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The cellular response to heat stress in the goby *Gillichthys mirabilis*: a cDNA microarray and protein-level analysis

Bradley A. Buckley*, Andrew Y. Gracey† and George N. Somero
Hopkins Marine Station, Stanford University, Pacific Grove, CA 93950, USA
*Author for correspondence (e-mail: brad.buckley@stanford.edu)
†Present address: Marine Environmental Biology, University of Southern California, 3616 Trousdale Parkway, #107, Mail Code 0371 Los Angeles, CA 90089, USA

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Summary

The cellular response to stress relies on the rapid induction of genes encoding proteins involved in preventing and repairing macromolecular damage incurred as a consequence of environmental insult. To increase our understanding of the scope of this response, a cDNA microarray, consisting of 9207 cDNA clones, was used to monitor gene expression changes in the gill and white muscle tissues of a eurythermic fish, *Gillichthys mirabilis* (Gobiidae) exposed to ecologically relevant heat stress. In each tissue, the induction or repression of over 200 genes was observed. These genes are associated with numerous biological processes, including the maintenance of protein homeostasis, cell cycle control, cytoskeletal re-organization, metabolic regulation and signal transduction, among many others. In both tissues, the molecular chaperones, certain transcription factors and a set of additional genes with various functions were induced in a similar manner; however, the majority of genes displayed tissue-specific responses. In gill, thermal stress induced the expression of the major structural components of the cytoskeleton, whereas these same genes did not respond to heat in muscle. In muscle, many genes involved in promoting cell growth and proliferation were repressed, perhaps to conserve energy for repair and replacement of damaged macromolecules, but a similar repression was not observed in the gill. Many of the observed changes in gene expression were similar to those described in model species whereas many others were unexpected. Measurements of the concentrations of the protein products of selected genes revealed that in each case an induction in mRNA synthesis correlated with an increase in protein production, though the timing and magnitude of the increase in protein was not consistently predicted by mRNA concentration, an important consideration in assessing the condition of the stressed cell using transcriptomic analysis.

Supplementary material available online at
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Key words: genomics, transcriptome, stress, fish, heat shock, goby, *Gillichthys mirabilis*.

Introduction

The coordinated and adaptive manipulation of the cellular mRNA pool, the transcriptome, is one of the most rapid and versatile responses available to organisms experiencing environmental stress. Transcriptional profiling, through the application of cDNA microarrays, has begun to elucidate those shifts in gene expression that are likely to be important in the stress response, whether the particular adaptive response is involved in acquired tolerance to a given stressor or in the initiation of repair-and-replacement strategies to return the cell to homeostasis after an exposure to environmental insult (Gracey and Cossins, 2003). In a small number of model species, defined as those species for which substantial genomic information is available, this approach has identified conserved cassettes of genes that appear to be important to both general and stressor-specific responses [e.g. in yeast (Gasch et al., 2000; Gasch et al., 2001, Chen et al., 2003), in *Drosophila* (Girardot et al., 2004), in human cells (Murray et al., 2004), in *E. coli* (Riehle et al., 2005)]. However, the conservation of these transcriptional patterns across diverse taxa remains to be demonstrated. To this end, transcriptomic analyses are beginning to be applied to non-model species of particular ecological or evolutionary interest to investigate their ability to adjust to changes in their surroundings and to describe broad evolutionary similarities in the stress response (Gracey et al., 2001; Gibson, 2002; Feder and Mitchell-Olds, 2003; Gracey et al., 2004; Podrabsky and Somero, 2004; Krasnov et al., 2005; Sneddon et al., 2005; Vornanen et al., 2005). Here, we continue this effort, using a cDNA microarray to investigate the impact of

acute heat shock on the profile of expressed mRNA in the eurythermic goby fish, *Gillichthys mirabilis.*

Many aspects of the physiology and ecology of fishes promote them as potentially useful models for studying the genomic basis of stress tolerance and response (see Cossins and Crawford, 2005). As poikilotherms that often live in thermally variable environments, fish must adjust phenotypically to rapid changes in body temperature. As vertebrates, fish share many developmental and physiological pathways with mammalian model species, so abundant sequence information exists for conserved genes involved in these processes. In addition, the recent sequencing of the genomes of zebra- and pufferfish has made fish species increasingly tractable systems in which to study broad-scale transcriptional responses to environmental change. Furthermore, any observed differences between the transcriptional responses of fish and those of birds and mammals may be helpful in understanding the evolutionary changes that underpinned the development of avian and mammalian endothermic homeothermy.

Gillichthys mirabilis (Cooper), a species of estuarine goby that tolerates broad temperature fluctuations in its natural habitat on both daily and seasonal timescales (Buckley and Hofmann, 2002), displays rapid responses to heat (Buckley and Hofmann, 2004) and other stressors (Gracey et al., 2001). A cDNA microarray consisting of thousands of unique genes was generated for *G. mirabilis* and has been used previously to describe the response to hypoxia in this species (Gracey et al., 2001). The version of the array used in the current study includes several thousand newly generated clones collected from heat shocked individuals, and therefore represents a broad complement of the stress-responsive genome of *G. mirabilis*.

An important caveat often applied to these types of transcript profiling studies is that in the absence of measurements of protein concentrations or activities, the link between mRNA levels and changes at the protein level can only be inferred. To address this problem, western blotting was used to quantify the concentrations of products from a set of genes that displayed dramatic transcriptional responses to temperature in gill, muscle or both, in the microarray analyses. Our results reveal the scope of phenotypic plasticity to changing temperature found in this highly eurythermic species and show that whereas common stress responses occur in different tissues, distinct tissue-specific transcriptional responses also occur.

Materials and methods

Animal collection and maintenance

Specimens of *Gillichthys mirabilis* (Cooper) were collected by baited minnow trap from the lagoon on the campus of the University of California at Santa Barbara. Fish were transported to Hopkins Marine Station in coolers and maintained in flowing seawater aquaria for 4 weeks. For the first 3 weeks of laboratory acclimation, fish were fed trout pellets (Bio-Oregon, Warrenton, OR, USA) every other day. Fish were not fed during the final 7 days of the acclimation period, to normalize nutritional state. Mortality during acclimation was 0%. Fish used in experiments were selected for size (8–10 cm total length). The genders of the specimens were not determined because this species does not display gender-specific external morphology and no ripe gonads were observed.

Heating regime

A total of 48 individuals were used in the following experiments. Three 400 l, aquaria filled with re-circulating constantly aerated seawater were outfitted with data loggers set to record temperature every minute. Twelve individual fish were held in the control tank at 18±0.5°C (see Fig. 1). Twenty-four individuals were held in a second tank, which was ramped from 18°C to 32°C at a rate of 0.08°C min^{-1}. The chosen heating rate is similar to that seen during the course of a day in this species' natural habitat (see Buckley and Hofmann, 2002). A final group of 12 fish were held in a third tank which was ramped to 32°C; after 2 h at 32°C, this tank was ramped back down to 18°C (as in the first tank, ramping rate, both up and down, was 0.08°C min^{-1}) and maintained at that temperature for the duration of the experiment (a total of 480 min). Four individuals were removed from the control tank at several time points (listed below) and killed by cervical transection, wrapped in aluminum foil and flash frozen in liquid nitrogen. Time points of collection from this tank were 0, 240 and 480 min. Sampled time points in the second tank were 180, 240, 270, 300, 360 and 480 min. Finally, in the third tank, in which the fish were cycled back down to 18°C after heat shock, fish were sampled at 300, 360 and 480 min, in an effort to assess recovery condition.

cDNA microarray preparation

A description of the construction of the *G. mirabilis* microarray used in these experiments is reported elsewhere

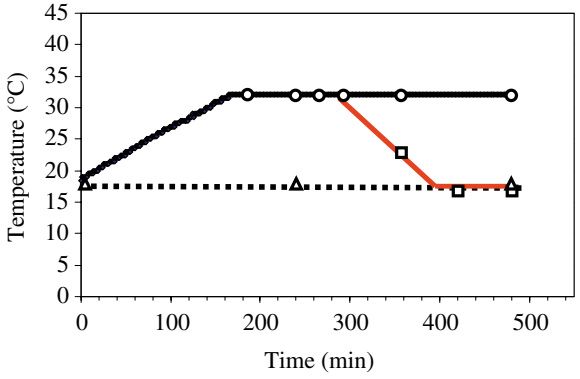

Fig. 1. Experimental heating regime. Control fish were held at 18°C for 480 min; fish were sampled at three time points (triangles) from the control tank at 0, 240 and 480 min. Another group of fish was ramped to 32°C, and sampled at six time points (circles). A third group was ramped to 32°C then ramped back down to 18°C and allowed to recover. Samples were taken from this group at 360, 420, 480 min (squares). *N*=4 for all samples.

(Gracey et al., 2001). The microarrays prepared for the current study, however, included additional clones not described in the earlier publication; these clones were generated from heat shocked gill and muscle tissue, and therefore represent sub-libraries enriched for expressed sequences from heat stressed individuals. Briefly, RNA from heat shocked gill and muscle tissue was reverse transcribed to cDNA and the resulting cDNAs were directionally cloned into pTriplEx2 vector (Clontech, Mountain View, CA, USA). Plasmid libraries were transformed into *E. coli* and bacterial colonies were picked at random into 384-well microtiter plates and grown overnight at 37°C. cDNA inserts were amplified by PCR using 1 μl of bacterial suspension in 50 μl standard PCR reactions with vector specific primers. After ethanol precipitation and one round of washing in 70% ethanol, PCR products were prepared in 3× sodium citrate (SSC) and 1.5 mol^{-1} betaine, for spotting onto Fisher Scientific (Hampton, NH, USA) Gold Seal glass microscope slides. Slides were coated with poly-L-lysine and prepared for spotting according to established methods (see www.microarrays.org). PCR products were applied to slides by a printing robot in the laboratory of Dr Gary Schoolnik, at the Stanford University School of Medicine, according to the procedure outlined elsewhere (Gracey et al., 2001). The slides printed here contained 9207 features, each representing a cloned gene. Repeated subtractive hybridization was used to reduce redundancy of spotted clones (see Gracey et al., 2001); sequencing revealed that approximately 84% of the features on the array represent unique genes (this estimate is based only on annotated features).

RNA extraction for hybridization

Gill and white muscle tissue were removed from unthawed fish and homogenized with silicone pestles in RNA extraction solution [38% v/v phenol in diethyl pyrocarbonate (DEPC)-treated H_2O, 0.1 mol l^{-1} sodium acetate pH 5.0, 0.8 mol l^{-1} guanidine isothiocyanate, 0.4 mol l^{-1} ammonium thiocyanate, 5% v/v glycerol]. Homogenates were spun at 12 000 ***g*** at 4°C for 10 min. Supernatants were removed and added to 0.2 ml of chloroform. Tubes were inverted several times and incubated at room temperature for 10 min, then spun at 12 000 ***g*** at 4°C for 10 min. The aqueous fraction was removed and added to 0.5 ml of isopropanol. Tubes were inverted and left to stand at room temperature for 10 min, then spun at 12 000 ***g*** at 4°C for 10 min. Supernatant was discarded and pellets washed 2× in 80% ethanol (ETOH). Dried pellets were resuspended in 0.1 ml of 10 mmol l^{-1} Tris–HCl and 1.0 mmol l^{-1} EDTA (TE). 0.3 ml of 6 mol l^{-1} guanidine HCl and 0.2 ml of 100% ETOH were added and the entire volume was loaded onto a spin column (Ambion, Austin, TX, USA) and spun for 1 min at 12,000 ***g*** at 4°C. Flowthrough was discarded, and filters were washed 2× with 0.2 ml 80% ETOH. RNA was eluted off of filters with 0.1 ml of TE buffer. 0.1 vol of 3 mol l^{-1} sodium acetate (pH 5.0) and 2.5 vol of 100% ETOH were added to eluate, and the contents mixed by inversion of tubes. RNA was precipitated for 1 h at –80°C. After this period, tubes were spun at 12,000 ***g*** for 20 min at 4°C. Pellets were washed 2× with 80% ETOH and resuspended in 50 μl of TE buffer.

Profiling gene expression with cDNA microarrays

Equivalent amounts of total RNA from four individuals (determined by A_{260} absorbance) from each time point were pooled (RNA from gill and muscle was treated separately). Total RNA was also collected from the gill and muscle of five reference individuals, which were tank-acclimated at 18°C for 4 weeks but otherwise untreated, and pooled for use as a source of reference RNA. It was against this reference sample that the values from the experimental (both control and heat shocked) samples were normalized (see Podrabsky and Somero, 2004). Ten μg of total RNA from each sample was reverse transcribed (RT) to cDNA, using oligo(dT_{15}) and pdN_6 random hexamer primers and amino-allyl UTP. The RNA template was removed from the RT reactions by incubating at 65°C for 30 min in 0.2 mol l^{-1} NaOH and 0.1 mol l^{-1} EDTA. Single stranded cDNA was labeled with either Cy3 (reference samples) or Cy5 (control or heat treatment samples) cyanine monoreactive dye (Amersham, Piscataway, NJ, USA), prepared in 100 μl of dimethylsulfoxide. cDNA was incubated with 5 μl of either Cy3 or Cy5 for 1 h at room temperature. The fluorescently labeled cDNAs were cleaned over PCR purification columns (Qiagen, Valencia, CA, USA) and eluted in 10 μl of dH_2O. Samples were brought to a final volume of 40 μl in 25 mmol l^{-1} Hepes, 0.75 mg ml^{-1} tRNA (Sigma, St Louis, MO, USA), 3× SSC, and 0.2% SDS. Samples were boiled for 1 min, allowed to cool to room temperature for 5 min, and then applied to microarray slides. Hybridizations were conducted overnight at 65°C in Genetix (Boston, MA, USA) hybridization chambers. After hybridization, slides were washed gently by immersion in 0.6× SSC and 0.03% SDS for 1 min to remove unbound dye. Slides were then dipped repeatedly in 0.6× SSC wash solution and dried by centrifugation. The slides were scanned on an AXON GenePix 4000B microarray scanner (Axon Instruments, Molecular Devices, Sunnyvale, CA, USA).

Analysis of microarray data

Data from the 24 arrays used in this experiment were extracted using GenePix Pro 4.0 software and the ratio of Cy5 to Cy3 fluorescence was quantified for each spot on the arrays (Axon Instruments). This ratio reflected the relative intensity from either a control or heat shocked time point *versus* that of the reference sample. For each array, spatial and intensity based trends in the data were removed by Lowess normalization (GeneSpring, Agilent). The ratios of fluorescence intensities at a given heat shock time point were then normalized against the average ratio for that spot for the three control time points (t=0, t=240, t=480). This method of normalizing fluorescence values in time course experiments conforms to that of a previously published study on time course transcriptomic profiling in fish (Podrabsky and Somero, 2004); time course experiments have a clear starting condition (t=0) against which to compare the results from the treatment time

points. Furthermore, we chose to average the values from three control time points, spanning the 8-h experiment, to remove any natural cycling of gene expression that may have occurred during the 8-h duration of the experiment. The resulting ratio, which is actually a ratio of a ratio (Cy5 fluorescence from a heat shock time point/Cy3 from the reference divided by the average Cy5 fluorescence from the three controls/Cy3 from the reference), reveals those changes in gene expression related solely to temperature treatment. Only those genes that were up- or downregulated at least twofold, relative to the average of the three controls, for at least one time point were included in the analysis. The twofold change in expression as a threshold for consideration is a conservative convention employed in many transcript profiling studies (Gracey et al., 2001; Podrabsky and Somero, 2004; van der Meer et al., 2005), and was adhered to here to facilitate comparison of the findings from the current study with those of earlier reports. An additional statistical analysis was conducted on all genes, designed to detect the effect of 'treatment' on expression levels (see section on Statistical Analysis of Microarrays below and supplementary material). Genes with similar expression patterns were grouped according to a hierarchical clustering algorithm, using a Pearson correlation similarity measure (GeneSpring, Agilent).

DNA sequencing

Genes displaying twofold changes in expression were sequenced on a 3100 Genetic Analyzer DNA sequencer (Applied Biosystems, Foster City, CA, USA). PCR products generated from cDNA inserts isolated from the given clones were sequenced directly and all sequencing was done from the 5′ end using primers specific to the plasmid vector. Blastx searching was conducted against the NCBI public databases to identify sequenced genes. The blastx result with the highest homology to the *G. mirabilis* sequence was used to annotate the clones. A minimum e-value of $e=1.0\times10^{-5}$ was imposed as the requirement for annotation, although the majority of annotated clones had considerably more significant e-values (median e-value of sequenced clones=3.0×10^{-34}). Gene ontology (GO) classifications (see Harris et al., 2004) were assigned to all identified clones. All sequences have been entered into the GenBank database (accession numbers available in Tables S1 and S2 in supplementary material).

Quantification of protein concentration: solid-phase immunochemistry

Concentrations of specific proteins in gill and white muscle tissue were quantified using western blotting. Frozen tissue sections (taken from the same tissue samples used for the microarray hybridizations) of approximately 100 μg were thawed in 100 μl of homogenization buffer, containing 32 mmol l^{-1} Tris–HCl (pH 6.8) and 2% SDS. Homogenates were heated at 100°C for 5 min and centrifuged at 12 000 ***g*** for 10 min. Pellets were discarded, and total protein content of the supernatants was determined by Bradford assay (Pierce, Rockford, IL, USA).

The specificity of each antibody used was confirmed using western blotting. Only a single band was detected for all proteins, except for ubiquitin-conjugates, where multiple banding is expected. For western blots, 10 μg of total protein from each sample were separated on 10% gels by SDS-polyacrylamide gel electrophoresis (PAGE). After separation, proteins were transferred to nitrocellulose membranes by electroblotting at 30 V overnight at 4°C. Following transfer, membranes were dried at 70°C for 45 min. Final quantification of protein concentrations was accomplished by binding protein samples directly to nitrocellulose membranes with a BioDot® apparatus (Bio-Rad, Hercules, CA, USA). For the dot blotting analysis, 1 μg of total protein from each sample was brought to a final volume of 100 μl in 1× phosphate-buffered saline (PBS; 140 mmol^{-1} NaCl, 2.68 mmol^{-1} KCl, 5.4 mmol^{-1} Na_2HPO_4, 1.74 mmol^{-1} NaH_2PO_4) and was blotted directly onto nitrocellulose membranes using the BioDot® apparatus according to manufacturer's instructions. Following blotting, membranes were dried at 70°C for 45 min.

Blots were blocked for 1 h in 5% non-fat dry milk (NFDM) in 1× PBS under constant shaking. Blots were washed three times for 5 min in 1× PBS containing 0.01% Tween 20. Following washes, blots were incubated in primary antibody (1:1000 dilution in 1× PBS containing 5% NFDM). Primary antibodies used were: α-Hsp40 (SPA-400, StressGen, Victoria, BC, Canada), α-Hsp70 (SPP-832, StressGen), α-Hsp90 (SPA-891, StressGen), α-Protein disulfide isomerase (SPA-400, StressGen), α-actin (sc-10731, Santa Cruz Biotechnologies, Santa Cruz, CA, USA), and an α-ubiquitin-conjugated protein (generated by Lars Tomanek in the laboratory of Arthur L. Haas). All incubations in primary antibodies were conducted for 1.5 h, except for the incubation in α-protein disulfide isomerase antibody, which was conducted overnight to increase the intensity of the final blot. Following incubation in primary antibody, blots were washed 3× 10 min in 1× PBS with 0.1% Tween 20, then incubated in a secondary antibody (horseradish peroxidase-conjugated protein A, 170-6522, Bio-Rad) at a dilution of 1:5000 in 1× PBS containing 5% NFDM. Blots were washed 3× 5 min in 1× PBS with 0.01% Tween 20, and exposed to enhanced chemiluminescent reagent (ECL, Amersham, Piscataway, NJ, USA), for 5 min. Blots were wrapped in plastic wrap and exposed to X-ray film (XOMAT-AR film, Kodak, Rochester, NY, USA). Densitometry was conducted on dot blots using ImageMaster software (Amersham Pharmacia Biotech); all values were standardized to the control values.

Statistical analysis of microarray data

One-way ANOVA analysis was used to identify genes for which the expression patterns showed a significant effect of treatment (listed for gill and muscle data, respectively, in Tables S3 and S4 in supplementary material). Treatments used were: 'control', 'heat shock' and 'recovery'. Multiple time points within a treatment were treated as 'replicates'. As averaging the time points tends to underestimate the importance of heat shock duration in determining mRNA

levels, direct comparison of each experimental value against the normal distribution of the values of three control time points was also conducted for each time point independently. For each spot on the array, the average value of the three control time points (value=control channel Cy5/reference channel Cy3) and their standard deviation were calculated. Then, for each time point, the experimental value for each spot on the array (experimental channel Cy5/reference channel Cy3) was tested to see whether it fell outside two standard deviations of the mean for the three control time points. Those genes meeting this criterion are listed in Table S5 and S6 (for gill and white muscle, respectively) in supplementary material. Every gene that passed the twofold change in expression criterion outlined above (see Figs 1 and 2) also passed this analysis for at least one time point.

Statistical analysis of protein quantification data

The effect of 'time point' on protein concentration for N=4 individuals per time point was determined by one-way ANOVA using SysStat 10.0 software (SysStat Software, Inc., Point Richmond, CA, USA).

Results and discussion

The effect of heat shock on broad-scale patterns of gene expression in *Gillichthys mirabilis* was investigated to explore to what extent the environmental stress response (ESR), which to date has been best described in yeast (Gasch et al., 2000; Gasch et al., 2001), is conserved across diverse taxa. A second goal of this project was to correlate gene expression profiles for a sub-set of induced genes with profiles of protein concentration. A cDNA microarray consisting of 9207 features was used to generate transcriptomic profiles from individuals exposed to elevated temperature for up to 6 h. This time course analysis (Fig. 1) revealed numerous genes that were transcriptionally responsive to heat treatment: 476 and 339 clones in gill and white muscle, respectively, varied their expression by more than twofold. These clones were sequenced and comparison of the generated sequences with those in public databases successfully identified 283 clones from gill and 201 clones from white muscle. The decision to discuss those genes for which expression varied by ⩾twofold provides a context within which to discuss only the most markedly up- or downregulated genes. However, it should be noted that many genes altered their expression at lower fold-change levels (see Tables S5 and S6 in supplementary material) and these changes could have important biological effects. The majority of the affected genes displayed tissue specificity in their response to temperature, however, a group of 31 genes were heat-responsive in both tissues. Where multiple features on the array represented a single gene, the expression profiles for these features were always highly similar (e.g. for actin, which is represented by 15 spots on the array, across the time points the average standard error among the spots was 6% of the mean of their values), which is important platform validation for the *G. mirabilis* microarray.

The genes with expression profiles that changed ⩾twofold in response to temperature, in one or both tissues, were grouped into a wide variety of classes according to their gene ontology (GO) classification for 'biological process'; the GO database resource is an effort to establish codified functional descriptors for the growing number of gene sequences being generated through modern genomic applications [for further description of the GO database, see Harris (Harris, 2004)]. Here, the relevant processes are clustered as follows: (1) protein rescue and folding, (2) protein degradation, (3) protein synthesis, (4) proteolysis, (5) cell signaling, (6) cell proliferation and growth, (7) transcriptional regulation, (8) cytoskeletal structure and reorganization, (9) cell–cell or cell–matrix adhesion, (10) carbohydrate metabolism, (11) fatty acid metabolism, (12) transport, (13) apoptosis and (14) other functions. As many genes are integral to numerous cellular processes, where applicable, expression data from these genes will be discussed in the context of their possible interactions with members of other clusters.

It is worth noting that the majority of the clones on the array (~9000) did not alter their expression level, at the twofold change criterion, during thermal stress. Similar observations were reported for the annual killifish, *Austrofundulus limnaeus*, exposed for 14 days to either constant or fluctuating daily temperatures (Podrabsky and Somero, 2004). In that study, the expression of less than 10% of the features on a 5376-clone microarray varied with temperature. That such a small percentage (~4%) of the genes on the *G. mirabilis* microarray responded markedly to heat shock in the current study supports their active participation in responding to the deleterious impacts of acute thermal stress, rather than simply reflecting a general effect of heat on transcription.

Thermally responsive changes in gene expression

Cluster 1: protein rescue and folding

The most strongly inducible genes on the array, in both gill and muscle, were those of the molecular chaperones, including the heat shock proteins (Hsps). A hallmark of the phylogenetically conserved 'heat shock response' is the preferential production of Hsps over that of nearly all other classes of polypeptide (Lindquist, 1986). These and other chaperone proteins are pivotal in maintaining protein homeostasis during cellular exposure to proteotoxic stressors such as heat or heavy metals by interacting with stress-denatured proteins, preventing their aggregation and/or degradation (Parsell and Lindquist, 1993). Some Hsps, such as Hsp40 and Hsp70, interact with misfolded proteins, preventing them from forming aggregates (Fink, 1999) that can become cytotoxic (Bucciantini et al., 2002). Other members of the family, such as Hsp108, have the ability to disassociate proteins that have already begun to form aggregates (Parsell et al., 1994).

Every size class of Hsp present on the *Gillichthys* array was upregulated in response to heat shock (Figs 2, 3, Cluster 1). Hsp70 and Hsp90 were the most strongly upregulated and expression of these two genes remained elevated throughout

the heat shock and recovery. Hsp70 was more strongly induced in muscle than in gill, whereas the opposite was true for Hsp90. A fivefold increase in Hsp70 was measured in gill tissue, whereas a nearly 19-fold increase was measured in muscle. Hsp90 was induced 11-fold in gill and eightfold in muscle. Hsp108 was induced threefold in both tissues. A complex pattern was seen in the Hsp40 family in muscle; member 7 of subfamily C was downregulated, whereas member 1 of subfamily A was upregulated (Fig. 3, Cluster 1). All of the members of the Hsp40 family act as co-chaperones for Hsp70 but the multiple subfamilies are expressed in specific intracellular compartments (Rassow et al., 2005; Ohtsuka and Hata, 2000); the pattern of expression observed here may reflect location-dependent variability in the need for protein chaperoning. The small molecular mass Hsp, Hsp27, was induced in muscle only. This may reflect a particular focus on protecting cytoskeletal proteins in the muscle, as Hsp27 has been demonstrated to chaperone the structural components of the cytoskeleton (Liang and MacRae, 1997). Interestingly, Hsc71, the cognate form of Hsp70 that is expressed constitutively and is not inducible in most taxa, was induced in *G. mirabilis*. The upregulation of Hsc71 has also been shown in medaka (Arai et al., 1994) and Atlantic salmon (Lund et al., 2002). The induction of Hsc71 may be related to a stress-related increase in the requirement for chaperoning of nascent polypeptides during protein biosynthesis, a primary role of Hsc71 under normal conditions.

In addition to the heat shock genes, other genes with chaperone activity were induced in one or both tissues. In both tissues, peptidyl-prolyl isomerase was induced and in the gill only, protein disulfide isomerase (PDI), a chaperone involved in folding secreted proteins in the ER, was also upregulated. The chaperonin containing T-complex protein 1, a multi-subunit protein related to Hsp60 and involved in folding actin, tubulin and cyclin E, among other proteins (Kubota et al., 1999; Yokota et al., 2000), showed a complex expression pattern in gill, wherein some subunits were induced, whereas others were repressed during heat shock. This may reflect an effort to normalize cellular concentrations of the multiple subunits of this protein in order to produce a coherent intact polypeptide. Of all the gene clusters, the 'protein folding' cluster displayed the highest-fold changes in expression level in response to temperature, underscoring the importance of these genes in conferring cellular thermotolerance through protein chaperoning.

Cluster 2: protein degradation

The eukaryotic cell has two competing pathways for managing proteins abnormally denatured due to proteotoxic stress: the molecular chaperone pathway with Hsps performing the signal role in the eventual rescue and refolding of damaged polypeptides, and the ubiquitin-proteasome pathway, which results in the degradation of the abnormally folded protein (Ciechanover, 1998). Damaged proteins that do not enter the chaperone pathway are covalently tagged with multiple units of ubiquitin, a small (76 amino acid) protein which, when conjugated to a damaged polypeptide, targets it to the proteasome for proteolytic degradation. Here, various components of the ubiquitin-proteasome pathway, including ubiquitin itself and proteasome-associated proteins and subunits, were induced in response to heat (Figs 2, 3, Cluster 2), suggesting that despite the increased rescue of misfolded proteins through chaperoning, the cell was incurring sufficient levels of protein damage that an increase in protein degradation was also necessary. This is confirmed by our measurements of increased ubiquitylation of proteins in both tissues in response to heat stress (discussed below).

The expression patterns of the Cluster 2 genes were tissue-specific. Ubiquitin mRNA levels in gill tissue increased 4.2-fold immediately upon exposure to heat, whereas the induction in muscle was more moderate and slower (approx. twofold increase over control levels after 120 min of heat shock). Considered in light of the patterns of Hsp70 induction described above (with the magnitude of Hsp70 induction being greater in muscle than in gill), these data are consistent with a scenario in which strong protein chaperoning in the muscle resulted in a lesser requirement for the ubiquitylation of damaged protein whereas, in the gill, ubiquitylation was more extensive. These data perhaps nominate gill as a candidate tissue for investigating the energetic cost of heat stress on protein degradation and eventual replacement.

In addition to its role in protein biosynthesis, elongation factor 1-alpha (EF-1α) is an essential factor in releasing ubiquitin from multi-ubiquitin complexes, rendering ubiquitin conjugates susceptible to the degradative action of the 26S proteasome complex (Gonen et al., 1994). Therefore, its induction in gill (Fig. 3, Cluster 4) may be related to the mobilization of the ubiquitin-proteasome pathways. The repression of EF-1α in muscle (Fig. 3, Cluster 4) may be related to a lesser need for ubiquitylation and degradation of damaged proteins in this tissue, perhaps due to a stronger investment in chaperoning. EF-1α has recently been shown to interact with a non-coding RNA (HSR1) in a complex that is essential for activation of heat shock factor-1 (HSF1) in vitro (Shamovsky et al., 2006). Thus, induction of EF-1α may play multiple roles in the response to heat stress.

Two other genes associated with proteasomal degradation of protein were induced in muscle (Fig. 3, Cluster 2). That for Ecm29, a protein associated with the proteasome and proposed to link secretory compartments involved in protein quality control to centers of proteolysis (Gorbea et al., 2004), was induced after 270 min, after which, levels of message remained high for the duration of the experiment. In addition, an unidentified gene coding for a protein containing a HECT domain was transiently induced in late stage recovery. Proteins with HECT domains have been shown to have E3 ubiquitin-ligase activity, the final step in the linking of ubiquitin to damaged protein (Huang et al., 2000). These results support a delayed response of the ubiquitin-proteasome pathway in muscle, compared with a relatively more rapid response in gill tissue, consistent with the general trends in the Cluster 2 genes as a whole.

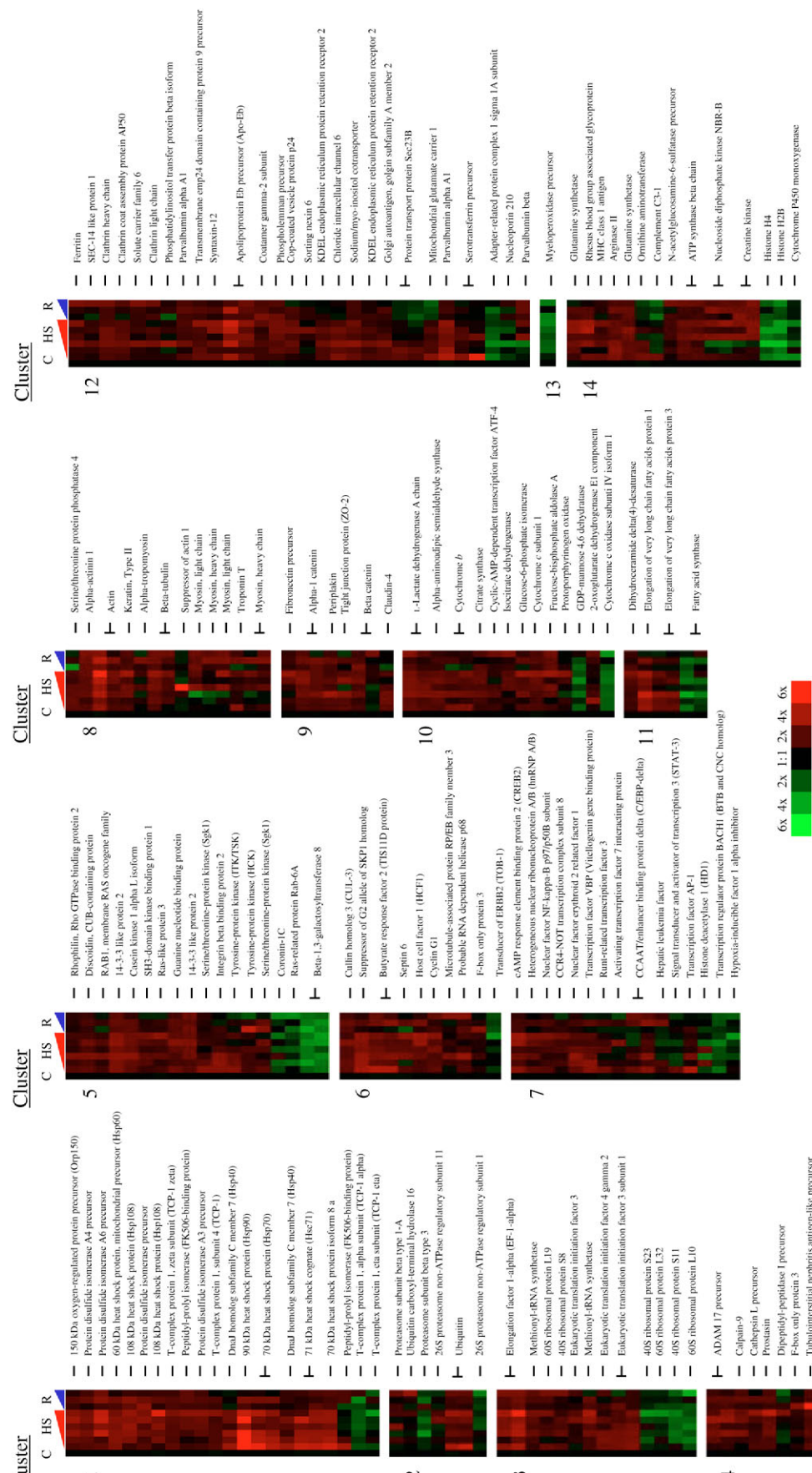

Fig. 2. Gene expression profiles in gill tissue from control and heat shocked *Gillichthys mirabilis*. Each row represents a single cDNA clone and each column represents a time point in the heat shock exposure. Genes depicted are those that were up- or downregulated at least twofold compared to the averaged values of the three control time points. Genes are clustered by cellular process, according to their gene ontology (GO) classification. Clustered processes are: (1) protein rescue and folding, (2) protein degradation, (3) protein synthesis, (4) proteolysis, (5) cell signaling, (6) cell proliferation and growth, (7) transcriptional regulation, (8) cytoskeletal structure and reorganization, (9) cell–cell or cell–matrix adhesion, (10) carbohydrate metabolism, (11) fatty acid metabolism, (12) transport, (13) apoptosis and (14) other functions. For clarity, only two rows are dedicated to genes represented by ⩾ two spots on the array. C, control; HS, heat shock, R; recovery.

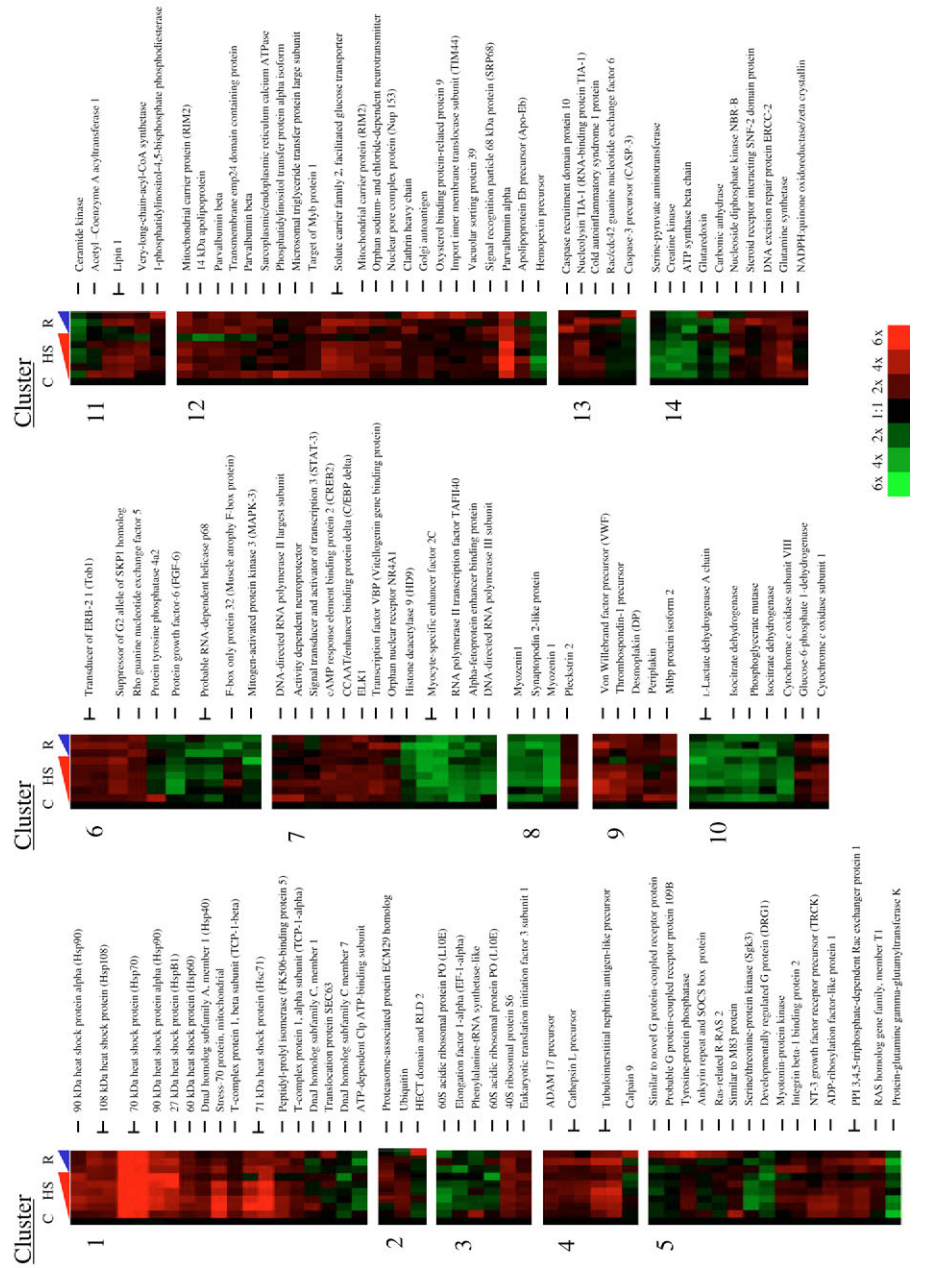

Fig. 3. Gene expression profiles in white muscle tissue from control and heat shocked *Gillichthys mirabilis*. Each row represents a single cDNA clone and each column represents a time point in the heat shock exposure. Genes depicted are those that were up- or downregulated at least twofold compared to the averaged values of the three control time points. Genes are clustered by cellular process, according to their gene ontology (GO) classification (classifications are numbered identically to those described in the legend to Fig. 2). For clarity, only two rows are dedicated to genes represented by ⩾two spots on the array. C, control; HS, heat shock; R, recovery.

Cluster 3: protein biosynthesis

It has been widely observed across taxa that exposure to sufficiently high temperatures can inhibit general protein synthesis, concomitant with the preferential production of specific stress response proteins (Lindquist, 1986) [in *G. mirabilis* (Buckley and Hofmann, 2002)]. Our understanding of this phenomenon comes primarily from measurements of protein production, but the effect of acute temperature stress on the transcriptional regulation of genes involved in protein synthesis remains relatively unexplored. In *G. mirabilis*, temperature-dependent changes in the expression of various components of the protein synthetic machinery were observed in both gill and white muscle (Figs 2, 3, Cluster 3).

In each tissue, the expression of several ribosomal proteins responded to thermal stress. In gill, large subunit proteins L10 and L19 and small subunit proteins S11 and S23 were repressed, whereas L19 and S8 were induced. In muscle, L10 was repressed as it was in gill, and S6 was induced. These proteins associate with and stabilize various sub-regions of the ribosome and we can conjecture that their expression or repression during heat stress may be an effort to protect ribosomal structure and/or function through replacement or substitution of these key structural components.

In both tissues, translation initiation factor 3 (as well as factor 4 in muscle) was immediately upregulated upon initiation of heat stress, suggesting a requirement for rapid translation of message, perhaps that of 'first responder' stress proteins such as Hsps. A tissue-specific response was observed for EF-1α, which was induced in gill but repressed in white muscle. EF-1α mediates polypeptide construction by binding aminoacyl-tRNAs to the ribosome in a GTP-dependent manner (Riis et al., 1990). The reason for the tissue-specific patterns of expression observed here remains to be explored. However, in gill, the induction of EF-1α coincided with the marked upregulation of methionyl-tRNA synthetase, which produces the initiating methionine for all polypeptides, suggesting that several components of the translational machinery were synthesized rapidly in response to heat, presumably priming the gill cell for rapid protein synthesis.

Cluster 4: proteolysis

Several genes associated with proteolysis, from diverse sub-cellular localizations and processes, were upregulated in response to heat (Figs 2, 3, Cluster 5). Interestingly, the same four genes that were upregulated in muscle were also upregulated in gill, suggesting that they are involved in a general cellular response to thermal stress. In both tissues the gene for the ADAM-17 precursor was induced; this metalloprotease-disintegrin cleaves soluble tumor necrosis factor-α (TNF-α) (Reddy et al., 2000). TNF-α is a cytokine that activates a large number of cellular signaling events that in turn affect processes that govern cell survival and death (Alikhani et al., 2004). The induction of ADAM-17, therefore, may be an upstream initiator of apoptosis. Also induced in both tissues were two genes involved in lysosomal proteolysis, cathepsin L and tubulointerstitial nephritis antigen-like precursor. In gill only, another lysosomal protease, dipeptidyl peptidase I precursor (Rao et al., 1997) was also induced. In addition, calpain 9, an intracellular, non-lysosomal cysteine protease with a broad suite of targets, was induced in both tissues. The targets of calpain 9 include several mitogenic and apoptotic factors such as FOS, JUN and p53, so it may play a role in mediating one or both of these processes. In general, the induction of these four proteases in both tissues may signal the need for increased intracellular digestion of macromolecules during heat shock.

Cluster 5: cell signaling

A key aspect of transducing extra-organismal signals to the cell and ultimately to the nucleus is the stimulation of cell signaling cascades. This could fairly be considered the primary cellular response to environmental stress, initiating and coordinating the subsequent actions taken to mediate the deleterious impacts of a given set of stressors. The relaying of signals through the activation of pre-existing molecules is thought to increase the rapidity of the cellular response to environmental insult. It is possible, however, that in some cases, the de novo synthesis of signaling molecules may attend cellular stress exposure. Here, transcription of several components of various signaling pathways responded to heat shock in both gill and muscle (Figs 2, 3, Cluster 5). Diverse classes of signaling molecules were affected, including tyrosine-protein kinases, G-protein-coupled receptor proteins, serine/threonine kinases, members of the RAS and RAB signaling pathways and 'scaffold' signaling molecules such as the 14-3-3 proteins, among others.

In gill tissue, the majority of genes in Cluster 5 were induced by heat. These genes included those of three kinases with diverse functions, serine/threonine-protein kinase (Sgk1), casein kinase 1, and tyrosine protein kinase (ITK/TSK or HCK), were upregulated in heat-shocked individuals (Fig. 2, Cluster 5). Sgk1 responds to a large number of extracellular signals and initiates several cellular responses to these cues (Lang and Cohen, 2001). Casein kinase 1 is involved in the Hedgehog signaling pathway (Jia et al., 2004). ITK/TSK is a non-receptor protein tyrosine kinase that participates in the intracellular signaling cascades that lead to T-cell activation (Brazin et al., 2002), but, in other cells, can generate secondary messengers involved in cytoskeletal reorganization (Tsoukas et al., 2001). It is possible that the induction of ITK/TSK was functionally related, if not directly causal, to the induction of cytoskeletal elements detailed below (see section on Cluster 8 genes).

Heat shock also induced the expression of RAB1 and repressed that of RAB-6A. The RAB pathway mediates various stages of intracellular transport, including vesicle formation and targeting (Zerial and McBride, 2001). RAB1 and RAB-6A are involved in tethering COP1-coated vesicles to the Golgi and in retrograde Golgi-ER transport, respectively. A suite of genes involved in transport through the Golgi was also induced in gill (see Cluster 12) and this may be correlated with the observed transcriptional regulation

of RAB1 and RAB-6A. Two members of the Ras and Rho-GTPase pathways, Ras-like protein 3 and rhophilin, were induced in heat-shocked gill. These molecules are upstream mediators of signaling pathways that regulate cell cycle progression (Coleman et al., 2004); the expression patterns observed here suggest they may play a role in controlling cell proliferation during heat stress. Thermally responsive expression patterns were also observed for genes whose products involved in relaying messages from cell–cell and cell–matrix interfaces to intracellular cascades; integrin β binding protein 2 was induced whereas a discoidin-motif-containing protein was repressed.

Interestingly, the sustained induction of a 14-3-3 gene was observed in gill. The sequence of this heat-inducible 14-3-3 gene shares the highest homology with the epsilon isoform, although it shares no exact homology with any extant sequence for a 14-3-3 isoform (data not shown). The 14-3-3 proteins act as signaling 'scaffolds', binding a wide variety of target proteins, which potentiates their phosphorylation (Takahashi, 2003). Through this single activity, 14-3-3 proteins affect a striking breadth of processes including cytoskeletal reorganization, cell division, apoptosis, gene expression, ion physiology and further signal transduction. A novel, osmoregulated isoform of 14-3-3 was detected in the teleost fish *Fundulus heteroclitus* (Kültz et al., 2001); it remains to be determined if the heat-inducible isoform identified here in *Gillichthys* gill tissue may represent a similarly novel coordinating nexus for cell signaling in ectothermic organisms.

The diversity of thermally regulated signaling genes was similar in muscle (Fig. 3, Cluster 5). Myotonin-protein kinase, NT-3 growth factor receptor precursor (TRCK) and tyrosine-protein phosphatase were induced. In late stage recovery, two G-protein-coupled receptor proteins were also induced. Upregulation of the cell cycle mediators R-RAS2, Ras homology gene family member T1 and PPI (phosphatidylinositol) 3,4,5-P_3-dependent Rac exchanger protein was also observed. Finally, two genes involved in mediating signals generated by cell–cell contact, M83 like protein (Motohashi et al., 2000), and integrin β binding protein 2 were induced. Downregulated were, serine/threonine kinase 3 (Sgk3), protein glutamine gamma-glutamyltransferase K and developmentally regulated G-protein (DRG1). As was the case in the gill, the diverse targets available to the products of each of these genes include members of many of the networks available for transducing signals through the environmentally stressed muscle cell.

Cluster 6: cell proliferation and growth

One strategy to deal with the energetic cost of the cellular stress response might be to conserve energy through inhibition of cell growth and proliferation. We postulated that this might be reflected in the expression of genes governing these processes, as was observed for *G. mirabilis* treated to hypoxia (Gracey et al., 2001). Indeed, numerous modulators of the cell cycle responded to heat shock (Figs 2, 3, Cluster 6); some genes were regulated in both tissues whereas others showed tissue-specific responses. Overall, the expression pattern for this gene cluster suggests cell proliferation was favored in the gill and suppressed in muscle.

In gill, the cell cycle regulators cyclin G1 and microtubule-associated protein RP/EB were induced. Also induced was F-box only protein 3. F-box proteins lend substrate specificity to SCF boxes (Skp1, Cul, F-box), complexes that are integral to orderly progression through the phases of the cell cycle (Reed, 2003). The induction of F-box protein 3 was concomitant with the induction of other elements of SCF boxes, cullin-3, and suppressor of Skp1. These components function in concert to promote cell cycle progression through the ubiquitylation of cyclin (Singer et al., 1999). Also upregulated was host cell factor 1 (HCF1), a promoter of cell growth and the butyrate response factor 2 (also termed TS11d). Although the role of TS11d continues to be clarified, it has been shown to respond to mitogenic signals and may promote cell proliferation through mRNA stabilization (Hudson et al., 2004). Finally, transducer of Erb-B2 (Tob1) an important antiproliferative gene whose product that interacts with the oncogene Erb-B2 to suppress cell growth by inhibiting the cell cycle (Matsuda et al., 1996) was repressed. Taken together, these results support active cell growth and proliferation occurring in the gill.

Conversely, in muscle, the pattern of gene expression suggests that an inhibition of the cell cycle may have occurred (Fig. 3, Cluster 6). First, in contrast to gill, the cell cycle inhibitor Tob1 was induced in muscle tissue. Second, protein growth factor-6, a promoter of cell growth, and two signaling molecules responsible for cell cycle regulation, protein tyrosine phosphatase 4a2 and mitogen-activated protein kinase 3 (MAPK3) were repressed. Third, the RNA-dependent helicase p68 was downregulated; these 'DEAD-box' proteins are thought to be involved in promoting cell growth through their control over RNA metabolism (Rocak and Linder, 2004). Finally, in muscle tissue an eightfold repression of myocyte-specific factor 2C (MEF2) and histone deacetylase 9 (HD9), two functionally linked transcriptional regulators that are key to cell proliferation (Han et al., 2004) was observed (see Fig. 3, Cluster 7). All of these gene expression changes are consistent with the inhibition of cell growth and proliferation in muscle.

Cluster 7: transcriptional regulation

It is perhaps not surprising, in light of the wide variety of genes that displayed heat-responsive patterns of expression, that thermal stress also affected the expression of numerous transcriptional regulators (Figs 2, 3, Cluster 7). In both tissues, upregulation was observed for transcription factors VBP, CREB2, C/EBP and STAT 3, which are important for responding to transduced extracellular signals to effect further gene expression. However, the majority of up- or downregulated transcription factors showed tissue-specific responses to temperature. As with the signaling genes, the breadth of targets available to the transcription factors involved here renders it problematic to speculate as to which cellular processes they were mediating, but it is interesting to note that

the induction or repression of the transcription factors themselves was clearly a part of the transcriptomic response to temperature stress. It is also important to note that the patterns presented here are only for those genes that displayed ⩾twofold changes in response to heat. In the case of transcription factors, which act as genetic switches, even smaller changes in their cellular concentration, say a 25% increase or decrease, may be expected to have profound effects on the transcriptome.

Cluster 8: cytoskeletal structure and organization

In the gill, genes encoding multiple structural components of the cytoskeleton were induced including actin, tubulin, myosin and keratin (Fig. 2, Cluster 8). In addition, genes for several cytoskeleton-associated proteins were induced: alpha-actinin, which links actin to various cellular structures, and the contractile proteins α-tropomyosin and troponin. An upregulation of serine/threonine protein phosphatase 4, which is involved in microtubule organization (Sumiyoshi et al., 2002), was also observed. Finally, as discussed above, both Sgk1 and ITK/TSK were induced in gill; Sgk1 and ITK can play important roles in adjusting cell volume through manipulation of transporters and cytoskeletal reorganization, respectively (Waldegger et al., 1997). The induction of such a broad suite of cytoskeleton-associated genes suggests at least two scenarios, which are not mutually exclusive. The first is that actin and other cytoskeletal components, whose functions require them to rapidly polymerize and depolymerize under normal conditions, are more susceptible to the perturbative effects of thermal stress than are other proteins. If so, the induction of these genes may reflect a replacement strategy by the cell, whereby new cytoskeletal proteins are generated to replace those that are degraded. A second scenario suggested by these data is that part of the gill's response to heat stress is extensive cytoskeletal reorganization. Osmotically stressed cells can undergo cytoskeletal reorganization to modulate cell volume and therefore the osmotic strength of the cytoplasm (Di Ciano et al., 2002). If osmotic shock were a secondary effect of heat stress, perhaps caused by temperature effects on the membrane, then a reorganization of the cytoskeleton may reflect an attempt to regain osmotic balance.

Support for the second scenario may be found in the data from muscle. In this tissue, the induction of multiple structural components of the cytoskeleton was not observed. Although an induction of the gene for pleckstrin 2, a protein involved in actin rearrangement (Hu et al., 1999), was observed in muscle (Fig. 3, Cluster 8), the genes for two other actinin bundling proteins, myozenin and synaptopodin 2-like protein, were repressed. Perhaps more importantly, there was no induction of the major cytoskeletal structural genes such as those encoding actin, tubulin, myosin or keratin. If the first scenario outlined above were correct, wherein actin and other polymeric proteins are inherently susceptible to thermal denaturation and the cellular strategy to deal with this problem was to stimulate the synthesis of replacement cytoskeletal proteins, then we would expect to observe this synthesis not only in gill but in muscle as well. However, if the second scenario was true, and heat stress caused a secondary osmotic shock in the gill, it is conceivable that a similar osmotic insult did not occur in the muscle, precluding the need for cytoskeletal reorganization. Therefore, we conclude that the data are more consistent with the second scenario and may provide a demonstration of the synergistic effects of a multi-stressor cascade, one beginning with heat stress and leading to osmotic stress and its attendant effects on cellular structure and function.

Cluster 9: cell adhesion

Tissue-specific expression profiles were observed for genes associated with cell–cell and cell–matrix adhesion (Figs 2, 3, Cluster 9). In the gill, genes for four important cell adhesion molecules, alpha- and beta-catenin, fibronectin and periplakin, were induced by heat (Fig. 2, Cluster 9). Two genes essential for tight junction integrity, ZO-2 and claudin-3, also were upregulated. Tight junctions in gill are critical for regulating paracellular transport; it is possible that the upregulation of ZO-2 and claudin-3 is associated with an effort to tighten the gill, which becomes 'leakier' at high temperatures because of the effects of heat on membrane fluidity. Careful regulation of paracellular transport at the gill may be of particular importance here as the need for increased irrigation of the gills with water (and increased internal perfusion with blood) are required to support the higher rates of respiration observed in fish at high temperature. As in gill, desmosome-associated elements, such as periplakin and desmoplakin, were induced in muscle (Fig. 3, Cluster 9). The regulation of these genes during heat stress may reflect the need to repair damage to cell–matrix and cell–cell adhesion centers through replacement of degraded components, or the restructuring of these centers.

Interestingly, the gene for muscle integrin binding protein (MIBP) was induced in heat shocked muscle. Myogenesis is tightly regulated by cell adhesion receptors such as the integrins and it has been shown that overexpression of MIBP, which interacts with β1 integrin, results in the suppression of myogenesis (Li et al., 1999; Li et al., 2003). The induction of MIBP here is consistent, therefore, with the stress-related inhibition of cell proliferation in muscle (and the results from Cluster 6).

Cluster 10: carbohydrate metabolism

The expression of genes involved in regulating metabolism may be expected to respond to heat shock, as cellular energy pools are accessed to fuel stress response and repair mechanisms. In fact, several genes involved in glycolysis, gluconeogenesis, the tricarboxylic acid cycle and the electron transport chain were induced or repressed in both gill and white muscle (Figs 2, 3, Cluster 10). In gill tissue, heat shock resulted in the immediate induction of several ATP-generating enzymes, including fructose-bisphosphate aldolase A, glucose-6-phosphate isomerase and L-lactate dehydrogenase A of the glycolytic pathway and the TCA cycle enzymes isocitrate dehydrogenase, 2-oxoglutarate dehydrogenase, and citrate synthase. Two drivers of gluconeogenesis, cyclic-AMP-dependent transcription factor

ATF-4 and GDP-mannose 4,6 dehydratase, were induced and repressed, respectively. Finally, four mediators of the electron transport chain, cytochrome b, cytochrome c subunit 1, protoporphrinogen oxidase and alpha-aminoadipic semialdehyde synthase were all induced, whereas another subunit of cytochrome c, subunit IV, was repressed. Overall, the impact of heat shock on the expression of genes involved in energy metabolism in the gill cell was stimulatory, immediate and sustained, suggesting a need for rapid production of ATP during heat stress. This may be due in part to the requirement for ATP of many molecular chaperones (Fink, 1999).

The transcription of ATP-generating genes also responded to heat in the muscle, although, in contrast to the pattern observed in gill, many of these genes were repressed and a cohesive metabolic response to heat was more difficult to discern. Isocitrate dehydrogenase, L-lactate dehydrogenase A, and phosphoglycerate mutase were all downregulated, whereas glucose-6-phosphate 1-dehydrogenase was induced. Cytochrome c oxidase subunit 1, which is involved in electron transport, was induced, as it was in gill, whereas subunit VIII was repressed.

Cluster 11: Fatty acid metabolism

Temperature can have profound effects on membrane integrity and the maintenance of membrane phase and viscosity depends on modifying the composition of lipid bilayers (Hazel, 1995). Therefore, genes involved in lipid biosynthesis were predicted to respond to heat shock. Two regulators of fatty acid metabolism, elongation of very long chain fatty acids protein and dihydroceramide delta (4)-desaturase, were induced in gill, whereas the fatty acid synthase was repressed (Fig. 2, Cluster 11). In muscle, acetyl-coenzyme A acyltransferase 1, lipin 1 and very-long-chain-acyl-CoA synthetase, 1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase were all induced, whereas ceramide kinase was repressed (Fig. 3, Cluster 11). Whether these changes in the expression of regulators of lipid biosynthesis were related to changes in the membrane composition due to thermal stress remains to be elucidated, as no clear saturation signal was observed.

Cluster 12: transport

Genes coding for proteins involved in transporting various molecules throughout the cell were grouped according to this shared functional property, but these proteins act in service to disparate cellular processes. Some of the gene products of Cluster 12 are tasked with transporting specific molecules such as lipids, proteins, carbohydrates, iron or calcium throughout the cell, whereas others mediate transport through the Golgi (Figs 2, 3, Cluster 12).

Genes involved in lipid transport were induced in gill and muscle. Apolipoprotein Eb precursor was induced in both tissues; in muscle only, microsomal triglyceride transfer protein large subunit and oxysterol binding protein-related protein 9 were upregulated. Likewise, genes encoding proteins involved in transporting the essential mineral iron were induced in both tissues. In gill, serotransferrin precursor and the functionally related transferrin were both induced. In muscle, hemopexin precursor, a beta-1B-glycoprotein involved in iron ion homeostasis, was the only gene from this cluster to be repressed by heat. Transporters of other ions such as chloride and calcium were also upregulated in both tissues; chloride intracellular channel 6 and phospholemman precursor were induced in gill and the critical calcium transporter and mediator of muscle contraction sarcoplasmic/endoplasmic reticulum calcium ATPase 1 was induced in muscle.

Additional genes involved in transport that were upregulated in muscle included those crucial to transport in the mitochondrion (import inner membrane translocase TIM44 and mitochondrial carrier protein RIM2) and the glucose transporter solute carrier family 2, perhaps reflecting a need to provide energy to the heat-stressed muscle.

In gill, nine genes associated with vesicle-dependent transport through the Golgi were induced during heat shock (Fig. 2, Cluster 12), those for clathrin light and heavy chain protein, clathrin coat assembly protein, coatamer gamma 2 subunit, Golgi autoantigen, cop-coated vesicle protein, the vesicle targeting molecule syntaxin 1, and two members of the retrograde transporter COPII coat complex, sec23B and Emp24 protein, were all upregulated in the gill. In muscle, heat shock induced the expression of clathrin heavy chain, vacuolar protein sorting 39, Emp24 protein, Golgi autoantigen and target of Myb protein 1 (Fig. 3, Cluster 12). This expression pattern suggests an increase in Golgi-mediated transport during heat shock.

Cluster 13: apoptosis

In some cases, sustained heat stress may cause significant enough damage to the macromolecular machinery of the cell that repair mechanisms are insufficient to prevent apoptosis. There were no gene expression changes associated specifically with apoptosis observed in gill; however, several apoptotic genes were upregulated in muscle (Fig. 3, Cluster 13). Caspase-3 precursor was induced, as was caspase recruitment domain protein 10. Three other pro-apoptotic genes, nucleolysin TIA-1, cold autoinflammatory syndrome 1 protein (also termed PYPAF1), and Rac/cdc42 guanine nucleotide exchange factor 6 were also upregulated. These data suggest that despite rapid and pronounced induction of chaperones, damage to muscle tissue was extensive enough that apoptosis may have been potentiated.

Cluster 14: other functions

In some cases, only one or two genes associated with given biological processes demonstrated thermally responsive changes in expression, although this does not preclude their playing an important role in the cellular stress response (Figs 2, 3, Cluster 14). In both tissues, the expression of genes associated with nucleotide biosynthesis responded to heat treatment; in gill, ATP synthase beta chain was repressed, whereas in muscle this gene was induced. In both tissues nucleoside diphosphate kinase NBR-B was induced.

In both tissues, glutamine synthase, a key enzyme for nitrogen metabolism was induced. The primary function of

glutamine synthase is tissue-specific in fishes (Walsh et al., 2003); it is possible that its upregulation here is related to the movement of nitrogenous waste products out of the muscle and ultimately their excretion at the gill. This is supported by the induction of the Rhesus blood group-associated glycoprotein, which has been identified as an ammonium transporter (Weihrauch et al., 2004).

Two genes associated with DNA repair, DNA excision repair protein ERCC-2 and steroid receptor interacting SNF-2 domain protein, were induced in muscle. No DNA repair genes were upregulated in the gill. These patterns, combined with the induction of apoptotic genes in the muscle only, suggest that genomic damage was more extensive in the muscle during heat stress and that DNA repair and apoptotic pathways were activated.

Shared gene expression response

As described above, the expression of 34 features, corresponding to 31 unique genes, varied with heat exposure in both gill and muscle tissue (Table 1). Of these, 26% were molecular chaperones, although genes associated with numerous cellular processes were represented. In nearly every case, the genes in question were induced. Of these 31 genes, six were found to be members of a eukaryotic 'minimal stress proteome' described in a recent multi-species analysis (Kültz, 2005). This group of six genes includes the chaperones Hsp40, Hsp 60, Hsp70 and peptidyl-prolyl isomerase, as well as the metabolic enzyme isocitrate dehydrogenase and nucleoside diphosphate kinase, which is involved in NTP biosynthesis. Kültz (Kültz, 2005) hypothesizes that isocitrate dehydrogenase (and other metabolic enzymes) may play a role in producing reducing equivalents (NADH, NADPH) that are necessary for combating oxidative stressors. Whether oxidative stress was a secondary effect of heat stress in the current study is unknown, although both PDI and peptidyl-prolyl isomerase also respond to changes in the redox state of the cell (Papp et al., 2003) and

Table 1. *Genes that responded to heat shock in both gill and white muscle, the biological process with which they are associated, and whether they were up- or downregulated*

Gene	Biological process	Direction of response in gill	Direction of response in muscle	Minimal stress proteome? (*sensu* Kültz, 2005)*
Hsp40	Protein folding	+	+/–	Yes
Hsp60	Protein folding	+	+	Yes
Hsp70	Protein folding	+	+	Yes
Hsc71	Protein folding	+	+	No
Hsp90	Protein folding	+	+	No
Hsp108	Protein folding	+	+	No
Peptidyl-prolyl isomerase	Protein folding	+	+	Yes
T-complex protein 1	Protein folding	+	+	No
Euk. translation initiation factor 3	Protein synthesis	+	+	No
Elongation factor 1α	Protein synthesis	+	+	No
Ubiquitin	Protein degradation	+	+	No
Cathepsin L	Proteolysis	+	+	No
Tubulointerstitial nephritis antigen	Proteolysis	+	+	No
ADAM 17	Proteolysis	+	+	No
Calpain 9	Proteolysis	+	+	No
RNA-dependent helicase p68	Cell proliferation	+	–	No
Suppressor of G2 allele of SKP1	Cell proliferation	+	+	No
Transducer of ERBB2	Cell proliferation	–	+	No
Integrin beta-1 binding protein	Cell adhesion	+	+	No
Periplakin	Cell adhesion	+	+	No
Isocitrate dehydrogenase	Carbohydrate metabolism	+	–	Yes
L-lactate dehydrogenase	Carbohydrate metabolism	+	–	No
Glutamine synthetase	Nitrogen metabolism	+	+	No
cAMP response element binding protein	Transcriptional regulation	+	+	No
C/EBP delta	Transcriptional regulation	+	+	No
VBP	Transcriptional regulation	+	+	No
STAT-3	Transcriptional regulation	+	+	No
Clathrin	Transport	+	+	No
Parvalbumin alpha	Transport	+	+	No
Parvalbumin beta	Transport	+	+	No
Nucleoside diphosphate kinase	NTP biosynthesis	+	+	Yes

+, upregulated; –, downregulated.
*Whether the gene is a member of the cross-taxa, minimum stress proteome proposed by Kültz.

were induced in both tissues. The postulated function of nucleoside diphosphate kinase in the minimal stress proteome is the provision of nucleotides for DNA repair (Kültz, 2005), an idea supported by the induction of genes involved in DNA repair in the muscle (Fig. 3, Cluster 14).

Ubiquitin-conjugation of damaged protein

As much of what is known about the cellular stress response demonstrates the need for protection of the protein pool against abnormal heat-denaturation (Fink, 1999; Kültz, 2003), it was desirable to relate any observed changes in gene expression to a direct measure of macromolecular damage. We chose to measure the concentration of proteins that were covalently conjugated to ubiquitin and, therefore, targeted for proteasomal degradation (Fig. 4). Tissue-specific increases in ubiquitylated protein were observed. Two separate peaks in ubiquitin-conjugates were measured in gill, with the first occurring 240 min after initiation of the experiment, and the second occurring after 480 min. There was also a significant increase in ubiquitin-conjugated protein in the recovery group at 420 min, after which levels returned to those at time 0. In muscle, there was only a single peak in ubiquitin-conjugated protein, observed at 360 min. Levels dropped back to those at time 0; levels also decreased immediately upon return to 18°C in the recovery group. The delayed peak in ubiquitin-conjugate levels in muscle, relative to the first peak observed in gill, may be related to the greater induction in Hsp70 observed in the muscle. Strong investment in protein chaperoning in this tissue may have precluded the need for a significant increase in protein degradation, until a threshold was reached at 360 min. The multiple peaks in gill tissue suggest that ubiquitylation can be rapidly followed by clearing of tagged protein at the proteasome and that this can be followed with additional rounds of ubiquitylation if needed.

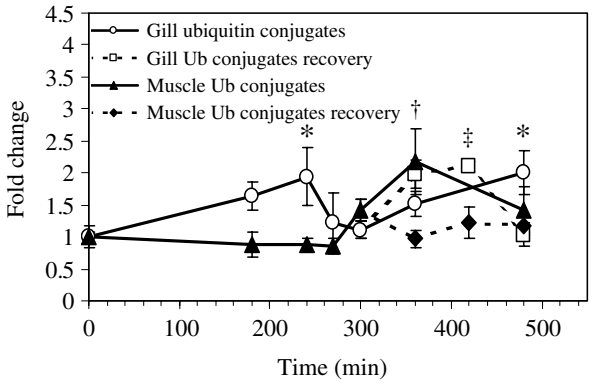

Fig. 4. Ubiquitin-conjugated proteins in gill and white muscle from *Gillichthys mirabilis*. For determination of the concentration of ubiquitin-conjugated proteins, thawed tissues were homogenized and dot-blotted onto nitrocellulose membranes. Following incubation in primary and secondary antibodies, blots were visualized with enhanced chemiluminescence reagent and exposed to X-ray film; exposed film was scanned and spot intensity quantified densitometrically. Average pixel intensity for each spot was normalized to the average for the four time 0 individuals. Values are means ± s.d. (N=4). *Heat shock time points, in gill tissue, that differ significantly (ANOVA, $P \leq 0.001$) from time 0; ‡recovery time points, in gill tissue, that differ significantly from time 0; †heat shock time points, in muscle tissue, that differ significantly ($P \leq 0.001$) from time 0. In some cases, error bars are contained within symbols.

Correlating gene expression with protein levels

An important consideration for cDNA microarray-based experimentation is that the transcriptomic response may not predict changes at the protein level, a concern based upon the fact that ultimately it is the stress proteome that does the work in protecting and repairing the cellular machinery during exposure to stress. To begin to link gene expression with protein production, we selected a group of genes whose expression profiles showed strong responses to temperature, and we quantified the concentration of their corresponding protein products using western blots. The genes selected were

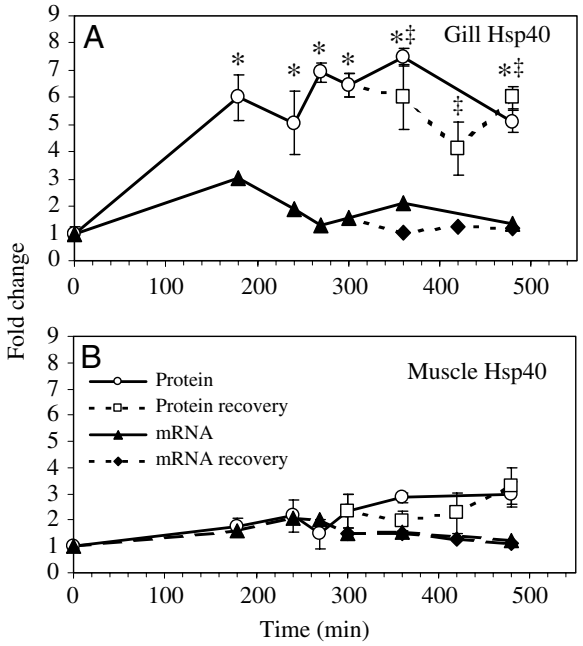

Fig. 5. Hsp40 mRNA (filled symbols) and protein (open symbols) in the (A) gill and (B) white muscle from *Gillichthys mirabilis*. Average protein concentrations and mRNA levels are shown for fish (N=4) from each time point, either during heat shock (solid lines; circles) or during recovery (broken lines; squares). At a given time point, fish were sacrificed and frozen immediately in liquid nitrogen. The mRNA values are taken from the microarray analyses (see Materials and methods and Figs 2, 3). For protein concentration determination, thawed tissues were homogenized and dot-blotted onto nitrocellulose membranes. Following incubation in primary and secondary antibodies, blots were visualized with enhanced chemiluminescence reagent and exposed to X-ray film; exposed film was scanned and spot intensity quantified densitometrically. Average pixel intensity for each spot was normalized to the average for the four time 0 individuals. For protein levels, values are means ± s.d. (N=4). *Heat shock time points that differ significantly (ANOVA, $P \leq 0.001$) from time 0; ‡recovery time points that differ significantly from time 0. In some cases, error bars are contained within symbols.

Hsp40, Hsp70, Hsp90, PDI and actin (Figs 5–9). We chose genes with similar responses in both tissues (Hsp70 or Hsp90) or tissue-specific patterns of expression (Hsp40, PDI and actin), to determine if these patterns would be mirrored in protein levels. Actin was included as it is often used as a control against which to compare the expression of stress-responsive genes. Here, we demonstrate that this 'housekeeping' gene is not only heat-inducible at the transcriptional level, but at the translational level as well.

In every case, an induction in message production was correlated with an increase in protein concentration (Figs 5–9) although the relative timing and magnitude of mRNA *versus* that of protein production varied from gene to gene. The production of Hsp40 mRNA and protein occurred rapidly upon initiation of heat shock (Fig. 5A,B), although, as discussed above, expression patterns differed among three Hsp40 subfamily members (expression of the only induced gene, subfamily A member 1, is depicted in Fig. 5). In gill, a threefold induction of message from this gene was correlated with an eightfold increase in the protein. In muscle, although there was a trend of increasing Hsp40 protein with increasing temperature, this trend was not statistically significant.

Hsp70 and Hsp90 were both rapidly induced in each tissue (Figs 6, 7). A given fold-change in mRNA levels, however, did not consistently predict fold-change in protein levels and the observed patterns for these two genes were tissue-specific. An approximately fivefold increase in Hsp70 mRNA in gill was followed by a 12-fold increase in protein levels. By contrast, in the muscle, the fold-increase in Hsp70 mRNA was much greater than that of the protein (18-fold and threefold, respectively). A similar relationship between mRNA and protein was observed for Hsp90. In gill, an approximately 12-fold increase in mRNA levels occurred concomitantly with a 15-fold increase in protein concentration, whereas in muscle, Hsp90 mRNA was induced eightfold, whereas increases in protein concentration were again more moderate (2-fold). The explanation for these patterns may lie in the relationship between pre-stress concentrations of a protein and the eventual amounts of this protein required for mounting an effective cellular stress response. It is possible that the upstream initiation of cell signaling events that result in the induction of a given gene may act independently of the secondary controls on translation that may respond directly to feedback mechanisms calibrated to the existing cellular concentration of the protein product of this gene.

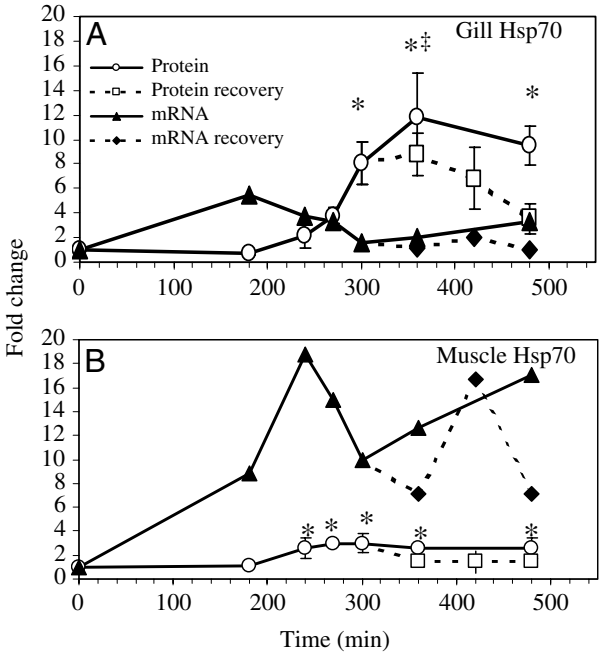

Fig. 6. Hsp70 mRNA (closed symbols) and protein (open symbols) in (A) the gill and (B) white muscle from *Gillichthys mirabilis*. Average protein concentrations and mRNA levels are shown for fish (N=4) from each time point, either during heat shock (solid lines; circles) or during recovery (dashed lines; squares). Methods were identical to those described in the legend to Fig. 5, except that antibodies used were specific to Hsp70. Average pixel intensity for each spot was normalized to the average for the four time 0 individuals. For protein levels, values are means ± s.d. (N=4). *Heat shock time points that differ significantly (ANOVA, $P \leqslant 0.001$) from time 0; ‡recovery time points that differ significantly from time 0. In some cases, error bars are contained within symbols.

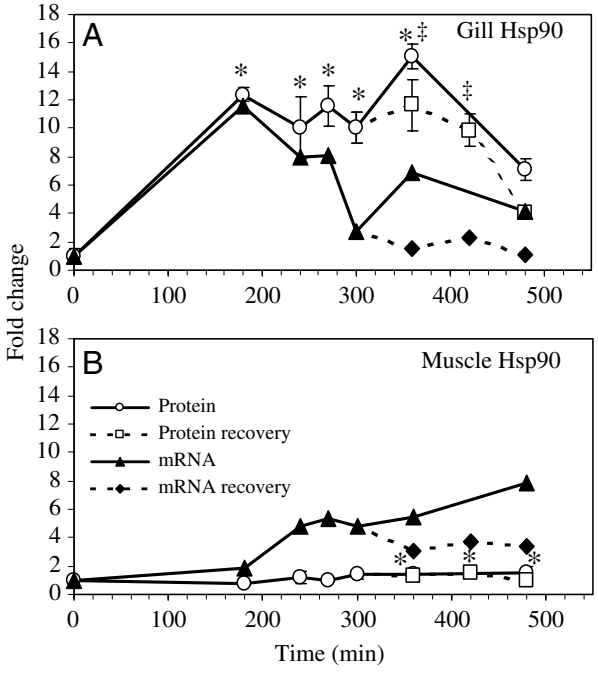

Fig. 7. Hsp90 mRNA (filled symbols) and protein (open symbols) in (A) the gill and (B) white muscle from *Gillichthys mirabilis*. Average protein concentrations and mRNA levels are shown for fish (N=4) from each time point, either during heat shock (solid lines; circles) or during recovery (broken lines; squares). Methods were identical to those described in the legend to Fig. 5, except that antibodies used were specific to Hsp90. Average pixel intensity for each spot was normalized to the average for the four time 0 individuals. For protein levels, values are means ± s.d (N=4). *Heat shock time points that differ significantly (ANOVA, $P \leqslant 0.001$) from time 0; ‡recovery time points that differ significantly from time 0. In some cases, error bars are contained within symbols.

Despite the absence of a tight one-to-one coupling of mRNA and protein concentrations for these induced genes, we consider it an important validation of the microarray platform that no increase in protein levels was observed without a correlated induction of mRNA and where no induction was observed, changes at the protein level were also absent. For example, the ER-localized chaperone PDI displayed a tissue-specific pattern of response, being strongly induced in gill only. Accordingly, an increase in PDI protein level was measured in this tissue, and not in muscle (Fig. 8). A similar tissue-specific response was observed for actin (Fig. 9), with actin mRNA being markedly induced in gill but not in muscle; these changes, too, were reflected at the protein level.

As the heat shock genes are among those most commonly nominated for use as biomarkers for environmental stress, it is clear from the data presented here that using mRNA values alone may not properly describe the condition of the organism in the field and that protein levels may be more prolonged and appropriate for assessment of stress exposure.

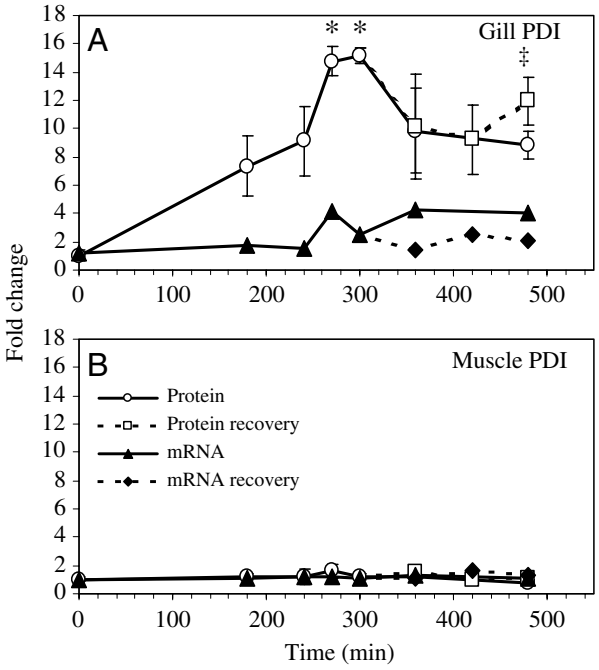

Fig. 8. Protein disulfide isomerase (PDI) mRNA (filled symbols) and protein (open symbols) in the (A) gill and (B) white muscle from *Gillichthys mirabilis*. Average protein concentrations and mRNA levels are shown for fish (N=4) from each time point, either during heat shock (solid lines; circles) or during recovery (broken lines; squares). Methods were identical to those described in the legend to Fig. 5, except that the antibodies used were specific to PDI. Average pixel intensity for each spot was normalized to the average for the four time 0 individuals. For protein levels, values are means ± s.d (N=4). *Heat shock time points that differ significantly (ANOVA, ($P \leq 0.001$) from time 0; ‡recovery time points that differ significantly from time 0. In some cases, error bars are contained within symbols.

Summary

Heat shock resulted in numerous effects on the transcriptome of *G. mirabilis*. In addition to the induction of molecular chaperones, which is the best-described reaction to cellular hyperthermia, the transcription of genes from many different functional classes was found to also respond to thermal stress. This may support a broader definition of the "heat shock response" at the transcriptional level than that which is traditionally applied to the translation level.

A recent study on the effects of progressive cooling on the transcriptome of the carp *Cyprinus carpio* (Gracey et al., 2004) revealed that the changes in gene expression associated with the response to cold were considerably more extensive, in terms of the numbers of genes involved, than the changes observed in the current study on heat stress. Twenty-five percent of the genes on the carp microarray, nearly 3400 unique cDNAs, responded to cooling; this number is an order of magnitude higher than the number of genes that varied here in response to heat exposure. The difference may stem from the fact that the cooling study was a test of acclimation to lowered temperature over the course of 22 days, whereas the heat stress regime employed here was in the order of hours.

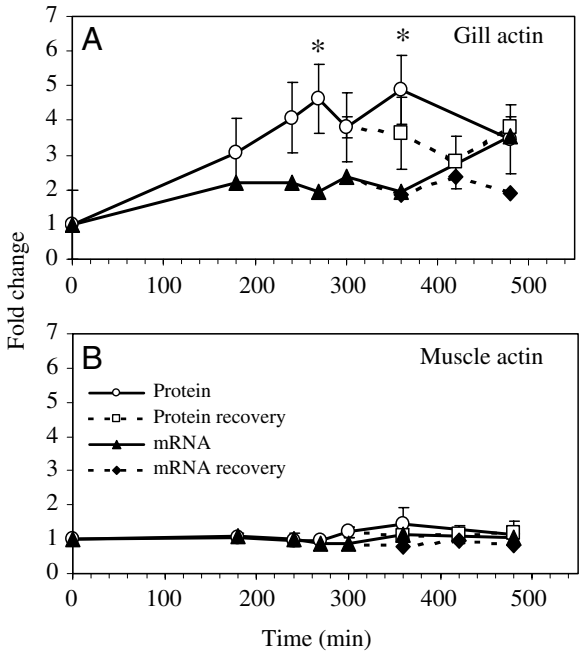

Fig. 9. Actin mRNA (filled symbols) and protein (open symbols) in the (A) gill and (B) white muscle in from *Gillichthys mirabilis*. Average protein concentrations and mRNA levels are shown for fish (N=4) from each time point, either during heat shock (solid lines; circles) or during recovery (broken lines; squares). Methods were identical to those described in the legend to Fig. 5, except that the antibodies used were specific to actin. Average pixel intensity for each spot was normalized to the average for the four time 0 individuals. For protein levels, values are means ± s.d. (N=4). *Heat shock time points that differ significantly (ANOVA, $P \leq 0.001$) from time 0; ‡recovery time points that differ significantly from time 0. In some cases, error bars are contained within symbols.

The changes in gene expression that underpin the compensatory adjustments made during long-term acclimation to the metabolically depressive effects of cold may be more extensive than those associated with the immediate damage prevention and repair mechanisms required for mediating the negative impacts of acute heat shock. Evidence for a two-stage stress response, recently described by Kültz (Kültz, 2005), supports this conclusion. It appears that the early reaction to environmental stress, termed the 'cellular stress response (CSR)', involves the protection and repair of macromolecules such as membranes and proteins. Critical for immediate cellular survival, the CSR can be measured in the order of minutes to hours. The subsequent initiation of a longer-term response, the 'cellular homeostatic response (CHR)' involves a suite of additional changes at the molecular changes that function to restore the cell to homeostasis, within the context of new environmental conditions. The timescale of the current study was short enough that perhaps only the CSR was observed, whereas the carp study almost certainly was measuring aspects of the CHR.

It is probable that, in many cases, the gene expression changes measured by microarray analyses represent just 'the tip of the iceberg' in terms of the subsequent cellular responses these changes promote. For instance, the moderate induction or repression of cell signaling genes may have profound effects on the cell as their protein products proceed to alter the activity of other pre-existing molecules. It may be the activity of such molecules that determines the fate of the stressed cell. Likewise, a relatively minor induction of a transcription factor, a 25% increase for example, may initiate the transactivation of numerous genes involved in a variety of response and repair processes. The utility of the cDNA microarray, therefore, is to focus attention on those genes that are directly responsive to stress, with further molecular, biochemical and physiological analyses being the next logical arena of experimental pursuit.

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