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Constitutive roles for inducible genes: evidence for the alteration in expression of the inducible *hsp70* gene in Antarctic notothenioid fishes

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Place, Sean P., Mackenzie L. Zippay, and Gretchen E. Hofmann. Constitutive roles for inducible genes: evidence for the alteration in expression of the inducible *hsp70* gene in Antarctic notothenioid fishes. *Am J Physiol Regul Integr Comp Physiol* 287: R429–R436, 2004. First published April 29, 2004; 10.1152/ajpregu.00223.2004.—Previous research on the Antarctic notothenioid fish *Trematomus bernacchii* demonstrated the loss of the heat shock response (HSR), a classical cellular defense mechanism against thermal stress, characterized by the rapid synthesis of heat shock proteins (Hsps). In the current study, we examined potential mechanisms for the apparent loss of the HSR in Antarctic notothenioids and, in addition, compared expression patterns of two genes from the 70-kDa Hsp family (*hsc71* and *hsp70*) in tissues from *T. bernacchii* to expression patterns in tissues of two closely related temperate notothenioid fishes from New Zealand, *Bovichtus variegatus* and *Notothenia angustata*. The results showed that transcript for both the constitutive and inducible genes in the Hsp70 gene family were expressed in detectable levels in all three species. However, only the cold-temperate New Zealand fishes displayed the ability to upregulate the inducible transcript, *hsp70*. Although *hsp70* was present in detectable levels in several tissues of the Antarctic notothen *T. bernacchii*, in vitro thermal stresses failed to produce a significant increase in mRNA levels. In all species, the expression of the constitutive transcript *hsc71* was variable and nonresponsive to temperature increases, even at temperatures as high as 10°C above the ecologically relevant range for the species under study. Field-collected tissues from *T. bernacchii* (sampled immediately after capture) indicated that *hsp70* mRNA was expressed at high levels in field-acclimatized fishes. Thus upregulation of molecular chaperones suggested that low-temperature stress may be significantly denaturing to cellular proteins in Antarctic fish, an observation that was supported by elevated levels of ubiquitin-conjugated protein.

heat shock response; molecular chaperones; heat shock proteins; gene expression; cold adaptation; *hsp70* messenger ribonucleic acid

THE PHYSIOLOGICAL MECHANISMS that underlie an organism's ability to cope with rapidly changing environmental conditions remains an area of strong scientific interest for many researchers. Variation in the expression of specific subsets of genes in concert with changing physiological or cellular conditions is one approach employed by organisms to maintain homeostasis (e.g., 9, 13, 32). One of the widest studied of these mechanisms focuses on the rapid change in gene expression patterns associated with the upregulation of several classes of molecular chaperones known as heat shock proteins (Hsps). Hsps have long been understood to be preferentially transcribed in response to multiple perturbations of cellular homeostasis, including exposure to thermal stress, heavy metals, hypoxia, and toxins (for reviews, see Refs. 8, 31). This cellular response, termed the heat shock response (HSR), is highly conserved across all taxa, with a general eukaryotic model of transcriptional activation involving the trimerization and localization of a heat shock factor (HSF1) to the nucleus of stressed cells. Trimerized HSF further binds to the heat shock element (HSE), a DNA promoter element located upstream of heat shock genes, resulting in the preferential transcription of these genes (29, 30, 41).

The HSR has been described in every organism studied to date with the exception of two, a freshwater hydra, *Hydra oligactis* (3), and the Antarctic notothenioid *Trematomus bernacchii* (16). The mechanistic explanation for the inability of the Antarctic notothenioid to mount a typical HSR remains unknown. Three possible hypotheses exist for this occurrence: *1*) the HSR is absent in these fish because the mRNA is never transcribed due to upstream interruptions such as the lack of functional HSF1 or the presence of a pseudogene, *2*) the HSR is absent in these fish because the mRNA is unstable and is never translated, or *3*) the HSR is absent because the gene is present but functional only in a constitutive role. Initial data indicating the presence of an *hsp70*-related sequence in several Antarctic species of fish were reported by Maresca et al. (26). These data suggest the gene is present and the transcriptional machinery, such as HSF1, is present and functional in Antarctic fish. Furthermore, studies of the freshwater hydra determined the *hsp70* transcript, one of the most strongly induced size classes of proteins expressed during the HSR, was unstable and readily degraded before translation could ensue, resulting in the absence of a detectable increase in heat shock protein levels in response to thermal stress (10, 4). These data are consistent with the hypothesis that the lack of a HSR in *T. bernacchii* may be a result of the expression of unstable mRNA. Therefore, the major goal of this study was to determine if a similar occurrence, i.e., unstable *hsp70* transcript, was responsible for the loss of a typical HSR in these cold-adapted fishes.

In this study we examined the expression of *hsp70* mRNA as a function of temperature exposure from differentially adapted notothenioids to examine a possible lesion in the manifestation of a characteristic HSR in *T. bernacchii*. We compared the relative quantity of mRNA for the constitutively expressed *hsc71* and the inducible *hsp70* in segments of gill and liver tissue from two New Zealand notothenioid species known to elicit a characteristic HSR, *Notothenia angustata* and *Bovichtus variegatus* (Hofmann, unpublished observations) and the Antarctic notothenioid *T. bernacchii*. To control for the effects

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of handling and captivity stresses, we measured the expression patterns in tissues from field-acclimatized fish sampled immediately after capture. We further measured the concentration of ubiquitin conjugate in the same tissues to determine the relative levels of denatured proteins in these fishes. Results from this study support *hypothesis 3*, indicating the inducible *hsp70* gene may have been converted to a constitutive role.

MATERIALS AND METHODS

Collection of study organisms. Specimens of the benthic Antarctic notothenioid *T. bernacchii* (Boulenger, 1902) were collected in McMurdo Sound, Antarctica (77°53′S, 166°40′E), in October to December of 2001 and 2002. Specimens of *T. bernacchii* were obtained by hook and line as well as baited traps and maintained in flow-through aquaria near ambient seawater temperatures (−1.5°C) for 48 h before they were killed. The study was carried out in accordance with the American Physiological Society's "Guiding Principles for Research Involving Animals and Human Beings" (1a).

Specimens of the two non-Antarctic notothenioids were collected at Portobello Marine Laboratories (University of Otago) on the Otago Peninsula of the South Island, New Zealand (45.50°S, 170.38°E) in August to September of 2003. *N. angustata* (Hutton, 1875), the black cod, is a benthic nearshore species and was caught in traps placed on the substrate at depths of 2–10 m. The tidepool thornfish *B. variegatus* (Richardson, 1846) was collected by hand in pools located near Portobello at Allans Beach and Blackhead Beach. After capture, fish were maintained in flow-through aquaria at ambient seawater temperatures (9°C) for 48 h before they were killed.

Probe development. For the New Zealand species, a 242-bp constitutive (*hsc71*) probe and a 350-bp inducible (*hsp70*) probe were constructed by RT-PCR amplification of mRNA isolated from non-heat shocked white muscle of *B. variegatus*. For *T. bernacchii*, a 241-bp *hsc71* probe and a 350-bp *hsp70* probe were constructed by RT-PCR amplification of mRNA isolated from non-heat shocked gill tissue of *T. bernacchii*. For all probes, reverse transcription was performed using random oligo(dT) primers, and subsequent PCR amplification was performed with consensus primers: *hsc71*, forward primer 5′-TAAGATTCTGGAGAAGTGCAA-3′ and reverse primer 5′-TCAATGGTTGGTCCAGATGAT-3′; *hsp70*, forward primer 5′-CACAAGAAGGACATCAGCCAG-3′ and reverse primer 5′-GGGTTGATGCTCTTGTTCAG-3′ (S. Lund and A. Whitmer, unpublished observations). PCR products were inserted into a pCR 2.1-TOPO vector and chemically transfected into *E. coli* using a TOPO TA Cloning kit (Invitrogen, K4500−01).

In vitro incubation of tissues across thermal gradients. For all experiments, fish were anesthetized by immersion in seawater containing 2 mM MS-222 for 10–15 min at 9°C for *N. angustata* and *B. variegatus* or −1.5 °C for *T. bernacchii* before they were killed. After being anesthetized, samples of gill and liver tissue were removed and equally segmented; one tissue segment was immediately frozen in liquid nitrogen as a control [*time 0* (t_0)], and the rest were placed into open-capped microcentrifuge tubes containing 0.4 ml MEM that had reached thermal equilibrium with the indicated temperatures. The osmolarity of the MEM was adjusted to the measured osmolarity of each species blood plasma, 340 mosM for *N. angustata*, 380 mosM for *B. variegatus*, or 560 mosM for *T. bernacchii*, before use. Tissues were incubated at the indicated temperatures for 1 h. After the 1-h thermal treatment, the tissues were immediately removed from the MEM and frozen in liquid nitrogen.

Total RNA extraction and Northern blotting. All tissue samples were held at −80°C before extraction of total RNA. Total RNA was extracted in 500 μl TRIzol for gill tissue or 750 μl TRIzol for liver tissue according to manufacturer's instructions (TRIzol, Invitrogen Life Technologies). RNA concentration and purity were determined by UV absorption (260:280 nm) using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). RNA extracts were stored at −80°C before use in Northern blotting. For Northern blotting, 10 μg total RNA was denatured with glyoxal/DMSO and fractionated by electrophoresis on a 1% agarose gel in 10 mM sodium phosphate buffer (pH 7.0) at 100 V with continuous recirculation of buffer. After electrophoresis, the fractionated RNA was vacuum transferred to Zeta Probe nylon membrane (Bio-Rad) under 5 in. Hg for 2 h using 10× standard saline citrate (SSC). The membranes were UV cross-linked once at 120,000 μJ/cm^2 using a CL-1000 UV cross-linker (UVP) before hybridization with probes.

The probes were labeled with [α-^{32}P]dCTP (1×10^{-6} Ci/ng DNA, specific activity 3,000 Ci/ mmol) using the Ready-to-Go labeling system (Amersham Pharmacia BioTech). Glyoxal adducts were removed from the membranes by incubation in 20 mM Tris·HCl (pH 8.0) at 65°C for 5 min immediately before prehybridization. Membranes were prehybridized in 20 ml Church's buffer (0.5 M sodium phosphate, 10 mM EDTA, 7% SDS) at 60°C for 3 h, followed by hybridization with labeled probe at 60°C for 18 h. After hybridization, membranes were washed twice at room temperature for 15 min with a low-stringency wash buffer [1× SSC/0.1% SDS] and once at 60°C for 20 min with a high-stringency wash buffer [0.25× SSC/0.1% SDS]. After washing, membranes were wrapped in Saran wrap and exposed to a phosphor storage screen (Molecular Dynamics) for 12–18 h. Phosphor storage screens were scanned using the Bio-Rad Personal FX imager, and densitometry was performed with Quantity One software (Bio-Rad). Relative levels of mRNA were background corrected and standardized across successive Northern blots.

Immunochemical analysis of ubiquitin conjugates. Concentrations of conjugated ubiquitin protein were determined using immunochemical analysis of gill and liver tissue collected from all three species immediately after capture using modified methods outlined in Hofmann and Somero (17). Ubiquitin conjugate standards were prepared following the methods of Haas and Bright (14). Equal amounts of protein (0.5 μg) from each sample were blotted onto prewetted nitrocellulose membrane (MSI NitroBind, 0.45 μm) in triplicate along with a dilution series of ubiquitin-conjugated protein of known concentration by gravity filtration using a BioDot dot blotter (Bio-Rad). Wells were washed three times with 200 μl of Tris-buffered saline Tween-20 (TBST) wash buffer (20 mM Tris·HCl, 140 mM NaCl, 0.01% Tween-20, pH 7.6) and heat fixed at 50°C for 20 min. Immunochemical detection was performed using an enhanced chemiluminescent protocol. After heat fixing, the membrane was blocked for 1 h in blocking solution (5% nonfat dry milk in TBST). After three 5-min washes in TBST, the membrane was incubated with a ubiquitin conjugate specific antibody for 2 h (diluted 1:5,000 in blocking solution), followed by three 5-min washes in TBST. The presence of primary antibody was detected by a 1-h incubation in horseradish peroxidase-conjugated protein A (diluted 1:2,000 in blocking solution) followed by three 5-min washes in TBST. The membrane was developed using the chemiluminescent substrate SuperSignal West Dura Extended Duration Substrate, following manufacturer's instructions (Pierce). Chemiluminescence was detected using a VersaDoc imager and quantified using Quantity One software (Bio-Rad). A linear regression was performed for the standards, and concentrations were determined using the first-order equation for the regression.

Statistical analysis. All ANOVA measurements were performed using JMP 5 software (SAS Institute) for Windows. We verified that the ANOVA assumptions of normality were met by testing the fit distribution of each dataset before performing a one-way ANOVA with a Tukey's honestly significantly different test, to test for significant differences, $P < 0.05$, between treatments. All data are presented as means ± SE unless otherwise stated in the figure legend.

RESULTS

Probe specificity. Our probes hybridized as expected and showed a high degree of specificity, differentiating between the

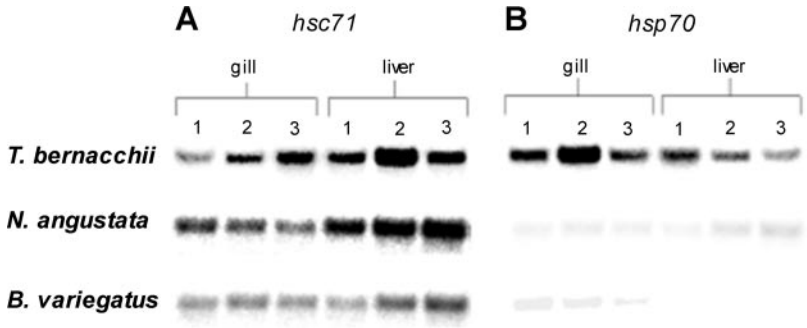

Fig. 1. Northern blot of gill and liver total RNA hybridized with *hsc71*-specific (*A*) or *hsp70*-specific (*B*) probes. *Lanes 1–3* correspond to individual field-acclimatized fish whose tissues were sampled immediately after capture and frozen in liquid nitrogen before RNA extraction. *T. bernacchii, Trematomus bernacchii; N. angustata, Notothenia angustata; B. variegatus, Bovichtus variegatus.*

constitutive and inducible transcripts in both gill and liver tissue from all species. Northern blotting performed with *hsc71*-specific probes hybridized to a single size of transcript corresponding to 2.49 kb (data not shown). Northern blotting performed with *hsp70*-specific probes hybridized with a single size of transcript corresponding to 2.67 kb (data not shown). Transcript size determination was performed by plotting the log of the molecular weight of known DNA fragments vs. the distance traveled.

mRNA levels in tissues of field-acclimatized fishes. Figure 1 shows a representative Northern blot of total RNA extracted from field-collected tissues hybridized with an [α-^{32}P]dCTP-labeled cDNA probe specific for *hsc71* (Fig. 1*A*) or *hsp70* (Fig. 1*B*). The two New Zealand species, *N. angustata* and *B. variegatus*, showed detectable levels of *hsc71* mRNA in both gill and liver sampled from fish immediately after capture (Fig. 1*A*). However, *hsp70* mRNA was not detectable above background in either tissue (Fig. 1*B*). The Antarctic notothen *T. bernacchii* displayed detectable levels of *hsc71* mRNA as well as significant levels of *hsp70* mRNA in both gill and liver tissues from wild caught fish (Fig. 1). Densitometric analysis of the Northern blots revealed the relative density of *hsc71* mRNA was over fivefold higher than the relative levels of *hsp70* mRNA in gill and liver tissues from both *N. angustata* and *B. variegatus* wild caught specimens (Fig. 2, *A* and *B*). While *hsc71* levels were comparable in the Antarctic and New Zealand species, the relative levels of *hsp70* mRNA in *T. bernacchii* were equivalent to relative levels of *hsc71* mRNA in liver tissue and twofold higher than the relative level of *hc71* mRNA in gill tissue from wild caught fish (Fig. 2, *A* and *B*).

In vitro incubation of tissues across temperature gradients. Figures 3*A* and 4*A* show a representative Northern blot of gill tissue from *N. angustata* and *B. variegatus* subjected to a temperature gradient in vitro for 1 h and probed with *hsc71*- or *hsp70*-specific cDNA, respectively. Densitometric analysis indicated levels of *hsc71* mRNA in gill tissue varied between temperature treatments when normalized to the relative density of the t_0 sample for both *N. angustata* and *B. variegatus* species, with no statically significant increases in detectable mRNA after a 1-h incubation across the temperature gradient (Fig. 3*B*, ANOVA). In contrast, for *hsp70* mRNA, increased expression was detected in both *N. angustata* and *B. variegatus* within 1 h of incubation at 18.8°C (Fig. 4*A*). When normalized to the relative density of the t_0 sample, statistically significant increases in relative *hsp70* mRNA levels are detected at 20.9°C for *N. angustata* gill tissue and 23.0°C for *B. variegatus* gill tissue (Fig. 4*B*). Relative levels of *hsp70* mRNA remained elevated up to 25.3°C, with *hsp70* mRNA levels declining after a 1-h incubation at 27.7°C (Fig. 4*B*).

Representative Northern blots of liver tissue from *N. angustata* and *B. variegatus* subjected to a temperature gradient in vitro for 1 h and probed with *hsc71*- or *hsp70*-specific cDNA are shown in Figs. 5*A* and 6*A*, respectively. Levels of *hsc71* mRNA in liver tissue varied between temperature treatments when normalized to the relative density of the t_0 sample for both *N. angustata* and *B. variegatus* species, with no signifi-

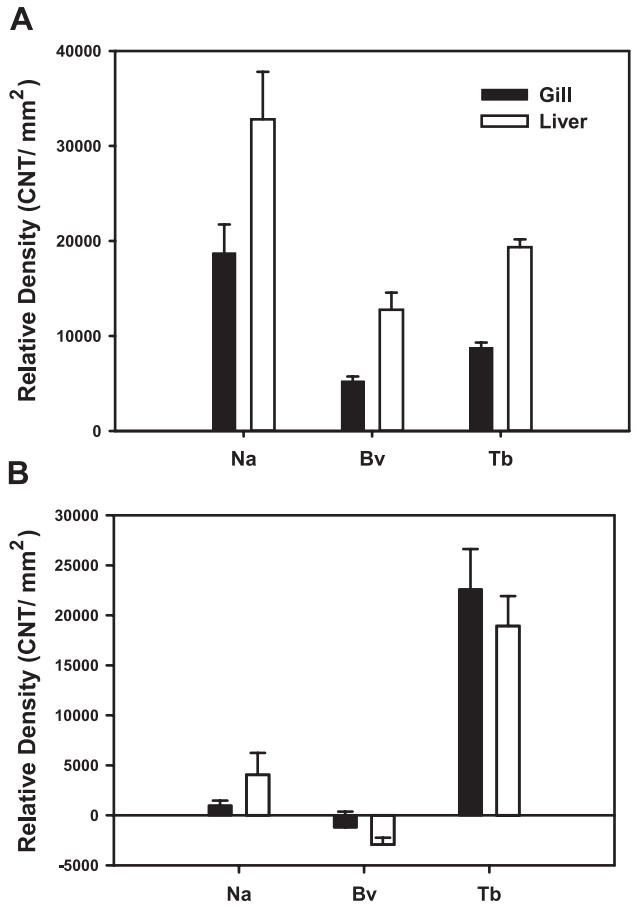

Fig. 2. *A*: densitometric analysis of relative *hsc71* mRNA levels in gill and liver tissues sampled from field-acclimatized fish immediately after capture. *B*: densitometric analysis of relative *hsp70* mRNA levels in gill and liver tissues sampled from field-acclimatized fish immediately after capture. Bars represent relative mean density ± SE for $n = 3$ fish for *N. angustata* (Na) and *B. variegatus* (Bv) and $n = 10$ fish for *T. bernacchii* (Tb).

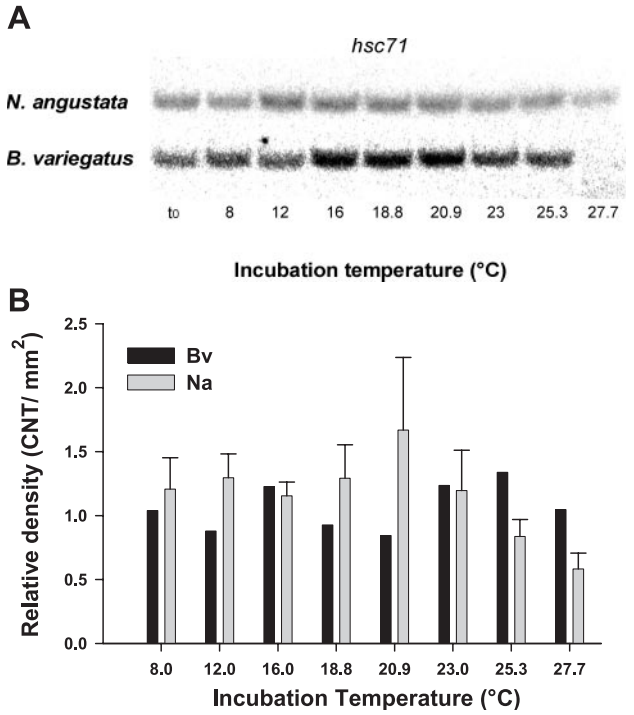

Fig. 3. *A*: Northern blot of total RNA extracted from gill tissue excised from *N. angustata* and *B. variegatus* and incubated in MEM for 1 h at the indicated temperatures and hybridized with *hsc71*-specific probe. *B*: densitometric analysis of relative *hsc71* mRNA levels in gill tissue from *N. angustata* and *B. variegatus* after a 1-h incubation at the indicated temperature. All values were background corrected and normalized to *time 0* (t_0) mRNA levels. Bars represent relative mean density ± SE for $n = 5$ fish.

cant increases in detectable mRNA after a 1-h incubation across the temperature gradient indicated in Fig. 5*B*. Increased expression of *hsp70* mRNA in *N. angustata* was detected within 1 h of incubation at 16.0°C and between 16 and 18.8°C for *B. variegatus* (Fig. 6*A*). When normalized to the relative density of the t_0 sample, significant increases in relative *hsp70* mRNA levels were detected at 18.8°C for *N. angustata* liver tissue and at 20.9°C for *B. variegatus* liver tissue (Fig. 6*B*).

Northern blots of gill and liver tissue isolated from *T. bernacchii* and subjected to a temperature gradient ranging from −2.0°C to +10°C for 1 h in vitro are shown in Figs. 7*A* and 8*A*. Northern blots in Fig. 7*A* were hybridized with *hsc71*-specific probe, and blots in Fig. 8*A* were hybridized with *hsp70*-specific probe. Relative expression of *hsc71* and *hsp70* was variable and temperature insensitive across the indicated temperature range in both gill and liver tissue of *T. bernacchii* (Figs. 7*A* and 8*A*). Densitometric analysis of Northern blots indicated a similar relative density profile of *hsc71* mRNA across the indicated temperature range in *N. angustata* and *B. variegatus* (Fig. 7*B*). However, Fig. 8*B* showed no significant increase in relative density of *hsp70* mRNA in response to increasing temperatures, indicating relative temperature insensitivity in both gill and liver tissue of *T. bernacchii*.

Ubiquitin conjugate measurements. Figure 9, *A* and *B*, shows densitometric analysis of dot blots of gill and liver tissue from all three species detected with an antibody specific to ubiquitin conjugates. Gill tissue collected from *T. bernacchii* and *B. variegatus* immediately after capture displayed similar concentrations of ubiquitin conjugates, 0.27 and 0.328 pmol/μg protein, respectively (Fig. 9*A*). In contrast, concentrations of ubiquitin conjugate in gill tissue from *N. angustata* were significantly elevated at 0.603 pmol/μg protein (Fig. 9*A*, ANOVA; $P < 0.0001$). Liver tissue from *N. angustata* also displayed elevated levels of ubiquitin conjugates (0.584 pmol/μg protein) compared with *B. variegatus* (0.092 pmol/μg protein) or *T. bernacchii* (0.275 pmol/μg protein); however, the Antarctic notothenioid also displayed significantly higher concentrations of ubiquinated proteins compared with *B. variegatus* (Fig. 9*B*, ANOVA; $P < 0.005$).

DISCUSSION

In this study, the expression of constitutive (*hsc71*) and inducible (*hsp70*) mRNA in differentially adapted notothenioid fishes was characterized in an effort to better understand the underlying mechanisms involved in the loss of the HSR in the Antarctic notothenioid *T. bernacchii* previously described by Hofmann et al. (16). Relative mRNA levels were measured in gill and liver tissue from wild caught fish and in tissues that had undergone an in vitro temperature treatment from *T. bernacchii* and two cold-temperate adapted notothenioids known to elicit a typical HSR, *N. angustata* and *B. variegatus* (Hofmann, unpublished observations). Northern blotting indicated both the constitutive and inducible forms of mRNA were expressed in all three species (Fig. 1). Despite the presence of significant

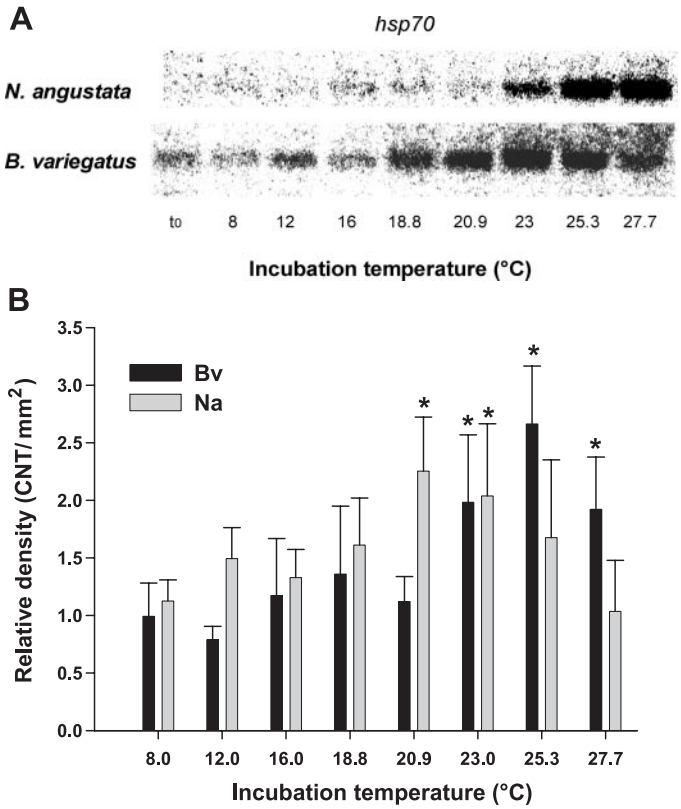

Fig. 4. *A*: Northern blot of total RNA extracted from gill tissue excised from *N. angustata* and *B. variegatus* and incubated in MEM for 1 h at the indicated temperatures and hybridized with *hsp70*-specific probe. *B*: densitometric analysis of relative *hsp70* mRNA levels in gill tissue from *N. angustata* and *B. variegatus* after a 1-h incubation at the indicated temperature. All values were background corrected and normalized to t_0 mRNA levels. Bars represent relative mean density ± SE for $n = 5$ fish. * Statistically significant increases in relative mRNA levels above the control temperature (8°C) ($P < 0.05$).

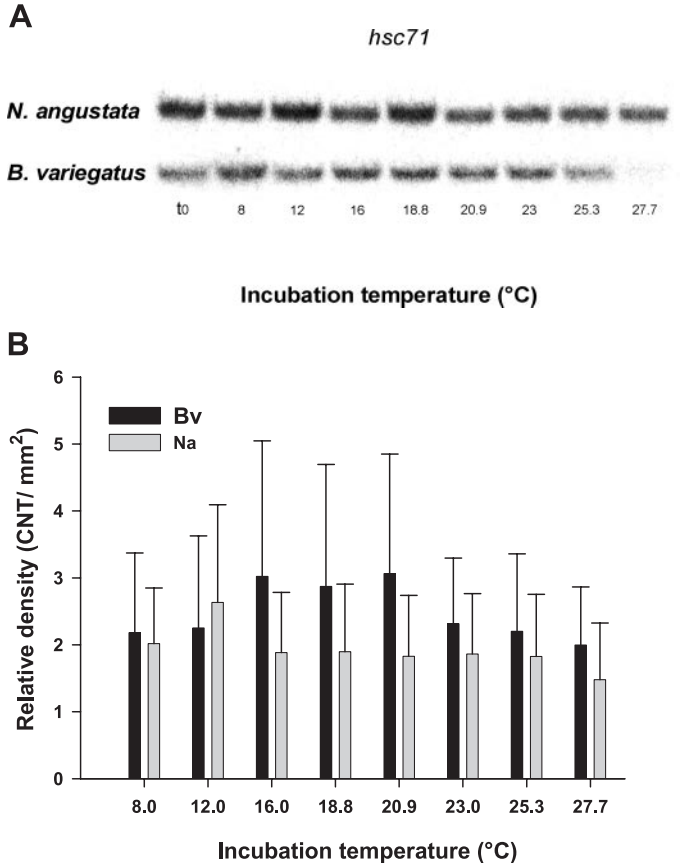

Fig. 5. *A*: Northern blot of total RNA extracted from liver tissue excised from *N. angustata* and *B. variegatus* and incubated in MEM for 1 h at the indicated temperatures and hybridized with *hsc71*-specific probe. *B*: densitometric analysis of relative *hsc71* mRNA levels in liver tissue from *N. angustata* and *B. variegatus* after a 1-h incubation at the indicated temperature. All values were background corrected and normalized to t_0 mRNA levels. Bars represent relative mean density ± SE for $n = 4$ fish.

interindividual variation in levels of mRNA, definitive trends in gene expression were detected among differentially adapted notothenioids. This study highlighted three salient findings: *1*) New Zealand notothenioids, but not the Antarctic species, maintained the ability to upregulate the *hsp70* gene in response to thermal stress; *2*) the *hsp70* gene was expressed at levels equivalent to the constitutive *hsc71* gene in wild caught specimens of *T. bernacchii*; and *3*) concentrations of ubiquitin conjugates in gill and liver tissue of *T. bernacchii* were equivalent and, in one case, significantly higher than those in tissues of the New Zealand notothenioids.

In these studies, probes developed to differentiate between *hsc71* and *hsp70* functioned as expected, hybridizing to a single size class of mRNA corresponding to 2.49 kb for *hsc71* and 2.67 kb for *hsp70* (data not shown). These transcripts were within the expected size range, with previous studies reporting *hsc70* mRNA from the zebrafish *Danio rerio* to be ~2.6 kb (35). The degree of sequence conservation in these genes between distant species allowed us to develop probes with high target specificity, further increasing our confidence that these probes hybridize with the expected transcript. In addition, these probes were tested in several species of fish and were able to differentiate between *hsc71* and *hsp70* in all species regardless of phylogenetic distance, further confirming their specificity (data not shown). In all three species used in this study, detectable levels of both transcripts were present in gill and liver tissue, indicating functional transcriptional machinery for the inducible *hsp70* gene is present in these fish (Fig. 1).

The expression of *hsc71* was variable in tissue segments of gill and liver sampled from all three species (Figs. 3*A*, 5*A*, and 7*A*). While relative levels of expression varied between individuals, the expression of the constitutive transcript did not increase in a temperature-sensitive manner (Figs. 3*B*, 5*B*, and 7*B*). Although *hsp70* mRNA was detectable in both the New Zealand and Antarctic species, only the New Zealand notothenioids have retained the ability to upregulate this gene in response to thermal stress, further highlighting the loss of the HSR in *T. bernacchii* (Figs. 4*A* and 6*A*). Both New Zealand notothenioids displayed the ability to upregulate the *hsp70* gene at 16–18.8°C, temperatures near the upper limits of their ecological range. Levels of *hsp70* mRNA increased as much as twofold at 20.9°C in gill tissue of *N. angustata* and at 23.0 °C in gill tissue of *B. variegatus* (Fig. 4*B*). Liver tissue of *N. angustata* displayed over a twofold increase in the relative *hsp70* mRNA levels at temperatures as low as 16°C (Fig. 6*B*). Relative levels of inducible transcript in gill and liver tissue from *T. bernacchii* were variable, yet insensitive to tempera-

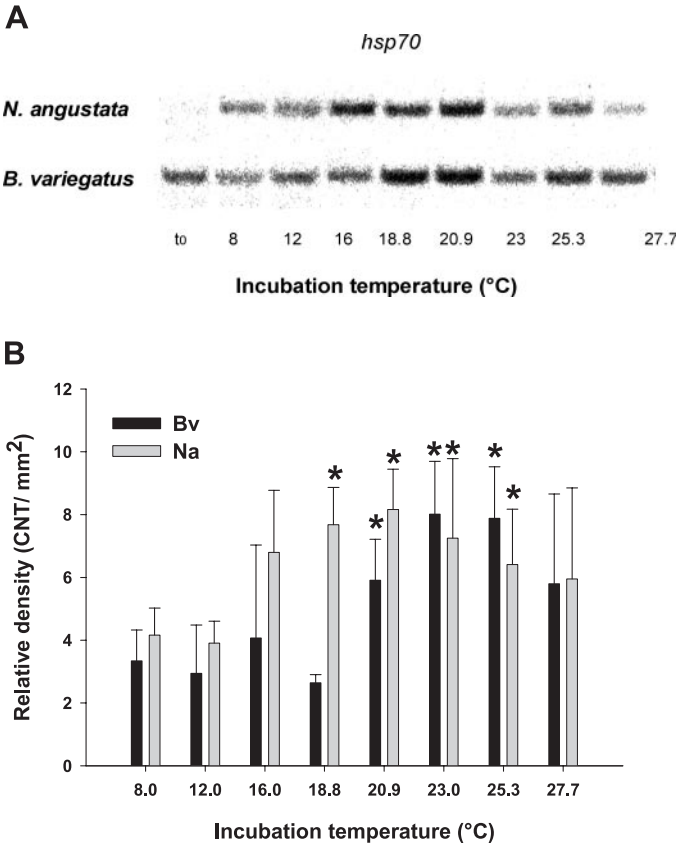

Fig. 6. *A*: Northern blot of total RNA extracted from liver tissue excised from *N. angustata* and *B. variegatus* and incubated in MEM for 1 h at the indicated temperatures and hybridized with *hsp70*-specific probe. *B*: densitometric analysis of relative *hsp70* mRNA levels in liver tissue from *N. angustata* and *B. variegatus* after a 1-h incubation at the indicated temperature. All values were background corrected and normalized to t_0 mRNA levels. Bars represent relative mean density ± SE for $n = 5$ fish. * Statistically significant increases in relative mRNA levels above the control temperature (8°C) ($P < 0.05$).

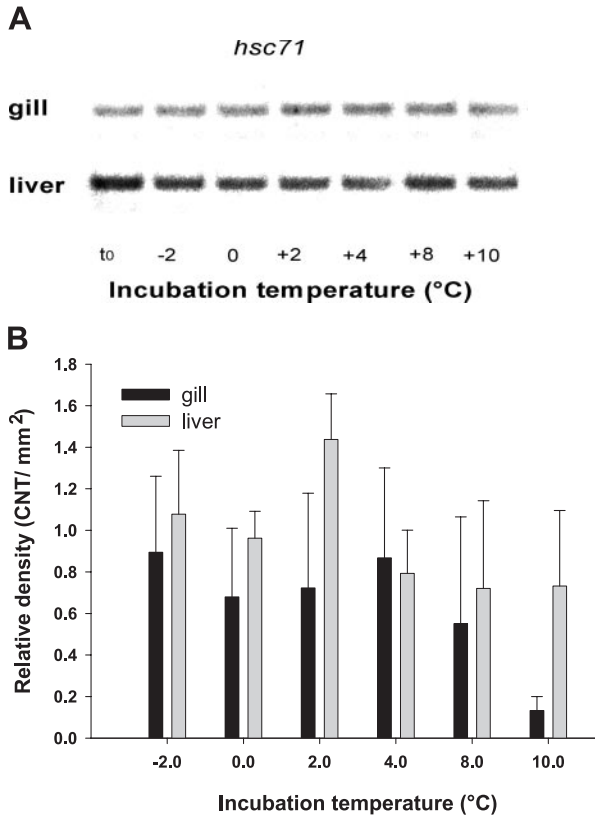

Fig. 7. *A*: Northern blot of total RNA extracted from gill and liver tissue excised from *T. bernacchii* and incubated in MEM for 1 h at the indicated temperatures and hybridized with *hsc71*-specific probe. *B*: densitometric analysis of relative *hsc71* mRNA levels in gill and liver tissue from *T. bernacchii* after a 1-h incubation at the indicated temperature. All values were background corrected and normalized to t_0 mRNA levels. Bars represent relative mean density ± SE for $n = 5$ fish.

ture increases across the temperature range of −2 to +10 °C (Fig. 8, *A* and *B*). As with the constitutive transcript, *hsp70* mRNA showed no significant upregulation in response to elevated temperature, even when assayed at temperatures 10°C above the ecologically relevant range for this species. Our findings are in agreement with earlier studies reporting the ability of *T. bernacchii* to maintain levels of Hsp70 protein pools equivalent to temperate notothenioids (5) but an inability to increase these pools after a significant heat shock event (16).

Antarctic notothenioids have been previously shown to manifest other potentially deleterious phenotypes, such as the loss of hemoglobin and myoglobin in some Antarctic notothenioids, which have persisted in the uniquely cold and stable environment inhabited by these fishes (7, 36, 38). The loss of a measurable HSR in *T. bernacchii*, however, does not appear to be the result of a persisting change in gene structure such as the presence of a pseudogene or the deletion of the gene itself. The expression of both forms of transcript in multiple tissues of *T. bernacchii* suggests the *hsp70* gene is present and functional. Results of this study suggest a possible change in the general expression of the *hsp70* gene may have occurred during the unique evolutionary history of this species. In the New Zealand species, the expression of *hsc71* was over fivefold higher than that of *hsp70* in wild caught specimens of *N. angustata* and *B. variegatus*. However, mRNA levels of *hsp70* in *T. bernacchii* were comparable to *hsc71* mRNA levels in New Zealand and Antarctic wild caught specimens (Fig. 2, *A* and *B*). These results indicate the inducible *hsp70* gene may be continuously expressed in *T. bernacchii*.

The mechanism for this constitutive expression is still not fully understood. However, there are two likely mechanisms for this occurrence: *1*) a change in the promoter region that has led to the inability of the cellular feedback mechanisms to terminate transcription, or *2*) the existence of an environmental or physiological signal that maintains the *hsp70* gene in a constant "on" state to maintain homeostasis of the cell. A wide range of seemingly unrelated stimuli can give rise to the activation of Hsp genes, and a universal consequence of all these stimuli, the build up of abnormal proteins, has been singled out as a major signal for the activation of these genes (2, 40). In an attempt to determine the prevalence of denatured or misfolded proteins in natural populations of Antarctic notothenioids, we measured the concentration of ubiquitin conjugates, a common indicator of protein damage within a cell (for reviews, see Refs. 6, 11, 19, 33). Immunochemical analysis of gill and liver tissue collected from all three species immediately after capture was performed with an antibody specific for conjugated ubiquitin molecules. Ubiquitin conju-

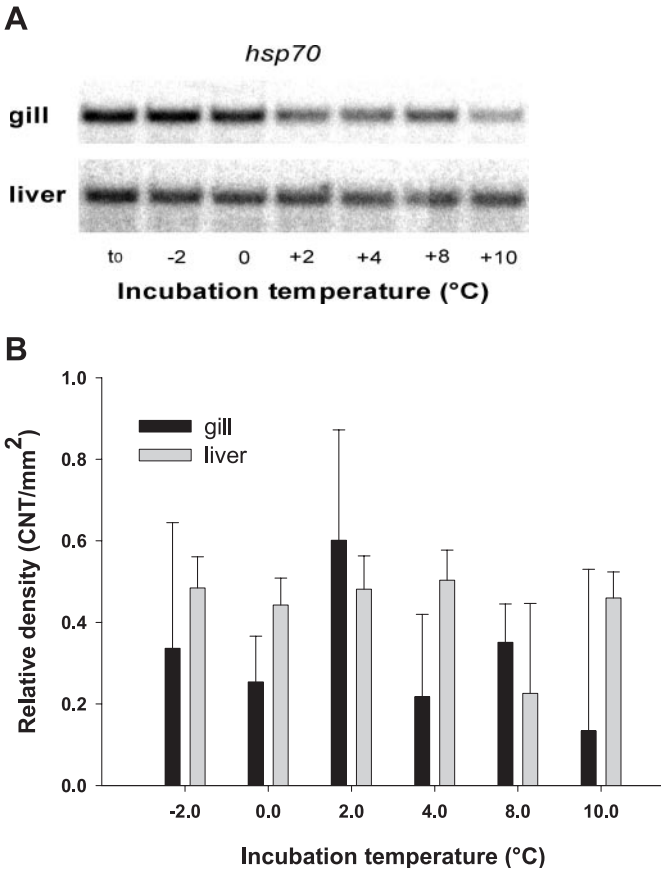

Fig. 8. *A*: Northern blot of total RNA extracted from gill and liver tissue excised from *T. bernacchii* and incubated in MEM for 1 h at the indicated temperatures and hybridized with *hsp70*-specific probe. *B*: densitometric analysis of relative *hsp70* mRNA levels in gill and liver tissue from *T. bernacchii* after a 1-h incubation at the indicated temperature. All values were background corrected and normalized to t_0 mRNA levels. Bars represent relative mean density ± SE for $n = 5$ fish.

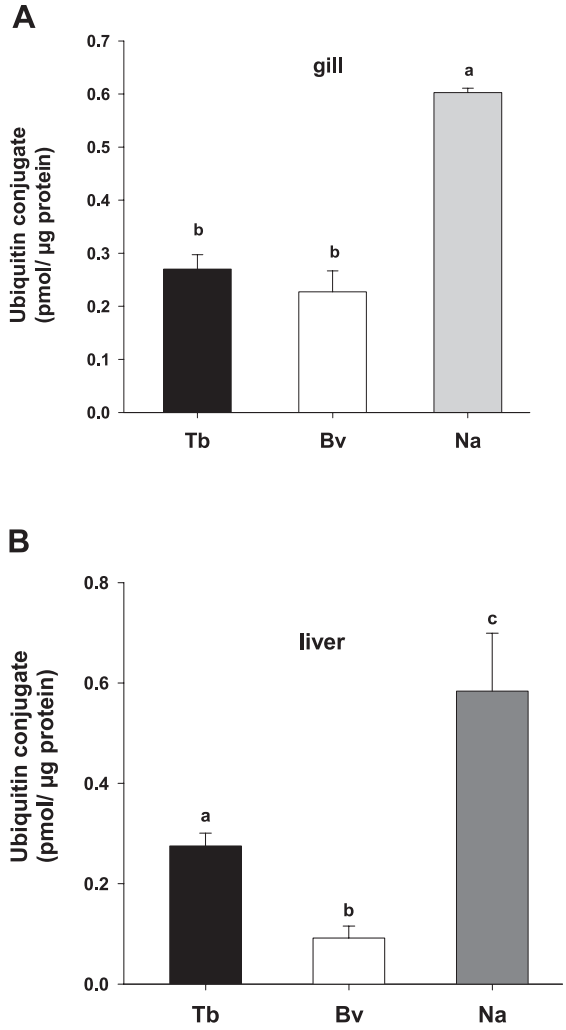

Fig. 9. *A*: concentration of ubiquitin conjugates (pmol/μg total protein) in gill tissue collected from field-acclimatized *T. bernacchii*, *B. variegatus*, and *N. angustata* immediately after capture. Bars represent pmol ubiquinated conjugates per μg total protein ± SE for $n = 10$ fish (*T. bernacchii*) or $n = 3$ fish (*B. variegatus* and *N. angustata*). Levels not connected by the same letter are significantly different (ANOVA; $P < 0.001$). *B*: concentration of ubiquitin conjugates (pmol/μg total protein) in liver tissue collected from field acclimatized *T. bernacchii*, *B. variegatus*, and *N. angustata* immediately after capture. Bars represent pmol ubiquinated conjugates per μg total protein ± SE for $n = 10$ fish (*T. bernacchii*) or $n = 3$ fish (*B. variegatus* and *N. angustata*). Bars not connected by the same letter are significantly different (ANOVA; $P < 0.005$).

gate concentrations, significantly higher in liver tissue of *T. bernacchii* than the New Zealand notothenioid *B. variegatus*, indicate these fish may be experiencing equivalent if not higher levels of denatured proteins in the wild than similar sized New Zealand notothenioids (Fig. 9, *A* and *B*). Specimens of the New Zealand notothenioid *N. angustata* showed significantly higher concentrations than the other species used in this study; however, the reason for these differences is not yet known. Furthermore, analysis of ubiquitin conjugate concentrations in gill and liver tissue of a second Antarctic notothenioid, *Pagothenia borchgrevinki*, also showed elevated levels of protein damage in field-collected specimens relative to *T. bernacchii* and *B. variegatus*, further suggesting an increased need for continuous expression of molecular chaperones in Antarctic notothenioids (data not shown). In addition, because molecular chaperones are known to assist in protein degradation via the ubiquitin-proteasome pathway (12, 15, 23, 28), constitutive recruitment of *hsp70* may be driven by these relationships. For example, Hsp70 has been shown to be directly involved with the ubiquitin proteasome pathway through the directed interactions of BAG-1, a ubiquitin related cochaperone of Hsp70 (25).

Overall, our data suggest the subzero environment inhabited by Antarctic notothenioids may be more perturbing to the formation and maintenance of native protein structures than once thought; however, to what extent extreme cold temperatures are denaturing to native protein conformations is still debated among biochemists. As early as 1930, scientists recognized that extreme cold temperatures can perturb the quaternary and tertiary structure of proteins, as seen with the increased rate of chemical denaturation of ovalbumin when performed at 0°C (18, 37). While the mechanism of cold denaturation is still not fully understood, temperatures at or near those encountered by Antarctic notothenioids have been shown to perturb protein structure for numerous proteins, especially those known to form multimers (for reviews, see Refs. 21, 27, 34). In addition to biochemical studies of structure-function relationships, several physiological studies have begun to show Hsps can be induced by an acute cold stress in response to increased denaturation and misfolding of proteins (for a review, see Ref. 39). For example, human cells subjected to a 4°C cold shock upregulated *hsp70* transcript after returning to 37°C for recovery. In addition, the onset, magnitude, and duration of induction were directly related to the severity of the cold shock (24). In another mammalian system, *hsp70* mRNA was induced in cultured neonatal rat cardiomyocytes subjected to a 4°C cold shock for 1 h and resulted in a sixfold increase in Hsp70 protein levels in protein extracts (22). More recently, Ali et al. (1) reported in carp acclimated to 12°C that a temperature drop of 7 °C for 1 h resulted in the upregulation of the inducible *hsp70* transcript in both liver and white muscle. Finally, yeast have been shown to upregulate a variety of molecular chaperones (in this case, Hsp104, Hsp42, Hsp12, and Ssa4) after exposure to near freezing temperatures (20).

Studies reporting the denaturing effects of subzero temperatures along with our current findings indicate the Antarctic notothenioid *T. bernacchii* may be living in an environment perturbing to native protein structure and therefore would be subject to high levels of denaturing or misfolded proteins. This increased need for molecular chaperone function in vivo, coupled with the removal of the selective pressures of thermal variation, may have led to the conversion of the inducible *hsp70* gene to a constitutive role. Analysis of the promoter region in these genes is necessary to verify the mechanism for this observed change in expression. In addition, similar analysis in other species as well as other molecular chaperone families such as *hsp90* and *hsp40* will help determine the extent of this potentially adaptive response in Antarctic notothenioids.

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