of
oxygen uptake, osmotic and ionic regulation, acid-base regu-
lation, and excretion of nitrogenous wastes in fishes (17).
Furthermore, previous studies of gill tissue have revealed rapid
and widespread changes in gene expression in response to
stress (9, 22, 34), illustrating that this tissue is an appropriate
study system for the questions we addressed.

MATERIALS AND METHODS

Animals

*Gillichthys mirabilis* were collected from an estuarine lagoon at the
University of California, Santa Barbara (34.39°N, 119.81°W) using
baitated minnow traps. Fish were transported to Hopkins Marine Sta-
tion, Pacific Grove, CA, in insulated containers and held in flow-
through seawater aquaria at ambient temperature (~13–14°C) for 1
wk. Eighteen individuals were killed after this initial period to make
up the reference pool of total RNA (see below). Fish were killed by
cervical transection and dissected immediately. Gill tissues were
placed in 1.5-ml Eppendorf microcentrifuge tubes and flash frozen in
liquid nitrogen for subsequent RNA extraction.

Following the week of holding at ambient seawater temperature,
three groups of 30 individuals were then randomly assigned to
temperature-controlled, recirculating 40-liter acclimation tanks
(AquaLogic, San Diego, CA), where they were held for 4 wk at either
9 ± 0.5, 19 ± 0.5, or 28 ± 0.5°C. Crushed coral (with some zeolite
and carbon pellets) lined the bottoms of the tanks and was sandwiched
by a carbon filter pad and Poly Filter pad (Poly-Bio-Marine, Reading,
PA). Plastic gridding was placed on top of the filters. Rocks were used
to hold the filters in place and provide some shelter to the fish. The
initial water temperature for the 28°C acclimation tank was 19°C.
Temperature was increased at a rate of 2°C per day until it reached
28°C, at which time the acclimation period began. The other two
acclimation groups started immediately at 9 or 19°C because the
temperature difference from the flow-through seawater (~13–14°C)
seemed too small to create significant stress for this eurythermal
species.

One-half of the volume of water in each tank was exchanged for
fresh filtered seawater once per week. Dissolved oxygen (YSI52,
Yellow Spring, OH), ammonia, and nitrite/nitrate levels (Quick Dip
Test Strips, Jungle Laboratories, Cibolo, TX) were measured weekly
to ensure adequate water quality. Animals were fed on a commercial
fish diet (Bio-Oregon, Warrenton, OR) three times per week. Animals
were not fed in the 48 h before the end of the acclimation period to
normalize for nutritional status. Sex could not be determined (*G.
mirabilis* does not display sex-specific external morphology, and ripe
gonads were not observed). The animal care and use protocol for these
experiments was approved by the Stanford Institutional Animal Care
and Use Committee (IACUC protocol ID 12972).

Acute heat stress. To expose fish to an environmentally relevant
heat ramp, we used an experimental heat-ramp tank identical to the
acclimation tanks, except for the addition of a computer-controlled
heating system. Before each experiment, all fish at a given acclimation
temperature were transferred into a bucket to control for handling
effects. Control fish were then placed back into the acclimation tank,
and experimental fish were placed into the heat-ramp tank at their
respective acclimation temperature. The temperature in the heat-ramp
tank was increased at 4°C/h until loss of equilibrium (LOE) occurred,
defined by the failure of the fish to maintain dorsoventral orientation
for at least 1 min (12). This heating rate is similar to that seen during
the course of a day in this species’ natural habitat (10).

Fish were sampled at ±4°C intervals, beginning at their acclima-
tion temperature (Fig. 1). At each sampling time point, three experi-
mental fish and one control fish were removed from the tanks. Gill
tissue was immediately dissected and flash frozen in liquid nitrogen
for subsequent RNA extraction. Three deviations from this regime
occurred over the course of the experiment. First, the initial exper-
imental sampling time point for the 9°C acclimation group was 17°C,
because we did not have sufficient fish to sample every 4°C (Fig. 1).
Second, fish in the 9°C acclimation group began to experience LOE
after the 33°C sampling time point, but before they reached the next
+4°C interval. Therefore, we sampled fish as soon as LOE began, at
36°C. The 9°C-acclimated fish were not expected to reach the next
time point, given that their previously measured LOE was 34.4 ±
0.15°C (SE) (34). The 28°C fish were last sampled at 40°C and had
not yet reached LOE [their previously measured LOE was 39.7 ±
0.05°C (SE)]; LOE for 19°C-acclimated fish was not measured in that
experiment (34). Third, our sampling scheme was modified on the day
of the experiment to ensure enough individuals to sample at all time
points, a problem arising from the fact that a few fish successfully
“hid” in the tanks and evaded detection and capture. Thus, at the
following four sampling points, only two fish were sampled: 36°C in
Fish were sampled at acclimation tank, and experimental fish were placed into a heat ramp tank at handling effects. Control fish were then placed back into the steady-state experiment, acclimated fish were transferred into a bucket to control for were previously acclimated to 9, 19, and 28°C for 1 mo. Before each experimental group (Fig. 1).

**Condition index.** To assess whole organism condition following acclimation, we measured the weight and length of all fish. The ratio of weight to length cubed (W/L^3) is called the condition index (CI), which can be a proxy for “well-being” (7). We calculated whether the ratios were significantly different between fishes acclimated to different temperatures using an ANOVA implemented in Prism 5 (GraphPad Software).

**Total RNA extraction and cDNA microarray analysis.** The design of the G. mirabilis cDNA microarray used in these experiments is reported elsewhere (9, 24). The slides printed for this study contained 9,207 features and were printed in a single print run on a printing robot in the laboratory of Dr. Gary Schoenlk at the Stanford University School of Medicine.

Our hybridization protocol followed that of Logan and Somero (34). Briefly, total RNA was extracted from gill tissue and inspected for quantity and integrity. Fifteen micrograms of total RNA from each sample were reverse transcribed with 3 μl pdN9 random hexamer primers, and amino-allyl modified dNTPs. RNA samples were removed from the RT reactions, and cDNA was precipitated and resuspended in 8 μl of nuclease-free water and 2 μl of fresh 0.5 M sodium bicarbonate. Single-stranded cDNA was labeled with either Cy3 (reference samples) or Cy5 (experimental samples) cyanine monoreactive dye (Amersham, Pis cataway, NJ), prepared in 80 μl of dimethylsulfoxide. After dye reaction termination, reference and experimental labeled cDNAs were mixed together in equal quantity and cleaned. Samples were brought to a final volume of 40 μl in 0.8 μl 1 M HEPES (pH 7.0), 1.7 μl RNA (Sigma, St Louis, MO), 3 × SSC, and 0.2% SDS. After samples were boiled and cooled, they were quickly applied to microarray slides. Hybridizations were conducted overnight (16–18 h) at 65°C in Genetix (Boston, MA) humid hybridization chambers. After hybridization, slides were washed, dried, and scanned on an AXON GenePix 400B microarray scanner in GenePix 4.0 (Axon Instruments, Molecular Devices, Sunnyvale, CA).

**Data normalization and filtering.** We used a “reference design” to compare the relative amount of each transcript to a common reference pool. Raw data for each feature (median pixel intensity of each spot with background correction) were imported from GenePix 4.0 into GeneSpring GX (Agilent Technologies, Santa Clara, CA) for subsequent analysis. We normalized the data using intensity-dependent, locally weighted linear regression (lowess) transformation to correct for spatial variation in the array. The remaining normalization and filtering was performed in GeneSpring GX. First, we discarded all features that were flagged as irregular by Genepix 4.0 software or by hand (irregularities are generally due to specks of dust or scratches on slides). Next, we calculated the ratio of the background-corrected median intensities of each feature for each wavelength (635 nm/532 nm), which normalizes the experimental samples to the reference (cy5/cy3). Next, we grouped arrays by time point/temperature and used a Cross-Gene Error Model (CGEM) based on replicates to provide a more accurate estimate for the precision of the expression data. The CGEM combines measurement variation and between-sample variation information by assuming that variability between replicates is similar for all genes with similar measurement level. GeneSpring recommends such an error model when the number of replicates is less than three. Finally, we normalized each time point/temperature to the control samples for each respective acclimation group (note: each control animal was hybridized to a single array and then values were pooled). This normalized value was log (base 2) transformed (“normalized log; ratio”) to provide a continuous and proportional scale centered on zero, with +1 equal to a twofold upregulation, and −1 equal to a twofold downregulation. All subsequent analyses were performed on this filtered list containing 7,488 features.

**ANOVA analysis for gene list extraction.** We performed three separate one-way ANOVAs to identify probes that were differentially expressed within each experiment (17). The CGEM provided standard deviations and standard errors and used all available error estimates for each ANOVA. In all cases, we used a Benjamini and Hochberg false discovery rate cutoff of 0.05 to correct for multiple comparisons (17). A post hoc Tukey’s test was used in conjunction with the ANOVA to determine which time points were statistically different from the acclimation controls. These analyses were performed in GeneSpring GX.

**cDNA sequencing and annotation.** Significant features on the array that had not been previously sequenced were reamplified using methods described in Gracey (24) and Logan and Somero (34) and sequenced on a 3100 Genetic Analyzer DNA sequencer (Applied Biosystems, Foster City, CA). We sequenced from the 5’ end using primers specific to the plasmid vector. New sequences were deposited in GenBank under accession numbers FG227819-FG228196, FG528590-FG528666, FK829270-FK829413, GE766558-GE766578, GE843185-GE843306, GH27286-GH727394, GH296564-GH296594, and GH552108-GH552143. All sequenced features (5,301/7,488) were exported from National Center for Biotechnology Information (NCBI) into the program PartiGene version 2.2 (39) to cluster redundant sequences. PartiGene generated 3,895 nonredundant consensus sequences that were imported into Blast2GO version 2.4.0 (15) for annotation. Homology searches were made against the SwissProt database in February 2010 using Blastx with a minimum E value of 1 × 10^−6, as described in Logan and Somero (34). Annotations and expression data were deposited in the Minimum Information About a Microarray Experiment-compliant NCBI Gene Expression Omnibus platform with Liflichthys mirabilis platform GPL10169, series GSE22462, with 61 samples (GSM557943-GSM558004). Unless otherwise referenced, additional functional information about specific genes was found in the Gene Ontology (GO) Consortium and/or the manually annotated UniProtKB/Swiss-Prot databases (3, 43).

**Fig. 1.** Sampling scheme for G. mirabilis acute heat stress experiments. Fish were previously acclimated to 9, 19, and 28°C for 1 mo. Before each experiment, acclimated fish were transferred into a bucket to control for handling effects. Control fish were then placed back into the steady-state acclimation tank, and experimental fish were placed into a heat ramp tank at their respective acclimation temperature. Temperature was increased at 4°C/hr. Fish were sampled at +4°C intervals from their starting acclimation temperature (n = 3 solid circles; n = 2 shaded circles). At each experimental time point, one control fish was also sampled.

**Experimental Procedures**
The common acute heat stress response. We identified a list of significant genes that were common among the responses to acute heat stress in all three acclimation groups (hereafter, "common genes"). To identify the function of these genes, we used biological processes GO annotations described above, as well as relevant scientific literature.

Tiered response. For initial examination of a tiered response to increasing heat stress, GO annotations were used to identify genes that encode proteins in the following categories: protein folding, proteolysis, cell cycle, and apoptosis. However, many of the proteins identified in these categories are involved in more than one of these processes. Therefore, we examined the timing of expression for three specific genes that are characteristic of protein folding (HSP70), ubiquitin-dependent proteolysis (UBIQ), and cell cycle arrest (CDKN1B). A unique characteristic gene indicating signs of apoptosis could not be identified among significant genes. For HSP70, UBIQ, and CDKN1B, transcriptional changes have been shown to correlate with protein levels (8, 46). However, transcript regulation often does not directly map onto protein expression in a simple manner (9). For example, CDKN1B is regulated by its concentration, subcellular localization, and phosphorylation status (13). Thus analysis of a potential graded response must be done with these caveats in mind.

RESULTS

CI

CI serves as an indicator of overall physiological state, with a higher CI indicating better condition (7). Fish acclimated to 9°C had a significantly higher CI (0.0104 ± 0.0002 SE) than either the 19°C (0.0091 ± 0.0002) or 28°C (0.0087 ± 0.0001) acclimation groups (ANOVA, P < 0.0001), which were not significantly different from each other (Tukey honestly significant difference). Thus, according to this metric, the 9°C-acclimated fish were in better condition than fish acclimated to 19 or 28°C.

28°C-Acclimated Acute Heat Stress Experiment

Two hundred ninety-three probes were significantly different in expression during acute heat stress in 28°C-acclimated fish (Supplemental Table S3 and Fig. S2). A heat map of all significant probes summarizes dominant expression patterns during acute heat stress in 28°C-acclimated fish (Supplemental Fig. S1). Of these, 45% (133) were unique and could be annotated with GO information. These 133 significant genes represent 8% of the unique annotated genes on the array (133/1,607).

Gene Expression Patterns

To better visualize the T_on at which differential expression began during acute heat stress, we used the results from Tukey’s post hoc tests to plot the number of significant genes that were differentially expressed between the starting acclimation temperature and each acute temperature sampling point (Fig. 2). We plotted absolute and relative temperature (equal to degrees above the acclimation temperature) vs. the percentage of total significant genes that changed during a given experiment. For 9°C-acclimated fish, a large number of genes began changing in expression at ~16°C above the acclimation temperature, as measured by the pronounced inflection point in the curve. For 19 and 28°C-acclimated fish, this inflection point...
was observed at \( \sim 8 \) and \( \sim 4^\circ C \) above the acclimation temperature, respectively. In absolute terms, the inflection point occurred at \( \sim 25^\circ C \) for the 9°C group, at \( \sim 27^\circ C \) for the 19°C group, and \( \sim 32^\circ C \) for the 28°C group (Fig. 2, top). The curve clearly shifts to the right as acclimation temperature increases, showing the plasticity that exists in the \( T_{on} \) of acute heat stress-responsive genes with increasing acclimation temperature (Fig. 2, top). Because of the experimental design, the total time spent under rising temperature differed among the three groups, and this difference in cumulative time at rising temperatures could have influenced the induction patterns shown in Fig. 2. However, we emphasize that the onset of significant expression changes in the 9°C group occurred near 25°C, a temperature below the acclimation temperature of the most warm-acclimated group. Thus we conjecture that the acclimation process, rather than the cumulative time spent at rising temperatures in the acute stress protocol, is the principal cause of the differences among acclimation groups in the temperature at which significant changes in expression were observed.

### Comparing Responses Between Acclimation Groups

We identified 52 genes with significant expression differences during acute heat stress that were common to all three acclimation groups (hereafter, “common genes”). Of these common genes, 21 were unique and annotatable. We created heat maps for the last four acute temperatures sampled in each group to visualize similarities and differences in expression patterns for the common genes (Fig. 3). The last four sampled temperatures are shown because all significant changes in expression of the common genes occurred during this period. The large majority (49 of 52) of the common genes were upregulated with acute heat stress.

To better examine how acclimation affects \( T_{on} \) for these common genes, we plotted time course expression profiles for the thematic heat-responsive genes (Fig. 4, A and B). These included all of the common molecular chaperones (HSP70, HSP90B1, HSP40, HSP60, HSPA9, HSP27, and PDIA4) (Fig. 4A), as well as genes involved in the ubiquitin-proteasome pathway (UBIQ, UBE2, SUGT1, LONRF1) (Fig. 4B). We also examined expression profiles for genes involved in cell cycle regulation or apoptosis (CDKN1B, SGK1), and the single identifiable downregulated gene (HUR), which is involved in mRNA stabilization (Fig. 4C). In cases where multiple probes represented the same gene (i.e., identical Uniprot Accession Number), the probe with the lowest \( P \) value is displayed. Normalized log₂ ratios are listed in Tables S1–S3 (genes displayed in Fig. 4 are listed in bold).
Fig. 4. Normalized log2 ratio expression profiles for notable genes that were commonly expressed during acute heat stress in all three acclimation groups in gill tissue of *G. mirabilis* (means ± SD). The last four time points are shown, since no significant expression changes in these genes occurred in earlier time points. Absolute sample temperatures (°C) are indicated along the x-axis, and relative temperatures (°C) are indicated just below in italics. Solid bars indicate an expression level that significantly differs from the control (one-way ANOVA, false discovery rate < 0.05, Tukey’s honestly significant difference). A: molecular chaperones. B: ubiquitin-dependent proteolysis. C: cell cycle, apoptosis, and mRNA stabilization.
**Tiered Response**

We examined the timing of expression for genes characteristic of protein folding (HSP70), ubiquitin-dependent proteolysis (UBIQ), and cell cycle (CDKN1B). In all three acclimation groups, HSP70 was upregulated at the last three time points (Fig. 5). UBIQ was significantly upregulated at the last time point for all acclimation groups, and in the second to last time point in 28°C-acclimated fish (Fig. 5). CDKN1B upregulation was not significant until the most extreme sampled temperature was reached for all acclimation groups (Fig. 5).

**DISCUSSION**

The Ton of the heat shock response has previously been shown to vary with acclimation in G. mirabilis. The plasticity of this response has been described for two HSPs, HSP70 and HSP90 (10, 11, 19, 20). Dietz and Somero (20) found that winter-acclimatized fish increased synthesis of HSP90 at 28°C, whereas summer-acclimatized fish showed a significant increase in synthesis at 32°C. Buckley and Hofmann (10) found that Ton for HSP70 synthesis shifted by up to 10°C in 21 vs. 28°C-acclimated fishes (22–26 vs. 32°C, respectively). Ton likely reflects temperatures at which the intrinsic thermal sensitivities of the cell (e.g., protein thermal stability) lead to sufficient thermal damage that requires activation of stress-related genes. Through mechanisms that remain unknown, acclimation to higher temperatures can lead to some increase in Ton. The present study extends this body of knowledge and shows that Ton is phenotypically plastic not only for HSP70 and HSP90, but also for a whole suite of genes regulated during acute heat stress (Fig. 2). It appears that, on average, Ton for a significant change in transcription shifts up-

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**Fig. 5.** Normalized log$_2$ ratio expression profiles for genes characteristic of different types of molecular damage prevention or repair: protein folding (HSP70), ubiquitin-dependent proteolysis (UBIQ), and cell cycle arrest (CDKN1B) for the 9°C (A), 19°C (B), and 28°C acclimation group (C) (means ± SD). Solid bars indicate an expression level that significantly differs from the control (one-way ANOVA, false discovery rate < 0.05, Tukey’s honestly significant difference).
upregulated during acute heat stress in all acclimation groups (Fig. 4A). However, as a group, these genes did not exhibit a uniform expression pattern. HSP70 was upregulated relatively early in all acclimation groups (Fig. 4A) and induced more strongly than any other gene (21-, 35-, and 40-fold higher than controls in 9, 19, and 28°C-acclimated fish, respectively). The differences in fold-change are noteworthy because HSP70 fold-change is indicative of stress severity (44). The differences we observed in fold-change are not due to different constitutive levels of transcript during steady-state acclimation; no differences in transcript levels of HSP70 were observed among the three acclimation groups (34). However, this does not preclude the possibility that constitutive differences exist at the protein level. Higher standing-stock concentrations of HSP70 could affect, i.e., increase, the Ton of increased gene expression.

The gene for mitochondrial stress-70 protein (HSPA9) encodes a constitutively expressed and heat-inducible heat shock cognate protein that is also thought to function in negative regulation of apoptosis and cell proliferation (28, 45). HSPA9 showed the second highest fold-change in expression after HSP70 (9-, 18-, and 14-fold higher than controls in the highest acute temperature for 9, 19, and 28°C-acclimated fish, respectively), and a steady increase in expression level with increasing acute heat stress. HSPA9 was significantly upregulated at the last two acute time points for all acclimation groups.

HSP90B1, the endoplasmic reticulum (ER) localized member of the HSP90 family (also known as endoplasmin), is a molecular chaperone that functions in the processing and transport of secreted proteins (12, 21). Its upregulation is often used as a hallmark of ER stress (21). HSP90B1 also is a major calcium binding protein in the ER (21). Changes in the flux of calcium in and out of the ER are involved in many signaling pathways and physiological responses. HSP90B1 may also be a key ER-associated degradation sensor protein in response to misfolded proteins (21). Thus this protein may play a number of roles in the CSR. Unlike the aforementioned HSPs, HSP90B1 was not significantly upregulated in the warmest acclimation group until 36°C, compared with earlier Ton values of 29 and 31°C, respectively, in 9 and 19°C-acclimated fish. Steady-state levels of this transcript did not differ with acclimation (34). Transcripts for the cochaperone HSP40 and the chaperonin HSP60 showed similar patterns of expression in all acclimation groups during acute heat stress. Both genes were significantly upregulated in the final two acute time points for all acclimation groups. Unlike HSP40 and HSP60, the protein disulfide isomerase family member, PDIA4, had different patterns of expression in all three acclimation groups. In 9°C-acclimated fish, PDIA4 was significantly upregulated, beginning at 29°C and every acute time point thereafter; in 19°C-acclimated fish, upregulation began at 35°C; and in 28°C-acclimated fish, significant upregulation did not occur until 40°C. PDIA4 was also expressed significantly higher with each increase in steady-state acclimation temperature (34). Hence, lower constitutive levels in cold-acclimated fish may explain earlier onset of expression during acute heat stress.

The small HSP27 has several functions, including its role as an ATP-independent chaperone. It is known to function in cytochrome c-mediated apoptosis, prevention of filament aggregation and preservation of cytoskeleton structure, and...
activation of the proteasome (2). It also plays a role in translation inhibition during heat shock (17). HSP27 was significantly upregulated in the most extreme time points in the 19 and 28°C acclimation groups, but earlier in the 9°C group. Under extreme heat stress, ectotherms have been shown to inhibit protein synthesis, possibly to limit accumulation of unfolded proteins that might damage cells (23). The extent to which HSP27 may be involved in translational arrest in this study is not known. However, previous work does show that translational arrest in *G. mirabilis* begins at 37°C for fish acclimated to 21°C, but is not affected by 39°C for fish acclimated to 28°C (10). We demonstrate that transcription itself is not inhibited at extremely high temperatures (Supplemental Fig. S1) and conjecture that the continuation of transcription may reflect the cell’s attempt to accumulate the types of mRNAs it will prefer to translate into proteins, once temperatures have fallen to values that permit effective translation and protein maturation.

Ubiquitin-dependent proteolysis. Thermal stress can cause irreversible damage to proteins that molecular chaperones cannot remedy, leading to proteolysis via the ubiquitin-proteasomal pathway (23). Four genes encoding proteins involved in this pathway were upregulated in response to acute heat stress in all acclimation groups. These include genes for ubiquitin (*UBE2*), ubiquitin conjugating enzyme E2 (*UBE2*), RING finger protein 19 (*LONRF1*), and suppressor of G2 allele of SKP1 homolog (*SUGT1*). Their combined upregulation provides evidence of increased ubiquitin-dependent proteolysis in gill tissue during acute heat stress.

The four genes associated with proteolysis showed considerable variability in their expression patterns in the steady-state acclimated fish (34) and in response to acute heat stress. *UBE2* had higher constitutive levels of expression with warmer steady-state acclimation temperatures (34). *UBE2* was only upregulated in the last acute time point during heat stress in all acclimation groups, except for the 28°C-acclimated fish, which showed significant upregulation at both 36 and 40°C. *UBE2* and *SUGT1* were upregulated in the final two time points for all acclimation groups. *SUGT1* was also significantly upregulated at the third to last time point in the 9°C-acclimated fish (29°C). *SUGT1* is involved in kinetochore formation during mitosis and plays a role in ubiquitination and subsequent proteasomal degradation of target proteins (29). *SUGT1* showed higher constitutive levels during steady-state acclimation with each increase in acclimation temperature (34). *LONRF1*, on the other hand, was not significantly upregulated until the last time point in all acute stress experiments, indicating its involvement during extreme stress. *LONRF1* has ATP-dependent peptidase activity and is a part of the ubiquitin ligase complex.

In summary, the upregulation of genes encoding proteins critical for proteolysis through the proteasomal system seen under steady-state acclimation to high temperature (34) and in response to acute heat stress emphasizes the importance of protein homeostasis in thermal relationships. If enhanced expression of genes associated with proteolysis is indicative of increased protein turnover at higher temperatures, then reallocation of energy toward protein homeostasis and away from processes like growth may be an important element in sublethal temperature stress.

Cell cycle and apoptosis. Two additional common genes were involved in cell cycle regulation or cell death via apoptosis. One encodes a known cell cycle inhibitor, CDKN1B (or p27); this gene was strongly upregulated in the final time point for all groups (Fig. 4C). This gene’s expression pattern may be indicative of late-stage cell cycle inhibition or apoptotic induction (16). CDKN1B had highest constitutive levels of expression in the steady-state 28°C-acclimated fish compared with the 9 or 19°C-acclimated fish (34). Serine/threonine-protein kinase Sgk1 (SGK1) is a protein kinase that plays an important role in the CSR and is a key regulator of transport, cell volume, and cell survival (41). It may be indirectly involved in negative regulation of apoptosis (32) and positive regulation of the cell cycle (41). SGK1 was upregulated in the final two time points for all acclimation groups (Fig. 4C). From the limited number of common genes related to cell cycle and apoptosis, it would appear that both positive and negative regulators of these processes are modulated during the CSR. However, direct involvement of CDKN1B in cell cycle repression implicates it may play a more dominant role in this function (13). Likewise another strong positive regulator of the cell cycle, G1/S specific cyclin D2, was strongly inhibited with increasing temperatures in the 9 and 28°C groups (data not shown), but not significantly so in the 19°C group.

Although some of the gene expression changes provide evidence of cell cycle repression with acute heat stress, it remains unclear if activation of apoptosis occurred as well. Absence of apoptosis during the short time frame of these acute stress experiments could, however, be a reflection of a postponement of this energy-demanding process until other cellular activities related to damage repair were initiated or completed. For example, some HSPs are inhibitors of apoptosis (5, 6), so, in light of the strong heat shock response noted in these studies, a delay in apoptosis might be expected until molecular chaperoning activities have been reduced. Additional studies involving extended recovery times after stress and more direct cellular examinations of apoptotic processes are needed to examine this possibility.

mRNA stabilization: HUR. The single downregulated transcript that we could identify among the common genes, HUR, encodes an RNA binding protein that is involved in posttranscriptional gene regulation by stabilizing mRNAs that contain AU-rich elements in their 3’ untranslated regions (8). AU-rich element-containing mRNAs encode a functionally important subset of early-response genes, including those encoding stress response and proliferative proteins (1). However, a few examples exist in which upregulation of HUR mediates repression of translation (36, 33). For example, HUR depletion is known to specifically increase CDKN1B translation, leading to cell cycle arrest (30). In human cells, previous work demonstrated that heat shock reduces levels of HUR protein via proteolysis, which enhances cell survival following heat stress, but did not affect HUR mRNA levels or stability (1). Our findings suggest that HUR may be regulated at the transcript level in *G. mirabilis*, and its downregulation may be linked to CDKN1B upregulation and cell cycle arrest. HUR was regulated similarly in the 9 and 19°C-acclimated fishes during acute heat stress, showing a gradual decrease in expression with increasing acute heat stress (Fig. 4C). In contrast, in the 28°C-acclimated fish, HUR did not show a significant decrease in expression until the final time point, at 40°C.
Defining Severity of Sublethal Stress

We predicted that severity of heat stress could be revealed by a graded expression of genes associated with processes reflecting different levels of cellular damage. Thus initial heat-induced, but reversible, unfolding of proteins would lead to increased synthesis of message for molecular chaperones. More extreme heat stress, sufficient to cause irreversible protein denaturation, would trigger synthesis of message for proteins involved in removing irreversibly denatured proteins from the cell through the ubiquitin-proteasomal pathway. We also hypothesized that cell cycle inhibition would occur at some level of stress, to redirect energy away from growth toward cellular repair. Last, we examined whether acute heat stress led to apoptosis at temperatures at which entire cells were irrevocably damaged. In our initial analysis of the gene expression data, we looked at all of the genes annotated according to these GO ontologies. Because we found significant overlap existed among genes that functioned in multiple categories, we chose to take an alternate approach, choosing well-characterized genes that represent these processes in a less ambiguous manner (14, 16, 23).

In all three acclimation groups, protein folding (indicated by increased expression of HSP70) was induced early, at the final three time points (Fig. 5). Ubiquitination (indexed by increased expression of UBIQ) was significantly induced at the last time point for 9 and 19°C-acclimated fish and at the last two time points in 28°C-acclimated fish. Cell cycle arrest (via CDKN1B) was not significant until the final time point in all acclimation groups. Thus changes in expression of these genes involved in three distinct aspects of the CSR provide at least an initial basis for characterizing a tiered response to increasing heat stress.

An alternative approach to defining severity of sublethal stress is to select candidate genes based on their Tm during acute heat stress. HSP70 expression was a good indicator of mild stress; no other identifiable genes showed consistent upregulation in the last three time points for all acclimation groups. Variation existed in expression of UBIQ, which means it may not represent the best marker of moderate stress (Fig. 5). Alternatively, genes encoding the molecular chaperones HSP40, HSP60, and HSPA9 consistently showed upregulation at the last two time points, making them more consistent indicators of moderate stress (Fig. 4A). Finally, CDKN1B expression appeared to be a good indicator of extreme stress in all acclimation groups, but so does the ATP-dependent peptidase LONRF1 (Fig. 4B). Likewise, some of the genes with unclassifiable expressed sequence tags (Fig. 3) may also be useful indicators of stress level.

Perspectives and Significance

Determination of acclimation capacity at the gene expression level and time course observations of transcriptional responses during acute heat shock provide new insights into phenotypic plasticity and the fundamental mechanisms of eurythermy. We have identified tiered, stress level-related gene expression patterns during acute heat stress in gill tissue of a highly eurythermal fish. To our knowledge, this is the first study to provide transcriptional evidence of a tiered stress response that is in line with predicted sequential activation of the processes of protein chaperoning, proteolysis, and cell cycle inhibition with increasing levels of acute heat stress (31). Based on this transcriptomic analysis, we postulate alterations in cellular function that warrant follow-up examination with proteomics and functional cellular assays. These genes and their related gene products provide a good starting point for development of thermal stress biomarkers in fish gill tissue. By looking at physiological indexes of stress, these biomarkers may serve as “physiological fingerprints” of health in individual organisms in their natural environments (27), an issue that is particularly relevant for environmental scientists examining the consequences of global warming. Whether these biomarkers and their cellular functions indicate similar levels of mild, moderate, and extreme stress in other tissues and fish species (especially stenothermal fish) remains to be investigated.

ACKNOWLEDGEMENTS

We thank Dr. G. Schoolnik of Stanford University for use of his laboratory for the printing of microarray slides. We also thank S. Simon (University of California, Santa Barbara) and colleagues for fish collection, J. Sanders for technical support in fish maintenance and microarray hybridization, and B. Lockwood for help performing fish dissections. We are additionally grateful to S. Holmes for helpful discussions regarding data analysis and interpretation. Present address of C. A. Logan: Atmospheric and Oceanic Sciences, Princeton University, Princeton, NJ 08540.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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