Transcriptional responses to thermal acclimation in the eurythermal fish *Gillichthys mirabilis* (Cooper 1864)

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Logan CA, Somero GN. Transcriptional responses to thermal acclimation in the eurythermal fish *Gillichthys mirabilis* (Cooper 1864). *Am J Physiol Regul Integr Comp Physiol* 299: R843–R852, 2010. First published July 7, 2010; doi:10.1152/ajpregu.00306.2010.—Thermal acclimation (acclimatization) capacity may be critical for determining how successfully an ectotherm can respond to temperature change, and adaptive shifts in gene expression may be pivotal for mediating these acclimatory responses. Using a cDNA microarray, we examined transcriptional profiles in gill tissue of a highly eurythermal goby fish, *Gillichthys mirabilis*, following 4 wk of acclimation to 9°C, 19°C, or 28°C. Overall, gill transcriptomes were not strikingly different among acclimation groups. Of the 1,607 unique annotated genes on the array, only 150 of these genes (9%) were significantly different in expression among the three acclimation groups (ANOVA, false discovery rate < 0.05). Principal component analysis revealed that 59% of the variation in expression among these genes was described by an expression profile that is upregulated with increasing acclimation temperature. Gene ontology analysis of these genes identified protein biosynthesis, transport, and several metabolic categories as processes showing the greatest change in expression. Our results suggest that energetic costs of macromolecular turnover and membrane-localized transport rise with acclimation temperature. The upregulation of several classes of stress-related proteins, e.g., heat shock proteins, seen in the species’ response to acute thermal stress was not observed in the long-term 28°C-acclimated fish. The transcriptional differences found among the acclimation groups thus may reflect an acclimation process that has largely remedied the effects of acute thermal stress and established a new steady-state condition involving changes in relative energy costs for different processes. This pattern of transcriptional alteration in steady-state acclimated fish may be a signature of eurythermy.

gene expression; gill; transcriptome; microarray; protein biosynthesis; transport

EUROPTHERAL ECTOTHERMS are distinguished by their capacities to acclimate (or acclimatize) their physiological processes to a wide range of environmental temperatures over a number of different timescales. Modulation of gene expression plays a central role in these acclimatory processes, albeit the types of transcriptional changes that occur vary with intensity and duration of thermal stress (19, 32). In response to acute temperature change, initial and rapid alterations in gene expression facilitate processes linked to repair of damage, a reaction termed the cellular stress response (30); somewhat slower transcriptional events foster restoration of cellular homeostasis, the cellular homeostatic response (30). Over longer timescales of days to weeks, acclimatory changes in gene expression facilitate remodeling of cells, tissues, and organs. This ability to acclimate, which is often referred to as an example of phenotypic plasticity, can allow an organism to shift its thermal optimum for numerous physiological activities to a new temperature range (20, 24). Over evolutionary time, adaptive modifications of gene regulatory systems that coordinate responses to changes in body temperature can lead to hard-wired or genetic shifts in thermal optima and tolerance ranges. An increased understanding of these three distinct temporal patterns of response of gene regulatory processes to changes in body temperature is needed to develop a mechanistic understanding of the effects of temperature change on ectotherms. Variations in temperature ranges over which adaptive changes in gene expression can occur during acclimation may also underlie the bases for the widely different thermal tolerance ranges of eurytherms and stenotherms.

Until recently, most of our knowledge about environmentally induced changes in gene expression came from studies of model organisms, such as yeast, which finely balance energy-efficient growth with the ability to rapidly adapt to sudden external challenges. This body of research has uncovered conserved basic principles by which cells are able to adjust their transcriptomes to respond to changing environments (18, 30, 32). In model organisms, -omics tools are now being applied at the proteome level as well, taking a systems biology approach to integrate across several levels of biological organization to better understand the regulation of physiological processes (37). Thus, while the transcriptome is an imperfect proxy for monitoring environmental physiology (due to changes in activity, regulation, and modification of proteins), it has proven to be very powerful in the discovery of candidate genes and hypothesis-independent driven elucidation of functional mechanisms (19). This is particularly true for ecologically important nonmodel species for which the absence of genome sequence data precludes fully exploiting the systems biology approaches readily available for model organisms. Nonetheless, by building on technological and empirical advances of studies of model organisms like yeast, comparative studies have begun to exploit genomic approaches to yield insights into how transcriptional responses in a variety of nonmodel animals, including several species of fish, contribute to abilities to cope with environmental change (19).

One species of fish that has proven highly suitable for analysis of transcriptional responses to a wide range of environmental conditions, including shifts in oxygen availability (22), salinity (17), and temperature (4) is a highly eurythermal goby fish, the longjaw mudsucker *Gillichthys mirabilis* (Cooper 1864). This species’ capacity to acclimate to a wide range of environmental factors makes it an excellent model for investigating the molecular basis of physiological plasticity (20). The biogeographic range of *G. mirabilis* extends from Tomales Bay, CA (38.1602°N, −122.8945°W) to both coasts of the Baja California peninsula and the northern coast of
mainland Mexico bordering the Gulf of California (38). In its shallow estuarine habitats, this species can experience one of the widest temperature ranges of any fish, from −5°C to 37°C (5). A number of studies have documented the phenotypic plasticity of this species in response to changes in temperature. At the level of the whole organism, lethal temperatures were shown to vary with acclimation history (50). At the level of protein expression, induction temperature of the heat-shock response shifts with acclimation temperature (5, 6, 13, 14). At the transcriptional level, acute thermal stress is known to cause changes in expression of several hundred genes (4).

In an effort to better understand the gene regulatory responses underlying this species’ broad thermal tolerance range and phenotypic plasticity, we used a complementary DNA microarray (20) to characterize the transcriptomes of gill tissue of fish acclimated for 4 wk to three temperatures (9°C, 19°C, and 28°C) that lie within the broad range of temperatures this species experiences over its biogeographic range. We chose gill tissue because of its multiple metabolic roles and demonstrated phenotypic plasticity (39). In teleost fish, gills are the primary site of oxygen uptake, osmotic and ionic regulation, acid-base regulation, and excretion of nitrogenous wastes (16), and have been shown to undergo large changes in gene and protein expression during acute heat stress in *G. mirabilis* acclimatized to ambient seawater temperatures (4). In the present study, our overall goal was to identify those genes that are responsible for sustaining cellular homeostasis following steady-state thermal acclimation, thereby elucidating the acclimatory changes in gene expression that may underlie this species’ remarkable level of eurythermy. Two specific issues were of particular interest within this broader framework. First, in the context of energy demands (costs of living) at different temperatures, we wished to determine whether the transcriptomes of warm- and cold-acclimated fish indicated different energetic requirements for critical, ATP-demanding processes like protein synthesis and osmotic/volume regulation. Second, we sought to determine whether gene expression patterns in long-term acclimated fish involved the continued upregulation of a suite of stress-related genes, for instance those that encode heat shock proteins (HSPs), which are known to be strongly induced in this species during acute stress (4, 34). Our results suggest that 19°C and 28°C-acclimated fish sustain higher energy costs than fish acclimated to 9°C, but the upregulation of stress-related genes seen during acute heat stress ceases during long-term acclimation. This suggests effective compensation during longer-term acclimation for some of the key forms of cellular perturbation caused by acute thermal stress.

**MATERIALS AND METHODS**

**Animals**

*G. mirabilis* were collected from an estuarine lagoon at the University of California, Santa Barbara, CA (34.39°N, 119.81°W) using baited minnow traps. Fish were transported to Hopkins Marine Station, 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 use of the reference pool of total RNA. Fish were killed by cervical transection and dissected immediately. Gill tissues were placed in 1.5-ml Eppendorf tubes and flash frozen in liquid nitrogen for subsequent RNA extraction. Following the week-long laboratory acclimation period, three groups of 30 individuals were then randomly assigned to temperature-controlled acclimation tanks for 4 wk at either 9 ± 0.5°C, 19 ± 0.5°C, or 28 ± 0.5°C in 40-liter recirculating seawater aquaria (AquaLogic, San Diego, CA). We chose acclimation temperatures that were about ± 2°C from the lowest and highest temperatures that fish had been previously acclimated to in the laboratory [7°C (29); 30°C (13)]. We chose 19°C because it was about halfway between 9°C and 28°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 at 9°C and 19°C since there was not a large temperature difference from the flow-through seawater (−13−14°C). Half of the volume of water in each tank was exchanged for fresh, filtered seawater once per week. Dissolved oxygen (cat. no. YSI52, YSI, Yellow Spring, OH), ammonia, and nitrite/nitrate levels (Quick Dip Test Strips; Jungle Laboratories, Cibolo, TX) were measured weekly. Animals were fed on a commercial fish diet (Bio-Oregon, Warrenton, OR) three times per week. Animals were not fed in the 48 h prior to the end of the acclimation period to normalize for nutritional status. Following the acclimation period, at least six individuals were killed from each tank (9°C: n = 9, 19°C: n = 6, 28°C, n = 7) for a combined total of 22 individuals. 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 (protocol no. ID 12972).

**Experimental Procedures**

**Critical thermal maximum.** To assess the differences in tolerance to acute thermal stress following acclimation, we performed a critical thermal maximum experiment on a different set of fish than those used for the gene expression measurements. These fish were collected at the same time but were held under the ambient flow-through seawater conditions for 2 mo prior to the acclimation period. Two groups of 12 individuals were then acclimated to 9°C or 28°C for 3 wk by using the same experimental setup described above. Fish were exposed to a temperature heat ramp at a rate of +4°C per hour from their starting acclimation temperature and observed for signs of loss of equilibrium. The critical thermal maximum is defined as the water temperature at which loss of equilibrium or the failure of a fish to maintain dorso-ventral orientation for at least 1 min is observed (11). No lethal effects occurred with our use of this thermal tolerance protocol.

**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 is called the condition index, which can be a proxy for well-being (2). We calculated whether the ratios were significantly different between fish 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 (4, 20). 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 Schoolnik at the Stanford University School of Medicine, Stanford, CA.

Total RNA was extracted from gill tissue using a TissueLyser for homogenization and an RNeasy Mini Kit (Qiagen, Valencia, CA). Total RNA was determined by A260 absorbance using a spectrophotometer (model ND-1000; Nanodrop, Wilmington, DE). Samples were run on 1% agarose gels to confirm RNA integrity. Then 15 μg of total RNA from each sample were reverse transcribed with 3 μl of AffinityScript enzyme (Agilent Technologies, Santa Clara, CA), 0.75 μl oligo(dT)15, and 0.75 μl dNTPs to random hexamer primers, and
amino-allyl modified 2-deoxynucleotide 5'-triphosphates. RNA templates were removed from the RT reactions by incubation at 65°C for 30 min in 0.2 M NaOH and 0.1 M EDTA. cDNA was precipitated at −80°C for 20 min in 4.5 μl 3M sodium acetate and 2.5 volumes on 100% ethanol. Samples were centrifuged at 10,000 rpm for 20 min at 4°C and resuspended in 8 μl of nuclease-free water and 2 μl of fresh 0.5 M sodium bicarbonate. Single-stranded cDNA was then labeled with either cy3 (reference samples) or cy5 (acclimation samples) cyanine monoreactive dye (Amersham, Piscataway, NJ), prepared in 80 μl of dimethylsulfoxide. cDNA was incubated with 5 μl of Cy dye for 1 h in the dark at room temperature. Reactions were terminated by incubation with 5 μl of 4 M hydroxylamine for an additional 15 min. Fluorescently labeled CDNAs were mixed together and cleaned over PCR purification columns to remove free dye (Qiagen) and eluted in 10 μl of dH2O. Samples were brought to a final volume of 40 μl in 0.8 μl 1M HEPES (pH 7.0), 1.7 μl rRNA (Sigma, St Louis, MO), 3× SSC, and 0.2% SDS. Samples were boiled for 2 min, allowed to cool to room temperature for 5 min, and then quickly applied to microarray slides.

Hybridizations were conducted overnight (16–18 h) at 65°C in GenePix (Boston, MA) humid hybridization chambers. After hybridization, slides were washed by repeated vigorous immersion in 0.5× SSC and 0.01% SDS for 2 min to remove unbound dye. Slides were then washed in 0.6× SSC on a shaker for 15 min and dried by centrifugation. The slides were scanned on an AXON GenePix 4000B 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 in each acclimation group to a common reference pool (see Animals). Raw data for each feature (median pixel intensity of each spot with background correction) were imported from GenePix 4.0 into GeneSpring GX 7.3.1 (Silicon Genetics, Redwood, 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 due to inconsistencies in printing pins, slide surface variability, and differences in local hybridization conditions across a slide. The remaining normalization and filtering was performed in GeneSpring GX 7.3.1. 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). This value was log (base 2) transformed to present a balanced view of up- and downregulation (Normalized Log-Ratio). Finally, we grouped arrays by acclimation temperature and removed features that showed standard deviations of > 1.0 among individuals within the same acclimation group. We filtered on standard deviation because genes whose expression level had large variability around the mean are less likely to be reliable. Thus, normalized Log-Ratio values represent the difference in expression between the experimental group and the reference pool. All subsequent analyses were performed on this filtered list containing 7,488 features.

ANOVA analysis for gene list extraction. We conducted a one-way ANOVA using a Benjamini and Hochberg false discovery rate cut-off of 0.05 to correct for multiple comparisons and to identify probes that were differentially expressed between acclimation groups (10). A post hoc Tukey’s test was used in conjunction with the ANOVA to determine which specific acclimation groups were statistically different from each other (9°C, 19°C, and 28°C). These analyses were performed in GeneSpring GX 7.3.1.

cDNA sequencing and annotation. Significant features on the array that had not been previously sequenced were reamplified using methods described in Gracey (20) 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-GE843305, GH272386- GH272394, GH296564-GH296594, and GH552108-GH552143. All sequenced features (5,301/7,488) were exported from NCBI into the program PartiGene v2.2 (41) to cluster redundant sequences. PartiGene generated 3,895 nonredundant consensus sequences that were imported into Blast2GO version 2.4.0 (9) for annotation. Homology searches were made against the SwissProt database in February 2010 using Blastx with a minimum E value of 1 × 10^-6. The manually curated SwissProt database is known for low levels of misannotation (45). Blast result accesses were used to retrieve gene names using NCBI mapping files (geneinfo and gene2 accession) in Blast2GO. Blast result GI identifiers were used to obtain Gene Ontology Consortium (GOC) accession numbers for biological process, molecular function, and cellular component from the GOC database. The 3,895 nonredundant consensus sequences were successfully annotated to 1,764 gene products. Of these, 1,607 were unique and had GOC information (in a small number of cases, multiple consensus sequences annotated to the same gene product). The average consensus sequence was 593 bp with an average minimum Blastx E value of 8 × 10^-13. Annotations and expression data were deposited in the MIAME-compliant NCBI Gene Expression Omnibus under Gillichthys mirabilis platform GPL10169, series GSE20849, with 22 samples (GSM521396-GSM521417). Only features that were sequenced and found to be unique and annotatable are used in the remaining analyses. In the cases where multiple features encoded the same gene, expression data for the feature with the lowest probability value are presented. Unless otherwise referenced, additional functional information about specific genes was found in the GOC and/or the manually annotated UniProtKB/Swiss-Prot databases (1, 51).

Expression profile clustering. We used the data generated from the Tukey’s post hoc test to manually cluster genes with similar expression patterns. Because the normalized Log-Ratio values represent the difference in expression between the acclimation group and the reference pool, this cluster analysis allows us to examine the relative differences in expression among acclimation groups. If expression levels for a given gene were significantly different between a pair of acclimation temperatures, we scored a 1 for that comparison, and if not significant, a 0. Each gene also received two additional scores to determine whether the change in expression was positive (0) or negative (1) with respect to acclimation temperature (e.g., is 9°C >19°C?). Using the combinations of the five scores, we placed each gene into one of eight expression profile clusters (Fig. 1).

Principal component analysis. Principal component analysis (PCA) is a statistical technique for determining the key variables in a multidimensional data set that explain the differences in observations. PCA can be used to simplify the analysis and visualization of microarray datasets after an ANOVA has removed uninteresting sources of variance (12). To identify expression patterns that explained the most amount of variation in the dataset, we performed a PCA on the significant genes using a Pearson correlation matrix implemented in JMP 8.0.1. The principal components (or eigenvectors) are derived from an eigenvalue decomposition of the correlation matrix. The shape of each eigenvector can be visualized as an eigenvalue decomposition of the correlation matrix. The matrix of each eigenvector can be visualized as an expression profile. The loading of each acclimation group indicates how much the group defines each principal component (the squares of the loadings indicate their percentage in the principal component). Pairwise Pearson’s correlation coefficients (r) between each pair of acclimation groups were calculated for all significant genes. A Student’s t-test was used to assess whether correlations were significant (t-test, P = 0.05).

Gene ontology analysis. Interpretation of gene expression data is greatly improved by identifying changes in individual genes within the context of a larger collection of curated gene sets (19). To identify functional categories that were enriched in our significant gene lists, we chose to visualize the biological processes within each gene list.
from each other. Thus, the 9°C-acclimated fish were in better condition than the 19°C or 28°C acclimation groups according to this metric.

The critical thermal maximum and condition index data thus support our conjecture, elaborated below, that a new homeostatic state is reached during the 3 or 4 wk acclimation periods, in part due to the transcriptional changes we now discuss.

**Overarching Gene Expression Patterns**

The number of genes showing significant changes in expression was a small fraction of the total number of genes on the array. Only 359 out of the 7,488 total genes on the array, i.e., ~4.8% of the genes, were significantly different in expression among the three groups of differently acclimated fish (see Table S.1). Supplemental data for this article are available online at the American Journal of Physiology–Regulatory, Integrative and Comparative Physiology website.) Of these 359 genes, 150 were unique and could be annotated with GOC information (hereafter referred to as significant genes). These 150 significant genes represent 9% of the unique annotated genes on the array (150/1,607). More than half of these genes (76/150) were significantly different in 9°C-acclimated fish compared with the other two groups, but were not significantly different between 19°C- and 28°C-acclimated fish (Table S.1). Thirty-nine genes were similarly expressed in the 9°C and 19°C groups, but were significantly different from the 28°C group. Seventeen genes were similarly expressed between the 9°C and 28°C groups, but were significantly different from the 19°C group. A heat map of the significant genes summarizes these expression patterns among the three acclimation groups and highlights the similarity in expression between the 19°C and 28°C groups, compared with the 9°C group (Fig. 2A).

To extend our analysis and initiate a physiological interpretation of the transcriptional differences among acclimation groups, we next clustered the significant genes by expression profile. Fig. 1 summarizes the eight possible patterns and the number of significant genes displaying each pattern. Clusters 1–3 include genes whose expression increased with temperature; these genes account for 58% of the significant genes. Clusters 4–6 include genes whose expression decreased with temperature; these genes account for an additional 30% of the significant genes. Clusters 7–8 include genes whose expression did not correlate consistently with temperature across the full acclimation temperature range. This smaller subset of genes accounts for only 12% of the significant genes. Together, clusters 2 and 6 comprised nearly half of the significant genes (76/150) and manifested expression patterns where genes in the 9°C-acclimation group are expressed either significantly lower or higher, respectively, than in the 19°C- and 28°C-acclimation groups, which are not significantly different from each other.

PCA revealed that 59.1% of the variation in expression could be described by eigenvector 1 (E1), 33.3% by eigenvector 2 (E2), and 7.5% by eigenvector 3 (E3) (Fig. 2B). Based on E1 and E2, the 19°C- and 28°C-acclimation groups are nearly indistinguishable from each other, but well separated from the 9°C group. E3 separates the 19°C and 28°C groups from each other. The loading plot of each acclimation group onto each principal component clearly shows this pattern (Fig. 2C). The eigenvector expression profiles (Fig. 2B) reflect the same expression patterns observed in clusters 2, 6 and 7, respectively (Fig. 1).

**RESULTS AND DISCUSSION**

**Critical Thermal Maximum and Condition Index**

Following 3 wk of acclimation to 9°C or 28°C, the critical thermal maximum differed by 5.3°C (Student’s t-test, \( P < 0.0001 \)). The 9°C-acclimated fish reached loss of equilibrium at 34.4°C ± 0.15 SE and the 28°C-acclimated fish, at 39.7°C ± 0.05 SE. Thus, thermal acclimation significantly alters whole organism tolerance to the effects of acute heat stress.

We calculated the condition index, or weight divided by length cubed, for fish in the critical thermal maximum experiment and for fish that were acclimated for the gene expression experiments. Condition index can be used as an indicator of overall physiological state, with a higher condition index indicating better condition (2). For the critical thermal maximum experiment, the 9°C-acclimated fish had a significantly higher condition index than the 28°C-acclimated fish (Student’s t-test, \( P = 0.0005 \)). For the gene expression experiments, the 9°C-acclimated fish had a significantly higher condition index than either the 19°C or 28°C acclimation groups (ANOVA, \( P = 0.003 \)), which were not significantly different
Thus, the multivariate PCA analysis agreed with the clusters derived from the post hoc Tukey’s test. Furthermore, correlation analysis on all significant genes showed that the 19°C- and 28°C-acclimation groups were significantly correlated to each other (r = 0.77; Student’s t-test, P < 0.0001) (Table 1).

**Gene Ontology Functional Analysis of Significant Genes**

Our gene ontology quantitative analysis of biological process revealed several interesting differences when a comparison was made between genes that were upregulated with increasing acclimation temperature (cluster 1–3) vs. those that were downregulated with increasing acclimation temperature (cluster 4–6). We identified gene ontology biological process terms using BLAST2GO at level 3 (less specific) and level 4 (more specific) that differed in response by at least 25% (Table 2). At level 3, differences between genes up- and downregulated with increasing acclimation temperature included cell communication and cell cycle. At the more specific level 4, differences existed in primary metabolic pathways, including lipid metabolic process, macromolecular metabolic process, phosphorus metabolic process, and regulation of growth. Other processes within this category included cell death, transport, gene expression, protein localization, and organelle organization.

**Gene Ontology Biological Processes with Similar Expression Patterns**

**Protein biosynthesis and turnover.** For some of the biological processes identified in the gene ontology analysis, we

### Table 1. Pairwise Pearson’s correlation coefficients (r) between each pair of acclimation groups for all significant genes

<table>
<thead>
<tr>
<th>Group</th>
<th>By Group</th>
<th>Correlation</th>
<th>Lower 95%</th>
<th>Upper 95%</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td>9°C</td>
<td>19°C</td>
<td>-0.0263</td>
<td>-0.1891</td>
<td>0.1378</td>
<td>0.7542</td>
</tr>
<tr>
<td>9°C</td>
<td>28°C</td>
<td>0.0446</td>
<td>-0.1198</td>
<td>0.2067</td>
<td>0.5954</td>
</tr>
<tr>
<td>19°C</td>
<td>28°C</td>
<td>0.7673</td>
<td>0.6904</td>
<td>0.8271</td>
<td>&lt;0.0001*</td>
</tr>
</tbody>
</table>

A Student’s t-test was used to test whether correlations were significant (Student’s t-test, P < 0.0001). *Genes in the 19°C- and 28°C-acclimated groups were the only pair that was significantly correlated.

![Image](https://example.com/image.png)
found a similar expression profile for all genes in that category. One example is the set of genes involved in translation, where our analysis identified 10 genes with highest expression at 19°C and 28°C, most reflecting a cluster 2 expression profile (Figs. 1 and 3A). These included genes encoding seven ribosomal proteins (RS4, RL28, RL6, RS30, RS19, RL34, and RLAO) and three elongation factors (EF1A3, EF2, and EIF1AX). This consistent pattern suggests that higher acclimation temperature fosters increased protein biosynthesis, perhaps to compensate for a higher rate of protein turnover at high temperatures.

Six genes involved in protein folding were also identified (Fig. 3B). These included genes encoding two members of the peptidyl-prolyl isomerase family (FKB10 and FKB18), two members of the protein disulfide isomerase family (PDIA3 and PDIA4), T-complex protein 1 (TCPB), and the constitutive HSP 90β (HSP90B). All of these proteins play roles in assisting the proper folding of proteins under housekeeping conditions and as part of the heat shock response (42). FKB10 and TCPB were expressed most highly at 9°C (cluster 6 profile), while the four others were expressed most highly at 28°C (cluster 1 or 2 profile) (Figs. 1 and 3B). Given the evidence that protein biosynthesis is increased at higher acclimation temperatures, it might be expected that constitutive protein-folding machinery would increase as well, and this is suggested by higher expression of genes for four of six proteins with chaperoning activities.

Making proteins is a costly process, and it has been theorized that the equivalent of four ATPs are required for each peptide bond synthesized (26). The aerobic cost of protein synthesis in fish gills is thought to be high. For example, measurements of oxygen consumption and protein synthesis before and after incubation with an elongation inhibitor revealed that 70% of oxygen consumption is used for protein synthesis in flounder gills (26, 35). In other fish tissues, this value ranged from 2% in trout hearts to 85% in trout hepatocytes (reviewed in Ref. 46). Given that oxygen uptake of gill tissue is estimated to be ~ 100 μl O₂g⁻¹ gill wt⁻¹ h⁻¹, roughly equivalent to almost twice the oxygen uptake of the intact fish on a per gram basis (28), gill tissue alone could sequester 7% of the fish’s total oxygen consumption for its own metabolism (39). Therefore, a substantial increase in branchial protein biosynthesis could be a significant energetic cost for the whole organism. However, the production costs of protein synthesis are known to decrease as protein synthesis rates increase (35); so direct measurement of oxygen consumption is needed to determine tissue and whole organism costs empirically.

Studies of temperature effects on expression of genes associated with protein synthesis in other fish species have shown both up- and downregulation at high temperature. Significant upregulation of genes encoding translational machinery in liver tissue has been observed following warm temperature acclimation in another highly eurythermal species, the annual killifish Austrofundulus limnaeus (43). In rainbow trout fry, an increase in water temperature from 5°C to 15°C brought about an increase in the rate of protein synthesis (36). In contrast, warm acclimation led to decreases in expression of genes encoding translational machinery in hearts of rainbow trout (52) and Pacific bluefin tuna (8) and in several tissues of carp (21). One of the contributors to the differences noted among these studies is the type of tissue used in the analyses. Gill tissue is known to be one of the most active tissues with respect to rates of protein turnover, second only to liver (35), so our results may reflect the choice of an especially metabolically active tissue.

Protein turnover is the balance between protein synthesis and degradation, so if protein synthesis is high, we might expect a concomitant increase in protein degradation. However, a closer examination of genes in the category of protein catabolism did not reveal a uniform increase in expression with increasing acclimation temperature. The two basic types of protein degradation are lysosomal degradation and selective protein destruction via the ubiquitin-proteasome pathway. Three genes that encode proteins involved in the ubiquitin cycle had higher expression in the warmer acclimation groups (cluster 2 or 3) (Figs. 1 and 3C). These included the genes encoding ubiquitin (UBIQ); suppressor of G2 allele of SKP1 (SUGTI), which may play a role in ubiquitination and subse-

Table 2. Biological processes that differ in number of genes between the upregulated (cluster 1–3) and downregulated (cluster 4–6) gene lists

<table>
<thead>
<tr>
<th>Biological Process</th>
<th>No. Genes Upregulated</th>
<th>No. Genes Downregulated</th>
<th>%Upregulated Gene List</th>
<th>%Downregulated Gene List</th>
<th>RPD</th>
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<tbody>
<tr>
<td>Cell communication</td>
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<td>6</td>
<td>26</td>
<td>13</td>
<td>33</td>
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<td>Cell death</td>
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<td>5</td>
<td>18</td>
<td>11</td>
<td>25</td>
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<tr>
<td>Regulation of growth</td>
<td>8</td>
<td>2</td>
<td>9</td>
<td>4</td>
<td>35</td>
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<tr>
<td>Organelle organization</td>
<td>15</td>
<td>4</td>
<td>17</td>
<td>9</td>
<td>32</td>
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<td>1</td>
<td>10</td>
<td>2</td>
<td>65</td>
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<tr>
<td>Transport</td>
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Relative %difference (RPD) was calculated by dividing the number of genes in a Gene Ontology Consortium (GO) category within a gene list by the total number of genes in the gene list and then calculating the %difference. Level 3 categories are more general than level 4 categories, generally representing Parent Terms of higher levels. RPD of > 25% are listed.
Temperature from left to right, 9°C, 19°C, and 28°C. Gene symbols are referred across rows. Acclimation groups are represented by columns, increasing in differences between acclimation groups can be visualized by comparing color in expression between the acclimation group and the reference pool. Relative to in RESULTS AND DISCUSSION and can be referenced to full gene name in Table S1.

Gene symbols are referred to in RESULTS AND DISCUSSION and can be referenced to full gene name in Table S1.

CNDP2)
CNDP2)
CNDP2)

**Ion and Active Transmembrane Transporters**

All five genes in the ion transport category had highest expression levels in the 28°C acclimation group, consistent with cluster 2 or 3 expression profiles (Figs. 1 and 3D). In fish gills, ion transport is critical for regulation of osmotic content/composition, cellular volume, and acid-base balance, as well as excretion of nitrogenous wastes (16). Two ammonium transporters (encoded by RHBG and RHAG) are likely involved in increased secretion of ammonia at 28°C. The metalloendopeptidase STEAP4 is involved in iron transport, using NAD⁺ as acceptor, and is also thought to play a role in systemic metabolic homeostasis. Transcription of the gene encoding the sodium/myoinositol cotransporter (SC5A3) is known to be stimulated in response to hypertonicity, leading to an increase in the activity of the cotransporter, which in turn drives accumulation of myoinositol inside the cell (44). Myoinositol can function as an osmoprotectant and protein stabilizer under thermal stress conditions and may be functioning as such under warm acclimation (53). Vacuolar type H⁺-ATPases couple the energy of ATP hydrolysis to proton transport across intracellular and plasma membranes. Vacuolar type H⁺-ATPases are thought to be important in osmoregulatory ion uptake across external epithelia of euryhaline fish adapted to life in fresh water (31). Here, VAOD1 may be expressed higher at the warmest acclimation temperature due to thermal enhancement of osmoregulatory demands.

In addition to VAOD1 and SC5A3, three other genes that encode ATP-utilizing active transmembrane transporters revealed higher expression at high temperatures: ATP-binding cassette subfamily D member 4 (ABCD4), tapasin (TPSN), and glutamate/H⁺ symporter (GHCI) (Fig. 3D). Together, the similar expression profiles of genes involved in ion transport, and those involved in active transmembrane movement of organic osmolytes, peptides, and fatty acids, is consistent with the possibility that transport activity and, hence, transport costs increase at high temperatures. It has been suggested that ion transport may account for up to 10% of a resting mammalian cell’s oxygen consumption (28). In view of the complex osmoregulatory and acid-base functions of fish gills, an even higher fraction of metabolism may be devoted to transport. Some of the increased costs at higher temperatures, notably those associated with inorganic ion transport, may reflect the effects of increased kinetic energy on transmembrane leakage of ions (25). Other transcriptional changes in transporter-encoding genes may reflect temperature-related differences in transport of metabolic substrates and other processes.

**Lipid Metabolism**

All genes in the lipid metabolism category were expressed most highly in the 9°C acclimation group, most reflecting the cluster 6 expression profile (Figs. 1 and 3E). Lipid metabolism is almost invariably found to change during thermal acclimation...
tion because of shifts in membrane composition and in fuel preferences for ATP production (23, 24). The changes in expression of genes related to lipid biosynthesis are consistent with the well-characterized changes in lipid metabolism that are critical for adjusting membrane fluidity and phase during thermal acclimation (24).

The maintenance of membrane fluidity (static order) at different acclimation temperatures (homeoviscous adaptation) is a common acclimatory response involving major alterations to membrane lipid composition (23, 24). We identified four genes that may contribute to homeoviscous adaptation: ceramide synthase 1 (LAG1), N-acetyl-β-glucosaminidase (HEXA), diaphorase (NB5R3), and fatty acid synthase (FAS). FAS is a multifunctional enzyme that catalyzes several reactions involved in lipid biosynthesis. NB5R3 functions in the desaturation and elongation of fatty acids and in cholesterol biosynthesis (33). LAG1 and HEXA are both involved in lipid membrane metabolic processes. HEXA is responsible for the degradation of glycosphingolipids and other molecules with terminal N-acetyl hexosamines. LAG1 regulates the synthesis of stearoyl-containing sphingolipids. Podrabsky and Somero (43) also found evidence for downregulation of genes involved in fatty acid biosynthesis following warm temperature acclimation in liver of killifish (Austrofundulus limbatus). Long-term exposure to different temperatures would be expected to lead to continuing differences in expression of genes related to lipid metabolism because of the reestablishment of new steady-state conditions of lipid biosynthesis and lipid catabolism.

Response to Stress

The broad category of response to stress includes proteins involved in restoring cellular homeostasis following cellular damage due to stress. One of the major types of cellular damage resulting from stress due to diverse abiotic factors, including temperature, is denaturation of proteins (30). Consequently, following stress, it is common to observe increased expression of HSPs. HSPs and other molecular chaperones interact with stress-denatured proteins to maintain or restore their native structures and prevent aggregation and degradation (42). Surprisingly, none of the genes encoding for HSPs were upregulated at the highest acclimation temperature, with the exception of the constitutive HSP90β (Fig. 3F). Acute heat stress has been shown to upregulate expression of HSP-encoding genes in G. mirabilis at temperatures several degrees below 28°C. For example, in 13°C-acclimated specimens, HSP70 mRNA in liver showed significant upregulation by 21°C (34). Increased synthesis of HSP70 protein in liver, observed with metabolic labeling, occurred by 22°C (5). Dietz and Somero (14) observed increased synthesis of HSP90 in brain of G. mirabilis when 18°C-acclimated individuals experienced an acute temperature ramp to 28°C. In a subsequent study, Dietz (13) showed enhanced synthesis of HSP90 at an acute onset temperature of 28°C in 20°C-acclimated fish and at 24°C in 10°C-acclimated fish in gill tissue.

The lack of a pronounced difference in expression of genes encoding HSPs following month-long acclimation stands in sharp contrast with the large fold changes (> 6-fold increase) exhibited by genes that encode HSPs (HSP27, HSP40, HSP60, HSP70, HSC71, and HSP108) during acute heat stress in G. mirabilis acclimated to ambient seawater temperatures (4) and in fish acclimated to 9°C, 19°C, and 28°C (C. A. Logan, unpublished observations). Molecular chaperones were the most strongly induced set of genes in any category under acute heat stress (Ref. 4 and C. A. Logan, unpublished observations). The absence of this type of response found in the month-long 28°C-acclimated fish may be a reflection of the effectiveness with which the immediate effects of thermal stress on protein homeostasis were overcome during acclimation. This ability to overcome acute stress may be a hallmark of eurythermy.

In contrast to the majority of genes encoding HSPs, HSP90β was expressed significantly higher with each increase in acclimation temperature (cluster 1). In unstressed cells, the constitutive HSP90β plays a number of important roles, which include assisting in folding, intracellular transport, maintenance, and degradation of proteins and in cell signaling. Thus, for HSP90β, it is difficult to assign the principal physiological role(s) that may be of increased importance at higher temperatures. Three other genes in the stress response category that were upregulated at 19°C and 28°C encode proteins involved in the immune response: the transcription factor ZN143, small-inducible cytokine A20 (CCL20), and tyrosine-protein kinase Lyk (ITK). Genes for serine/threonine-protein kinase (SGK3) and complement component 7 (C7) were upregulated most highly at 19°C; SGK3 phosphorylates several target proteins and has a role in neutral amino acid transport and activation of potassium and chloride channels. Cdk inhibitor p21 binding protein (BCCIP) is involved in DNA damage repair and was expressed most highly at 9°C and 19°C (35).

Magnitude of Transcriptional Changes: Acclimation vs. Acute Stress

One additional distinction between the expression patterns observed among acclimation groups and those found in acute heat stress experiments (4) lies in the magnitude of change in mRNA levels. In the present study, only 12/150 significant genes were differentially expressed greater than twofold between any two acclimation groups, whereas Buckley et al. (4) observed 283 annotated genes that changed greater than twofold between acutely heat-stressed G. mirabilis (32°C) and control fish (18°C). The small fold differences in gene expression for fish acclimated to a range of nearly 20°C, may be another reflection of the effectiveness with which this eurythermal fish can compensate for the effects of acute thermal stress.

Conclusion

The fact that the transcriptomes of the three acclimation groups were so similar shows that acclimation can substantially overcome the effects of acute thermal stress, which is marked by a large number of transcriptional changes, notably in genes encoding stress-related proteins like HSPs. This ability to overcome acute thermal stress may be a signature of eurythermy. Among the three groups, the 9°C-acclimated fish had the most divergent transcriptional profile, indicating that 19°C- and 28°C-acclimated fish use more similar homeostatic strategies.

Some of the most potentially important differences in gene expression were related to the processes of protein metabolism and active transmembrane transport, which are likely to be the most energetically costly processes in gill tissue (7, 49). Genes encoding proteins associated with both processes were upregu-
lated with increasing acclimation temperature, suggesting an even higher cost for these two activities in warm-acclimated individuals. The upregulation found in these genes associated with energy-demanding processes bears discussion in the context of temperature compensation of metabolism (24). A substantial fraction of the literature on temperature acclimation has focused on the question of whether acclimation to low temperatures leads to a compensatory upregulation of ATP-generating capacities, such that the acute effects of decreased temperature ($Q_{10}$ effects) are fully or partially compensated. Our data suggest that a full understanding of temperature compensation requires analysis of temperature-specific costs of living, not just responses that offset $Q_{10}$ effects. Thus, if enhanced demands for ATP occur at higher acclimation temperatures, as suggested by our data, temperature compensation in metabolic rates related to offsetting $Q_{10}$ effects would be, at most, only part of the suite of metabolic adjustments taking place during acclimation to different temperatures.

Several other cellular processes identified in our gene ontology functional analysis did not show consistent expression patterns among genes, including cell cycle, cell death, carbohydrate metabolism, transcription, and protein catabolism (see Extended Results and Discussion in Supplementary Materials). In some cases, this may be because a single gene plays multiple functional roles within the cell. In other cases, a consistent expression pattern would not necessarily be expected. This is true for transcription factors that can affect many downstream processes and for the cell cycle and carbohydrate metabolism categories with multiple levels of complex regulation. In these categories, we have proposed several hypotheses in regard to the regulation of these processes despite disparate expression profiles, and suggest follow-up work to confirm or refute these hypotheses.

**Perspectives and Significance**

In keeping with the exploratory role of transcriptomics studies (19), our results lead to a number of questions and hypotheses that merit further physiological analysis. The types of acclimatory adjustments that would obviate the need for continued HSP synthesis at high temperatures (28°C in the present case) is an important area of future work. Two potential mechanisms could lead to a more stable cellular protein pool following acclimation to high temperature. One is an increase in stabilizing osmolytes (47, 53), such that at a higher temperature the milieu in which proteins occur is more conducive to maintenance of native structure. The second is a readjustment of cellular and extracellular pH values, such that, through alphastat regulation, the appropriate pH for the new body temperature is achieved (47, 48). Alphastat pH regulation can ameliorate perturbation of protein function (e.g., ligand binding) and structure (e.g., subunit assembly) caused by acute shifts in intracellular pH (47, 48, but see also Ref. 15). The time course over which this key regulatory process can occur during thermal stress merits detailed analysis.

Another broad area for future inquiry lies in uncovering the mechanisms that underlie the different acclimatory capacities of eurytherms and stenotherms. Acclimation capacity may be critical in establishing the susceptibilities of ectotherms to global warming (7, 49). Our study reveals the pronounced capacity of *G. mirabilis* to thermally acclimate to a wide range of temperatures; however, we also reveal the possible energetic costs that may be associated with warm acclimation. A substantial change in gill respiration could affect whole organism energetics. And, assuming similar effects of high temperature in other tissues, the overall costs of protein biosynthesis and membrane transport at higher temperatures could be substantially greater than at low temperatures. To this end, future studies should 1) examine the energetic costs of warm acclimation at the whole organism level and determine whether a potential fitness trade-off exists in terms of allocating energy towards homeostasis vs. reproduction or growth; and 2) compare mechanisms of homeostasis in less thermally tolerant species to determine how past thermal history and differences in transcriptional regulation may contribute to establishing thermal limits and, thereby, sensitivities to climate change.

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**DISCLOSURES**

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

**REFERENCES**


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