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Physiology

The threshold induction temperature of the 90-kDa heat shock protein is subject to acclimatization in eurythermal goby fishes (genus *Gillichthys*)

(acclimation/heat shock response/temperature adaptation/thermal stress)

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ABSTRACT **Two extremely eurythermal goby fishes, *Gillichthys mirabilis* and *Gillichthys seta*, which encounter habitat temperature variations of ≈30°C, showed seasonal acclimatization of endogenous levels and of onset temperatures for enhanced synthesis of a 90-kDa-class heat shock protein (HSP90). Summer-acclimatized fishes had higher levels of HSP90 in brain tissue than winter-acclimatized specimens, as shown by Western blot analysis. For winter-acclimatized fishes, increased synthesis of HSP90 was observed when the temperature was raised from a control temperature (18°C) to 28°C. For summer-acclimatized fish, no significantly increased synthesis of HSP90 occurred until the experimental temperature was raised to 32°C. These data suggest that the threshold temperature at which enhanced expression of HSP-encoding genes occurs is not hard-wired genetically but may be subject to acclimatization. A causal relationship between seasonal changes in steady-state levels of HSP90 and the threshold temperature for enhanced HSP90 synthesis is discussed in terms of existing models for the regulation of HSP gene expression.**

Temperature regulation of gene expression is certain to be critical for the survival and success of ectothermic ("cold-blooded") animals living in variable thermal environments. Presently, however, little is known about the interactions between temperature and gene regulation in eukaryotic organisms. The only well-understood example of temperature control of gene expression is that of the heat shock proteins (HSPs), a group of proteins whose synthesis can be induced by increases in temperature and by exposure to a variety of other stresses (e.g., heavy metals and alcohols). In addition to being stress-inducible, HSPs (or their cognates) exist as normal cellular constituents. The HSPs and their cognates have been implicated in various roles, from assisting in protein folding and oligomerization to steroid receptor activation and inactivation (1, 2). Both the HSPs and the heat shock response are highly conserved among phylogenetically divergent organisms (3), which attests to the critical roles these molecules play in cellular function and adaptation.

HSPs may play a role in physiological adaptation to environmental temperature change (e.g., during seasonal or diurnal shifts in ambient temperature). A clear relationship exists between different species' normal body temperatures and the temperatures at which HSP synthesis is induced; in the species so studied, HSP induction occurs at temperatures 5–10°C above normal body temperature (3), with some exceptions (4). Thermal induction may involve either the expression of a type of HSP not previously expressed or the enhanced expression of a class of HSP already present in the cell at some constitutive level. The correlation between normal body (cell) temperature and HSP induction temperature suggests that the HSP gene regulatory processes of different species have different set points (temperature thresholds) for heat-triggered induction of enhanced HSP production. However, it is not known whether these thresholds for HSP induction are hard-wired genetically or are subject to acclimatization (e.g., for eurythermal ectotherms that encounter wide ranges of temperature seasonally and/or diurnally). It also is not known whether ectotherms vary the concentrations of HSPs in their cells on a seasonal basis, reducing these levels in winter and increasing them in summer.

The present study addressed these two questions by examining HSP induction temperatures and HSP concentrations in two extremely eurythermal teleost fishes, the gobies *Gillichthys mirabilis* and *Gillichthys seta*. Both the estuarine *G. mirabilis* and the intertidal *G. seta* encounter temperature changes of at least 30°C on a seasonal basis. Focusing on the 90-kDa HSP (HSP90) in brain tissue, we determined HSP induction temperatures and endogenous HSP levels in field-collected fish from winter and summer and in winter-collected fish acclimated to 26°C in the laboratory. Our data reveal a significant plasticity in the heat shock response.

METHODS

Animals. *G. mirabilis* were collected with baited minnow traps in a shallow estuary near San Felipe, Baja California, Mexico. *G. seta* were caught by hand in tide pools near Ensenada Blanco at San Felipe. Most fish were placed immediately into insulated containers filled with aerated seawater at ambient temperature. The water was held at this temperature during transport to the Scripps Institution of Oceanography, where experiments were conducted. Several fish from each collection were frozen on dry ice, either in the field or upon return to Scripps, for later antibody measurements of HSP90 levels. Fish were held in the laboratory at a temperature (control temperature) approximating the collection temperature (18°C for fish collected in February; 28°C for fish collected in July and August). A group of *G. mirabilis* collected in February was acclimated to 26°C for 5 weeks, a period shown to be adequate for thermal acclimation of several physiological functions in ectotherms (5). Fish were fed ad libitum on chopped squid. Survival of fish was almost 100%. Specimen weights ranged from ≈3 g to ≈20 g.

Heat Shock Protocol. Animals were placed into 2-liter glass bottles containing ≈750 ml of seawater at the relevant control temperature, 18°C (winter fish) or 28°C (summer fish and 26°C-acclimated winter fish). The water was aerated throughout the experiment. A bottle was placed into a water bath

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Abbreviations: HSP, heat shock protein; HSP70 and HSP90, 70-kDa and 90-kDa HSPs; ANOVA, analysis of variance; HSF, heat shock factor.

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equilibrated to the control temperature, and the bath temperature was then raised over a 1-h period to increase the temperature of the water in the holding bottle to the desired exposure temperature. In one series of experiments, the animals were placed directly into water at the exposure temperatures. No differences in induction temperature were noted between the two protocols. Exposure temperatures were 24°C, 28°C, 32°C, and 36°C for the winter-collected fish and were 28°C, 30°C, 32°C, and 36°C for the summer-collected fish and 26°C-acclimated winter fish. Fish were left at the exposure temperature for an additional 2 h, then immediately removed, and placed into a 2-liter glass jar containing ≈750 ml of aerated water at the appropriate control temperature. After 1 h of holding at the control temperature, the fish were injected interperitoneally with a mixture of [^{35}S] methionine and cysteine (Tran^{35}S-label; ICN) at 50 μCi/g (body weight) (1 Ci = 37 GBq) diluted in teleost Ringer's solution. After a 3-h incorporation of the labeled amino acids, the fish were sacrificed by cervical transection of the spinal column, and tissues were removed immediately and frozen on dry ice.

Preparation of Proteins. Brain tissue was used in all analyses reported herein. The frozen brains were placed in microcentrifuge tubes containing 100 μl of 2% (wt/vol) SDS/32 mM Tris Cl, pH 7.4/1 mM phenylmethylsulfonyl fluoride. The tubes were placed in a boiling water bath for 2 min. The tissue samples were then homogenized and boiled for a second 2-min period. Insoluble material was then removed by centrifugation at 16,000 × *g* for 15 min at room temperature.

Protein concentrations of the supernatants were measured using the Bradford dye binding method (Bio-Rad Protein Assay). Quantification of the radiolabeled amino acid incorporated into protein was by liquid scintillation counting. The protein samples were stored frozen at −20°C.

Gel Electrophoresis. Proteins were loaded to equivalent amounts of radioactivity (10^5 cpm) on SDS/7.5% polyacrylamide gels and subjected to electrophoresis (6). The gels were impregnated with EN^{3}HANCE (DuPont/NEN) by manufacturer's instructions, dried, and subjected to fluorography at −80°C.

Quantification and Statistical Analysis of HSP90 Induction. Visual inspection of the fluorograms revealed that synthesis of HSP90 varied considerably as a function of acclimatization and heat shock temperatures. To quantify these differences, the fluorograms were positioned over the dried gels treated with EN^{3}HANCE and regions on the gels corresponding to HSP90 were excised with a scalpel. The excised gel pieces were subjected to liquid scintillation counting, the radioactivity in each gel piece was measured for two 20-min periods, and the average cpm was recorded as an index of HSP90 synthesis during the 3-h synthesis period. Because the specific radioactivity of the methionine and cysteine used varied from experiment to experiment and as a function of the age of the gels prior to analysis by scintillation counting, we normalized each data set to the radioactivity found in the HSP90-containing gel section from the control fish from each experiment. The radioactivity in the HSP90 band of the control fish was given a value of 1.0, and the radioactivity in bands from the other exposure temperatures was expressed relative to the control value.

To determine the heat-shock threshold induction temperature, defined as the lowest exposure temperature at which the radioactivity in the HSP90-containing band was significantly ($P < 0.05$) higher than the control value (i.e., significantly greater than 1), we used a one-way analysis of variance (ANOVA) for each species. A two-way ANOVA was used to ascertain the significance of seasonal and exposure temperature effects on HSP90 synthesis (SYSTAT software). Because the responses to temperature of the two species did not differ, we combined data from both species in our final analysis of heat-shock threshold induction temperature. Assumptions of the ANOVA (normality, independence of error terms, and equal variances among groups) were tested. Evaluations showed that all assumptions except equivalence of variance were met.

Analysis with Antibodies of Endogenous Levels of HSP90. Three fish from each of the collections made in February and August and three February-collected fish that had been

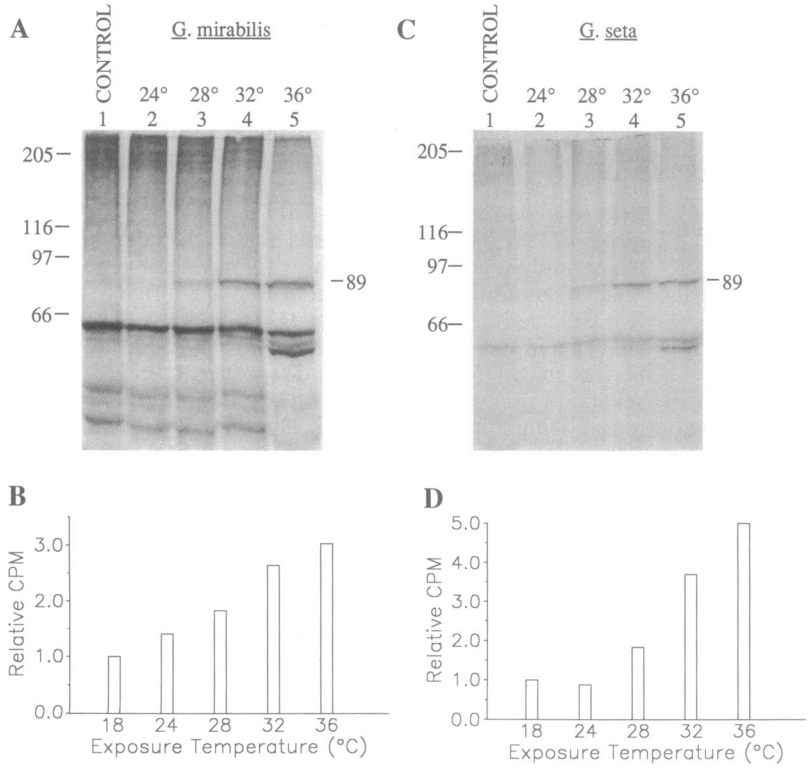

FIG. 1. HSP induction profile in brains of (February) collected *G. mirabilis* and *G. seta*. (*A*) Individual fish (*G. mirabilis*) were heat-shocked at the temperatures noted above each lane (in degrees Celsius). The control animal was maintained at 18°C. Molecular mass markers are noted to the left of the gel in kDa. The band corresponding to the 89-kDa heat-inducible protein is also indicated. (*B*) Relative incorporated radioactivity (cpm) of the 89-kDa band in *A*. Each bar is labeled to indicate heat shock temperature. (*C*) Same as *A* except that data are for *G. seta*. (*D*) Same as *B* except that data are for *G. seta*.

acclimated for 5 weeks to 26°C were frozen on dry ice and stored at −80°C. Brains were dissected from the fish while frozen, and proteins were prepared as described above. Proteins were resolved by gel electrophoresis as described, except that the gels were loaded for equivalent amounts of protein (75 μg per lane) rather than radioactivity. Western blot analysis was carried out as described by Towbin *et al.* (7), except 1% gelatin and 1% normal horse serum were used to block the nitrocellulose. The Western blot was incubated with a monoclonal antibody (AC19) against HSP90 from the water mold *Achlya ambi* (a gift from David Toft, Mayo Clinic, Rochester, MN). A biotinylated secondary antibody was used, and bands were visualized with diaminobenzoate and a Vectastain Elite kit (Vector Laboratories) by following the manufacturer's instructions.

RESULTS

To investigate whether acclimatization occurs in HSP induction temperature and HSP concentrations, we studied two extremely eurythermal gobies, *G. mirabilis* and *G. seta*, that encounter wide variations in temperature on daily and seasonal time scales. *G. mirabilis* is an estuarine species that occurs from Tomales Bay, CA to Baja California, Mexico (8, 9). In the estuary near San Felipe, Baja California, where specimens were collected, water temperatures in February varied from 9°C to 23°C during a single day. In July and August, daily water temperatures at this site ranged from 23°C to 36°C. *G. seta* occurs in the high rocky intertidal zone along the shores of the Sea of Cortez (10). Water temperatures in February varied from 16°C to 23°C; in July and August water temperatures ranged from 25°C to 36°C (T.J.D. and G.N.S., unpublished data). Thus, both species encounter fluctuations in habitat temperature of ≈30°C, which makes them well-suited for studies of acclimatization in the heat shock response.

Induction Temperatures for HSP90 Synthesis in Brain. Our initial metabolic labeling studies of temperature-induced changes in protein synthesis patterns revealed thermal induction of several size classes of proteins in the various tissues examined (brain, muscle, liver, gill, and skin). An especially clear and consistent temperature-induced change in synthesis was observed for an ≈90-kDa protein in brain tissue. We focused our study on this putative brain HSP90.

Fig. 1 illustrates the labeling pattern observed in winter-acclimatized fish of both species. The fluorogram reveals an exposure-temperature-related change in labeling of a protein of ≈90 kDa. Minimal labeling of this protein occurred in the 18°C (control) fish (lane 1) and increasing amounts of labeling were seen at higher exposure temperatures (lanes: 2, 24°C; 3, 28°C; 4, 32°C; and 5, 36°C). Molecular mass standards indicated that the band showing the large temperature-dependent increase in rate of synthesis was of 89 kDa. This band reacted strongly with a monoclonal antibody to HSP90 (data not shown; see Fig. 5). Therefore, we conclude that synthesis of a HSP of the HSP90 class is significantly increased in both species as exposure temperature is increased above a threshold value.

To quantify the response shown in the fluorograms, we excised the sections of the gels containing HSP90 and quantitated the radioactivity of each section. Fig. 1 *B* and *D* present the relative amounts of radioactivity corresponding to the HSP90 bands shown in the fluorograms of Fig. 1 *A* and *B*. The radioactivity, like the fluorograms, reveals a regular increase in HSP90 synthesis as the exposure temperature increases. For the winter-acclimatized fish, the increase in HSP90 synthesis relative to control (18°C) fish is significant by 28°C (see below). We conclude, therefore, that the HSP induction temperature of the fish collected in February is between 24°C and 28°C.

To determine if HSP induction temperatures shift as a result of adaptation to different temperatures, we acclimated *G. mirabilis* captured in February to 26°C for 5 weeks. The data in Fig. 2 suggest that the temperature at which synthesis of HSP90 is significantly increased is higher than for the winter-acclimatized fish [Fig. 2; compare lane 2 (28°C) to lane 3 (30°C)]. Thus, whereas winter-acclimatized fish exhibited an approximate doubling of HSP90 synthesis by 28°C (Fig. 1, lane 3), the 26°C-acclimated fish did not show a doubling of HSP90 synthesis until 30°C.

We next analyzed fish collected during July and August, when daily exposures to temperatures of ≈36°C were characteristic of both species. Consistent with the trends observed in the 26°C-acclimated fish, we found that the HSP90 induction temperature was significantly higher in these summer-acclimatized fish (Fig. 3). No significant increase in synthesis of HSP90 was found at exposure temperatures below 32°C (compare lanes 1–3 with lanes 4 and 5, and see below). Because there were no significant differences in HSP90 induction temperatures between the two species at either season, we grouped the data for both species and analyzed them with an ANOVA to determine when a statistically significant increase in HSP90 synthesis occurred in the winter- and summer-acclimatized fish. This analysis confirmed the conclusions reached from inspection of the fluorograms and the individual bar graphs derived from them: for winter-acclimatized fish, a significant increase in HSP90 synthesis was found by 28°C, whereas for the summer-acclimatized fish no significant increase in HSP90 synthesis occurred until 32°C (Fig. 4).

Parallel studies to the experiments just described were conducted with isolated brain slices *in vitro*. The majority of investigations into the heat shock response have been per-

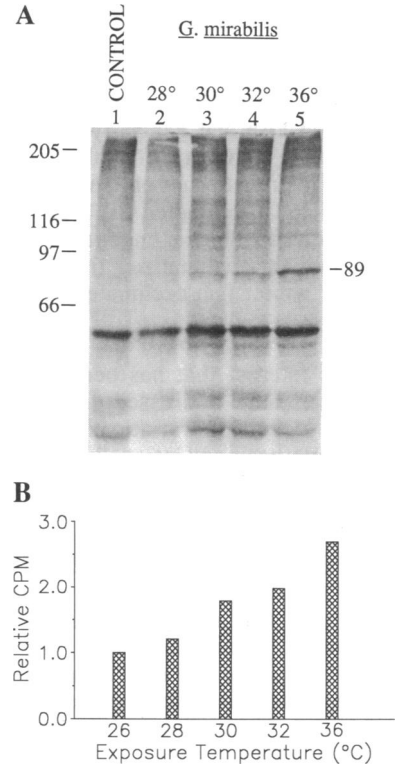

FIG. 2. HSP induction profile in brains of winter-collected *G. mirabilis* that were acclimated for 5 weeks to 26°C. (*A*) Individual fish were heat-shocked to the temperatures noted above each lane (in degrees Celsius). The control animal was maintained at 26°C. Molecular mass markers are noted (in kDa), as is the band corresponding to the 89-kDa heat-inducible protein. (*B*) Relative incorporated radioactivity (cpm) of the 89-kDa band in *A*.

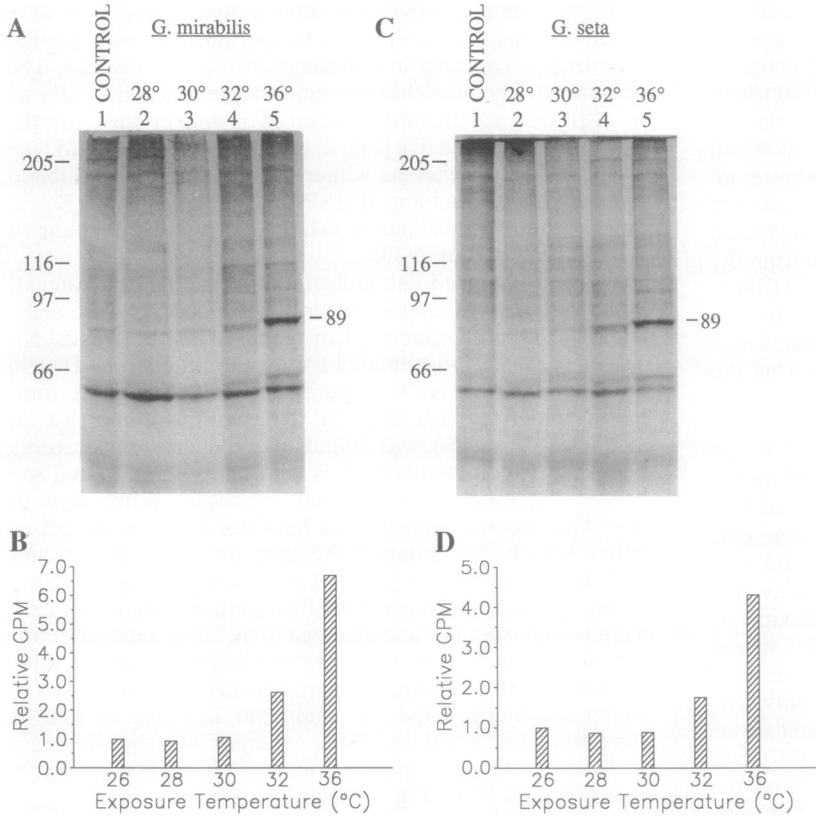

FIG. 3. HSP induction profile in brains of summer-collected *G. mirabilis* and *G. seta*. (*A*) Individual fish (*G. mirabilis*) collected in July were heat-shocked to the temperatures noted above each lane (in degrees Celsius). The control animal was maintained at 26°C. Molecular mass markers are noted (in kDa), as is the band corresponding to the 89-kDa heat-inducible protein. (*B*) Relative incorporated radioactivity (cpm) of the 89-kDa band in *A*. (*C*) Same as *A* except that data are for *G. seta*. (*D*) Same as *B* except that data are for *G. seta*.

formed either with isolated tissues or cultured cells, and we wished to determine if the responses seen in the whole organism were mirrored by isolated tissues. The same acclimatization of the HSP90 threshold induction temperature was observed in the experiments with brain slices (data not shown).

Endogenous Levels of HSP90 in Brains of Winter- and Summer-Acclimatized Fish and February-Collected Fish Acclimated to 26°C. Using Western blot analysis, we compared the levels of HSP90 in brain tissues of fish collected in winter and summer and of 26°C-acclimated fish from the February collection. Fig. 5 shows that higher endogenous HSP90 levels were present in summer-acclimatized fish than in winter-acclimatized fish or winter fish acclimated to 26°C.

DISCUSSION

Despite the intense interest that has been given to HSPs, extremely few studies have addressed questions about the effects of naturally occurring variations in environmental temperature on the synthesis and intracellular levels of HSPs. In fact, HSP induction and synthesis in whole animals has been studied in only a few ectothermic species. Bradley *et al.* (11) found that copepods raised at 4°C and 15°C produced different subsets of proteins than copepods raised at 20°C. These authors did not determine whether any of the HSPs common to all subsets had an alteration in their threshold induction temperature. To our knowledge, the results presented in this study provide the first example of seasonal changes in the temperature at which enhanced synthesis of a specific type of HSP is induced and of seasonal changes in endogenous levels of HSPs in cells.

The agreement we found in acclimatization-related differences in HSP induction temperatures by using whole fish and isolated brain slices suggests that freshly isolated tissues of differently acclimated fish can provide a valid picture of *in vivo* responses to temperature. However, it remains unclear whether cultured cells always will mimic accurately the effects of different acclimation or acclimatization temperatures on the whole organism. Using cultured hepatocytes from differently acclimated catfish, Koban *et al.* (12) concluded that no significant change in HSP induction temperature occurred after acclimation of fish to different temperatures. They concluded that HSP threshold induction temperature is genetically controlled rather than being subject to environmental regulation. It is unknown whether the lack of acclimation effect was due to the cell culture procedure used or was a reflection of differences between species or between liver and brain responses.

Our results have implications for the roles that HSPs play as elements in environmental adaptation processes in ectotherms and, further, provide evidence in support of one of the proposed mechanisms for regulating transcription of HSP-encoding genes. From the perspective of environmental adaptation, our results indicate that the heat shock response

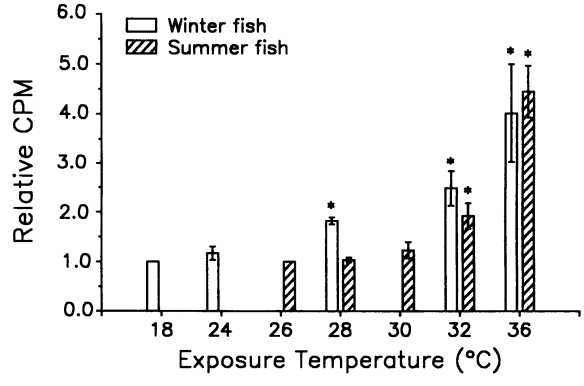

FIG. 4. Comparison of heat shock responses of winter- and summer-acclimatized *Gillichthys*. Data for both species are combined for this analysis. Five experiments were done with winter fish and seven experiments were done with summer fish. Error bars indicate the SEM. Asterisks indicate HSP90 synthesis is significantly ($P < 0.05$) higher than control rate (18°C for winter and 26°C for summer fish).

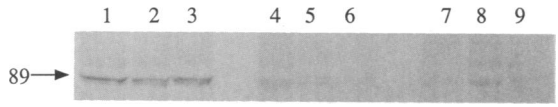

FIG. 5. Western blot analysis of endogenous levels of HSP90 in brains of winter- and summer-acclimatized and 26°C-acclimated winter specimens of *G. mirabilis*. Lanes 1–3 show endogenous HSP90 levels in three August-collected fish. Lanes 4–6 show endogenous HSP90 levels in three 26°C-acclimated winter fish. Lanes 7–9 show endogenous HSP90 levels in three February fish. The 89-kDa HSP90-class heat-inducible protein is labeled.

is not genetically hard-wired but is a modifiable property of cells, at least for HSP90 in brain. We did not examine acclimatization of the HSP90 response in other tissues. The similar changes associated with acclimation of February-collected fish to 26°C and seasonal acclimatization to summer temperatures under natural field conditions suggest that it is temperature rather than another seasonally varying environmental factor that is effective in altering the threshold induction temperature for HSP90 synthesis. We note the surprising lack of the induction of the 70-kDa HSP (HSP70) in brain. It is possible that fish brains contain only small amounts of heat-inducible HSP70.

The higher levels of HSP90 in summer-acclimatized fish may be adaptive in maintaining native protein structures under heat stress. Thus, what would seem to be required of the constitutive population of HSPs is that there be sufficient concentrations of HSPs to cope satisfactorily with the levels of aberrant proteins that are generated at any given body temperature. Higher constitutive levels of HSPs in eurythermal ectotherms during summer could provide cells with an adequate ability to process partially unfolded proteins. In winter, at lower cell temperatures, protein denaturation may occur less frequently, and lower concentrations of HSPs may be adequate.

The seasonal changes in HSP90 concentrations may also have a direct role in influencing the threshold induction temperatures for increased HSP synthesis. Using HSP70-class HSPs in their analysis, Craig and Gross (13) proposed that the level of free (not protein-bound) HSP70 in cells is of key importance in controlling the rate of transcription of the *hsp70* gene. In their model, HSP70 acts as a "cellular thermometer" by indicating to the regulatory elements of the *hsp70* gene the cells' requirements for HSP70 synthesis. Craig and Gross (13) propose that unliganded HSP70 binds to one or more modulators of *hsp* gene expression [e.g., the heat shock factor (HSF), a protein that is essential for expression of the *hsp70* gene]. When bound to HSP70, HSF is inactive. When temperature is elevated sufficiently to increase the rate of protein denaturation, HSP70 binds increasingly to denatured proteins, and HSF is increasingly free to trimerize into the form active in enhancing transcription of the *hsp70* gene (14, 15). If this model is correct, then higher endogenous levels of HSP70, or of any class of HSP for which the model is accurate, could shift the threshold induction temperature upward. That is, if the cell accumulates significantly higher levels of HSPs in summer to ensure that protein denaturation processes can be coped with adequately when temperatures reach peak values (i.e., ≈36°C for *G. mirabilis* and *G. seta*), then, at temperatures below which large-scale denaturation occurs, the increased pool of unliganded HSP may so fully bind HSF that the ability of HSF to induce *hsp* gene expression is significantly reduced. It should be recognized that HSF may not be the only mediator of HSP threshold induction temperature acclimatization. Levels of HSP90 synthesis are dependent upon transcriptional, posttranscriptional, and translational mechanisms, and the experiments described here do not indicate which of these processes is (are) affected by acclimatization.

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