Changes in gene expression associated with acclimation to constant temperatures and fluctuating daily temperatures in an annual killifish *Austrofundulus limnaeus*

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Summary

Eurythermal ectotherms commonly thrive in environments that expose them to large variations in temperature on daily and seasonal bases. The roles played by alterations in gene expression in enabling eurytherms to adjust to these two temporally distinct patterns of thermal stress are poorly understood. We used cDNA microarray analysis to examine changes in gene expression in a eurythermal fish, *Austrofundulus limnaeus*, subjected to long-term acclimation to constant temperatures of 20, 26 and 37°C and to environmentally realistic daily fluctuations in temperature between 20°C and 37°C. Our data reveal major differences between the transcriptional responses in the liver made during acclimation to constant temperatures and in response to daily temperature fluctuations. Control of cell growth and proliferation appears to be an important part of the response to change in temperature, based on large-scale changes in mRNA transcript levels for several key regulators of these pathways. However, cell growth and proliferation appear to be regulated by different genes in constant *versus* fluctuating temperature regimes. The gene expression response of molecular chaperones is also different between constant and fluctuating temperatures. Small heat shock proteins appear to play an important role in response to fluctuating temperatures whereas larger molecular mass chaperones such as Hsp70 and Hsp90 respond more strongly to chronic high temperatures. A number of transcripts that encode for enzymes involved in the biosynthesis of nitrogen-containing organic osmolytes have gene expression patterns that indicate a possible role for these 'chemical chaperones' during acclimation to chronic high temperatures and daily temperature cycling. Genes important for the maintenance of membrane integrity are highly responsive to temperature change. Changes in fatty acid saturation may be important in long-term acclimation and in response to fluctuating temperatures; however cholesterol metabolism may be most critical for short-term acclimation to fluctuating temperatures. The variable effect of temperature on the expression of genes with daily rhythms of expression indicates that there is a complex interaction between the temperature cycle and daily rhythmicity in gene expression. A number of new hypotheses concerning temperature acclimation in fish have been generated as a result of this study. The most notable of these hypotheses is the possibility that the high mobility group b1 (HMGB1) protein, which plays key roles in the assembly of transcription initiation and enhanceosome complexes, may act as a compensatory modulator of transcription in response to temperature, and thus as a global gene expression temperature sensor. This study illustrates the utility of cDNA microarray approaches in both hypothesis-driven and 'discovery-based' investigations of environmental effects on organisms.

Supplementary material available on-line

Key words: annual fish, *Austrofundulus limnaeus*, DNA microarray, ephemeral pond, eurytherm, gene expression, high mobility group proteins, transcription.

Introduction

The effect of temperature is manifested at every level of biological organization, from complex behaviors to molecular motion. Changes in temperature affect the fluidity of lipid membranes, the conformational mobilities and activities of proteins, and the stability of DNA duplexes (Hochachka and Somero, 2002). Because these thermal effects have such major consequences for cellular function, organisms typically manifest extensive evolutionary adaptations that establish distinct thermal optima and limits for physiological function, as well as capacities for altering the phenotype in response to changes in temperature that occur during the lifetime of an organism, e.g. on time scales from minutes to seasons.

Although targeted studies of thermal acclimation in individual physiological processes have revealed a number of important aspects of this type of phenotypic plasticity (Hochachka and Somero, 2002), little is currently known about the underlying shifts in gene expression that accompany thermal acclimation, a phenomenon first emphasized in a pioneering work by Hochachka (1967). Our most extensive knowledge about shifts in gene expression in response to changes in temperature comes from studies of the heat- and cold-shock responses (Feder and Hofmann, 1999; Fujita, 1999). However, these studies typically focus on a narrow range of high or low temperatures that are severe and induce a strong cellular stress response. Very few studies have addressed changes in gene expression associated with routine daily or seasonal temperature regimes experienced by the organism. In nature, most organisms experience moderate to large variations in temperature on a daily basis and must sense and react to continually changing temperatures. Likewise, seasonal changes in temperature dictate considerable modifications of the phenotype, which occur over time courses of days to weeks to months. How regulation of gene expression differs in response to daily and seasonal changes in temperature is virtually unknown. To investigate how these temporally disparate processes of thermal acclimation are mediated by shifts in gene expression we have employed cDNA microarray techniques to profile changes in gene expression associated with daily and multi-week temperature acclimation in the eurythermal annual killifish *Austrofundulus limnaeus*.

Annual killifish inhabit ephemeral pond environments in regions of Africa and South America characterized by distinct dry and rainy seasons (Myers, 1952; Bailey, 1972). *Austrofundulus limnaeus* occurs in small, isolated ponds in coastal desert and savanna regions of northern South America. In these habitats, *A. limnaeus* routinely experiences wide daily fluctuations in temperature, oxygen availability and pH (Podrabsky et al., 1998). Temperatures may change over 20°C on a daily basis and may reach highs above 40°C. In addition to these daily changes, temperatures are also likely to become increasingly hot as the dry season approaches and the ponds evaporate and, eventually, become completely dry. Because of its ability to thrive in this highly variable environment where daily and seasonal shifts are extreme, we reasoned that *A. limnaeus* would be an excellent study organism for examining shifts in gene expression in response to both rapid and long-term alterations in body temperature.

To this end, we created a cDNA microarray containing 4992 cDNAs isolated from liver tissue of *A. limnaeus*. This microarray was used to profile changes in gene expression associated with acclimation to constant temperatures spanning the species' environmental temperature range, and fluctuating, environmentally realistic daily temperature regimes in a laboratory setting. We wished to explore and characterize which parts of the known and well-described physiological responses to temperature acclimation were manifested at the transcriptional level, as well as to explore changes in gene expression associated with aspects of temperature acclimation

that had not yet been identified in physiological studies. We were especially interested in characterizing the differences in gene expression that (i) distinguish long-term thermal acclimation to stable temperatures from more rapid daily responses to temperature change and (ii) distinguish relatively rapid responses to thermally induced cellular damage from longer-term responses that restore cellular homeostasis.

Materials and methods
Treatment of fish and collection of samples

To investigate the effect of temperature on gene expression in *Austrofundulus limnaeus* Myers, we exposed fish to four laboratory temperature acclimation regimes (Fig. 1B): constant temperatures of 20°, 26° or 37°C or a daily cycling temperature regime from 20–37°C. The fluctuating temperature regime was designed to mimic the natural temperature variations experienced daily by *A. limnaeus* (Podrabsky et al., 1998;
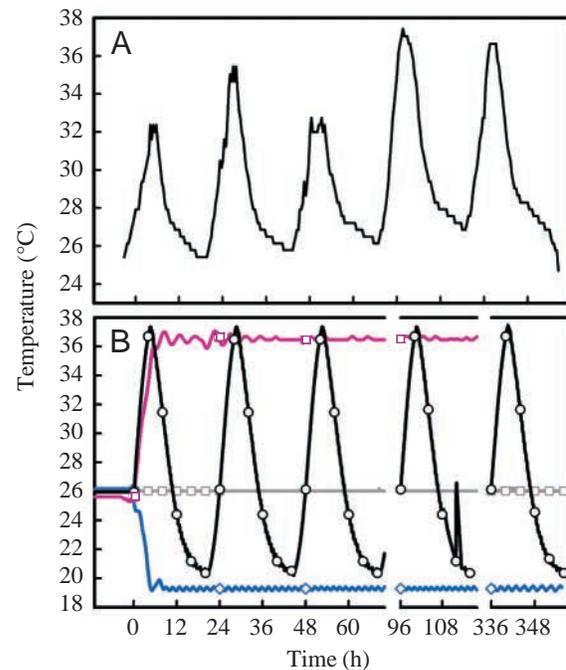

Fig. 1. Natural and laboratory temperatures experienced by adult *Austrofundulus limnaeus*. (A) Temperature record for 5 consecutive days in a typical ephemeral pond inhabited by *A. limnaeus* (Podrabsky et al., 1998). (B) Adult male fish were exposed to four different thermal acclimations. For the cycling temperature (20–37°C) treatment (black line) fish were collected (open circles) every 4 h for the first three daily cycles and then again every 4 h on day 5 and day 14. Control fish (gray line) kept at 26°C were collected (open squares) every 4 h on day 1 and day 14. Fish exposed to constant 20°C (blue line) were collected (open blue diamonds) at 24, 48, 72, 96, 168 and 336 h. Fish exposed to constant 37°C (pink line) were collected (open pink squares) as the 20°C fish, except for the 336 h time point. Time 0 for the temperature cycling is 12:30 h at a temperature of 26°C. Note the broken axis in B. While five temperature cycles were sampled during the 2-week acclimation, the temperature was continually cycling.

Fig. 1A). Adult male fish with a mean size of 2.1±1.1 g (mean ± S.D.) were used in this study to avoid complications associated with size and sex-specific gene expression. In total, 432 fish were sampled during the time-course sampling regime. Fish used in this experiment were produced from a laboratory stock that has been maintained for several generations at 26–28°C. The fish for each acclimation regime were housed in 210 l glass aquaria. Fish were fed daily during the duration of the experiment at 09:00 h with frozen blood worms (chironomid larvae). The experiment was started (*t*=0) at 12:30 h. Fish were sampled (Fig. 1B symbols) every 24 h for the 20° and 37°C acclimations and every 4 h during the fluctuating (20–37°C) and control (26°C) treatments. Four fish were removed from the acclimation tanks at each time point and flash frozen in liquid nitrogen. Fish were stored at –80°C for 2–4 weeks prior to extraction of total RNA from liver tissue.

Preparation of cDNA libraries

Livers were dissected from frozen fish and total RNA extracted using Trizol according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Total RNA from the livers of four fish was pooled for each time point. These pooled samples of total RNA were used to prepare cDNA for the production of the microarray and to profile changes in gene expression during temperature acclimation.

A cDNA library was prepared from total RNA pooled from all fish sampled during the acclimations. SMART cDNA technology (BD Biosciences, San Jose, CA, USA) was used to prepare the cDNA prior to cloning into the pλTriplEx2 directional cloning plasmid vector (ClonTech). Briefly, total RNA was used as a template to create a cDNA copy of each mRNA using reverse transcriptase (Powerscript, ClonTech). The single-stranded cDNA was then amplified *via* long-distance polymerase chain reaction (LD-PCR; Cheng et al., 1994). The amplified cDNA was then ligated into the cloning vector. Competent *E. coli* (DH10B cells, Invitrogen) were then transformed with the ligated plasmids *via* electroporation. Transformed cells were then selected for presence of a cDNA insert and ampicillin (100 µg l^{-1}) resistance *via* blue/white screening on LB/agar plates (Sambrook et al., 1989). A total of 4992 cDNA clones were isolated into 384-well microtiter plates and grown overnight in LB medium (Sambrook et al., 1989) supplemented with 10% glycerol. These plates were replicated and stored at –80°C.

Construction of the cDNA microarray

The cDNA inserts from each isolated clone in the cDNA library were amplified using polymerase chain reaction (PCR). A PCR master mix was prepared containing 1× Taq. Polymerase buffer (Promega, Madison, WI, USA), 0.2 mmol l^{-1} dNTPs and 0.2 µmol l^{-1} plasmid-specific primers. A single reaction volume was 25 µl. PCR master mix was inoculated with a small amount of a single bacterial broth culture from the 384-well plate using a 96-well plate replicator tool (Nalgene/Nunc, Rochester, NY, USA). The reactions were then heated to 95°C for 2 min to lyse the bacteria and release the plasmid DNA as template for the reaction. Reactions were then exposed to 40 cycles of amplification with a denaturing step of 95°C for 0.25 min, an annealing step of 56°C for 0.5 min, and an extension step of 72°C for 2.5 min. One row of reactions from each 96-well plate was randomly checked *via* agarose electrophoresis to assure proper amplification of the DNA.

A small amount (7 µl) of the PCR-amplified DNA was then transferred to a 384-well PCR plate corresponding to the original clone location used to seed the reaction. The salt concentration of the PCR reactions was brought to a concentration of 3× SSC (1× SSC contains 150 mmol l^{-1} NaCl, 15 mmol l^{-1} sodium citrate, pH 7.0) by adding 20× SSC directly to the PCR products. Microarrays were printed onto glass slides coated with poly-L-lysine according to standard protocols (www.microarray.org; specific protocols are also available at killifish.pdx.edu/protocols.htm). Poly-L-lysine coated microscope slides were produced using Fisher Scientific (Hampton, NH, USA) Gold Seal microscope slides and Sigma Chemical brand poly-L-lysine solution (see above websites). The slides were then stored in a desiccator for 3 weeks to allow the poly-L-lysine coat to age properly. Microarrays were printed using a homemade robot having a 16 pin configuration (TeleChem Chip Maker II pins, Telechem ArrayIt, Sunnyvale, CA, USA) and printing an 18×18 spot grid pattern with a spacing of 240 µm between spots for each pin. This configuration of pins and salt concentration in the DNA samples resulted in a typical spot size around 140–150 µm. Microarrays were post-processed according to standard procedures (see above web sites).

Profiling changes in gene expression using the cDNA microarray

Fluorescent-labeled cDNA probes were prepared from poly(A)$^{+}$-enriched RNA samples. poly(A)$^{+}$RNA samples were prepared from total RNA samples using an oligo-dT cellulose column. All poly(A)$^{+}$RNA samples are a pool of equal quantities of RNA from 4 individuals collected at each time point. 1 µg of the poly(A)$^{+}$RNA was used as template to make a single cDNA copy of the mRNA pool using reverse transcriptase (RT) and anchored oligo-dT$_{15}$ and pdN$_6$ random hexamer primers in the presence of amino-allyl dUTP (see above web sites). The RNA template was removed from the completed RT reactions by incubating at 65°C for 30 min in 0.2 mol l^{-1} NaOH and 0.1 mol l^{-1} EDTA. The single stranded cDNA was then covalently linked *via* the amino-allyl UTP to either a Cy3 or Cy5 monoreactive dye according to the manufacturer's instructions (Amersham, Piscataway, NJ, USA). The labeled cDNA probes were then cleaned using PCR purification columns according to the manufacturer's instructions (Qiagen, Valencia, CA, USA) except that the buffer PE was replaced with 80% ethanol. The probes were prepared for hybridization according to standard procedures (see above web sites). Briefly, the cleaned probes were brought to a final volume of 30 µl in a solution containing 25 mmol l^{-1}

Hepes buffer pH 7.0, 0.75 mg ml^{-1} tRNA (Sigma), 3× SSC and 0.2% SDS. The probes were boiled for 2 min and allowed to cool at room temperature for 5 min prior to starting the hybridizations. Probes were added to the microarray by placing the LifterSlip (15 mm×15 mm) coverslip over the array and using capillary action to draw the solution under the cover slip. Hybridizations were performed at 65°C overnight in Genomic Solutions (Ann Arbor, MI, USA) hybridization chambers. Each hybridization was performed once for each comparison. A total number of 55 hybridizations are represented in the data set. Two daily cycles, separated by 2 weeks, were sampled for control conditions (26°C). Five temperature cycles were sampled for the cycling temperature acclimation, resulting in a very consistent pattern of gene expression during multiple temperature cycles.

Following hybridization, the arrays were briefly and gently washed to remove any unbound dye and then rapidly dried by centrifugation (www.microarray.org). Briefly, the slides were placed flat into a solution of 0.6× SSC and 0.03% sodium dodecylsulfate (SDS) and the coverslip was gently washed off the slide. The slides were then transferred to a slide rack and gently dipped 10 times in a fresh 500 ml container of the same wash solution. The slides were then transferred individually and gently dipped 10 times in a second 500 ml of wash solution containing 0.06× SSC. Following this wash the slides were dipped 5 times into 500 ml of deionized water and then spun dry in a centrifuge at low speed. The washed slides were scanned using an Axon GenePix 4000B microarray scanner (Axon Instruments, Union City, CA, USA). Data were extracted from the scanned images using GenePix 4.0 software (Axon Instruments). The data were then entered into the Stanford Microarray Database (SMD). Data filtering and sorting were accomplished within the SMD database. Upon entry into the database, all data were normalized to balance the overall levels of Cy5 and Cy3 to a 1:1 ratio using a simple correction factor applied to the Cy5 data. For each spot on each array, data were selected for analysis only if they had a

Fig. 2. Replicate hybridizations and reciprocal dye labeling experiments. (A) Duplicate hybridizations were performed for each time point in the first two temperature cycles. Expression data were filtered by accepting only spots that changed over twofold in at least 1 time point. These data are not corrected relative to $t=0$, but they are median centered. Cy3 was used to label the reference sample while Cy5 was used to label the experimental sample in the forward (F) hybridizations. The dyes were reversed (R) in the second set of hybridizations. Visual inspection reveals a strong relationship between the two sets of hybridizations, especially for spots that change greater than twofold compared to the reference. (B) There is a strong correlation between the data for the forward and reverse hybridizations ($r=0.96$) for spots on the array that change more than twofold ($N=831$). The equation for the regression line on the graph is $y=1.061x+0.04056$. These data indicate that there is no significant dye bias in this data set. Further, it supports the conclusion that changes in gene expression observed in this study are not likely to be due to variation in hybridizations, but instead are biologically relevant.
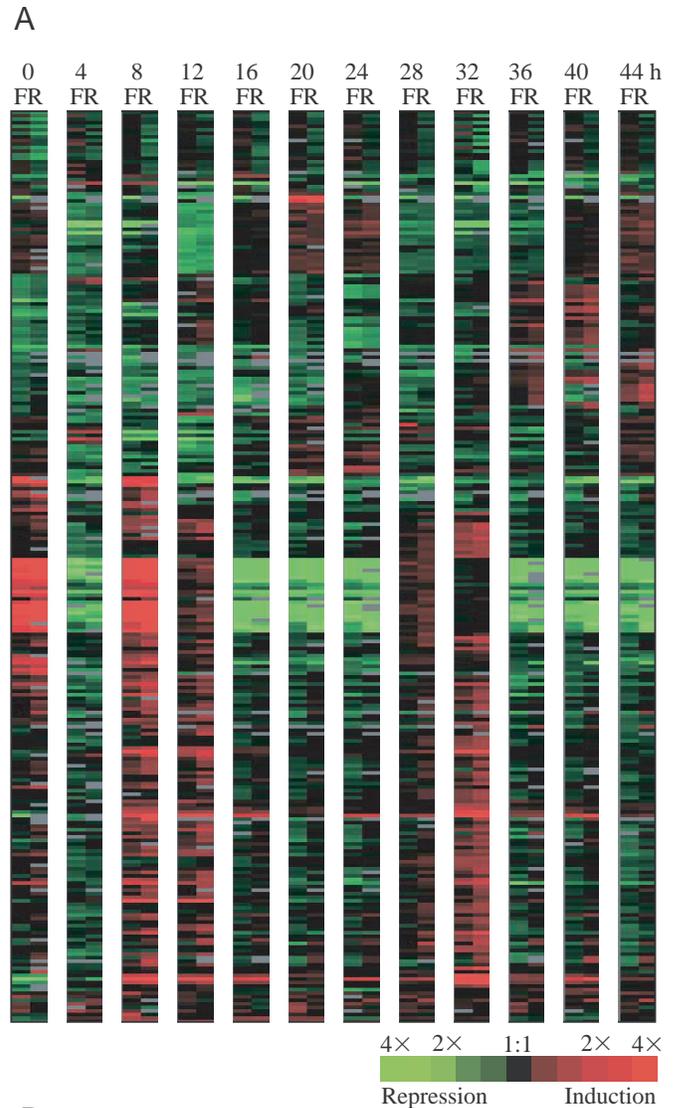
Cy5/Cy3 fluorescence regression coefficient of >0.6 and had a signal intensity of 2.5 times background. These spots are termed 'fair' data. This method of normalization and definition of 'fair' data were the default settings in SMD.

Data analysis and presentation

Gene expression levels were determined at each time point by comparing the amount of mRNA transcript present in the experimental sample (pooled from four fish at each time point) compared to a reference sample (pooled from over 400 fish used in the experiment). The use of a reference sample allowed the comparison of the relative amount of each transcript at each time point to a common sample. Reference samples were routinely labeled with Cy3 while experimental samples were labeled with Cy5. However, dye reversal experiments were performed for the first two daily temperature cycles (12 samples) to investigate possible Cy Dye bias in our data and to assess variation in the data (Fig. 2). These dye reversal experiments indicate a very high correlation between duplicate hybridizations (r=0.96), for cDNA clones that experience changes in gene expression greater than twofold compared to the reference samples (Fig. 2).

Although use of a reference sample provided a common basis of comparison for each experimental sample, the data expressed in this format are not necessarily biologically relevant because each comparison is of an experimental sample compared to the mean level of each transcript that was expressed during the entire experiment. Interpretation of these data is difficult and thus we chose to further adjust the data to a biologically relevant standard. There are several avenues for adjusting data for this purpose, each of which has its own strengths and weaknesses.

A commonly used technique is to adjust the gene expression data so that the median level of expression is equal to 0 on a $\log_2$ scale for each spot on the array across all treatments (i.e. a 1:1 ratio); this method is termed 'median centering' (Eisen et al., 1998). This method is logical for comparisons of different cell types or cells under different steady state conditions, but in our opinion was not suitable for presentation of time-course data because of the underlying assumption that the median expression level of a given gene should be 0 on a $\log_2$ scale. For instance, if a gene is strongly induced in all time points sampled, this strong induction would be represented as the median level of gene expression and would probably cause the true pattern of expression to be largely attenuated after median centering. We observed this type of pattern for many molecular chaperones in this study.

Another possibility, and perhaps the most logical for time-course data, is to adjust all of the expression ratios to be relative to t=0. This method assumes that the initial time point represents some steady state physiological condition in the organism that must be adjusted in response to the experimental treatment. However, gene expression data presented in this manner are dependent on high quality data for the t=0 time point. Additionally, if data are missing for the t=0 time point then it is impossible to adjust the other time points. These

problems can be largely avoided by replicating the t=0 hybridizations and using the average of these replicates to adjust the rest of the data set. In our case we used the average of two hybridizations from the control (26°C, t=0 and t=336 h) to adjust the data set relative to the initial condition. One additional problem with this method is that the effects of temperature are not separated from the effects of normal daily rhythms in the gene expression data for temperature cycling. In many cases the natural rhythms in gene expression under exposure to constant 26°C were strong and masked the effects of temperature on gene expression.

To control for daily changes in gene expression that were not associated with the temperature cycle, we normalized the temperature cycling data to be relative to the control daily patterns of gene expression (an average of two daily cycles at 26°C). This operation in effect 'subtracts' the daily rhythms in gene expression not due to temperature from the temperature cycling data. This operation was done by multiplying the Cy5:Cy3 ratio for each experimental time point in the cycling temperature treatment by the Cy5:Cy3 ratio for the corresponding control time point (e.g. cycling t=4 h multiplied by control t=4 h). We have named this permutation of the data 'the effect of temperature'. Data presented in this manner illustrate the net effect of temperature on the natural patterns of gene expression. However, these data may be misleading if not properly presented. For instance, if there is a strong circadian rhythm in gene expression that is not affected by temperature, this will result in a flat daily expression pattern when presented in this manner, even though the gene may be changing several-fold each day. Therefore, we have chosen to present the data in two formats in this paper, relative to t=0 and after subtraction of daily rhythms, because each method of data presentation has strengths and illustrates different components of the data set.

Cross correlation analysis (Chatfield, 1989) was used to determine if gene expression patterns were significantly correlated with the temperature cycle. Using this method it is possible to identify the phase shift in the data that yields the highest correlation coefficient, and thus the most significant relationship between gene expression patterns and temperature patterns. Correlation coefficients were identified as statistically significant at the level of 0.05.

The gene expression ratio data were clustered according to similarity in expression pattern using Cluster software (Eisen et al., 1998). Pearson non-centered, complete linkage hierarchical clustering was used to organize the data. Visualization of the clustered data was accomplished using TreeView software (Eisen et al., 1998).

DNA sequencing

Microarray spots with interesting expression patterns were identified by sequencing the cDNA insert isolated from plasmid DNA from the clone of interest. Sequencing was accomplished using an ABI 373 sequencer with dye terminator reaction mix. Plasmid-specific primers were used for the sequencing reactions. All cDNAs of interest were sequenced

from the 5′ end to maximize the possibility of identifying the transcript. Sequences were identified by homology to known sequences using an NCBI Blastx search of the GenBank database. The most significant or relevant results of these Blast searches as well as the GenBank accession numbers for sequenced cDNA clones presented in this paper are available in supplemental Table 1.

Results and Discussion
Overall patterns of gene expression

A total of 540 cDNA clones were identified as differentially expressed by filtering the data to include cDNA clones that changed their expression levels at least twofold compared to the appropriate reference sample in at least 1 time point (Figs 3–5). For most transcripts that were found to be responsive to temperature, shifts in gene expression were typically between two- and fourfold changes, although some transcripts (e.g. transcripts that encode for the high mobility group protein B1 and HMG-CoA synthase) experienced six- to tenfold changes in relative abundance. The use of an arbitrary twofold cut-off criterion is probably a conservative approach to identifying genes whose levels of expression change, because physiologically important effects may arise from shifts in gene expression that are less than twofold. We adopted this conservative approach because of the challenges faced in designing time series experiments of this nature that can be subjected to conventional statistical analysis. In designing these experiments, which involved sampling at many time points, we had no *a priori* basis for predicting the sampling times at which changes in gene expression would be greatest. Thus, we had to strike a compromise between obtaining data at a large number of time points with the need for large sample sizes at each time point. We reasoned that, even though our design precludes carrying out statistical analysis at all time points, if time-dependent trends were present, they would appear clearly in the data, as we in fact observed and discuss below. In this exploratory study, then, we discuss patterns in gene expression that reflect distinct temporal responses, in both cycling and steady state acclimation conditions, but we refrain from referring to these patterns as statistically significant. Nonetheless, the temporal changes in gene expression that we discuss (for instance, those for whose expression correlates with the daily cycling of temperature; see Fig. 4) manifest a regularity that cannot easily be explained as due simply to noise in the data. These cDNA clones represent genes involved in a wide variety of cellular and organismal functions (Fig. 5). In discussing these differentially expressed genes we first provide a general overview of thermal effects on gene expression and then examine the specific physiological systems that seem most responsive to temperature under either steady state or cycling conditions.

Changes in gene expression may arise for a variety of reasons, some of which may not be specifically associated with changes in the activity of gene products (e.g. proteins). For

Fig. 3. Genes with a cyclic expression pattern in response to temperature fluctuations. Gene expression profiles are organized by phase shift relative to the temperature cycle in the fluctuating temperature acclimation. Each phase shift cluster was organized with a complete linkage algorithm (Peterson, non-centered; Eisen et al., 1998). Each row represents a single cDNA clone and each column a time point in the acclimation time course. About 40% of the 540 cDNA clones that were differentially expressed have a cyclic expression pattern in response to temperature cycling as determined by cross correlation analysis. For the control daily cycles (26°C) and constant acclimation to 20°C and 37°C, expression patterns are corrected relative to t=0. For the fluctuating temperature treatment, the data are presented relative to t=0 as well as with circadian rhythms subtracted (labeled 'The effect of temperature'). Cycling temperature profiles presented with daily rhythms subtracted will have an expression pattern near a 1:1 ratio with controls if there is a negligible effect of temperature on gene expression (see Materials and methods for an expanded discussion). Letters on the right margin of the figure correspond to the line graphs presented in Fig. 4. For non-cyclical gene expression patterns see supplemental Fig. 1.

instance, transcripts that are highly labile or have a very short half-life may require constant transcription to maintain physiological transcript levels, and this may be exacerbated by changes in the physical environment that destabilize or stabilize the mRNA transcripts. It is also possible to have large changes in gene transcription without changing the level of expressed protein if changes in protein or mRNA stability are associated with the experimental treatment, or if the RNA is the active gene product (antisense RNA is an example). For example, changes in gene expression may simply represent the attempt to maintain the current level of protein in the cell in the face of changes in protein translation or degradation. In this situation, if the coupling of protein degradation and transcription is tight, there may be a large change in transcript abundance without any change in protein abundance. Although in this situation the amount of transcript does not parallel the changes in protein level, it still indicates an important cellular process that must be closely regulated to maintain cellular function. This type of gene regulation may be just as important, possibly even more important, than regulation that results in changes in the protein levels. We, therefore, argue that most changes in transcript abundance, whether they reflect effects of mRNA and protein stability or adaptive alterations in protein concentrations, are likely to be important in the context of temperature acclimation, especially during the initial phases of the process.

Transcript levels do not change in response to temperature in a global manner that would indicate large changes in rates of synthesis or degradation of mRNAs across all genes (Fig. 3). In fact, the majority of the cDNAs (>90%) changed less than twofold, if at all, in response to temperature, which indicates a very tight regulation of steady state levels of mRNA transcripts during large-scale temperature changes. Yet rates of transcription elongation in a hibernating mammal, as measured in nuclear run-on assays, have recently been shown to have a typical temperature sensitivity ($Q_{10} \approx 2$–3) (van Breukelen and
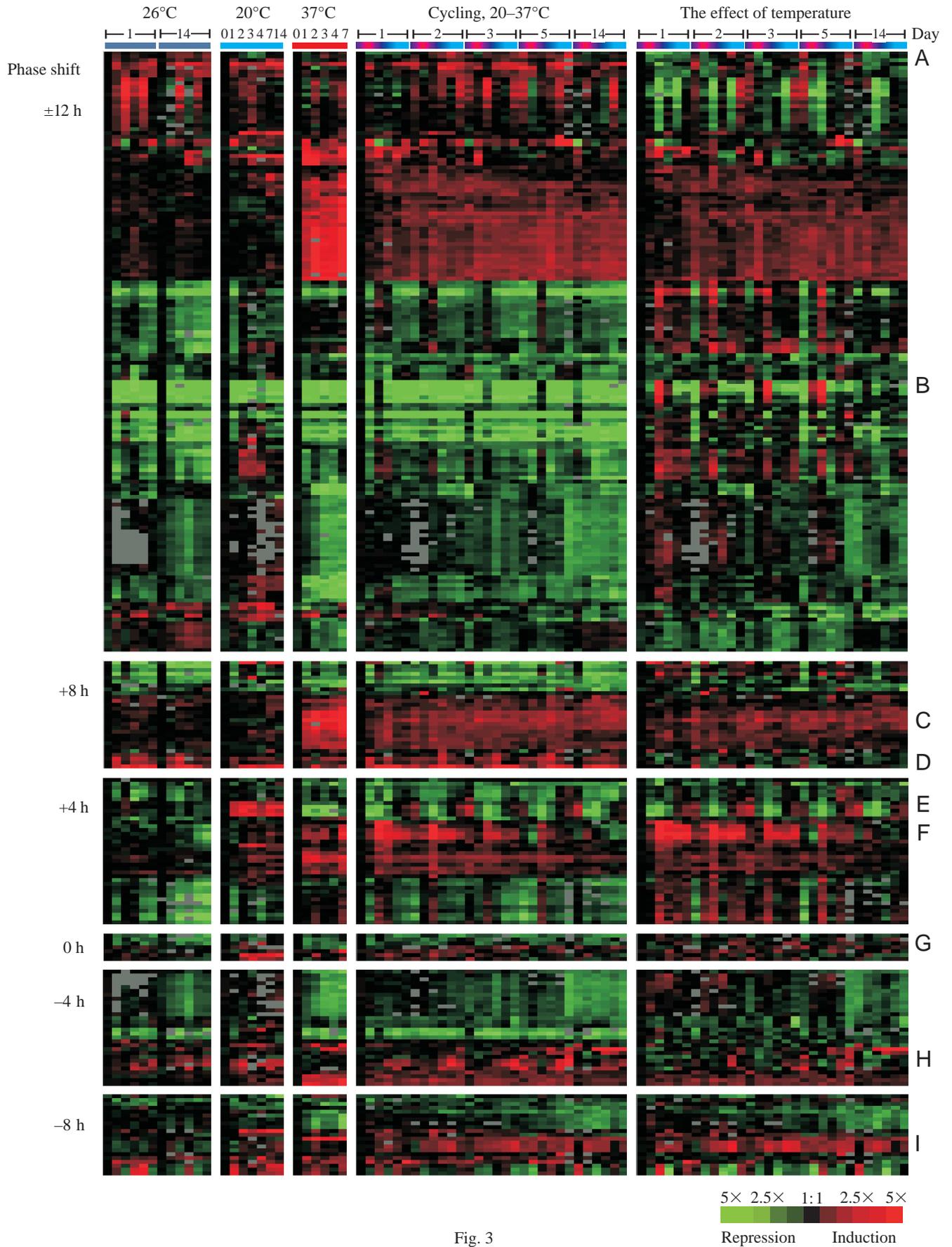
Fig. 3

Control

Cycling relative to *t*=0

The effect of temperature

Putative ER membrane protein

HMG-CoA synthase

Heat shock protein 70 kDa

Aquaporin-1

High mobility group protein b1

Unidentified clone LU05K02

Unidentified clone LU05B07

Unidentified clone LU07J19

Dihydropyrimidine dehydrogenase

Level of expression (log Cy5/Cy3)
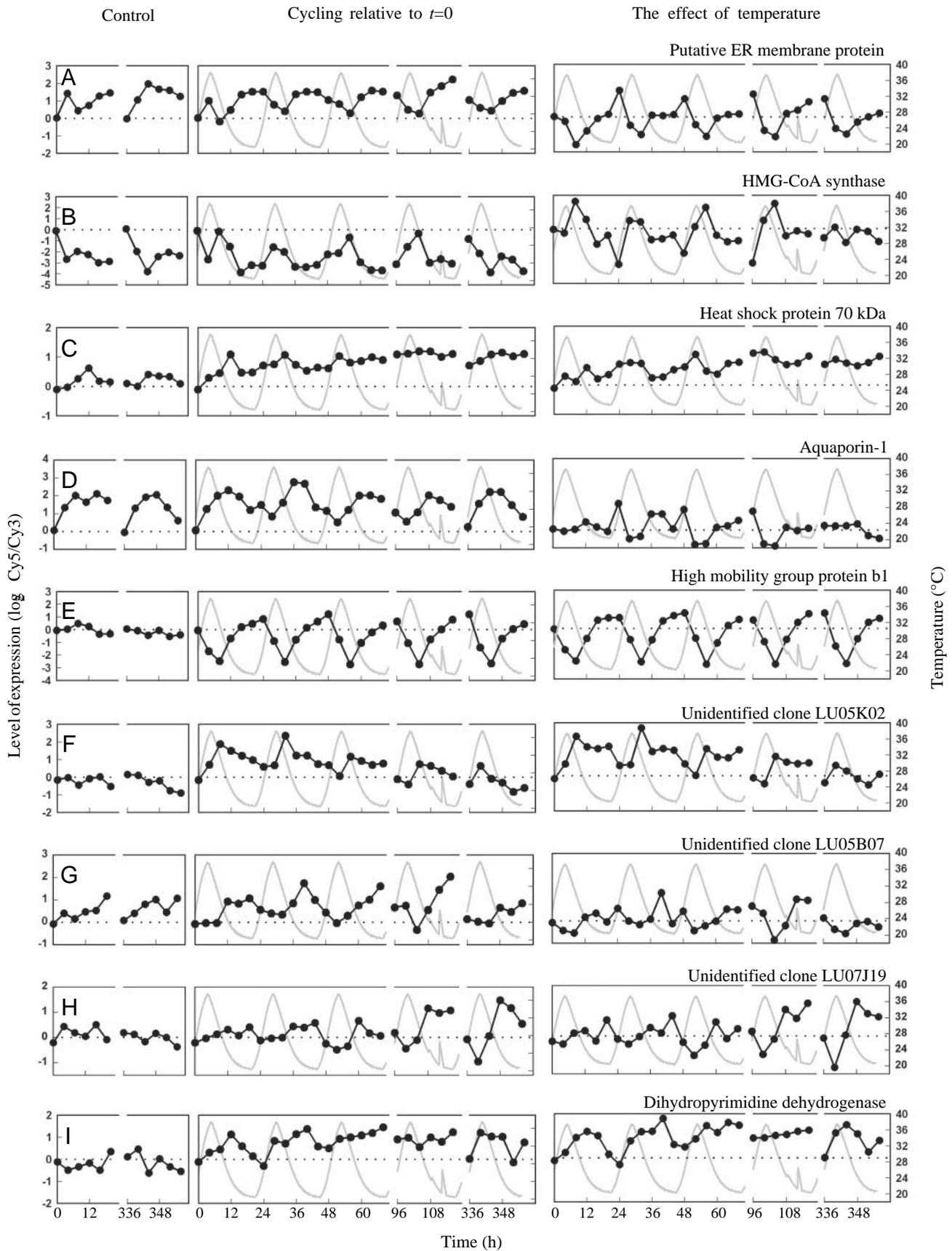
Temperature (°C)

Time (h)

Fig. 4

Martin, 2002). This suggests that temperature compensation in rates of transcription should occur at the level of either transcript initiation, degradation or both. We provide some evidence that global control of initiation through activities of high mobility group B1 proteins (HMGB1; see below, Fig. 3) may play an important role in the global regulation of transcription in response to temperature. However, it is important to note that changes in the abundance of mRNA transcripts in a cell can occur without alterations in rates of transcription. For instance, changes in mRNA turnover (e.g. stabilization or destabilization of a transcript) can cause changes in mRNA levels independent of rates of transcription. Thus, it is not safe to assume that changes in the relative abundance of mRNA transcripts are due to changes in rates of transcription. The nature by which specific mRNA levels are changed can only be addressed by more detailed investigation of each transcript species using conventional gene-specific approaches.

Diverse phase patterns in gene expression associated with the temperature cycle

There is a diversity of gene expression patterns associated with acclimation to cycling temperatures. To examine these patterns, the gene expression data expressed relative to *t*=0 were ordered according to their phase shift relative to the temperature cycle (Fig. 3). A clustering diagram of the genes with expression patterns found to have a statistically significant cross correlation with the temperature cycle is presented in Fig. 3. Representative expression patterns from each phase shift cluster are illustrated using line plots in Fig. 4. These patterns include changes in the phase pattern of gene expression as well as shifts in the amplitude and level of expression. The relative abundances of some transcripts are reduced when temperatures are high (ER membrane protein, HMGB1) while others such as hydroxymethyl glutaryl coenzymeA synthase (HMG-CoA synthase) increase. Some transcripts (myosin heavy chain) show a complex pattern with two negative peaks occurring as a result of temperature cycling. This pattern merges into a single negative peak associated with high temperatures after 2 weeks of continuous temperature cycling. Other transcripts show a clear cycling pattern with temperature and also show a shift in overall transcript abundance (complement protein C7, apolipoprotein E, the heat shock protein 70 kDa). Some transcripts show very little response during the first few temperature cycles and then develop a cycling pattern over time (unidentified clone LU07B24). Many transcripts that change in relative abundance in response to a cycling temperature regime return to the control pattern (e.g. unidentified clone LU05K02, HMG-CoA synthase) after 2 weeks of temperature cycling, while others develop new patterns (myosin heavy chain, ER membrane

Fig. 4. Diverse patterns of cyclic gene expression. The dotted line in each graph represents a 1:1 ratio relative to the appropriate control. Letters on the left margin of the figure correspond to the same letters in Fig. 3. The temperature cycle is represented by the light gray line.

protein). Surprisingly few genes are simply turned off or on in response to cycling temperatures and it appears that the expression of many genes may be altered on a hourly basis under temperature cycling conditions.

Temperature and daily rhythms

A number of transcripts found to have temperature-dependent patterns of expression also have strong daily rhythms under constant temperatures (Figs 3, 4). In almost all cases, these patterns are modulated by temperature in a consistent manner. For instance, the ER membrane protein and myosin heavy chain transcripts show opposite patterns of expression in response to temperature compared to the normal daily rhythm. These data indicate that natural daily rhythms of expression are likely to be strongly modulated by the temperature cycle in *A. limnaeus*. Some of the rhythms observed under control conditions may be a consequence of the feeding regime used during the study (fish were fed once daily at 09:00 h). This hypothesis is supported by the number of transcripts associated with energy metabolism (e.g. lipid and glucose metabolism) that exhibit strong daily rhythmic patterns under control and temperature cycling conditions (Fig. 5). It has recently been reported that circadian rhythms observed in mammalian liver can be entrained to the feeding cycle, independent of the light cycle (Stokkan et al., 2001). Our data suggest that daily rhythms of gene expression under natural conditions are likely to be the result of multiple environmental and nutritional inputs in the liver and that variation in environmental temperature is likely to have a strong influence on this integrated response.

Daily versus *long-term gene expression responses to temperature: general principles and background data*

The majority of laboratory temperature acclimation experiments force constant conditions upon organisms that typically occur in thermally variable environments in which daily changes in temperature occur. The need to study such short-term thermal fluctuations is apparent based on the findings of studies that have shown that acclimations to fluctuating and constant thermal environments result in different physiological phenotypes (Lowe and Heath, 1969; Feldmeth et al., 1974; Otto, 1974; Shrode and Gerking, 1977; Woiwode and Adelman, 1992; Heath et al., 1993). Most of these studies focused on thermal tolerance or thermal preference. In almost all cases, acclimation to fluctuating environments resulted in a higher thermal tolerance or an increased range of thermal tolerance compared to acclimation to constant conditions that approximated the mean temperature of the fluctuating acclimation. These data indicate that the physiological state of a fish exposed to fluctuating temperatures is indeed unique when compared to fish acclimated to constant environments. Gene expression data presented in this paper support the general conclusion that constant and fluctuating environments elicit different transcriptional and likely physiological responses.

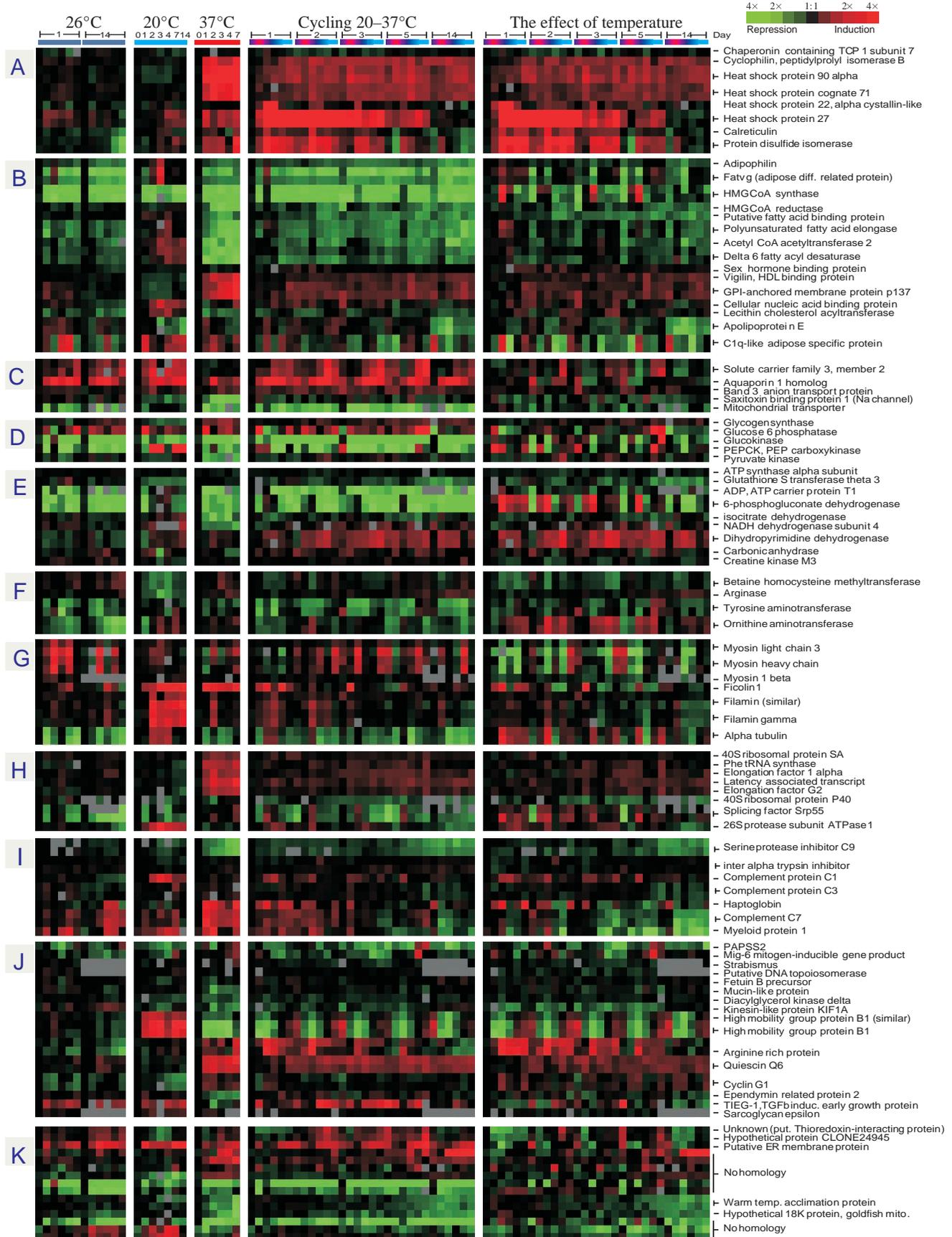The different responses in gene expression during

Fig. 5

Fig. 5. Gene expression patterns grouped according to cellular function. Data are presented as explained in Fig. 3. (A) Heat-shock proteins and molecular chaperones; (B) cholesterol and lipid metabolism; (C) solute carriers; (D) glycolysis/gluconeogenesis, blood glucose homeostasis; (E) intermediary metabolism; (F) nitrogen metabolism; (G) cytoskeletal elements; (H) protein turnover; (I) acute phase response and complement proteins; (J) cell growth and proliferation; (K) clones with unknown function or no homology to known sequences. Probable gene homologies were determined using Blast searches of the GenBank sequence database. The most significant or relevant results of these homology searches are listed in supplemental Table 1, as are the accession numbers for the DNA sequence of the cDNA clones presented in this paper.

acclimation to constant and fluctuating temperatures discussed below may have broad implications for other species of aquatic organisms that live in both constant and fluctuating environments. For instance, many marine intertidal organisms spend part of their day in the relatively thermally stable conditions of the ocean, and other parts of the tidal cycle exposed to air and more variable temperatures. Much of what is known about the temperature biology of these species is from acclimations to constant conditions, and a very different perspective might be gained from looking at acclimation to daily fluctuations in temperature. Additionally, the differences in transcriptional responses observed in constant *versus* fluctuating temperatures may indicate differences in organismal responses to daily *versus* seasonal changes in temperature. While there are likely to be daily fluctuations in temperature during all seasons, the mean temperature levels will be likely to change, as will the amplitude of the fluctuation. Constant acclimation regimes may mimic seasonal changes in daily mean temperature and thus may be eliciting changes in gene expression associated with adjustments needed for long-term survival or for seasonal changes in reproduction and feeding. In contrast, large-scale fluctuation in temperature on a daily basis may require more immediate changes in gene expression that are required for short-term survival and thus the fish respond *via* more temporary mechanisms that are not associated with long-term adjustments in physiology.

Gene expression grouped by cellular function

In order to explore the biochemical and molecular pathways that are affected by temperature on either daily or long-term time scales we organized the data according to cellular functions (Fig. 5). Fig. 5 illustrates the diversity of cellular pathways that are affected by temperature at the transcriptional level. For each cellular function we discuss the changes in transcription that we feel are most instructive for evaluating the effects of temperature on the function in question. By placing focus on a subset of the transcriptional changes we do not intend to imply that other changes in gene expression are without significance for the process in question.

Molecular chaperones

Upon initial exposure to temperature cycling, a number

of molecular chaperones are strongly induced (Fig. 5A). However, the transcript levels of most of these chaperones return to control levels after 2 weeks of temperature cycling. Transcripts of the small heat-shock proteins Hsp27 and Hsp22 are strongly induced (four- to fivefold induction) by the initial temperature cycles whereas the larger heat-shock proteins Hsc70 and Hsp90 are only mildly induced (>twofold) after several temperature cycles. However, transcripts encoding for Hsc70 and Hsp90 are strongly induced by chronically elevated temperatures. These differences in the kinetics of induction among different classes of heat-shock proteins indicate a complex transcriptional response to temperature cycling that is distinct from constant exposure to elevated temperatures. Transcripts for the other major types of molecular chaperones, such as protein disulfide isomerase (PDI) or calreticulin, which are initially induced by temperature cycling, are also induced and maintained by exposure to 37°C. These data taken together suggest that for *A. limnaeus* chronic high temperatures may be more 'stressful', i.e. cause more protein damage, than exposure to constantly changing temperatures. This conclusion is consistent with the variable natural habitat in which the fish are found to thrive (Fig. 1A). The apparent importance of the small heat-shock proteins to the survival of *A. limnaeus* in its thermally variable and extreme desert habitat is in agreement with the data of Hightower et al. (Norris et al., 1997; Hightower et al., 1999) for the survival of desert fishes from Mexico. These authors suggest that Hsp27 may play a role in signal transduction to the cytoskeleton during temperature stress (Norris et al., 1997).

Cholesterol and fatty acid synthesis – membrane structure

The maintenance of membrane integrity (homeoviscous and homeophasic adaptation) is known to be a crucial part of the acclimatory response to temperature and to involve major alterations in the lipid compositions of membranes (Hazel, 1995; Hochachka and Somero, 2002). Thus, we predicted that a number of genes related to lipid biosynthesis would alter their expression in response to fluctuating or long-term acclimatory temperatures. Indeed, this prediction was fulfilled (Fig. 5B). Two transcripts, one for a Δ6-fatty acyl desaturase and the other for a polyunsaturated fatty acid elongase, were found to be induced by constant acclimation to 20°C and repressed by chronic exposure to 37°C. If these changes in transcript levels are reflected in protein levels, then these data are completely consistent with homeoviscous adaptation theory, which predicts an increase in long-chain polyunsaturated fatty acids at lower temperatures. However, these two transcripts are not strongly regulated during exposure to cycling temperatures. It appears that membrane restructuring during temperature cycling may be accomplished by alternate means.

Insertion of cholesterol into lipid bilayers has multiple effects on membrane structure, and, in general, increased levels of cholesterol are associated with increased temperatures (Robertson and Hazel, 1997). The relative abundance of a transcript for an enzyme in the cholesterol biosynthetic pathway, 3-hydroxy-3-methylglutaryl-CoA synthase (HMG-

CoA synthase) (cytoplasmic form), is strongly and positively correlated with the temperature cycle (Figs 3–5). This suggests a role for cholesterol in the maintenance of membrane integrity during temperature cycling. After 2 weeks of cycling, the expression levels of the HMG-CoA synthase transcript return to control levels. This return to control levels of transcripts after 2 weeks of temperature cycling suggests that either a new steady state level of cholesterol has been achieved that is sufficient to maintain membrane integrity in the face of temperature cycling, or that other mechanisms, such as changes in the content of polyunsaturated fatty acids, may be employed during extended periods of exposure to cycling temperatures.

The pattern of HMG-CoA synthase transcript appears to have a daily rhythm that may be associated with the daily feeding pattern (see discussion above). HMG-CoA synthase transcript levels are highest about 3.5 h after feeding (at 12:30 h) under control conditions (26°C, Fig. 4). However, during temperature cycling, levels of this transcript peak strongly 8 h after feeding (4 h after peak temperature) and are highly correlated with the first four temperature cycles. It is worth noting that regulation of cholesterol biosynthesis is typically attributed not to HMG-CoA synthase but to HMG-CoA reductase. In this study transcript levels of HMG-CoA reductase are slightly induced during chronic cold acclimation (20°C) and repressed during acclimation to chronic temperature elevation (37°C), suggesting the lack of an appropriate transcriptional response to temperature that would be consistent with the role of cholesterol in current homeoviscous adaptation theory (Robertson and Hazel, 1997; Zehmer and Hazel, 2003). However, this enzyme is known to be regulated by allosteric and post-translational modifications and may not require a transcriptional response to effect a change in gene activity. The many biological roles of cholesterol in organismal physiology, and the complex control of sterol synthesis, suggest that the expression pattern of this gene is a result of the interplay between many converging pathways.

The expression pattern of the transcript encoding adipose differentiation-related protein (ADRP, Fig. 5B) provides additional evidence that cholesterol is probably important for the maintenance of membrane integrity during temperature cycling. When transcript levels of HMG-CoA synthase are high, ADRP transcript levels are low. Increased mRNA levels of ADRP are associated with increased storage of cholesterol and polyunsaturated fatty acids (PUFA) in lipid droplets of mammalian cells (Atshaves et al., 2001; Brown, 2001). ADRP binds tightly to cholesterol and is thought to be a critical regulator of cholesterol and PUFA storage and release from lipid droplets (Brown, 2001). Thus, decreased levels of ADRP mRNA transcripts would likely lead to the mobilization of cholesterol and PUFA stores for transport to the plasma membrane.

Recent studies suggest that the plasma membrane is organized into discrete lipid and lipid/protein domains. (Ikezawa, 2002; Morandat et al., 2002). The glycosylphosphatidylinositol (GPI)-anchored proteins are associated with specific plasma membrane subdomains called membrane 'rafts', which are rich in cholesterol and sphingolipids (Ikezawa, 2002; Morandat et al., 2002). These rafts are thought to be critical sites for many membrane-associated functions including cellular signaling. We observed a strong induction of transcripts for the GPI-anchored membrane protein p137 during exposure to chronically elevated temperatures, and a mild induction after several temperature cycles. These data suggest that increases in membrane raft proteins may play a role in the stabilization of the plasma membrane rafts and the maintenance of membrane functions during exposures to elevated temperatures. Recent studies suggest that homeoviscous adaptation of plasma membranes may occur primarily in these rafts and that cholesterol plays an especially important role in adjusting the physical properties of the rafts (Zehmer and Hazel, 2003). Thus our data support a role for both raft proteins and lipids in the adaptation of membranes to temperature.

Solute carriers

A number of transcripts that encode solute transporters and transmembrane proteins have strong daily expression rhythms that are not strongly modulated by temperature (Fig. 5C). These genes include aquaporin 1, and solute carriers found in the plasma membrane as well as in mitochondrial membranes. These genes seem to be temperature independent in their transcriptional regulation across a wide range of temperatures. The reason for this strong daily rhythm and temperature independence is not known, but may be due to action of the proteins encoded by these transcripts in the digestion and assimilation of food. For instance, aquaporins have recently been identified as being important for proper secretion of bile in hepatocytes (Huebert et al., 2002). The protein encoded by the gene for solute carrier family 3, member 2 (SLC3A2) is thought to be an amino acid transporter with high affinity ($\mu mol\ l^{-1}$ range) for dibasic and zwitterionic amino acids (Palacin et al., 1998). Anion transport function similar to that of the band 3 anion transporter from mammalian erythrocytes has been found in kidney tubules and may be important in solute transport across kidney tubules. Perhaps a similar function is required for transport and secretion of substances by hepatocytes during digestion.

Carbohydrate metabolism

Glucose is an important fuel source for many cell types in vertebrates and is supplied largely through the circulatory system. The liver plays an essential role in blood glucose homeostasis by balancing uptake and release of glucose (Nordlie et al., 1999). Changes in transcript levels for a number of genes critical for regulation of carbohydrate metabolism and blood glucose concentrations occur in response to temperature. Notably, glucokinase has a highly variable expression pattern that is nearly identical to that for another enzyme critical to gluconeogenesis, phosphoenolpyruvate carboxykinase (Fig. 5D). These genes are almost always expressed in parallel during temperature cycling and, importantly, are almost always

expressed in an opposite manner to glucose-6-phosphatase, the major regulator of glucose transport from cells into the bloodstream. Glucokinase has also been shown to play a role in the regulation of glucose metabolism and is often found in the nucleus of cells, where it is thought to act as a glucose sensor. The regulation of glucokinase and glucose-6-phosphatase is very complex and includes many effectors (Nordlie et al., 1999). Gene expression data from the present study indicate that blood glucose levels are likely to be highly responsive to nutritional status and strongly affected by temperature.

Two additional transcripts that encode for enzymes that are important regulators of glucose and glycogen metabolism, glycogen synthase and pyruvate kinase, show different responses to acclimation to chronic high temperatures, but do not exhibit strong changes in transcript levels during temperature cycling. The increase in glycogen synthase transcripts and decrease in pyruvate kinase transcripts during acclimation to 37°C would indicate that glycogen synthesis should be favored during acclimation to chronic high temperatures. The absence of a strong transcriptional response in these transcripts during temperature cycling is not surprising considering the many post-translational mechanisms for regulating these proteins on short time scales.

Intermediary metabolism

The transcript levels of carbonic anhydrase and creatine kinase genes appear to be affected by temperature in a similar manner (Fig. 5E). These proteins have both been implicated in the regulation of cellular energetics through their contributions to phosphotransfer networks that can act to couple spatially separated ATP-consuming and ATP-producing metabolic pathways (Dzeja and Terzic, 2003). The transcripts for these genes are both mildly upregulated after the coldest part of the temperature cycle (near $t=0$, ~26°C), when compared to the normal circadian expression pattern. This may indicate a need to increase the capacity of the phosphotransfer networks during cold periods in the face of continually changing environmental temperatures in order to maintain tight coupling of catabolic and anabolic processes.

The pentose phosphate shunt enzyme 6-phosphogluconate dehydrogenase appears to be strongly affected by temperature cycling during the first three temperature cycles. This transcript is upregulated when compared to the normal daily expression pattern at 26°C, but is downregulated during acclimation to 37°C (Fig. 5E). Previous studies indicate that the activity of this enzyme is responsive to temperature acclimation, being upregulated during exposure to reduced temperatures (Seddon and Prosser, 1997). Increased activity of the pentose phosphate shunt would be expected to support increased biosynthesis of fatty acids by providing reducing equivalents for biosynthetic reactions. Others have suggested a link between pentose phosphate shunt activity and antioxidant protection *via* glutathione (Winkler et al., 1986). However, in our study, expression of at least one enzyme involved in glutathione-based detoxification of oxygen radicals, glutathione-s-

transferase, is not similar to that for 6-phosphogluconate dehydrogenase, which suggests that expression of the latter is more likely to be involved in control of biosynthesis and overall redox balance in the cytoplasm and not responsive to oxidative damage *per se*. Additional support for this conclusion comes from expression patterns of the cytosolic isoform of isocitrate dehydrogenase. This enzyme has been found to play an important role in defense against oxidative stress in cultured NIH3T3 cells (Lee et al., 2002). Transcript levels for this enzyme are downregulated during temperature cycling and during acclimation to 37°C, and upregulated during acclimation to 20°C. This transcriptional response is much more consistent with temperature compensation of metabolic function than a need to cope with oxidative damage.

Dihydropyrimidine dehydrogenase catalyzes the rate-limiting step in pyrimidine catabolism, converting uracil to 5,6-dihydrouracil (KEGG website, http://www.genome.ad.jp/kegg/). This conversion eventually yields β-alanine or a number of other pyrimidine-derived metabolites. The transcript level of dihydropyrimidine dehydrogenase is upregulated strongly after repeated temperature cycling (Fig. 5E). The upregulation of this transcript could indicate an increased need for the metabolism of pyrimidines due to increased turnover of RNA and DNA, perhaps even due to cell damage after repeated temperature cycling. An alternative hypothesis is that temperature cycling induces the production of β-alanine as an organic osmolyte. β-alanine is a known organic osmolyte in prokaryotes and various animal lineages, including marine elasmobranchs (Hochachka and Somero, 2002). It is possible that accumulation of organic osmolytes (see discussion below) could help to mediate temperature stress by stabilizing protein structure in the face of fluctuating temperatures.

Nitrogen metabolism

A number of transcripts that encode for proteins in nitrogen metabolism are differentially regulated during acclimation to constant and fluctuating temperatures (Fig. 5F). The transcript for betaine homocysteine methyltransferase is downregulated under constant cold temperatures and upregulated under constant warm temperatures (Fig. 5F). Levels of this transcript are highly variable under temperature cycling conditions. These data may indicate that methylamine metabolism is important for temperature acclimation, and suggest that levels of methylamines may be elevated at high temperatures and reduced at low temperatures, which would be consistent with data emerging that indicate that methylamines, especially glycine betaine, can act as 'chemical chaperones' and have a stabilizing effect on proteins during exposures to combined high salt and temperature stress in *E. coli* (Diamant et al., 2001). These authors suggest that organic osmolytes, especially glycine betaine and, to some extent, proline, may regulate the activity of macromolecular chaperones such as the major heat-shock proteins. Interestingly, levels of transcripts for the proteins arginase and ornithine aminotransferase are also elevated during acclimation to 37°C and temperature

cycling. These enzymes are both involved in proline biosynthesis (KEGG website, http://www.genome.ad.jp/kegg/). We predict, based on these data, that proline and glycine betaine levels are likely to increase during acclimation to chronic high temperatures and cycling temperatures. Elevated levels of organic osmolytes may help to offset the need for molecular chaperones on a long-term basis and may also explain why transcript levels of heat-shock proteins return to control values after several temperature cycles. The use of organic osmolytes to enhance protein stability would seem to be an economical way to deal with variable environmental conditions without continually mounting a heat-shock response. We note that protein-stabilizing methylamine solutes have been shown to accumulate to high concentrations in deep-sea fishes and invertebrates and to be effective in counteracting the destabilizing effects of high pressure on protein structure and function (Yancey et al., 2002).

Cytoskeletal elements and contractile proteins

A number of genes that encode cytoskeletal proteins and proteins involved in contractile functions are variably expressed in relation to temperature (Fig. 5G). The mRNA transcript for α-tubulin appears to be especially variable during temperature cycling. Transcripts for myosin heavy chain and light chain are also strongly affected by temperature and share almost identical expression patterns. The reasons for these expression patterns are not yet clear, but may be related to a need to stabilize the cytoskeleton in response to changing temperatures. This hypothesis is supported by the increased levels of expression for ficolin 1 and other actin binding proteins as well as microtubule associated proteins. These data should indicate that normalization of mRNA levels to cytoskeletal genes such as tubulin, a standard procedure, can likely result in misleading or false interpretation of mRNA levels in situations of fluctuating temperatures.

Protein turnover

Acclimation to constant low or high temperatures appears to induce alterations in transcription that may affect levels of protein synthesis and degradation (Fig. 5H). Two translation elongation factors, a tRNA synthase, and at least one ribosomal protein all have increased levels of expression, while there is a slight decrease in the amount of transcript for a regulatory subunit of the 26S proteosome (part of the ubiquitin-dependent proteolysis system) in response to chronic elevated temperatures. These data suggest an increase in protein turnover during exposure to chronic high temperatures, and an attempt by the organism to maintain protein levels by increasing the capacity for protein synthesis and decreasing the capacity for protein degradation. During acclimation to cold conditions, there is a strong upregulation of the regulatory subunit of the 26S proteosome, but transcript levels for proteins involved in the protein synthetic machinery remain unchanged or are slightly downregulated after 2 weeks of acclimation. Temperature cycling appears to elicit a very weak induction of transcripts for proteins involved in protein

synthesis. In contrast, transcript levels of regulatory subunit for the 26S proteosome appear to be highly responsive to temperature cycling, and may indicate that regulation of protein degradation is critical during short-term fluctuations in temperature. These data on key components of the protein turnover machinery indicate that certain aspects of protein turnover are likely to be regulated at the transcriptional level during temperature acclimation, and the response to constant conditions is unique when compared to those for exposures to temperature cycling.

The acute phase response, complement and innate immunity

Several transcripts that encode for proteins involved in the acute phase response are differentially regulated during temperature acclimation (Fig. 5I). Many of the components of the acute phase response are initially upregulated during temperature cycling and then downregulated after 5 days of cycling. These transcripts are also strongly upregulated by exposures to chronic high temperatures and more weakly to acclimation to 20°C. While these results may appear inconsistent initially, upon closer examination they are consistent with what is known about the acute phase response and, in particular, what is known about the complement proteins in fish.

The complement proteins are a major part of the innate immune system of all vertebrates (Sunyer and Lambris, 1998). Complement proteins are known effectors of the inflammation response to tissue damage and infection, and activation of the complement pathway results in the marking of target cells (opsonization) with complement proteins, activation of leucocytes, and lysis of target cells *via* the formation of a membrane attack complex (MAC) comprising complement proteins that self-insert into the plasma membrane (Roitt et al., 1993). The activity of complement proteins and the marking of target cells are largely regulated by the activation of the C3 complement protein through two alternative pathways. One of these pathways, the alternate pathway, is regulated by the hydrolysis of a thiol-ester bond within the C3 protein itself, by the C3 convertase enzyme. This bond is known to react with water spontaneously at a low level, so that this pathway is always slightly activated (Roitt et al., 1993). The activation of C3 and thus the complement pathway are amplified by a positive feedback loop that is regulated largely by the presence or absence of a non-self surface for opsonization. Complement proteins can bind to both self and non-self surfaces but are retarded from binding to self surfaces by specific proteins. The activation of the complement pathway has been shown to contribute to tissue damage after ischemic injury to cardiac tissues (Roitt et al., 1993), which indicates that the complement pathway must be carefully regulated to function in the immune response without causing major damage to self tissues.

The initial upregulation of transcripts encoding several complement proteins upon exposure to high or cycling temperatures may be the result of an increased activation of the complement pathway *via* spontaneous activation of the C3 protein due to increased thermal energy, or *via* signals

originating from tissue damage. In either case, the response would be counterproductive in the long-term, and is downregulated after several temperature cycles in the fluctuating acclimation regime. However, high levels of transcript are maintained during exposure to chronic high temperature, which may be a sign of continued tissue damage, or a maladaptive immune response similar to that observed following ischemia (Roitt et al., 1993). The slower upregulation kinetics of complement protein transcripts during cold acclimation is probably a temperature compensatory mechanism to boost innate immune function, which is consistent with earlier findings that the classical immune response (antibody mediated) is attenuated in fish at low temperatures (Bayne and Gerwick, 2001).

There is also evidence suggesting that gene expression patterns associated with liver regeneration and the acute phase response are similar (Milland et al., 1990). It is possible that the initial exposure to temperature cycling and chronic exposure to high temperatures induced cellular damage and cell death in a subpopulation of liver cells and the activation of complement proteins is in response to cellular damage. Other evidence supporting the possibility of conditions causing cell damage is the upregulation of haptoglobin gene expression, which is known to be important in binding and scavenging hemoglobin from ruptured red blood cells (Dobryszycka, 1997).

Cell growth and proliferation

A number of transcripts that encode proteins that regulate cell growth and proliferation have changing expression patterns during acclimation to constant and cycling temperatures (Fig. 5J). For instance, two tumor suppressor genes, arginine-rich protein (ARP; Shridhar et al., 1997) and quiescin Q6 (Coppock et al., 1998), are induced by high temperatures, with ARP strongly induced during the initial temperature cycles and quiescin Q6 slowly induced after several cycles. Quiescin Q6 is strongly induced during exposure to chronic high temperatures, while ARP is only weakly induced. Quiescin Q6 has been shown to be strongly induced during entry into reversible cellular quiescence in mammalian cells and is expressed at very low levels in actively proliferating cells (Coppock et al., 1998). These data suggest that cell proliferation is probably arrested during temperature cycling and during exposure to chronic high temperatures, but perhaps through different pathways. The transcript for a putative oncogene, Mig-6 (Makkinje et al., 2000; Tsunoda et al., 2002), known to be critical to stress-activated protein kinase signaling (SAPK/JNK; Makkinje et al., 2000) also appears to be regulated by temperature. Mig-6 is induced during the first temperature cycle, but has a highly variable expression pattern in general. There is evidence that Mig-6 may be involved in the sustained activation of SAPK/JNK-induced changes in gene expression (Makkinje et al., 2000). This sustained activation leads to cellular hypertrophy due to increased cell growth but not cell division in some mammalian chronic diseases. The variable expression pattern of the Mig-

6 transcript during temperature cycling may be a way to carefully titer the activity of the SAPK/JNK signaling pathways and thus avoid the problems associated with chronic activation of this pathway. The relative abundance of TIEG-1 mRNA, which encodes a TGF-β early response gene (Cook and Urrutia, 2000; Hefferan et al., 2000) decreases during the initial temperature cycles. TIEG-1 mRNA has been shown to be rapidly induced by TGF-β signaling and is generally associated with a decrease in cell growth and proliferation in pancreas cells (Cook and Urrutia, 2000). The data presented here indicate that TGF-β signaling is probably not initially activated by temperature cycling, but may be activated by chronic exposure to cold temperatures (20°C) and during the cold parts of the daily temperature cycle (Fig. 5J). An integrated view of the above data suggests that temperature cycling activates the SAPK/JNK pathway and represses TIEG-1 expression, leading to increased cellular growth, while induction of ARP and quiescin Q6 indicates a cessation of cellular proliferation. These data suggest that different parts of the cell growth and proliferation cycle may be entrained to temperature. The partitioning of different parts of the cell cycle into different temperature conditions may have profound influences on the energetics and physiology of organisms exposed to cycling temperatures in nature, and may dictate when complex behaviors such as gamete synthesis or reproduction are favorable.

Unknown transcripts

A number of cDNAs that have no homology to known sequences or have unknown function were identified as responsive to temperature acclimation in this study (Fig. 5K). These cDNAs may represent critical parts of the acclimation response that have not yet been identified or studied. Further interrogation of these sequences and their expression patterns in response to temperature and other environmental factors is likely to result in the discovery of new genes and gene functions that are critical for survival of environmental variation.

Global regulation of transcription

The high mobility group protein HMGB1 transcript exhibits one of the most striking patterns of gene expression associated with cycling temperatures (Figs 3, 4, 5J). The relative abundance of this transcript is highly negatively correlated with temperature (Fig. 4). The HMGB1 transcript changes over tenfold during the daily temperature cycle and both the pattern and magnitude of the expression are consistent over the entire 2-week period of temperature cycling. Further, this transcript does not show any changes in expression on a daily basis under constant temperature conditions. The unique properties of the HMGB1 protein, coupled with the expression pattern presented in this paper, lead us to propose that the HMGB1 protein may be a critical part of a compensatory transcriptional response to temperature and may indeed be a highly sensitive temperature sensor.

The HMGB1 protein is in many ways the perfect candidate

as an immediate effector of transcription in response to temperature. HMGB1 is a small (28 kDa) and abundant protein that is highly conserved and ubiquitous among the vertebrates (Wolffe, 1999; Thomas and Travers, 2001). HMGB1 can bind DNA in a structure-specific manner with a preference for single stranded, bent or supercoiled structures (Hamada and Bustin, 1985; Stros, 2001; Thomas and Travers, 2001). It has been shown to partner with many important transcription factors such as p53, HoxD9 and steroid hormone receptors through specific interaction domains (Wolffe, 1999; Thomas and Travers, 2001). The protein is also very 'sticky' in nature and is able to bind to a variety of other proteins, including cytoskeletal elements and extracellular matrix proteins, and to various classes of lipids (Einck and Bustin, 1985). It is generally agreed that HMGB1 has an integral role in assembly of numerous nucleoprotein complexes that are critical to cell function such as V(D)J recombination and the formation of enhanceosome complexes (Stros and Reich, 1998; Wolffe, 1999; Ellwood et al., 2000; Thomas and Travers, 2001). HMGB1 is known to increase the affinity of the TATA binding protein TBPII for the TATA box by over 20-fold (Das and Scovell, 2001). Overexpression of HMGB1 in mammalian cell lines results in a global stimulation of transcription (gene- and polymerase-independent) that is associated with a generalized decondensation of chromatin structure (Aizawa et al., 1994; Ogawa et al., 1995). Additionally, injection of antibodies against HMGB1 inhibits transcription in *Xenopus* oocytes (Ogawa et al., 1995). These data taken together indicate a key role for HMGB1 in the global regulation of transcription.

The thermal stability and biochemical properties of HMGB1 protein suggest that the protein should be very sensitive to temperature *in vivo*. HMGB1 has a very broad thermal melting curve under dilute acidic conditions (Ramstein et al., 1999). The protein begins to melt at 20°C and is not completely denatured until 65°C, giving the protein a melting range of over 40°C. This broad melting range is likely due to the differing thermal stabilities of different domains within the protein (Ramstein et al., 1999). This point is critical because it indicates that one part of the protein may be stable and functional while the other part is denatured at physiologically relevant temperatures near 37°C. Additionally, these proteins may be post-translationally modified (Einck and Bustin, 1985) by the addition of several moieties, and the thermal stability of the protein can be modulated by as many as 5°C by these modifications (Stemmer et al., 2002). This property would allow for seasonal adjustments in the thermal stability of the HMGB1 protein. We hypothesize that the melting of this protein at physiologically relevant temperatures disrupts the ability of the HMGB1 protein to maintain the nucleoprotein complexes associated with transcription initiation and causes a global change in the rate of transcription, especially for those genes that contain a TATA box in their promoter. This mechanism is hypothesized to be important for modulating the level of transcription in a very general manner, while still allowing for specific changes in gene expression to be induced or repressed by regulatory transcription factors or enhancers and silencers. The patterns of transcript abundance presented in this report are consistent with this theory if the *HMGb1* gene is autoregulated by its own activity. This is likely to be the case since the hypothesized mechanism is global and not gene specific. Additionally, in order for this model to work, *HMGb1* transcripts need to have a very short half-life in the cell. Our data are consistent with a high turnover rate for this transcript *in vivo*, simply because a stable transcript would probably not show such large fluctuations in relative abundance on an hourly basis. Interestingly, the 3′UTR of the HMGB1 transcript is highly conserved among all vertebrates (Bustin et al., 1990), which strongly suggests a regulatory role because 3′UTR regions are classically highly variable.

We have identified the HMGB1 protein as a putative global regulator of transcription in response to temperature. If this hypothesis is supported by additional studies of protein function, this model could help resolve many unexplained phenomena associated with temperature acclimation. For example, seasonal shifts in thermal tolerance may be associated with changes in post-translational modification of the HMGB1 protein. The replacement of the linker histone H1 with HMGB1 during early development (Nightingale et al., 1996; Ner et al., 2001) may also explain the extreme temperature sensitivity of early embryos due to a loss of chromatin architecture, as HMGB1 proteins are easily denatured at biologically relevant temperatures. We believe that further investigations into the role of HMGB1 in temperature acclimation are likely to lead to a new understanding of how eukaryotic cells maintain homeostasis in the face of an ever-changing thermal environment.

In conclusion, these studies illustrate the utility of cDNA microarray approaches in both hypothesis-driven and 'discovery-based' experimentation in environmental physiology (Gracey and Cossins, 2003). In the former context, microarray studies can unravel the alterations in gene expression that are conjectured to underlie known physiological responses to temperature, e.g. in expression of heat-shock proteins and in the restructuring of cellular membranes. Of equal, if not greater, importance in microarray studies is the discovery of new facets of physiological responses that are not anticipated by the investigator, for instance potential global regulators of environmentally induced gene expression and interplay between normal circadian patterns of gene expression and alterations in gene expression made in response to fluctuations in temperature. Microarray technologies thus allow a type of exploration in 'molecular natural history' (Brown and Botstein, 1999) that seems certain to open up critical new areas for study in ecological and evolutionary physiology (Feder and Mitchell-Olds, 2003).

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