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HEAT-SHOCK PROTEIN EXPRESSION IS ABSENT IN THE ANTARCTIC FISH *TREMATOMUS BERNACCHII* (FAMILY NOTOTHENIIDAE)

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Summary

The heat-shock response, the enhanced expression of one or more classes of molecular chaperones termed heat-shock proteins (hsps) in response to stress induced by high temperatures, is commonly viewed as a 'universal' characteristic of organisms. We examined the occurrence of the heat-shock response in a highly cold-adapted, stenothermal Antarctic teleost fish, *Trematomus bernacchii*, to determine whether this response has persisted in a lineage that has encountered very low and stable temperatures for at least the past 14–25 million years. The patterns of protein synthesis observed in *in vivo* metabolic labelling experiments that involved injection of ^{35}S-labelled methionine and cysteine into whole fish previously subjected to a heat stress of 10 °C yielded no evidence for synthesis of any size class of heat-shock protein. Parallel *in vivo* labelling experiments with isolated hepatocytes similarly showed significant amounts of protein synthesis, but no indication of enhanced expression of any class of hsp. The heavy metal cadmium, which is known to induce synthesis of hsps, also failed to alter the pattern of proteins synthesized in hepatocytes. Although stress-induced chaperones could not be detected under any of the experimental condition used, solid-phase antibody (western) analysis revealed that a constitutively expressed 70 kDa chaperone was present in this species, as predicted on the basis of requirements for chaperoning during protein synthesis. Amounts of the constitutively expressed 70 kDa chaperone increased in brain, but not in gill, during 22 days of acclimation to 5 °C. The apparent absence of a heat-shock response in this highly stenothermal species is interpreted as an indication that a physiological capacity observed in almost all other organisms has been lost as a result of the absence of positive selection during evolution at stable sub-zero temperatures. Whether the loss of the heat-shock response is due to dysfunctional genes for inducible hsps (loss of open reading frames or functional regulatory regions), unstable messenger RNAs, the absence of a functional heat-shock factor or some other lesion remains to be determined.

Key words: heat-shock protein, Antarctic fish, teleost, *Trematomus bernacchii*, hepatocyte.

Introduction

Antarctic fish of the teleost suborder Notothenioidei are extreme stenotherms that live in the cold, thermally stable waters of coastal Antarctica, where temperatures range from +0.3 °C to −1.86 °C (Eastman, 1993). Although the precise geological time at which the Southern Ocean reached its current temperature range remains under debate, it is certain that these highly endemic fish have been isolated in extremely cold water for many millions of years (Eastman, 1993). Extensive cooling of Antarctic waters probably began when the Drake Passage opened approximately 25 million years ago, allowing circumpolar circulation of deep water and preventing mixing of Antarctic waters with the warmer waters of the temperate regions (Denton et al., 1991). Current estimates suggest that the Antarctic notothenioid stock has evolved in an extremely cold and stable thermal environment for approximately 14–25 million years (Eastman, 1993; Clarke and Johnston, 1996).

Given this extensive period of isolation, Antarctic notothenioids provide a unique opportunity to study evolutionary adaptation to stable, subzero temperatures. As a consequence of having undergone 14–25 million years of evolution in extreme cold, notothenioids are distinguished by a number of physiological adaptations to low temperatures, including antifreeze glycoproteins that prevent freezing of tissues in the subzero, ice-filled waters (DeVries, 1988), tubulin molecules that polymerize into microtubules at low temperatures (Detrich, 1991) and enzymes with unusually high catalytic efficiencies that offset the effects of low temperature

on metabolic rates (Fields and Somero, 1998). In addition to gaining novel physiological attributes for coping with low temperatures, some Antarctic notothenioids have been shown to have lost traits during evolution at subzero temperatures, for instance, the ability of fishes of the family Channichthyidae to express hemoglobin and myoglobin proteins (Cocca et al., 1997; Sidell et al., 1997; Somero et al., 1998). The loss of these respiratory proteins may reflect the absence of positive selection for their functions because of the high solubility of oxygen at low temperature and the relatively sluggish swimming behavior of channichthyid fishes.

Evolution at the low and stable temperatures of the Southern Ocean might also permit the loss of traits whose function is to increase heat tolerance or to facilitate acclimatization to short-term changes in temperature. One such trait is the heat-shock response, a cellular process in which organisms subjected to a heat stress increase the synthesis of a set of molecular chaperones termed heat-shock proteins (hsps). Under normal non-stressful conditions, molecular chaperones assist in the routine folding and compartmentation of newly synthesized proteins (Ellis, 1990; Hartl, 1996; Fink, 1999). During thermal stress, heat-induced chaperones, hsps, bind to thermally denatured proteins, thereby preventing their aggregation and providing an opportunity for them to re-fold into native, functional states following restoration of normal body temperatures. The heat-shock response is thought to be nearly universal among organisms, in only one case has the heat-shock response not been detected (*Hydra oligactis*: Bosch et al., 1988), and hsps themselves are highly evolutionarily conserved in sequence across phyla (Lindquist, 1986; Parsell and Lindquist, 1993; Feder and Hofmann, 1999). Although the temperatures that trigger expression of hsps vary among species according to their adaptation and acclimation temperatures (Hofmann and Somero, 1996; Feder and Hofmann, 1999; Tomanek and Somero, 1999), patterns of expression of hsps show striking levels of conservation among species.

To determine whether the capacity to elicit the heat-shock response has remained in Antarctic notothenioid fishes, we studied populations of *Trematomus bernacchii* (Family Nototheniidae) from McMurdo Sound, the southernmost region of the world ocean, where water temperatures year-round remain within a few hundredths of a degree Celsius of the freezing point of sea water, −1.86 °C (Eastman, 1993). We employed *in vivo* metabolic labelling of whole fish (injection of ^{35}S-labelled methionine and cysteine during heat stress) to determine whether sub-lethal heat stress triggered the synthesis of hsps. We performed metabolic labelling studies with isolated hepatocytes to determine the effects on patterns of protein synthesis after heat stress and exposure to the heavy metal cadmium, which is known to induce hsp synthesis. We used solid-phase antibody (western) analysis of molecular chaperones of the 70 kDa class (hsp70 isoforms) to determine whether stress-induced or constitutively expressed chaperones of this class were present and, if so, whether acclimation of fish to 5 °C, the highest temperature to which *T. bernacchii* can be acclimated, changed the level of expression of hsp70 isoforms. Although constitutively expressed isoforms of hsp70 were present in *T. bernacchii*, neither heat nor cadmium stress led to the induction of a heat-shock response.

Materials and methods

Specimen collection and acclimation conditions

Trematomus bernacchii (Boulenger) were collected in McMurdo Sound, Antarctica (77°53′S, 166°40′E), in January 1995, 1996 and 1999 using hand lines and baited fish traps. Specimens were either held in flow-through seawater aquaria maintained at the ambient seawater temperature (−1.86 °C) or placed into heated non-flowthrough aquaria for acclimation at 5 °C. Specimens maintained at −1.86 °C ranged in mass from 22.5 to 300 g. Specimens used in the acclimation studies ranged in mass from approximately 30 to 150 g. No attempt was made to distinguish gender before use; approximately equal numbers of male and female specimens were employed. In the acclimation study, fish (N=3 at each time point) were killed after 5, 11, 15 and 22 days of acclimation. Fish were killed by cervical transection and anesthetized with MS-222 dissolved in filtered sea water at −1 °C.

Heat-shock protein induction experiments: in vivo *metabolic labelling with ^{35}S-labelled amino acids*

The induction of hsps was tested in whole fish using *in vivo* metabolic labelling following the protocol of Dietz and Somero (1992, 1993). Fish were maintained at −1.86 °C for 3–5 days prior to the induction experiments to minimize the effects of capture and handling stress. For the induction experiments, individual fish were injected intraperitoneally with a ^{35}S-labelled methionine/cysteine amino acid mixture (NEN; ^{35}S Express label) at 4×10^6 Bq g^{-1} body mass. Smaller fish, ranging in body mass from 22.5 to 50.0 g, were used to economize on amounts of isotope. The amino acid mixture was diluted 1:1 with nototheniid Ringer's solution (570 mosmol l^{-1}) composed of 311 mmol l^{-1} NaCl, 5 mmol l^{-1} KCl, 2.5 mmol l^{-1} $MgCl_2$, 3.0 mmol l^{-1} $CaCl_2$, 2.5 mmol l^{-1} $NaHCO_3$, 2.0 mmol l^{-1} NaH_2PO_4 and 5.0 mmol l^{-1} glucose (J. Eastman, personal communication) prior to injection. The fish were maintained in ambient temperature sea water during the injection procedure to avoid prematurely heat-shocking the specimens. Following the injection, fish were transferred to 4 l vessels containing continuously aerated sea water equilibrated to the desired exposure temperature. For the heat-shock treatment, fish (N=4) were exposed to 10 °C for 2 h and then transferred to −1.5 °C for a 6 h recovery period. The median survival time of *T. bernacchii* at 10 °C is approximately 140 min (Somero and DeVries, 1967), so the 2 h heat shock at this temperature was predicted to be severe, but not rapidly lethal. Fish in the control group (N=3) were maintained at −1.5 °C for 8 h. For heat shock and control groups, fish were placed directly into water at the exposure temperature after the injection of ^{35}S-labelled amino acids. At the end of the 8 h temperature exposure period, the fish were killed, and samples of brain, heart, liver, white

skeletal muscle, gill and spleen were dissected (note that insufficient label was incorporated into white muscle to allow autoradiographic analysis). Tissues were immediately frozen on dry ice and stored at −80 °C. Because of restrictions on modes of shipment of radiolabelled materials from Antarctica, tissue samples were returned to the USA aboard ship (transit time, approximately 3 months), where electrophoresis and autoradiography were performed.

In preparation for SDS–PAGE, tissue samples were homogenized with a Teflon pellet pestle in 200 µl of SDS homogenization medium (32 mmol l^{-1} Tris-HCl, 2 % SDS, 1 mmol l^{-1} phenylmethylsulfonylfluoride, pH 6.8). Following homogenization, tissue extracts were first boiled for 5 min, then centrifuged for 15 min at 16 000 ***g***, and the resulting supernatant was removed and stored at −20 °C prior to electrophoresis.

Suspension culture of hepatocytes

Hepatocytes were isolated according to the two-step collagenase perfusion method of Seglen (1976), as modified by Råbergh et al. (1992), with additional adjustments as follows. Briefly, fish were anesthetized in MS-222 at −1 °C, and the portal vein was cannulated to perfuse the blood from the liver. Both fish and perfusion solution (290 mmol l^{-1} NaCl, 2 mmol l^{-1} KCl, 10 mmol l^{-1} Hepes, 0.5 mmol l^{-1} EGTA, 25 mmol l^{-1} Tricine, pH 7.8) were kept on ice during the procedure. The liver was transferred to a Petri dish, and the gall bladder was removed. Then, the liver was cut into small pieces and digested with suspension buffer (292.5 mmol l^{-1} NaCl, 5.0 mmol l^{-1} KCl, 2.5 mmol l^{-1} $MgCl_2$, 3.0 mmol l^{-1} $CaCl_2$, 2.0 mmol l^{-1} $NaHCO_3$, 2.0 mmol l^{-1} NaH_2PO_4, 5 mmol l^{-1} glucose, 50 mmol l^{-1} Hepes, pH 7.8) containing 5 units ml^{-1} collagenase type 1A, type IV or type L (Sigma Chemical Co.) for 1 h at 4 °C. Dispersed hepatocytes were filtered through a nylon filter (100 µm) and washed twice with the perfusion buffer. Cells were resuspended in the suspension buffer at a density of 2×10^6 cells ml^{-1} and allowed to recover with constant shaking at 0 °C for 30 min prior to use in experiments. Viability was measured by Trypan Blue exclusion (0.4 % w/v) before, during and after the exposures. Viability exceeded 85 % in all temperature-treated samples; cadmium treatment appeared to lower the viability (to 62–97 %).

Metabolic labelling of hepatocytes and gill cells

For the heat-shock experiments, cell suspensions (3×10^6 per tube) were routinely exposed to the treatment temperature for 1 h followed by a 4 h metabolic labelling step at 0 °C. Cells were incubated in suspension buffer containing 4×10^6 Bq of ^{35}S-labelled methionine/cysteine amino acid mixture (NEN ^{35}S Express Label). During experiments employing cadmium, cells were incubated for 1 h at 0 °C in cadmium chloride concentrations ranging from 5 to 150 µmol l^{-1}. Cells were washed twice to remove the cadmium, and metabolic labelling in cadmium-free buffer was carried out as above.

Gill cells were isolated from the temperate, eurythermal goby fish *Gillichthys mirabilis* following the protocol given in Kültz and Somero (1995).

Electrophoresis, fluorography and analysis of protein expression patterns

Patterns of protein synthesis were examined using SDS–polyacrylamide gel electrophoresis in combination with fluorography, as described by Hofmann and Somero (1996). Prior to electrophoresis, tissue extracts were analyzed using liquid scintillation counting. Samples from each tissue extract were then diluted into SDS sample buffer (62.5 mmol l^{-1} Tris-HCl, pH 6.8, 10 % glycerol, 2 % SDS, 5 % 2-mercaptoethanol and 0.01 % Bromphenol Blue) and loaded to equivalent amounts of radioactivity per lane. The standardized amount of radioactivity loaded per lane varied for each tissue to ensure sufficient radioactivity for autoradiography while not overloading the gel with respect to protein. Protein samples were subjected to electrophoresis on a 10 % polyacrylamide gel for 3.5 h at 20 mA. Following electrophoresis, the gels were treated with EN^{3}HANCE, an autoradiographic enhancer, according to the manufacturer's instructions (DuPont NEN). Fluor-impregnated gels were dried at 60 °C for 2.5 h, exposed to X-ray film (Kodak X-OMAT AR5) at −70 °C for an empirically determined period and developed. For tissues from the whole fish experiments returned by ship to the USA, the decay of ^{35}S label during the 3–4 month period following initial labelling required lengthy exposure times of X-ray film, approximately 4–6 weeks, depending on the intensity of the residual radioactivity.

Immunochemical analysis of hsp70 in T. bernacchii *tissue*

Western blot analysis and scanning densitometry were used to compare the levels of hsp70 in *T. bernacchii* brain and gill at sampling intervals during the 5 °C acclimation experiment, according to the methods described by Hofmann and Somero (1995). Equal amounts of protein (25 µg) were separated on 7 % gels and transferred to nitrocellulose using semi-dry electrophoretic transfer; the resulting blots were stained using an enhanced chemiluminescence (ECL) protocol as the final detection step. The primary antibody used was an anti-hsp70 rat monoclonal antibody (Affinity BioReagents; MA3-001) that recognizes both cognate and heat-inducible forms of hsp70. Protein determinations were made on the tissue extracts using a Coomassie Plus protein assay (Pierce Chemical Co.).

After the ECL western blot procedure had been completed, scanning densitometry was used to determine the relative absorbance of each band. Data are expressed as optical density×area (mm^2). The amount of protein used in each lane of the gel was optimized to be within the linear range of detection for the X-ray film.

Results

Whole-organism induction experiments

The fluorograms of radiolabelled proteins in tissues of *T. bernacchii* sampled after *in vivo* metabolic labelling of whole fish provided no evidence for a heat-shock response (Fig. 1). After a 2 h heat-shock treatment at 10 °C followed by 6 h of recovery at −1.5 °C, no size class of protein exhibited enhanced

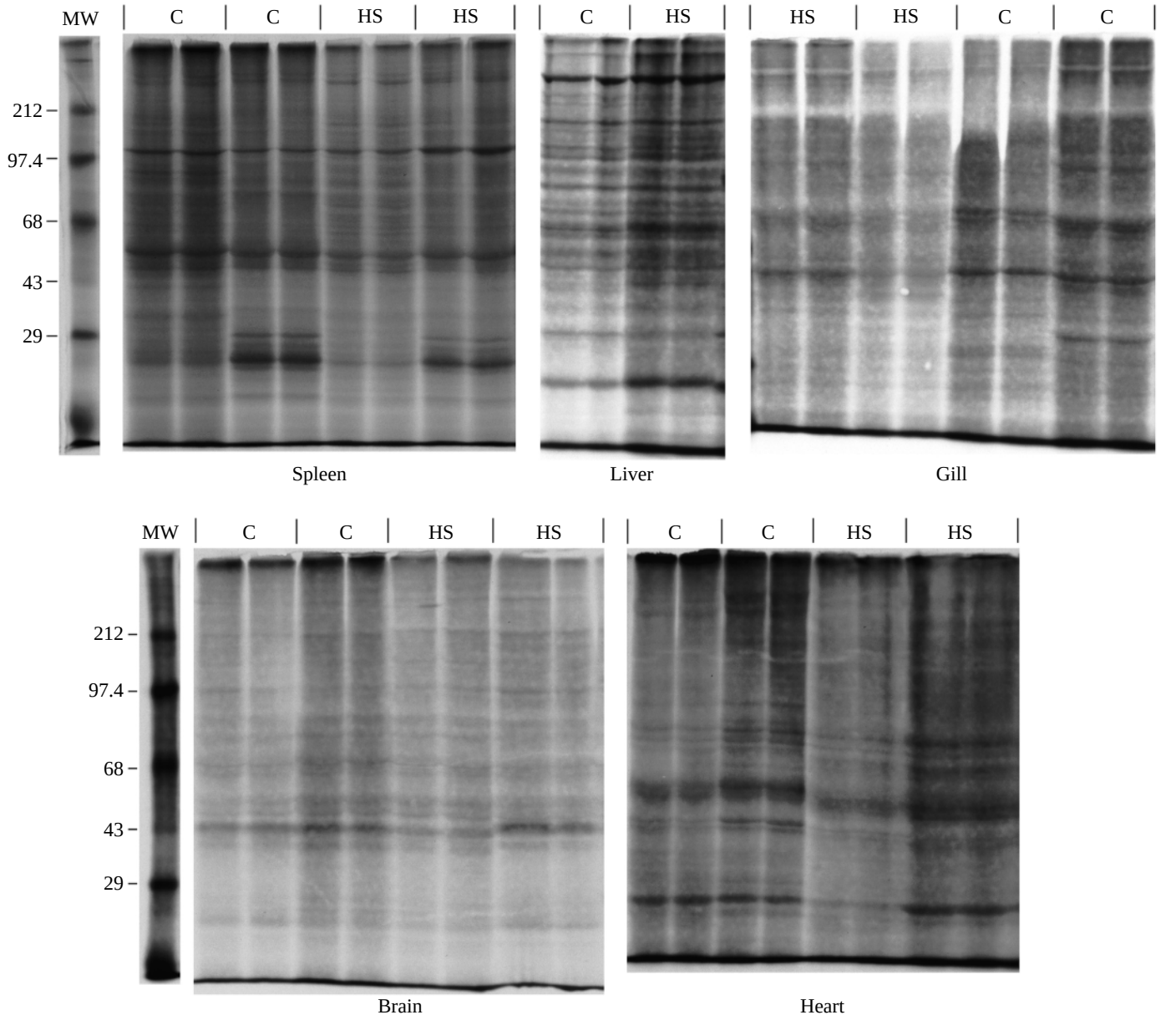

Fig. 1. Whole-organism heat-shock induction experiments on the Antarctic notothenioid *Trematomus bernacchii*. The fluorograms show the protein synthesis pattern in five different tissues from control (C) and heat-shocked (HS) fish. Individual fish were injected with 4×10^6 Bq g^{-1} body mass and exposed to either a control or heat-shock treatment (see Materials and methods for experimental protocol). Proteins were separated on 10 % SDS–polyacrylamide gels; individual lanes were loaded with equivalent amounts of radioactivity, and individual samples were run in duplicate. ^{14}C-labelled protein molecular mass markers (MW) are shown in kDa.

synthesis in any of the five tissues examined. However, adequate labelling of proteins was observed, indicating that the radiolabelled amino acids were efficiently incorporated into protein during the labelling period despite exposure to a temperature that is lethal after a period of 2–3 h (Somero and DeVries, 1967).

Metabolic labelling of isolated hepatocytes

The patterns of protein synthesis observed in isolated hepatocytes stressed with high temperature (Fig. 2A) or cadmium treatment (Fig. 3) also provided no evidence of a heat-shock response. After 1 h incubations at 5, 8 or 10 °C, followed by metabolic labelling at 0 °C, heat-shocked cells were viable and capable of synthesizing proteins (Fig. 2A). Visual inspection of the autoradiograms demonstrated that the translational capacity of heat-shocked cells was equivalent to that of control cells (Fig. 2A). The pattern of protein expression in heat-shocked cells was essentially identical to that observed in control cells. Although translation started to be reduced at 15 °C (data not shown), in all experiments conducted on hepatocytes (N=7 individual cell preparations from seven livers), there was no detectable induction of hsps,

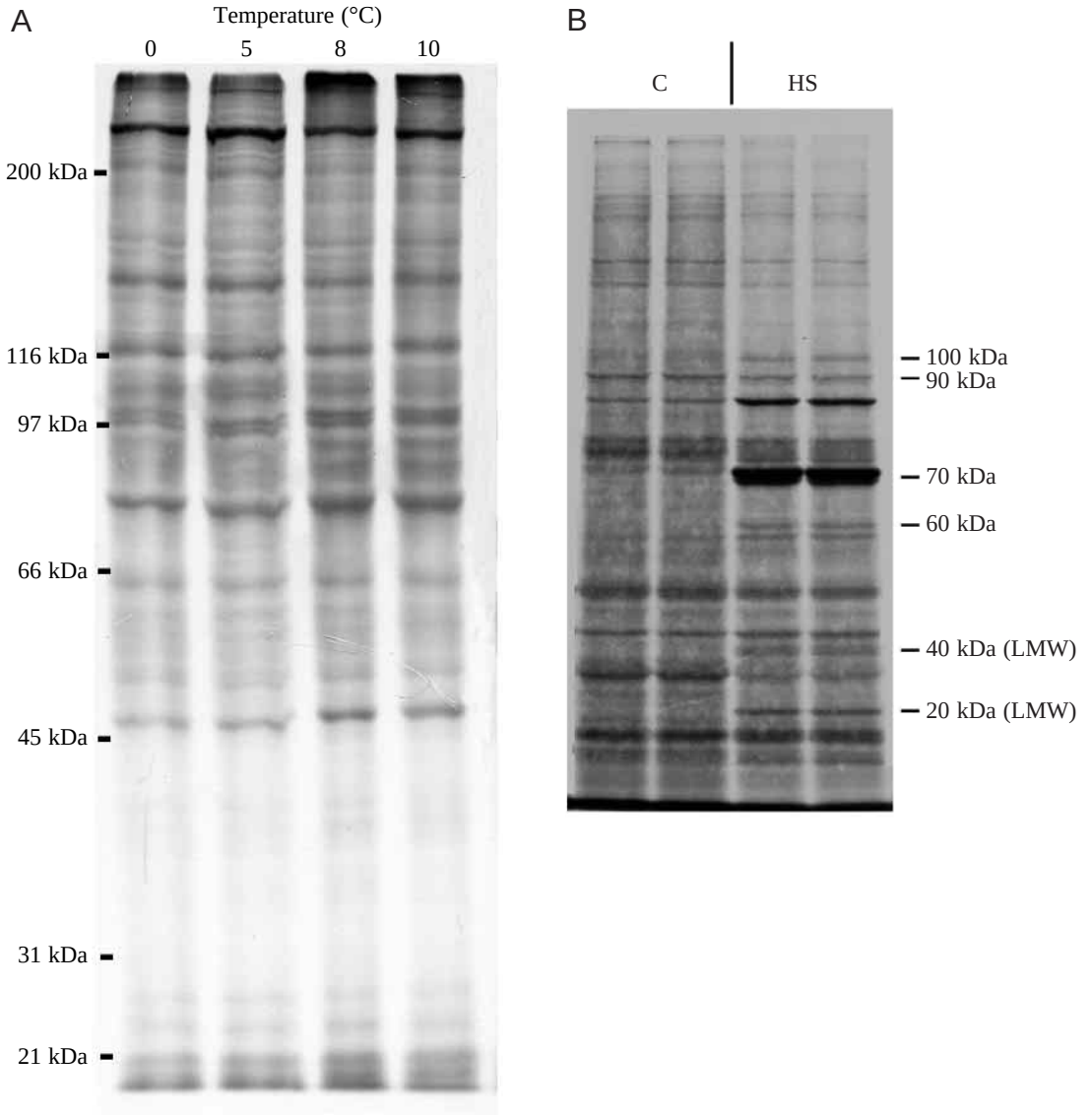

Fig. 2. (A) Effects of temperature on protein synthesis patterns of hepatocytes isolated from *Trematomus bernacchii*. Hepatocytes were exposed to the temperature indicated above each lane for 1 h. Following metabolic labelling for 4 h at 0 °C, proteins in cell lysates were separated using SDS–polyacrylamide gel electrophoresis; equivalent counts (300 000 cts min^{-1}) were applied to each lane. Positions of protein molecular mass standards are shown on the left. (B) Heat-shock induction experiment with isolated gill cells from the eurythermal goby *Gillichthys mirabilis*. Control (C) and heat-shocked (HS) cells were incubated for 1 h at 23 °C and 37 °C, respectively, in 2×10^6 Bq of ^{35}S-labelled methionine/cysteine amino acid mixture. Arrows indicate the molecular masses of the different classes of heat-inducible heat-shock proteins. LMW, low molecular weight chaperone.

despite heat-shock temperatures that exceeded the ecologically relevant temperature of *T. bernacchii* by over 15 °C.

For purposes of comparison and to validate the protocol used with the hepatocytes of *T. bernacchii*, Fig. 2B shows an autoradiogram from an experiment on isolated gill cells from the eurythermal goby *Gillichthys mirabilis*. These data show the classical heat-shock response of cells exposed to elevated temperatures, using the same protocols employed to study hepatocytes of *T. bernacchii*.

Concentrations of cadmium known to induce hsps in other cell types did not result in hsp synthesis in *T. bernacchii* hepatocytes (Fig. 3). In all cases, the hepatocytes exhibited high levels of protein synthesis; thus, the lack of hsp induction could not be attributed to poor incorporation of radiolabelled amino acids into newly synthesized proteins.

Detection of 70 kDa hsp isoforms in T. bernacchii *tissue*

Constitutively expressed 70 kDa hsps were immunochemically detected in tissues of *T. bernacchii* caught in water at −1.86 °C and never exposed to elevated temperatures (Fig. 4). Using western analysis, isoforms of the 70 kDa hsp family were detected in gill (Fig. 4) and brain (data not shown) of freshly caught, non-heat-shocked individuals. One-dimensional gel electrophoresis revealed

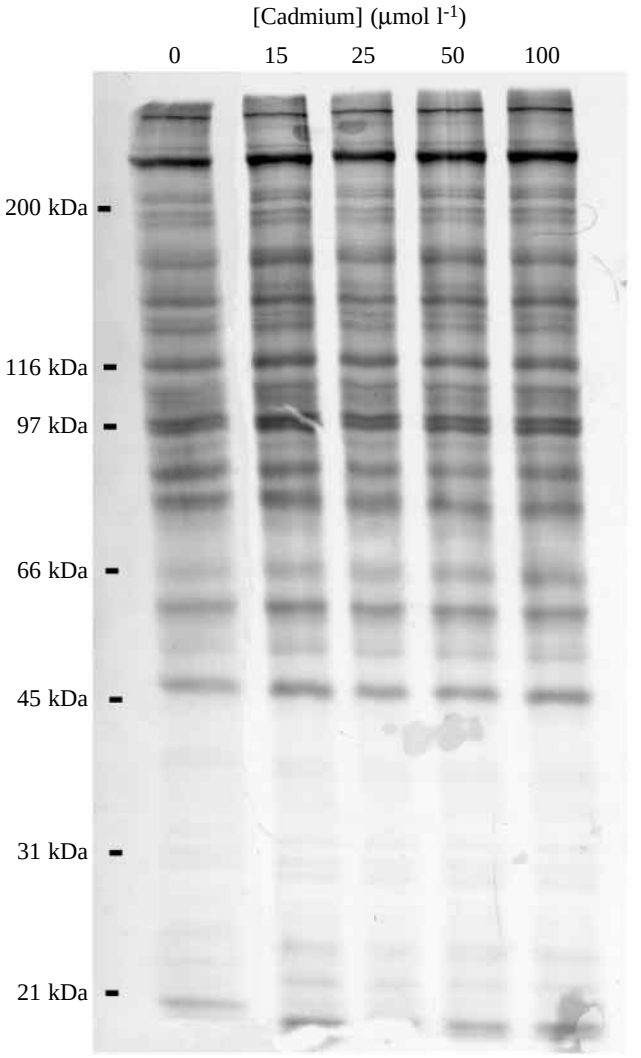

Fig. 3. Effects of cadmium on heat-shock protein induction in isolated hepatocytes. Cells were incubated in the indicated concentration of cadmium chloride for 1 h at 0 °C. Following the cadmium treatment, cells were washed twice with cadmium-free suspension buffer and then radiolabelled at 0 °C as described in Materials and methods. The positions of protein molecular mass standards are shown on the left.

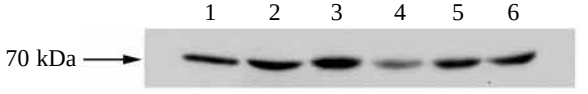

Fig. 4. Western-blot detection of constitutively expressed isoforms of the 70 kDa heat-shock protein (hsp) gene family in gill tissue from field-acclimatized *Trematomus bernacchii*. Samples of gill from five different fish were screened for 70 kDa hsp isoforms using a rat monoclonal anti-hsp70 antibody. Each lane contains 25 μg of total protein; the standard shown on the left (lane 1) is bovine brain heat-shock cognate 70 (hsc70) (from StressGen). Lanes contained the following samples: lane 1, bovine brain hsc70 (0.1 μg); lanes 2–6, gill tissue from individual specimens of *T. bernacchii*.

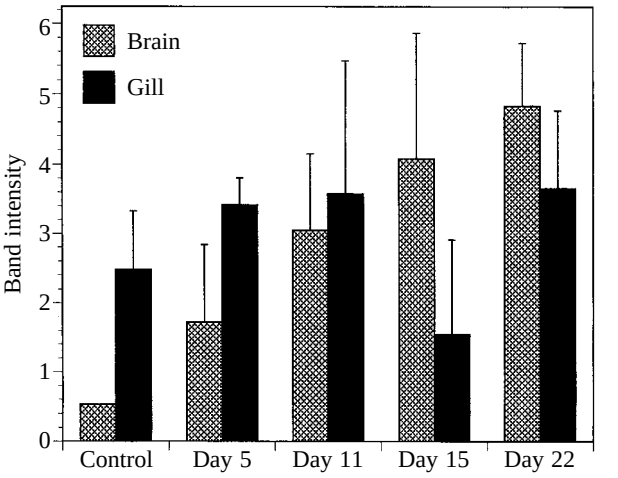

Fig. 5. Effects of acclimation to 5 °C on endogenous concentrations of 70 kDa heat-shock proteins (hsps) in gill and brain of *Trematomus bernacchii*. The bar graphs show the amount of 70 kDa hsp isoforms detected relative to a standard amount of bovine brain heat-shock cognate 70 (0.1 μg) run on each gel. Each column represents the mean + S.E.M. for N=3 individual fish (error bars lie within the column for control brain data). Analysis of variance revealed a significant interaction between acclimation time and band intensity (optical density×area) in brain (P<0.0080).

one predominant band with an apparent molecular mass of 72 kDa.

Effect of acclimation on cellular concentrations of 70 kDa hsps

Gill and brain tissues were analyzed for changes in levels of 70 kDa hsp isoforms during a 22-day acclimation period (Fig. 5). Acclimation to 5 °C led to an increase in the levels of constitutively expressed forms of the 70 kDa hsp family in brain (Fig. 5; ANOVA, P<0.0080) but, in accordance with the results of the metabolic labelling studies, there was no indication of induction of heat-induced isoforms. Unlike brain, gill tissue did not exhibit a statistically significant increase in hsp70 levels during acclimation to 5 °C (Fig. 5).

Discussion

To determine whether a heat-shock response has been retained in Antarctic notothenioid fishes during their 14–25 million years of evolution in a cold and thermally stable environment, we employed well-established methods that have been used to study the induction of hsps in other organisms. Metabolic labelling protocols using whole fish (Dietz and Somero, 1992, 1993) and isolated hepatocytes (Koban et al., 1987; Airaksinen et al., 1998) have proved to be highly effective means for detecting the induction of new synthesis of hsps following heat stress. Although metabolic labelling yielded clear evidence for induction of hsps in gill cells from *Gillichthys mirabilis* (Fig. 2B), none of the five tissues of *T. bernacchii* examined in metabolic labelling experiments with whole fish heat-shocked at 10 °C (Fig. 1) and in hepatocytes

(Fig. 2A) subjected to heat-shock at temperatures up to 10 °C showed any indication of induction of synthesis of hsps. Therefore, we conclude that *T. bernacchii* has lost the heat-shock response.

Because hsps can be induced by a number of physical and chemical factors that have in common the ability to denature proteins, the failure of heat stress to induce synthesis of hsps does not, in and of itself, prove that all types of stress-induced induction of hsps has been lost in these fishes. Thus, we tested the effects of a well-established chemical inducer of hsps, the heavy metal cadmium, on patterns of protein synthesis (Fig. 3). As in the case of heat stress, no changes were observed in the patterns of proteins newly synthesized in cadmium-stressed cells. We conclude, then, that the ability to induce hsps of all size classes following either physical (temperature) or chemical stress is absent in this species.

The loss of ability to induce synthesis of stress-induced chaperones in *T. bernacchii* represents an additional example of how the absence of positive selection has allowed physiological capacities of notothenioid fishes to be lost during their radiation in the Southern Ocean. The loss of an ability to synthesize hemoglobin in all species of the Family Channichthyidae ('icefishes'; Cocca et al., 1997) and the absence of myoglobin in several icefishes (Sidell et al., 1997) may reflect in part the high concentrations of dissolved oxygen in sea water and body fluids at temperatures near 0 °C. The loss of the heat-shock response, which is more generally termed the 'stress response' to indicate that hsps are induced by a suite of protein-denaturing physical and chemical factors (Hightower, 1980), may reflect the absence of heat stress and the low levels of chemical stressors such as heavy metals found in Antarctic waters.

While extremely unusual, the absence of a heat-shock response has been observed previously. The freshwater cnidarian *Hydra oligactis* was unable to synthesize hsps of any size class in response to thermal stress, even though a congener, *Hydra vulgaris*, synthesized hsps of molecular masses 23, 70 and 80 kDa (Bosch et al., 1988). *Hydra oligactis* is found exclusively in cold, thermally stable environments, but it is not known whether the loss of the heat-shock response occurred during evolution under these thermal conditions or, alternatively, whether the loss of the heat-shock response relegated this species to habitats with stable low temperatures. To our knowledge, *Hydra oligactis* is the only species other than *T. bernacchii* in which the heat-shock response has not been detected.

The apparent lack of induction in *T. bernacchii* of any size class of hsp in response to heat stress or exposure to cadmium suggests that one or more types of lesion have occurred either in the hsp-encoding genes themselves or in the complex regulatory mechanisms that govern hsp synthesis. It is conceivable that the reading frames of all hsp-encoding genes are disrupted, such that even if transcription of these genes were to occur, no functional message would result and no translation of hsps would occur. It is also possible that the hsp-encoding genes persist as intact reading frames, but mutations in gene regulatory regions block transcription (see below). Another lesion leading to the loss of expression of hsps might involve an unstable hsp-encoding mRNA. Petersen and Lindquist (1989) have identified a number of factors that affect the stability of hsp-encoding mRNAs, and variation in the 5′ untranslated region of hsp70 mRNA has been shown to inhibit translation (Hess and Duncan, 1996). In *Hydra oligactis*, failure to translate mRNA is conjectured to account for the absence of hsp70 synthesis (Gellner et al., 1992).

The complete failure to induce synthesis of any type of hsp in *T. bernacchii* might be explained most economically by a mechanism involving one or more transcription factors that control the expression of all classes of hsp-encoding genes. An essential step in the induction of synthesis of hsps is the interaction between a transcription factor, the heat-shock factor (HSF), and highly conserved regulatory DNA sequences termed heat-shock elements (HSE) found in the promoter regions of heat-shock genes (Morimoto, 1998). Different transcriptional factors control the activation of inducible and constitutively expressed hsp genes (for reviews, see Wu, 1995; Morimoto, 1998), and these distinct HSFs are responsive to different cellular cues (Morimoto, 1998). In eukaryotes, only one HSF, designated HSF1, is sensitive to thermal stress and induces hsp expression in response to heat shock (Morimoto, 1993, 1998; Jedlicka et al., 1997; Zhong et al., 1998). The actions of the different HSFs are so specific that the activity of HSF2, the factor that controls constitutive expression, will not rescue the function of HSF1 when HSF1 has been deleted from the genome (McMillan et al., 1998; Jedlicka et al., 1997). The mechanisms by which HSF1 is activated and binds to HSE regions is not fully understood, but direct temperature control of HSF1 activity (Zhong et al., 1998) and control by phosphorylation and several regulatory molecules including molecular chaperones have all been proposed (Morimoto, 1998; Shi et al., 1998, Zou et al., 1998; Bharadwaj et al., 1999). If one or more of these mechanisms governing HSF1 function is dysfunctional in notothenioid fishes, then expression of hsps could be eliminated. Mutations involving the activity of HSF1 seem a more likely basis for explaining the loss of expression of multiple size classes of hsps than, for instance, the loss of hsp synthesis through mutations in the regulatory regions or reading frames of all individual hsp genes. The detection of abundant levels of a constitutively expressed chaperone belonging to the 70 kDa hsp family (Fig. 4; Carpenter and Hofmann, 1999) indicates that the regulatory factors controlling expression of the constitutive isoforms of this family have remained intact during the evolution of this lineage, as would of course be predicted on the basis of the need for molecular chaperones in protein biosynthesis under all physiological conditions. The occurrence of an increased level of constitutively expressed hsp70 in brain (but not in gill) following acclimation to 5 °C (Fig. 5) may simply be a reflection of enhancement of protein synthesis at elevated temperatures in the warm-acclimated specimens. Because the heat-shock response is an acute response that occurs within minutes of heat stress (Lindquist, 1986), the gradual and

continuing rise in chaperone concentrations found in brain are not indicative of a true heat-shock response.

To increase our understanding of the evolution of the heat-shock response in notothenioids, it will be important to examine other Antarctic notothenioids to establish whether the loss of this response is found throughout the suborder and to determine whether the cold-temperate members of this suborder possess a heat-shock response. If the ancestors of contemporary cold-temperate notothenioids were themselves cold-temperate species, then we predict that the heat-shock response will be found in non-Antarctic notothenioids. We base this assumption on the observation of a heat-shock response in all cold-temperate fishes examined to date (see Dietz and Somero, 1993). However, if cold-temperate notothenioids are found to lack the heat-shock response, this would be evidence that their ancestors were Antarctic species that successfully re-colonized cold-temperate habitats.

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