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Some like it hot, some like it cold: the heat shock response is found in New Zealand but not Antarctic notothenioid fishes

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Abstract

Previous research on Antarctic notothenioids has demonstrated that cells of cold-adapted Antarctic notothenioids lack a common cellular defense mechanism called the heat shock response (HSR), the induction of a family of heat shock proteins (Hsps) in response to elevated temperatures. The goal of this study was to address how widespread the loss of the HSR is within the Notothenioidei suborder and, specifically, to ask whether cold temperate non-Antarctic notothenioids possess the HSR. In general, Antarctic fish have provided an important opportunity for physiologists to examine responses to selection in the environment and to ask whether traits of the notothenioids represent cold adaptation, or whether the traits are related to history and are characteristics of the notothenioid lineage. Using in vivo metabolic labeling, results indicate that one of the two New Zealand notothenioids possess an HSR. The thornfish, *Bovichtus variegatus* Richardson, 1846, expressed heat shock proteins (Hsp) in response to heat stress, whereas the black cod, *Notothenia angustata* Hutton, 1875, did not display robust stress-inducible Hsp synthesis at the protein-level. However, further analysis using Northern blotting clearly demonstrated that mRNA for a common Hsp gene, *hsp70*, was present in cells of both New Zealand species following exposure to elevated temperatures. Overall, combined evidence on the HSR in notothenioid fishes from temperate New Zealand waters indicate that the loss of the HSR in Antarctic notothenioid fishes occurred after the separation of Bovichtidae from the other Antarctic notothenioid families, and that the HSR was most likely lost during evolution at cold and constant environmental temperatures.

Keywords: Antarctica; *Bovichtus variegatus*; Heat shock proteins; Heat shock response; Notothenioidei; *Notothenia angustata*

1. Introduction

Increasingly, the fish fauna of coastal Antarctica are being recognized as an important example of adaptive radiation (Johns and Avise, 1998; Eastman, 2000; Eastman and McCune, 2000; Bargelloni et al., 2000; Montgomery and Clements, 2000; Clarke and John-

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ston, 1996; Eastman, 1993). A single group of highly endemic marine fishes, the Antarctic notothenioids (suborder Notothenioidei), dominates the Antarctic coastal shelf waters and the ecological success of the radiation is thought to be related to the evolution of antifreeze glycoproteins (Eastman, 1993; Chen et al., 1997). While the Antarctic notothenioids are exemplary of the action of positive selection, these fish also provide examples for the evolutionary loss of gene function under a particular selection regime (Somero et al., 1998; Montgomery and Clements, 2000). Most notably, the loss of haemoglobin and myoglobin expression in the icefish (Family Channichthyidae) are examples of traits that were lost during evolution at subzero water temperatures (Ruud, 1954; Hamoir, 1988; Cocca et al., 1995; Zhao et al., 1998; Sidell et al., 1997; Moylan and Sidell, 2000; Montgomery and Clements, 2000). In addition to the loss of oxygen-carrying blood proteins, members of the Antarctic notothenioids lack a heat shock response (HSR), the highly coordinated transcriptional activation of a set of genes that encode the heat shock proteins (Hsps), in response to high temperature stress (Hofmann et al., 2000; Buckley et al., 2004; Place et al., 2004; Place and Hofmann, 2004). The goal of the current study was to address how widespread the loss of the HSR is within the Notothenioidei suborder and, specifically, to ask whether cold temperate non-Antarctic notothenioids possess the HSR. At present, there is no indication as to whether the lesion in gene expression is an ancestral condition, or whether the event occurred in the Antarctic members of the Notothenioidei after they were isolated in the subzero waters of the Southern Ocean by the formation of the Antarctic Polar Front (see Eastman, 1993).

What phylogenetic evidence supports the selection of the comparative non-Antarctic species that would address the question of how the HSR is distributed throughout the phylogeny of the notothenioid fishes? Currently, the taxonomy, systematics and phylogenetics of notothenioid fishes are active areas of research with new species being discovered every year (e.g., Eastman and Eakin, 2000; Eastman, 2000; Eastman and McCune, 2000; Bargelloni et al., 2000; Balushkin, 2000). However, although the systematics of the suborder Notothenioidei is itself evolving (Dettaï and Lecointre, 2004; Near, 2004), strong support can be found for choosing two New Zealand species as appropriate comparative study organisms. These fish are: the black cod *Notothenia angustata*

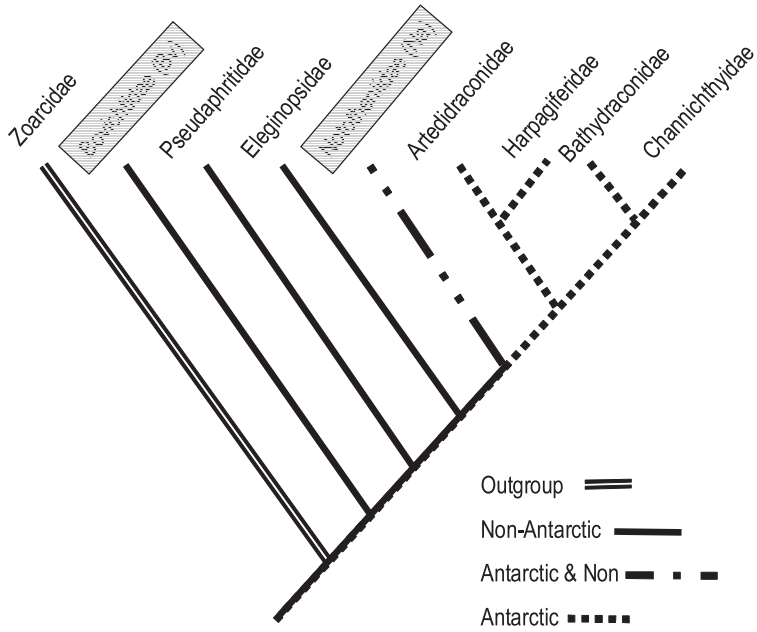

Fig. 1. Phylogenetic relationships of the suborder Notothenioidei. Redrawn from Lecointre et al. (1997). Shaded boxes indicate the family classification and abbreviation of the two New Zealand study species; Bv=*B. variegatus*, Na=*N. angustata*.

Hutton, 1875 and the thornfish *Bovichtus variegatus* Richardson, 1846 (Balushkin, 2000). These two species are among several non-Antarctic notothenioids that occupy the waters of New Zealand, Australia and South America where water temperatures are at least 5 °C warmer than those encountered by Antarctic species (Johnston et al., 1998). In this study, the New Zealand species were selected for their informative phylogenetic position and because they were readily collected in areas that would allow experimental lab work to be performed (Fig. 1).

N. angustata, the black cod, occurs in coastal waters of the South Island of New Zealand (Ayling and Cox, 1982; Fig. 2) and has a completely non-Antarctic distribution. *N. angustata* is a member of the family Nototheniidae, the most speciose family in the suborder in which most of the members have Antarctic distributions (Balushkin, 2000). The intertidal thornfish, *B. variegatus*, has a distribution similar to the black cod (Ayling and Cox, 1982; Fig. 2) but is classified in Bovichtidae, a family that has a completely non-Antarctic distribution (Balushkin, 2000). Notothenioid molecular phylogenetics place *B. variegatus* as a distant relative of the other notothenioid families (Fig. 1). Some recent research suggests that the thornfish may not be any more closely related to the Antarctic notothenioids than are other outgroup perciform fish (Lecointre et al., 1997). However, *N. angustata* has recently been placed well within the Nototheniidae (Cheng et al., 2003). Despite

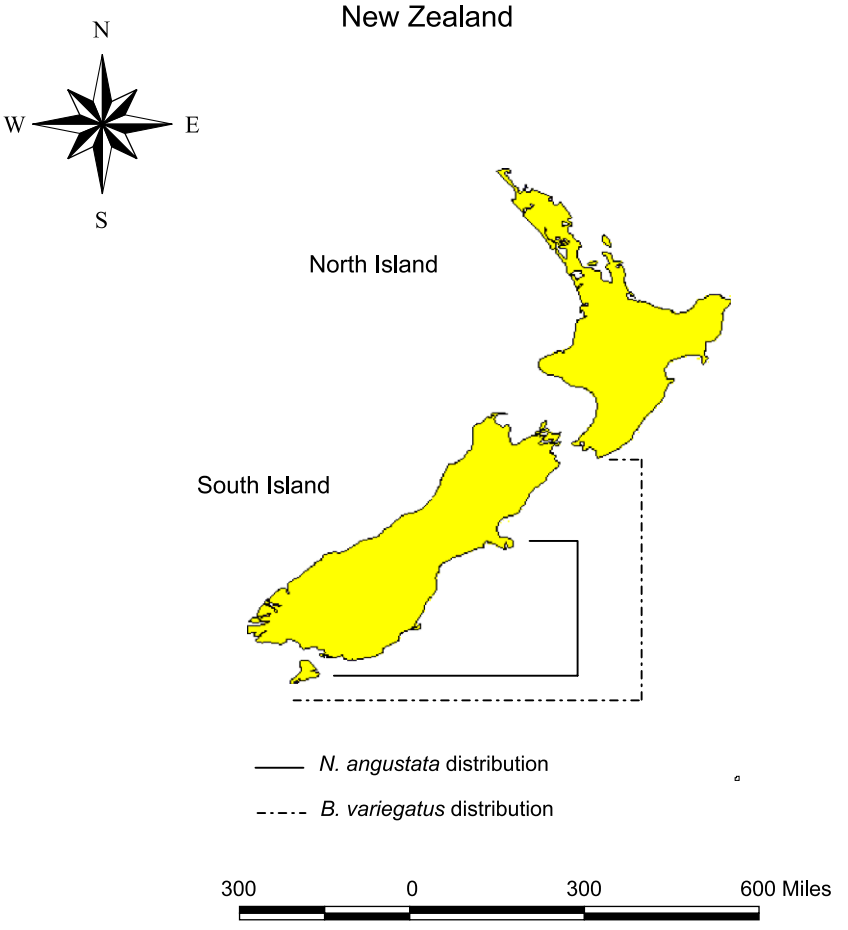

Fig. 2. Latitudinal distribution of the black cod, *N. angustata* (solid line), and the thornfish, *B. variegatus* (broken line), on the South Island of New Zealand. Both species are found in the coastal waters of the sub-antarctic islands to the south of New Zealand (not shown on this map; Ayling and Cox, 1982).

different phylogenetic positions in the notothenioid tree, both of the New Zealand notothenioids are cold temperate species that experience a fluctuating thermal environment as compared to the Antarctic notothenioids; New Zealand nearshore seawater temperatures range from 8 to 15 °C, whereas coastal Antarctic waters rarely vary from the freezing point of seawater, −1.86 °C.

Despite the fact that the Antarctic seawater is not particularly stressful, in terms of high heat content, it is very unusual to discover an organism that does not synthesize Hsps in response to heat stress. The HSR is a ubiquitous gene activation event that occurs in almost every organism examined (Parsell and Lindquist, 1993) and, to date, few other organisms have been shown to lack this cellular mechanism. A freshwater hydra, *Hydra oligactis*, has been found to fail to synthesize Hsps in response to high temperature stress (Bosch et al., 1988). Similarly, La Teraza et al. (2001) reported the Antarctic ciliate *Euplotes focardii* did not show appreciable activation of the *hsp70* gene at the transcriptional level in response to thermal stress. All the evidence suggests that the HSR is part of a larger cellular defense mechanism that enables organisms to tolerate fluctuations in the thermal environment, regardless of the absolute temperatures that are experienced (for a review see Feder and Hofmann, 1999). In general, Hsps are activated at environmentally relevant temperatures and this gene induction tends to occur at 5–10 °C above the average environmental temperature for that particular animal (Parsell and Lindquist, 1993). Although the activation of Hsp genes in response to elevated temperatures is a universal gene activation event in organisms, significant plasticity has been observed in studies of natural populations of ectothermic animals. Patterns have emerged that correlate Hsp induction temperatures with the thermal history of the organism on many time scales, including short-term acclimation (Dietz, 1994; Hofmann and Somero, 1996; Roberts et al., 1997; Yu et al., 1998; Tomanek and Somero, 1999; Wood et al., 1999; Currie et al., 2000; Buckley et al., 2001; Tomanek and Somero, 2002; Buckley and Hofmann, 2002), seasonal variation as experienced in the field (Dietz and Somero, 1992; Fader et al., 1994; Hofmann and Somero, 1995; Roberts et al., 1997; Chapple et al., 1998; Buckley et al., 2001) and on evolutionary time-scales as indicated by species' differences (Dietz and Somero, 1992; Hofmann and Somero, 1996; Tomanek and Somero, 1999). Thus, the Antarctic notothenioids represent the most extreme case of plasticity of the HSR: it has been lost altogether.

The specific objective of this study was to determine whether the New Zealand notothenioid fishes synthesized Hsps in response to heat stress. This was assessed using metabolic labeling techniques that allowed the detection of novel proteins, presumably Hsps that were synthesized in response to heat stress, and Northern analysis to detect the presence of mRNA for specific Hsps. One of the predictions of the study was that the New Zealand fish would induce Hsps in response to heat stress since they do encounter moderate fluctuation in environmental temperature and probably have done so for millions of years.

2. Materials and methods

2.1. Collection of study organisms

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). *N. angustata* Hutton, 1875, the black cod, is a benthic inshore species and was caught in traps placed on the substrate at depths of 2 to 10 m. The tidepool thornfish, *B. variegatus* Richardson, 1846, was collected by hand in pools located near Portobello at Allans Beach, Blackhead, and Mapoutahi in Purakanui Bay. Following capture, fish were maintained in aquaria at ambient seawater temperatures (~9 °C) for several weeks prior to sacrifice.

2.2. Hsp induction experiments

Hsp induction experiments were conducted according to previously published protocols (see Hofmann et al., 2000) with some modification. Prior to dissection, fish were anesthetized in MS-222 (~0.1 g/g body mass) dissolved in 8 °C seawater. Once the fish were anesthetized, samples of gill and liver tissue were removed and placed into microcentrifuge tubes containing 0.4 ml chilled (4 °C) minimum essential

medium (MEM) with an osmolarity adjusted to 340 mosM for *B. variegatus*, and 380 mosM for *N. angustata*, the concentrations equivalent to the osmolarity of the fish plasma. Tissue segments were metabolically labeled for 2 h in 50 μCi of ^{35}S methionine/cysteine cell labeling mixture (Easytag; NEN) at temperatures that ranged from 14 to 32 °C. Following the 2 h labeling period, the tissue segments were washed twice with 1.0 ml of chilled MEM and homogenized in buffer (50 mM, pH 6.8, 4% SDS) containing a protease inhibitor cocktail (complete™; Boehringer Mannheim). Homogenates then were heated at 100 °C for 5 min and centrifuged for 15 min at 12,000×*g*. The resulting supernatant was removed and stored at −20 °C prior to SDS-polyacrylamide electrophoresis.

2.3. SDS-PAGE and autoradiography

De novo synthesized radioactive proteins were separated on 10% SDS-polyacrylamide gels. Each separate sample was loaded at equivalent amounts of radioactivity, approximately 300,000 cpm. Prior to drying at 80 °C for 1 h, the gels were treated with an autoradiographic enhancer according to the manufacturer's instructions (Amplify™, Amersham Pharmacia). Dried gels were exposed to X-ray film (Kodak XAR 5) at −70 °C for 48 h.

2.4. RNA extraction and quantification

After the fish were anesthetized as previously described, samples of gill tissue were removed and one tissue segment was immediately frozen in liquid nitrogen as a control (t_0), the rest were placed into microcentrifuge tubes containing 0.4 ml chilled (4 °C) minimum essential medium (MEM) with an osmolarity adjusted to 340 or 380 mosM as described above. Gill tissues were heat shocked for 1 h at 23 °C. Following the 1 h heat shock, the tissues were immediately removed from the MEM and total RNA was extracted in 500 μl TRIzol™ according to manufacturer's instructions (Invitrogen life technologies). RNA concentration and purity was determined by UV absorption at 260:280 using a NanoDrop ND-1000 spectrophotometer (NanoDrop technologies). RNA extracts were stored at −70 °C prior to use in Northern blotting.

2.5. Probe development

Hsp probes were generated using first strand cDNA synthesized from total RNA extracted from non-heat shocked *B. variegatus* white muscle tissue using M-MuLV reverse transcriptase and random primers. Hsp70 forward and reverse primers were designed by determining highly homologous regions from the alignment of Hsp70 genes of 5 fish species (*O. tschawytscha*, *D. rerio*, *X. maculatus*, *O. latipes* and *O. mossambicus*) with the Hsp70 genes of three mammals (*M. musculus*, *R. norvegicus* and *H. sapiens*), all obtained from GenBank and aligned using Clustal W (version 1.8). The Hsp70 probe was a 350 bp internal segment of the *B. variegatus* Hsp70 coding region resting between nucleotides 752 and 1101 of the *O. mossambicus* Hsp70 gene (GenBank accession no. AJ001312; Molina et al., 2000). This probe was amplified by PCR at an annealing temperature of 60 °C using the forward primer 5′-CAC AAG AAG CAC ATC AGC CAG and the reverse primer 5′-GGG TTG ATG CTC TTG TTC AG. Primers used to amplify the 242 bp heat shock cognate (Hsc71) probe were as previously described for rainbow trout Hsc71 (Currie et al., 1999). The Hsc71 probe was amplified by PCR at an annealing temperature of 50 °C, followed by a second PCR under the same conditions using 1 μl of the first reaction as a template. All PCR reactions involved an initial denaturation (94 °C×30 s) followed by 30 cycles of: 94 °C, 30 s; annealing temperature, 1 min; 72 °C, 1.5 min, followed by a final extension for 15 min at 72 °C. The resulting PCR products were ligated into pCR 2.1 (Invitrogen) and sequenced. The identities of the probe sequences were confirmed through NCBI BLAST analysis and Northern blotting using RNA extracted from control and heat shocked liver tissue from *Gillichthys mirabilis*, a fish known to exhibit a characteristic Hsp70 response under heat shock conditions (see Table 1 for data on probe hybridization performance).

2.6. Northern blotting

A 10 μg aliquot of extracted RNA was fractionated by glyoxal/dimethylsulfoxide (DMSO) denaturing electrophoresis on a 1% agarose gel and vacuum transferred to Zeta probe nylon membrane (BioRad)

Table 1
Target species in which Hsp70 and Hsc71 probes (generated through PCR using *B. variegatus* white muscle tissue cDNA) hybridized with extracted RNA examined by Northern analysis, and the presence (+) or absence (−) of an inducible Hsp/Hsc mRNA response for each species

Probe	Target species	Cross-reactivity	Inducibility
B. variegatus Hsp70	*G. mirabilis*	+	+
	B. variegatus	+	+
	N. angustata	+	+
	T. bernacchii	+	−
B. variegatus Hsc71	*G. mirabilis*	+	−
	B. variegatus	+	−
	N. angustata	+	−
	T. bernacchii	+	−

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) prior to hybridization with probes.

The probes were labeled with [^{32}P]-dCTP [1×10^{-6} Ci/ng DNA, specific activity 3000 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 prior to prehybridization. Membranes were prehybridized in 20 ml Church's buffer [0.5 M $NaPO_4$, 10 mM EDTA, 7% sodium dodecylsulfate (SDS)] at 60 °C for 3 h, followed by hybridization with labeled probe at 60 °C for 18 h. Following 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]. Following washing, membranes were wrapped in Saran Wrap® and exposed to a phosphor screen for 12–18 h. Phosphor screens were scanned using the BioRad Personal FX imager and densitometry was performed with Image-Quant software (BioRad).

3. Results

3.1. Hsp induction experiments: in vitro metabolic labeling of fish tissues

Assessment of the presence or absence of a heat-shock response is often made using metabolic labeling with radiolabeled amino acids (Lindquist, 1986) and this method has been successfully used for isolated tissue preparations from fish (see Buckley and Hofmann, 2002). Using these methods, evidence for heat stress-inducible synthesis of Hsps was found in tissues of *B. variegatus* but the apparent synthesis of Hsps was not as evident in *N. angustata*. Fig. 3 shows the protein synthesis patterns in gill and liver in the two non-Antarctic notothenioids. For two specimens of *B. variegatus* gill, novel proteins were synthesized at 26 °C with additional bands appearing at 30 °C (Fig. 3A). Novel bands were also induced in *B. variegatus* liver, but this pattern was more difficult to discern due to the high fat content of the liver tissue (Fig. 3A). No unique bands were observed in gill from *N. angustata* (Fig. 3B), although there is the suggestion of a novel band at 30 °C, and a slight increase in the synthesis of a few prominent bands on the autoradiographs (possibly due to induction or to the direct effects of temperature on translation). Similarly, no pattern of Hsp induction was observed in liver from *N. angustata* (Fig. 3B). Although the liver preparations exhibited a few new proteins during metabolic labeling, the number of proteins and the pattern was not as profound as was observed in gill tissue.

3.2. Northern analysis

Another technique used to assess the presence or absence of a heat shock response is Northern blotting. This method allows for detection of low-level heat shock protein transcript that may or may not lead to protein translation. Representative Hsc71 and Hsp70 Northern bands from experiments exposing isolated *B. variegatus* and *N. angustata* gill tissue to both control (9 °C; C) and heat shock (23 °C; HS) conditions are shown in Fig. 4. These Northern bands display the characteristic Hsc71 and Hsp70 mRNA-level responses that would typically be seen during control and acute heat shock conditions in tissues isolated from more temperate-ranging fish (Dietz and Somero, 1993; Currie et al., 2000; Lund et al., 2002, 2003). These bands show that Hsc71 mRNA levels in the livers of both New Zealand species are unaltered by a 1-h heat shock. In contrast to the metabolic labeling experiments, the Northern results for both *B.*

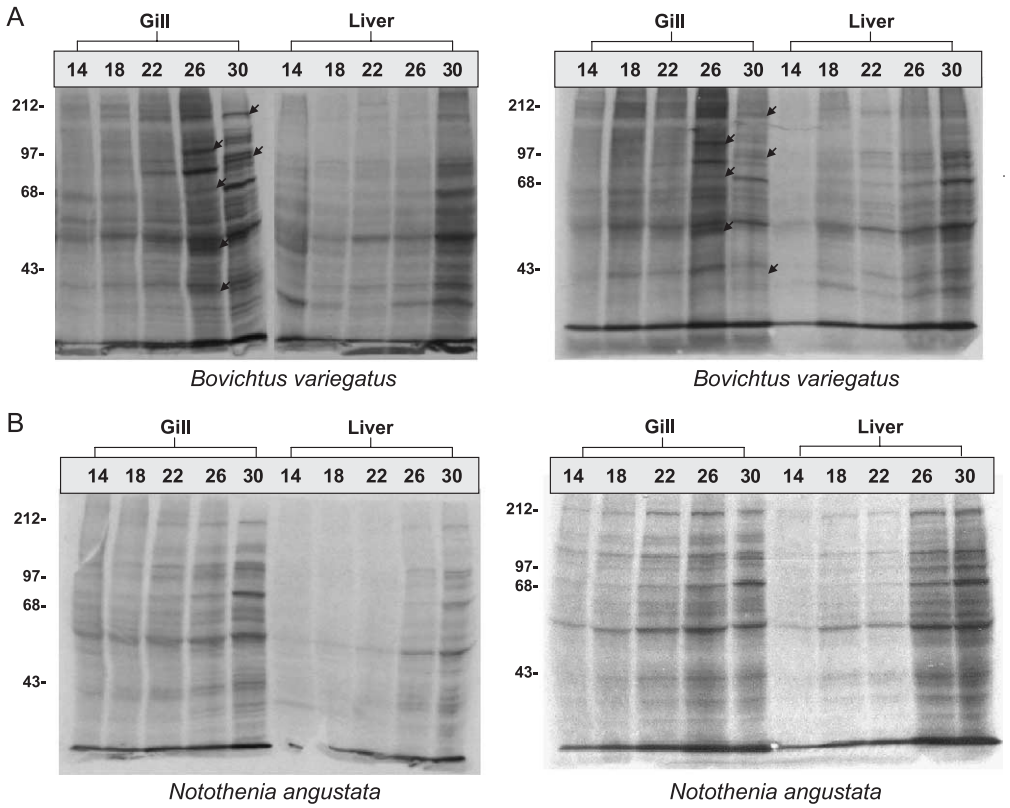

Fig. 3. Autoradiograms of representative protein synthesis patterns in liver and gill tissue of the New Zealand notothenioid fishes, *B. variegatus* (A) and *N. angustata* (B). Proteins were radiolabeled during in vitro metabolic experiments with ^{35}S methionine/cysteine at the temperatures (°C) shown in the shaded box above each lane. After radiolabeling, proteins in tissue extracts were separated on 10% SDS-polyacrylamide gels; gels were treated with an autoradiographic enhancer, dried and exposed to X-ray films. Molecular mass markers are denoted on the left in kDa. Novel bands induced with increasing heat shock temperature in *B. variegatus* gill tissue are indicated by arrows.

variegatus and *N. angustata* show a definitive and marked increase in levels of Hsp70 mRNA, in comparison to control samples, over a 1 h heat shock at 23 °C.

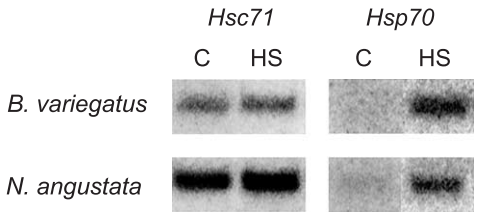

Fig. 4. Northern blot analysis of Hsc71 and Hsp70 mRNA in gill from *B. variegatus* and *N. angustata* during both control (9 °C, C) and heat shock (23 °C, HS) conditions.

4. Discussion

A working hypothesis that resulted from prior research was that the Antarctic species had experienced an evolutionary loss of the HSR under the cold and stable environmental temperature regime of the Antarctic marine ecosystem (Hofmann et al., 2000; Place et al., 2004; Buckley et al., 2004; Place and Hofmann, 2004). It was predicted that the New Zealand notothenioid fishes, not having been exposed to the same selection pressures as the Antarctic fish and living in a comparatively thermally heterogeneous environment, would retain the HSR. Furthermore, in keeping with the observation that all organisms induce Hsps as a cellular stress response and that induction occurs at temperatures 5–10 °C above average habitat

temperatures, it was predicted that the New Zealand species would synthesize Hsps in response to temperatures of approximately 20 °C. On the whole, this prediction was largely supported by the results of the present study.

The salient findings were as follows: Hsps were synthesized on the transcript level in tissues from both *B. variegatus*, the most distantly related species, and *N. angustata*, a more closely related species that is within a predominantly Antarctic notothenioid family (see Fig. 4). In contrast, metabolic labeling studies showed that while *B. variegatus* exhibited a classic heat-stress induced synthesis of Hsps, this response appeared to be significantly attenuated in the tissues of *N. angustata* that were examined (see Fig. 3). These results are significant in that they support the idea that Antarctic fish lost the HSR after evolution at constant and cold temperatures.

What are the biological consequences of not having a HSR? The HSR is an emergency gene induction that directly increases the pool of molecular chaperones in response to thermal stress (Lindquist, 1986; Feder and Hofmann, 1999). Since proteins are known to unfold in response to elevated temperatures, the presence of additional Hsps helps cells cope with the elevated demands of protein chaperoning (for reviews see Parsell and Lindquist, 1993; Fink, 1999; Hartl and Hayer-Hartl, 2002). The Hsps are also important because of the tendency of proteins to aggregate when they unfold, or are in nonnative conformations (Jaenicke and Seckler, 1998; Buccantini et al., 2002). Thus, under heat stress there is a dual driving force that endangers proteins—the tendency to unfold and the elevated chance of aggregation. Since Antarctic fish are removed from the thermal denaturation of proteins, the requirement for an inducible gene activation may not be necessary as there are numerous other cognate chaperones that are present in Antarctic species (Carpenter and Hofmann, 2000). These chaperones are significant because, given that most new proteins require some chaperone assistance to fold properly (Frydman, 2001), Antarctic fish still require cognate members of the HSP gene families in order to perform protein biosynthesis.

Mechanistically, it is not clear how the Antarctic fish lost the HSR, or how the exact gene expression mechanisms in the New Zealand fish differ from those of the Antarctic species. There are, however, several possible mechanisms. One possibility is that *hsp* transcript is unstable and has a very short half-life. This situation has been observed in *H. oligactis* where the loss of the induction of a 70 kDa class Hsp was attributed to unstable Hsp mRNA (Gellner et al., 1992). Whether this mechanism also accounts for the loss of Hsp synthesis in cells of both the Antarctic notothenioids and the New Zealand relative, *N. angustata*, is unknown but under investigation. An alternative explanation to loss of transcript is that transcriptional activation is abnormal in these fish. This mechanism may involve the loss of the activity of heat shock factor 1 (HSF1), the transcription factor that controls the expression of all the stress-inducible Hsps. Preliminary data suggest that HSF1 is present in cells of Antarctic fish (Buckley et al., 2004) and that HSF1 may not respond to heat stress as the factor does in other fish cells (see Buckley and Hofmann, 2002). Finally, evolution at cold temperatures may have altered the promoter architecture of the *hsp70* gene in the Antarctic fishes as compared to the New Zealand notothenioids. We are currently exploring this avenue of exploration to determine the mechanism underlying the differences in expression of stress-inducible genes within the Notothenioidei. Interestingly, La Terza et al. (2004) has examined the promoter region of the *hsp70* gene from the Antarctic ciliate *E. focardii* and reported that the gene contained both *cis*-acting regulatory elements involved in the thermal response of these molecular chaperone genes, however, the functionality of these elements had not been determined.

This investigation of the absence of the HSR and the question of the state of this trait in non-Antarctic notothenioids joins a cluster of studies focused on the evolution of function in Antarctic fish and the timing at which these traits evolved relative to the speciation events of the suborder. Most of this interest has been driven by a desire to understand the nature of cold-adaptation in Antarctic fishes. From a practical standpoint, the availability of comparative species that evolved at very different temperatures has made this an outstanding study system. Most notable among the cold-adaptation studies are the examinations of antifreeze proteins (e.g., DeVries, 1988; Chen et al., 1997), metabolism (e.g., Johnston et al., 1998), and cardiovascular function (e.g., Egginton et al., 2001). Since there is no fossil record for this group, all estimates are based

upon molecular phylogenies for the notothenioids. Inferences about when the Antarctic notothenioids may have lost the HSR can be garnered from the molecular phylogenies that exist for the suborder Notothenioidei. Most of the notothenioid taxa are of recent origin and are thought to have appeared subsequent to the isolation of the Antarctic continent, about 25–22 MYA (Eastman, 1993; Bargelloni et al., 2000). The geographic and oceanographic isolation of Antarctica is thought to have provided ancestral notothenioids with a unique cold and stable environment that fostered the notothenioid radiation (Eastman, 1993).

The most current molecular phylogenies support this hypothesis and estimate that the ancestral notothenioid stock was split by the formation of the Polar Front and the separation of continents (25–22 MYA) with support for a recent diversification of most notothenioids at 12–5 MYA (Bargelloni et al., 1994, 2000). Mapping of the presence or absence of the HSR onto the notothenioid phylogeny shows that *B. variegatus* retained the HSR, suggesting it never lost the mechanisms of Hsp gene activation. Similarly, the black cod has maintained the ability to synthesize *hsp70* mRNA in response to heat stress, and possesses the ability to synthesize Hsps. Although we did not detect this clearly in the experiments reported here, previous research using Western blotting has shown that *N. angustata* does indeed contain an expected contingent of 70 kDa molecular chaperones (Carpenter and Hofmann, 2000). Interestingly, the black cod is considered to be an 'Antarctic escapee' and most likely originated inside the Polar Front and, during an interglacial period, for example during the Pliocene (4.8–2.5 MYA), migrated north and occupied the ice-free temperate waters of coastal New Zealand (Cheng et al., 2003). In fact, *N. angustata* retains some of the characteristics that reflect its polar origins. For example, Southern blotting has shown that *N. angustata* possesses functional antifreeze glycoprotein genes, supporting the close phylogenetic relationship to the members of Family Nototheniidae (Cheng et al., 2003). Other studies have noted that *N. angustata* possessed features that are similar to those found in Antarctic notothenioids (Eastman and DeVries, 1986; D'Avino and di Prisco, 1997; Egginton et al., 2001). Thus, these results—that the New Zealand notothenioid fishes possess an HSR—support the scenario that the HSR, the up-regulation of heat stress sensitive genes, was lost after evolution in the subzero, stenothermic environment of Antarctic waters during the period of non-bovichtid notothenioid radiation of approximately 5–12 MYA.

Other comparisons of Antarctic and non-Antarctic notothenioids have suggested that life at subzero temperatures has eliminated the need for the physiological plasticity required of species occupying more variant environments (e.g., Egginton et al., 2001). In the Antarctic notothenioids, the HSR may have been eliminated because during evolution the fish were no longer exposed to temperature variation that would illicit protein damage and induce Hsps. A functional HSR has been observed in at least one Antarctic organism, an alga (Vayda and Yuan, 1994) and the absence of stress-inducible Hsp expression may be a situation unique to the notothenioid fish. Presumably, one consequence of evolution for the ancestral notothenioid stock at low and constant temperatures was the loss of traits that function to permit heat tolerance or acclimation to shorter-term variations in environmental temperatures, a situation that still exists for the New Zealand notothenioids in the current study.

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