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ORIGINAL PAPER

Is cold the new hot? Elevated ubiquitin-conjugated protein levels in tissues of Antarctic fish as evidence for cold-denaturation of proteins in vivo

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Abstract Levels of ubiquitin (Ub)-conjugated proteins, as an index of misfolded or damaged proteins, were measured in notothenioid fishes, with both Antarctic (*Trematomus bernacchii*, *T. pennellii*, *Pagothenia borchgrevinki*) and non-Antarctic (*Notothenia angustata*, *Bovichtus variegatus*) distributions, as well as non-notothenioid fish from the Antarctic (*Lycodichthys dearborni*, Family Zoarcidae) and New Zealand (*Bellapiscis medius*, Family Tripterygiidae), in an effort to better understand the effect that inhabiting a sub-zero environment has on maintaining the integrity of the cellular protein pool. Overall, levels of Ub-conjugated proteins in cold-adapted Antarctic fishes were significantly higher than New Zealand fishes in gill, liver, heart and spleen tissues suggesting that life at sub-zero temperatures impacts protein homeostasis. The highest tissue levels of ubiquitinated proteins were found in the spleen of all fish. Ub conjugate levels in the New Zealand *N. angustata*, more closely resembled levels measured in other Antarctic fishes than levels measured in other New Zealand species, likely reflecting their recent shared ancestry with Antarctic notothenioids.

Keywords Antarctic fish · Protein denaturation · Ubiquitin · Cold adaptation · Notothenioid

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Introduction

Low temperature places fundamental constraints on physiological processes through its reduction in chemical reaction rates, reduction in membrane fluidity, increase in viscosity of biological fluids and when temperatures drop below 0°C, through the threat of freezing. Over the past 50 years there has been a substantial amount of research documenting the biochemical and physiological adjustments of polar organisms to counteract the temperature constraints of inhabiting a sub-zero environment. Evolution in the sub-zero Antarctic marine environment has resulted in a series of physiological and biochemical adaptations that include antifreeze protein production (DeVries 1983; Fletcher et al. 2001), elevated blood osmotic concentrations (Dobbs and DeVries 1975; O'Grady and DeVries 1982), decreases in haematocrit (Wells et al. 1980; Egginton 1996), mitochondrial proliferation (Johnston et al. 1998; Johnston 2003) and thermal compensation of metabolic activity (Fields and Somero 1998; Crockett and Sidell 1990; Hardewig et al. 1999; Kawall et al. 2002). An emerging area of investigation suggests that protein homeostasis—the maintenance of a functional cellular protein pool—is proving to be yet another process that is impacted by evolution at sub-zero temperatures.

Previous research on Antarctic notothenioid fishes has shown that these cold-adapted species lack a common cellular defense mechanism called the heat shock response (HSR), the highly conserved and coordinated induction of a family of heat shock proteins (Hsps) in response to elevated temperatures (Hofmann et al. 2000; Buckley et al. 2004; Place et al. 2004; Place and Hofmann 2005). Although these fishes are unable to mount a typical HSR, they do not lack the ability to synthesize Hsps. Instead, Antarctic fishes appear to maintain high constitutive levels of a normally

inducible Hsp isoform, Hsp70. Acting as molecular chaperones, Hsps play a critical role in maintaining the integrity of the cellular protein pool by promoting proper folding of nascent polypeptides, by preventing aggregation of misfolded or denatured proteins and by working in cooperation with degradation pathways to recycle irreversibly damaged proteins (for review see Lindquist 1986; Hightower 1991; Hartl and Hayer-Hartl 2002. For review in fishes see Iwama et al. 1998; Basu et al. 2002). The accumulation of abnormal proteins is the major signal for the activation of *hsp* genes (Ananthan et al. 1986) and therefore the presence of high constitutive levels of inducible Hsps in Antarctic fishes may be evidence of unusually high levels of misfolded or damaged proteins. Subsequent studies have demonstrated that constitutive expression of Hsp70 is not limited to Antarctic notothenioids but is also present in an Antarctic eelpout, *Lycodichthys dearborni* (Place and Hofmann 2005) as well as the larvae of an Antarctic midge (Rinehart et al. 2006). It is interesting to note that while the Antarctic midge larvae constitutively express *hsp70*, *hsp90* and a *small hsp*, the adult midge, which inhabit a much more thermally variable habitat, subject to frequent freeze-thaw episodes do not and instead have the capacity to induce *hsp* expression in response to thermal stress (Rinehart et al. 2006). Taken together, these findings suggest that the cold and thermally stable Antarctic environments may be more perturbing to the formation and maintenance of native protein structures than once thought and that molecular chaperones facilitate the proper folding of proteins at low temperatures.

There has been a substantial amount of research documenting the effect of cold temperature on protein structure and function (for review see Jaenicke 1990; Fujita 1999); however, much of this work has been done on in vitro systems that do not adequately represent protein thermal stability under in vivo conditions in the cell. These studies highlight cold denaturation of proteins but whether this translates into higher levels of damaged proteins in vivo in organisms that are cold-adapted is unclear. In addition to its potential effects on protein stability, low temperatures will also affect the rates of protein folding. As nascent polypeptides undergo folding events that lead to the native state, there exists the possibility that these proteins could fold incorrectly or interact with other nascent polypeptides forming protein aggregates (Hochachka and Somero 2002). It is possible that the slow protein folding associated with the cold temperatures of the polar marine environment could allow for more "non-productive" folding and as a result disrupts protein homeostasis in these organisms.

Maintaining protein homeostasis is a fundamental physiological process in all living organisms. An animal's proteome is in a constant state of flux and reflects a dynamic balance in synthetic and degradation processes. While proteins are essential to the proper functioning of the cell, the accumulation of damaged or misfolded proteins is cytotoxic, interfering with the function and viability of the cell (Sherman and Goldberg 2001). There are two fates for these non-native proteins: remodeling back to their native state by molecular chaperones or degradation by proteases, primarily by the ubiquitin (Ub)-proteasome pathway (Wickner et al. 1999). Protein degradation via the Ub-proteasome pathway involves two distinct and successive steps: (1) tagging of the misfolded or damaged protein by multiple Ub molecules and (2) degradation of the tagged protein by the 26S proteasome complex (Glickman and Ciechanover 2002; Goldberg 2003). Levels of Ub-conjugated proteins in the cell have been shown to be a good indicator of the integrity of the cellular protein pool, with high levels representing a high degree of protein damage (Hofmann and Somero 1995; Wing et al. 1995; van Breukelen and Carey 2002; Velickovska et al. 2005). An initial examination of Ub-conjugated protein levels in notothenioid fishes provided some evidence that the low temperatures of the Antarctic environment may be significantly perturbing to cellular proteins (Place et al. 2004); however, a more thorough examination is necessary to begin to understand the physiological constraints of maintaining protein homeostasis in these cold-adapted polar species.

The Antarctic fish fauna is dominated by the perciform suborder Notothenioidei (Eastman 1993; Clarke and Johnston 1996). Notothenioids represent 55% of the fish species of the Southern Ocean and in many coastal shelf areas represent over 90% of the fish biomass. Although largely endemic to the Antarctic region, there are several non-Antarctic notothenioid species that inhabit the southern coastal waters of New Zealand, Australia and South America. Unlike their Antarctic relatives that inhabit waters that rarely vary from the freezing point of seawater (near −1.9°C), these fishes experience a fluctuating thermal environment that likely rarely drops below 5°C. Antarctic notothenioids, through comparison with their cold temperate non-Antarctic relatives, provide physiologists with an ideal opportunity to determine if a physiological trait of these fishes represents an adaptation to the sub-zero Antarctic environment or whether the traits are related to history and are characteristic of the notothenioid lineage (Fig. 1, for review see Coppes Petricorena and Somero 2007).

The specific objective of this study was to determine whether Antarctic fishes naturally possess higher levels of damaged proteins when compared to fish from more temperate environments. This was assessed by measuring levels of Ub-conjugated proteins in a variety of tissues of field-collected fish. By examining Ub conjugate levels in a diversity of notothenioid fishes, with both Antarctic and non-Antarctic distributions, as well as a couple of non-notothenioid

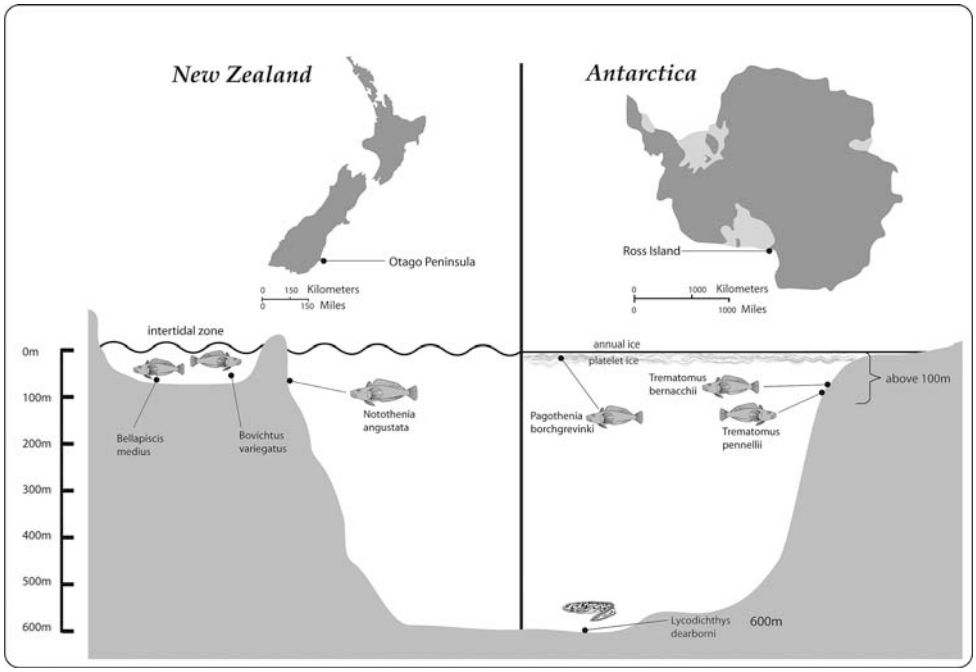

Fig. 1 Habitat distribution of New Zealand and Antarctic fish species examined in this study. *Dots* indicate the typical habitat of these species; however, many of the species inhabit a considerable range of depths

fish species from the Antarctic and New Zealand, this study starts to address what impact inhabiting the sub-zero Antarctic environment has on the integrity of the cellular protein pool.

Materials and methods

Fish collection

Specimens of three Antarctic notothenioids (Family Nototheniidae): *Trematomus bernacchii*, *T. pennellii*, *Pagothenia borchgrevinki* and a single zoarcid species, the eelpout *L. dearborni* were collected in McMurdo Sound, Antarctica (77°53′S, 166°40′E) during October and November 2005 ($n = 10$ for all species). Benthic nearshore specimens of *T. bernacchii* and *T. pennellii* were obtained by hook and line from depths of 20–30 m. Cryopelagic specimens of *P. borchgrevinki* were obtained by hook and line directly below the brash ice. Specimens of *L. dearborni* were caught in traps that were placed on the substrate at depths of 500–600 m. After capture, these fish were maintained in flow-through aquaria near ambient seawater temperature (−1.5°C) for 48 h before they were sacrificed. Specimens of two New Zealand notothenioids: *Notothenia angustata* ($n = 6$) and *Bovichtus variegatus* ($n = 7$) from the families Nototheniidae and Bovichtidae, respectively as well as a single triplefin species (Family Tripterygiidae), the twister *Bellapiscis medius* ($n = 6$) were collected in areas around the Portobello Marine Laboratory (University of Otago) on the Otago Peninsula of the South Island, New Zealand (45°50′S, 170°38′E) during March 2006. The tidepool thornfish *B. variegatus* and twister *B. medius* were collected using hand nets and the black cod, a benthic nearshore species, was caught in traps placed on the substrate at depths of 5–10 m. Following collection, these fish were maintained in flow-through aquaria at ambient seawater temperatures (12°C) for 48 h before they were sacrificed. An illustrative diagram of the habitats of the New Zealand and Antarctic fishes can be found in Fig. 1.

Tissue sampling

Fish were netted and rapidly anaesthetized with a high dose of MS-222 (0.2 g MS-222/l of water) and following onset of anaesthesia, the spinal cord was severed. Liver, heart, spleen and gills were then rapidly excised, snap-frozen in liquid nitrogen and stored at −80°C until further analysis.

Sample preparation for total protein and dot blot analyses

Frozen tissue samples were homogenized with a Teflon pellet pestle (Kontes, USA) in homogenization buffer [4% SDS (w/v), 1 mM EDTA and a protease inhibitor cocktail (Complete Protease Inhibitor Cocktail Tablets, Roche, USA) in 50 mM Tris–HCl buffer, pH 6.8], at a ratio of 200 mg tissue to 1 ml of buffer. Homogenates were then boiled for 5 min to denature all proteins and then centrifuged at 12,000*g* for 15 min. Supernatant was transferred to a fresh microcentrifuge tube and total protein concentration of the tissue homogenate was determined using the Bradford protein assay (Bradford 1976). The remaining tissue homogenate was stored at −20°C until dot blot analysis.

Dot blot analysis

Levels of Ub-conjugated protein were measured using immunochemical analysis of gill, liver, heart and spleen using modified methods outlined in Hofmann and Somero (1995). Equal amounts of total protein (0.5 μg) from each sample were blotted onto pre-wetted nitrocellulose membrane (Bio-Rad, 0.2 μm pore size) in triplicate by gravity filtration using a BioDot dot blotter (Bio-Rad). Wells were washed twice with 200 μl of Tween-20 Tris-buffered saline (TTBS) (20 mM Tris–HCl, 140 mM NaCl, 0.01% Tween-20, pH 7.6) and then heat fixed at 65°C for 20 min. After heat fixing, the membrane was blocked in 5% nonfat milk powder in TTBS for 1 h. Following blocking, membranes were rinsed once and washed three times in TTBS for 5 min. Membranes were then soaked in an Ub conjugate specific primary antibody (1:5,000, rabbit polyclonal antibody produced by Cocalico Biologicals Inc.) in 5% nonfat milk for 1.5 h. Following three 5 min washes in TTBS, membranes were soaked in horseradish peroxidase-conjugated protein A secondary antibody (1:5,000, Bio-Rad) in 5% nonfat milk for 1 h. After three 5 min washes in TTBS, the membrane was developed using the chemiluminescent substrate SuperSignal West Dura Extended Duration Substrate following manufacturer's instructions (Pierce, USA). Chemiluminescence was detected using a VersaDoc imager and quantified using Quantity One software (Bio-Rad). Values were standardized using dot intensity values from an individual sample of *T. pennellii* gill homogenate that was run in triplicate concurrently on each gel (referred to as "Internal Std" in figures).

Statistical analysis

Two-way analysis of variance (ANOVA) was used to determine significant ($P < 0.05$) differences in Ub-conjugated protein levels where fish species and tissue type were used as independent categorical variables, and Ub-conjugated protein level was used as the dependent variable. Mean values were compared using the post-hoc Student–Newman–Keuls multiple comparison test ($P < 0.05$). All data were tested for normality (Kolmogorov–Smirnov test) and homogeneity of variance (Levene Median test). In cases where these assumptions were not met, values were log-transformed and the statistical analysis was repeated. Results for Ub-conjugated protein are reported as mean ± SEM.

Results

Many size classes of proteins can be tagged by Ub for degradation by the Ub-proteasome pathway. We confirmed this by western blot analysis of representative spleen homogenates from *T. bernacchii*, *N. angustata* and *B. variegatus* (Fig. 2). Visual inspection of these protein "smears" suggested that *T. bernacchii* had the highest Ub-conjugate levels, that *B. variegatus* had the lowest levels of Ub-conjugated protein and that *N. angustata*'s levels were intermediate between the two.

For quantitative purposes, Ub-conjugated proteins were analysed using dot blot analysis. Overall, there were significant differences in the levels of Ub-conjugated proteins between the Antarctic and New Zealand species. Results of 2-way ANOVA on Ub-conjugated protein levels showed that there was a significant effect of fish species ($P < 0.001$), a significant effect of tissue type ($P < 0.001$) and a significant interaction between fish species and tissue type ($P < 0.001$). The highest measured Ub-conjugate levels were found in the Antarctic, cyropelagic notothenioid *P. borchgrevinki* and the deep water Antarctic eelpout, *L. dearborni*. The next highest levels of Ub-conjugated protein were measured in the two shallow, benthic Antarctic notothenioids, *T. bernacchii* and *T. pennellii*, and the temperate New Zealand notothenioid, *N. angustata*. The lowest Ub-conjugate levels were found in the intertidal New Zealand fish, with levels in the thornfish, *B. variegatus* being higher than the twister, *B. medius*. In general, spleen tissue levels of Ub-conjugated proteins were the highest amongst all the tissues and liver levels were the lowest. Gill Ub-conjugate levels were higher than levels measured in the heart.

Tissue-specific differences in Ub conjugates

Within the gill, Ub-conjugate levels were highest in *P. borchgrevinki*, although these levels were not significantly elevated above branchial levels in the eelpout, *L. dearborni*

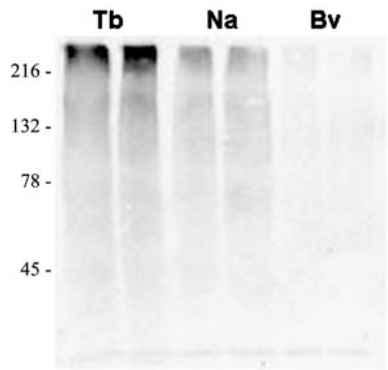

Fig. 2 Western blot of ubiquitin-conjugated proteins in the spleen tissue of three notothenioids: *T. bernacchii* (*Tb*), *N. angustata* (*Na*) and *B. variegatus* (*Bv*). The position of molecular weight markers is shown on far *left*. Molecular masses are given as kilo Daltons

(Fig. 3a). The other Antarctic notothenioids, *T. bernacchii* and *T. pennellii*, along with the New Zealand notothenioid, *N. angustata*, had significantly lower levels of Ub conjugates in the gills when compared to *P. borchgrevinki*. The lowest levels of branchial Ub conjugates were measured in the New Zealand thornfish, *B. variegatus*, and twister, *B. medius*.

Similar to what was measured in the gill, the highest Ub-conjugate levels measured in the liver were found in *P. borchgrevinki*, and these levels were significantly higher than the levels measured in the other Antarctic fish species, *T. bernacchii*, *T. pennellii* and *L. dearborni*, as well as the non-Antarctic notothenioid *N. angustata*, which did not differ significantly from one another (Fig. 3b). Liver Ub-conjugate levels were significantly lowest in *B. variegatus* and *B. medius*.

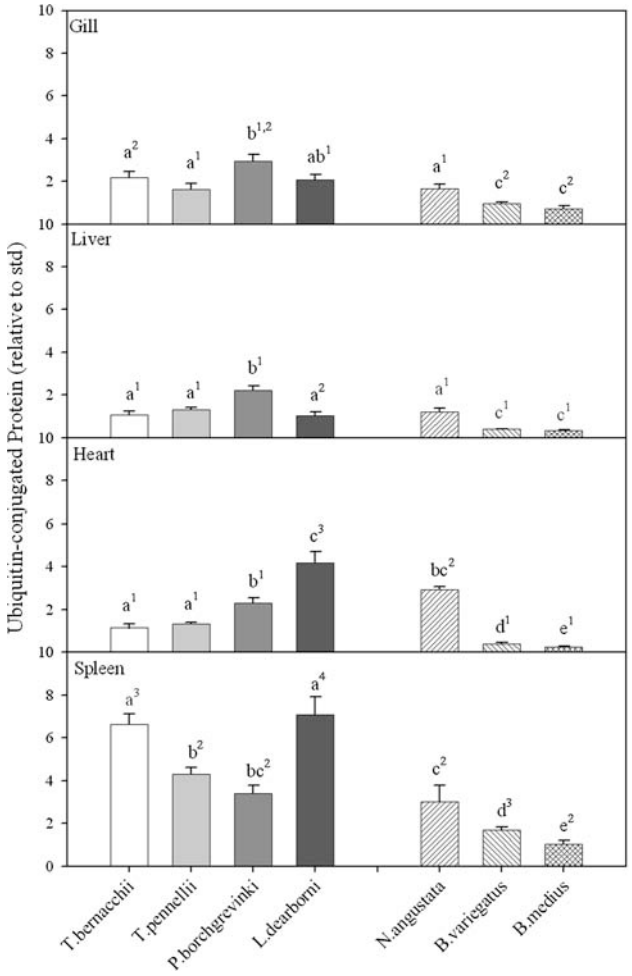

Fig. 3 Ubiquitin-conjugated protein levels in the gill, liver, heart and spleen tissues collected from wild caught fish 48 h after capture. Specimens of *T. bernacchii*, *T. pennellii*, *P. borchgrevinki* and *L. dearborni* were collected in McMurdo Sound, Antarctica ($n = 10$ for all species). Specimens of *N. angustata* ($n = 6$), *B. variegatus* ($n = 7$) and *B. medius* ($n = 6$) were collected on the Otago Peninsula of the South Island, New Zealand. Ubiquitin-conjugated protein levels are shown as relative values based on dot intensities standardized against an individual sample of *T. pennellii* gill homogenate (mean ± SEM, $n = 10$ for each fish). *Different letters* denote significant differences between fish species within a specific tissue type ($P < 0.05$). *Different numbers* denote significant differences between tissue levels of ubiquitin conjugates for a particular fish species ($P < 0.05$)

Within the heart, Ub-conjugate levels were highest in the eelpout, *L. dearborni*, although these levels did not differ significantly from those measured in the New Zealand black cod *N. angustata* (Fig. 3c). Ub-conjugate levels in the heart of *P. borchgrevinki* did not differ significantly from *N. angustata*, but were significantly elevated above the other Antarctic notothenioids, *T. bernacchii* and *T. pennellii*. The lowest heart Ub-conjugate levels were measured in the New Zealand intertidal fish; however, levels were significantly higher in *B. variegatus* when compared to *B. medius*.

Spleen Ub-conjugate levels were highest in *T. bernacchii*, an Antarctic notothenioid, as well as the Antarctic eelpout, *L. dearborni* (Fig. 3d). The next highest Ub-conjugate levels were measured in the spleens of *T. pennellii* and *P. borchgrevinki*; however, spleen Ub-conjugate levels in the New Zealand notothenioid *N. angustata* did not differ significantly from *P. borchgrevinki*. Similar to what was seen in the other tissues, spleen Ub-conjugate levels were lowest in the New Zealand intertidal fish species, with *B. variegatus* having significantly higher levels than *B. medius*.

Species-specific differences in Ub conjugates

Within the Antarctic notothenioid *T. bernacchii* the highest Ub-conjugate levels were measured in the spleen, followed by the gills, which were significantly higher than levels measured in both the heart and the liver (Fig. 3). The highest tissue Ub-conjugate levels in *T. pennellii* were measured in the spleen, and these levels were significantly elevated above levels measured in the heart, liver and gill, which did not differ significantly from one another (Fig. 3). Within *P. borchgrevinki*, the highest tissue Ub-conjugate levels were measured in the spleen and gill, although levels in the gill were not significantly elevated above levels measured in the heart and liver. Ub-conjugate levels measured in the Antarctic eelpout, *L. dearborni*, were highest in the spleen, followed by the heart, which were significantly higher than the gills. The lowest tissue levels of Ub conjugates in *L. dearborni* were in the liver.

Ub-conjugate levels in the New Zealand black cod, *N. angustata*, were highest in the spleen and heart and these levels were significantly higher than levels measured in the liver and gill. Within the thornfish, *B. variegatus*, Ub-conjugate levels were highest in the spleen, followed by the gill, which were significantly higher than levels measured in both the heart and the liver. Ub-conjugate levels in the spleen and gills of the twister, *B. medius*, were significantly higher than levels measured in the liver and heart tissue.

Discussion

In this study, the levels of Ub-conjugated proteins in cold-adapted notothenioid fishes were measured in an effort to better understand the effect that inhabiting a sub-zero environment has on maintaining the integrity of the cellular protein pool. Relative levels of Ub conjugates were measured by dot blot analysis in the spleen, heart, gill and liver from three wild caught Antarctic notothenioid fishes and compared to levels present in two cold-temperate New Zealand notothenioids. In addition, Ub conjugate levels in two non-notothenioid fishes, one each from the Antarctic and New Zealand, were also compared to provide some information on the importance of environment versus phylogeny in determining levels of non-native proteins in notothenioid fishes. This study highlighted three salient findings: (1) overall, levels of Ub-conjugated proteins in cold-adapted Antarctic fishes were significantly higher than New Zealand fishes ($P < 0.001$); (2) there were significant differences in tissue levels of Ub-conjugated proteins measured in all fishes, with levels being highest in the spleen ($P < 0.001$) and (3) Ub conjugate levels in the New Zealand black cod, *N. angustata* more closely resembled levels measured in other Antarctic fishes than levels measured in other cold temperate New Zealand species.

Ub-conjugated proteins are higher in Antarctic fishes

Higher levels of Ub-tagged proteins, as a measure of non-native proteins, in Antarctic notothenioids as well as the Antarctic eelpout, *L. dearborni*, suggest that the sub-zero Antarctic marine environment places considerable physiological constraints on maintaining proteins in their native state in these fishes. Mechanistically, it is not clear whether these elevated levels of Ub conjugates are the direct denaturation of proteins at sub-zero temperatures or a more cellular mechanism where the process of protein homeostasis is affected due to cold adaptation of the machinery. These data are however consistent with previous work that demonstrated that Antarctic fishes maintain high levels of a regularly inducible molecular chaperone (Hsp70) on a constitutive basis suggesting that these organisms require additional help in chaperoning proteins along productive paths in the cell (Place et al. 2004; Place and Hofmann 2005).

An obvious possibility for the high levels of Ub-conjugated proteins is that proteins in general could be more sensitive to denaturation or misfolding under the near-freezing conditions of the Antarctic environment resulting in a higher number of cellular proteins targeted for degradation by the proteasome. There has been a considerable amount of work investigating the effects of low temperature on proteins in vitro and the modifications of cold-adapted proteins that allow them to function at subzero temperatures (for review see Jaenicke 1990; Somero 1995; Hoyoux et al. 2004; Siddiqui and Cavicchioli 2006). Recent studies on Antarctic microorganisms suggest that these structural adaptations have not only made these enzymes more heat labile but also more susceptible to cold denaturation (D'Amico et al. 2003); however, whether these denaturing conditions would be experienced in their natural environment is unknown. Antarctic notothenioids have evolved under stable sub-zero conditions for the last 10–14 MY and as a result have had appropriate time to adapt to this particular thermal niche. It therefore seems unlikely that these cold-adapted proteins would not have undergone appropriate modification to maintain stability under natural conditions. There is appropriate evidence in the literature to suggest that these elevated levels of misfolded or damage could be a result of the temperature constraints on protein synthesis and degradation and reflect the cold adaptation of the protein homeostasis machinery to compensate for these effects.

There has been a significant contribution to understanding the efficiency and cost of protein synthesis in Antarctic invertebrate and fish species (for review see Pörtner 2006). Whole body protein synthesis rates of Antarctic invertebrate species in vivo are cold compensated but are still lower than those measured in comparable temperate species of isopods (Whiteley et al. 1996; Robertson et al. 2001), sea urchins (Marsh et al. 2001) and limpets (Fraser et al. 2002), and this is in accordance with lower metabolic rates. These organisms have been shown to maintain elevated RNA:protein ratios, through increased RNA levels, and it has been suggested that this is required to offset the low RNA translational efficiency (mg protein synthesized μg^{-1} RNA day^{-1}) brought about by the cold temperatures. Studies investigating the energetic cost of protein synthesis in Antarctic invertebrates compared to their temperate counterparts have been variable in their conclusions (Smith and Haschemeyer 1980; Whiteley et al. 1996; Marsh et al. 2001); however, more recent work using an in vitro cell-free lysate system has eloquently shown that the energetic cost to synthesize proteins is comparable in the gill tissues of an Antarctic and temperate scallop (Storch and Pörtner 2003; Storch et al. 2003). A similar study has been conducted on Antarctic and temperate zoarcid fishes, demonstrating that the Antarctic eelpout (*Pachycara brachycephalum*) maintains higher protein synthesis capacity by reducing activation energies of protein synthesis and by increasing RNA translational capacity (Storch et al. 2005). These adaptations are thought to counterbalance the decrease in reaction rates by low temperature. Taken together, these results suggest temperature compensation of the protein synthesis capacity in Antarctic species; however, it is still unclear how efficient these polar organisms are at synthesizing and folding functional proteins.

Although the capacity of Antarctic fishes to properly fold newly synthesized proteins has yet to be characterized, elevated levels of Ub-conjugated proteins could reflect greater inefficiencies in protein maturation at sub-zero temperatures. Low temperatures will affect the kinetics of protein folding and there is the potential for nascent polypeptides undergoing folding events under low "operating temperatures" to misfold and form protein aggregates as they interact with other non-native proteins (Hochachka and Somero 2002). Schubert et al. (2000) found that 30% or more of newly synthesized proteins are routinely degraded within 10 min of synthesis by the proteasome in HeLa cells at 37°C, suggesting that naturally there is some inherent inefficiency in nascent polypeptide folding as well as multimer assembly. The polar marine environment may exacerbate this "non-productive" folding, further disrupting protein homeostasis in these organisms and resulting in the high levels of Ub-conjugated proteins measured in Antarctic fishes in this study.

Alternatively, as low temperature is known to place fundamental constraints on the rates of all physiological processes, the high levels of Ub-conjugated proteins may reflect a reduction in the activity of the 26S proteasome with cold adaptation. As a result, there would be a build-up of Ub-tagged proteins if these proteins were being generated faster than they could be degraded. Metabolic studies of enzyme activities in polar organisms demonstrate that there is a certain degree of upward adjustment or compensation to offset the effects of cold (Fields and Somero 1998; Crockett and Sidell 1990; Hardewig et al. 1999; Kawall et al. 2002; Marx et al. 2007). As a result, enzyme activities of polar organisms are higher than what would be predicted when the rates of temperate or tropical species are extrapolated down to subzero temperatures. To date, there have been no studies documenting the effects of low temperature on the activity of the 26S proteasome, yet thermal compensation of this process seems likely. It is becoming clear that a cost intensive pattern of metabolic adaptation is required for successful living in polar environments and that this is ultimately reflected in low mean annual growth rates (Pörtner 2006). The Antarctic notothenioids, through comparisons with their temperate New Zealand relatives, offer an excellent study system to examine whether any thermal compensation of 26S proteasome activity exists in cold-adapted fish.

Tissue differences in Ub conjugate levels

The inter-tissue differences in levels of Ub-conjugated proteins suggest that there are factors in addition to temperature that are responsible for the variations in Ub-conjugates seen in these fishes. In the fish species examined in this study, Ub conjugate levels were found to be highest in the spleen. In *T. bernacchii*, *T. pennellii*, *L. dearborni* and *B. variegatus* Ub-conjugated proteins were significantly higher in the spleen, compared to the other tissues. The abundance of ubiquitinated proteins in *P. borchgrevinki* and *B. medius* were found to be highest in the spleen and the gills, which did not differ significantly from each other. Finally, in *N. angustata*, the highest levels of Ub conjugates were measured in the spleen and heart tissue. There is preliminary evidence to suggest that Antarctic notothenioids accumulate endogenous ice crystals in the spleen that are growth arrested due to their adsorption to antifreeze glycoproteins (A. L. DeVries, personal communication). It is unknown what effect this has on protein degradation and whether turnover of anti-freeze glycoproteins (AFGPs) is significant enough to account for the higher splenic levels of Ub conjugates. Although the Antarctic fishes, along with *N. angustata*, have significantly higher overall levels of Ub-conjugated proteins, the two New Zealand intertidal fishes also have relatively high splenic levels of Ub conjugates, indicating that this trait may not be limited to cold-adapted fishes. The fish spleen has important functions in erythrocyte sequestering, processing, storage, release and degradation as well as immune responses (Fänge and Nilsson 1985). It is possible that the high levels of Ub conjugates in the spleen of these fish reveal that this tissue is particularly metabolically active in terms of protein breakdown, for example in erythrocyte turnover or haemoglobin recycling. Although lower than the spleen, levels of ubiquitinated protein were relatively high in the gill tissue. In particular, the gills of *P. borchgrevinki* had the highest level of Ub-conjugates for this tissue. Living in the brash ice directly under the sea ice, this fish might have a particularly high protein turnover associated with ice-induced tissue damage, especially in the gills that are in constant contact with ice. While there has been little research documenting Ub-conjugate levels in fish and the associated activity of the Ub-proteasome pathway, the results from the present study indicate that the metabolic activity and protein turnover rate of the specific tissues need to be considered in addition to the effect of temperature in cold-adapted fishes.

Ub conjugate levels in *N. angustata* more closely resemble levels in Antarctic rather than New Zealand fishes

While overall levels of ubiquitinated proteins are higher in the Antarctic fishes examined in this study, levels measured in the temperate notothenioid *N. angustata* are statistically more similar to their Antarctic relatives than their New Zealand counterparts. In all four tissues examined, the black cod, *N. angustata*, possessed significantly higher levels Ub conjugates than the thornfish, *B. variegatus*, and the twister, *B. medius*. When compared to the Antarctic fishes,

in the gill and liver tissue, only the cryopelagic *P. borchgrevinki* had significantly higher levels of Ub compared to *N. angustata*. Similarly, in the heart, only the Antarctic eelpout, *L. dearborni*, had significantly greater levels of Ub-conjugated proteins than the black cod. Within the spleen, Ub conjugates of *N. angustata* were not significantly different than *P. borchgrevinki*, but were significantly lower than the other three Antarctic fishes. The similarity in Ub conjugate levels of the New Zealand black cod to the other Antarctic notothenioids likely reflects the evolutionary history of these fishes and their shared ancestry. Based on analysis of the mtDNA ND2 gene of notothenioids, Cheng et al. (2003) estimate that the ancestor of *N. angustata* diverged from the rest of the Antarctic notothenioids 11 MYA, millions of years after the isolation of Antarctica and following the establishment of the Antarctic Polar Front (25–22 MYA), which led to the current sub-zero conditions around the mid-Miocene (10–15 MYA) (Eastman 1993; Anderson 1999). From these estimations, it is widely believed that the ancestor of *N. angustata* inhabited the sub-zero Antarctic environment for some time and therefore had many of the cold-adapted physiological traits of the other Antarctic notothenioids. As a result, the New Zealand black cod is often referred to as an "Antarctic escapee."

The shared evolutionary history between Antarctic notothenioids and the more temperate *N. angustata* is evident in a number of physiological and anatomical similarities in the two groups. New Zealand black cod resemble their Antarctic relatives in their possession of functional AFGP genes (Cheng et al. 2003; Cheng et al. 2006), the conserved sequence identity and multiplicity of haemoglobins (D'Avino and di Prisco 1997) and the presence of a paucciglomerular kidney, phylogenetically intermediate in structure between the aglomerular kidney of Antarctic notothenioids and the fully glomerular kidney of the other non-Antarctic notothenioids (Eastman 1993). It is the presence of functional AFGPs genes in black cod that provide the most compelling evidence of an ancestral Antarctic existence and the hypothesis that they developed many cold-adapted traits before "escaping" to temperate waters. As *N. angustata* does not experience sub-zero conditions in its present day marine environment, and therefore has no need for these proteins, the presence of AFGPs in black cod is likely an artifact of a historic sub-zero existence. The high level of Ub conjugates in *N. angustata* provides further evidence that the predecessor of this fish inhabited the sub-zero waters of Antarctica and that the New Zealand black cod have retained a trait that reflects a cold adaptation strategy adopted millions of years ago. In the case of *B. variegatus*, low levels of Ub conjugates is consistent with the hypothesis that the Bovichtidae is a basal group that evolved under temperate conditions.

Concluding remarks

In summary, fish living at sub-zero temperatures exhibit high levels of Ub-conjugated proteins suggesting that the cold adaptation of Antarctic fishes to the polar marine environment likely occurred with some trade-offs in their capacity to maintain protein homeostasis. Interestingly, the "Antarctic escapee", *N. angustata*, displayed levels that more closely matched their Antarctic relatives than their temperate New Zealand counterparts. Currently, one explanation is that these high levels of Ub conjugates in *N. angustata* reflect an evolutionarily conserved intrinsic mechanism of cold adaptation of the protein homeostasis machinery; however, this has yet to be investigated. Future studies are necessary to examine the efficiency of protein folding as well as whether there is any thermal compensation of the protein degradation pathways in cold-adapted animals. Finally, further investigations are warranted to determine whether other Antarctic animals experience similar difficulties in the formation and maintenance of native protein structures.

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References

Ananthan J, Goldberg AL, Voellmy R (1986) Abnormal proteins serve as eukaryotic stress signals and trigger the activation of the heat-shock genes. Science 232:522–524

Anderson JB (1999) Antarctic marine geology. Cambridge University Press, Cambridge

Basu N, Todgham AE, Ackerman PA, Bibeau MR, Nakano K, Schulte PM, Iwama GK (2002) Heat shock protein genes and their functional significance in fish. Gene 295:173–183

Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye-binding. Anal Biochem 72:248–254

Buckley BA, Place SP, Hofmann GE (2004) Regulation of heat shock genes in isolated hepatocytes from an Antarctic fish, *Trematomus bernacchii*. J Exp Biol 207:3649–3656

Cheng C-HC, Chen L, Near TJ, Jin Y (2003) Functional antifreeze glycoprotein genes in temperate-water New Zealand nototheniid fish infer an Antarctic evolutionary origin. Mol Biol Evol 20:1897–1908

Cheng C-HC, Cziko PA, Evans CW (2006) Nonhepatic origin of notothenioid antifreeze reveals pancreatic synthesis as common mechanism in polar fish freezing avoidance. Proc Natl Acad Sci USA 103:10491–10496

Clarke A, Johnston IA (1996) Evolution and adaptive radiation of Antarctic fishes. Trends Ecol Evol 11:212–218

Coppes Petricorena ZL, Somero GN (2007) Biochemical adaptations of notothenioid fishes: comparisons between cold temperate South American and New Zealand species and Antarctic species. Comp Biochem Physiol A 147:799–807

Crockett EL, Sidell BD (1990) Some pathways of energy metabolism are cold adapted in Antarctic fishes. Physiol Zool 63:472–488

D'Amico S, Marx J-C, Gerday C, Feller G (2003) Activity-stability relationships in extremophilic enzymes. J Biol Chem 278:7891–7896

D'Avino R, di Prisco G (1997) The hemoglobin system of Antarctic and non-Antarctic notothenioid fishes. Comp Biochem Physiol A 118:1045–1049

DeVries AL (1983) Antifreeze peptides and glycopeptides in cold-water fishes. Annu Rev Physiol 45:245–260

Dobbs GH, DeVries AL (1975) Renal function in Antarctic teleost fishes: serum and urine composition. Mar Biol 29:59–70

Eastman JT (1993) Antarctic fish biology-evolution in a unique environment. Academic, San Diego

Egginton S (1996) Blood rheology of Antarctic fishes: viscosity adaptations at very low temperatures. J Fish Biol 48:513–521

Fänge R, Nilsson S (1985) The fish spleen: structure and function. Experientia 41:152–158

Fields PA, Somero GN (1998) Hot spots in cold adaptation: localized increases in conformational flexibility in lactate dehydrogenase A(4) orthologs of Antarctic notothenioid fishes. Proc Natl Acad Sci USA 95:11476–11481

Fletcher GL, Hew CL, Davies PL (2001) Antifreeze proteins of teleost fishes. Annu Rev Physiol 63:359–390

Fraser KPP, Clarke A, Peck LS (2002) Low-temperature protein metabolism: seasonal changes in protein synthesis and RNA dynamics in the Antarctic limpet *Nacella concinna* Strebel 1908. J Exp Biol 205:3077–3086

Fujita J (1999) Cold shock response in mammalian cells. J Mol Microbiol Biotechnol 1:243–255

Glickman MH, Ciechanover A (2002) The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiol Rev 82:373–428

Goldberg AL (2003) Protein degradation and protection against misfolded or damaged proteins. Nature 426:895–899

Hardewig I, Van Dijk PLM, Moyes CD, Pörtner HO (1999) Temperature-dependent expression of cytochrome-c oxidase in Antarctic and temperate fish. Am J Physiol 277:R508–R516

Hartl FU, Hayer-Hartl M (2002) Molecular chaperones in the cytosol: from nascent chain to folded protein. Science 295:1852–1858

Hightower LE (1991) Heat shock, stress proteins and proteotoxicity. Cell 66:191–197

Hochachka PW, Somero GN (2002) Biochemical adaptation. Mechanism and process in physiological evolution. Oxford University Press, New York

Hofmann GE, Somero GN (1995) Evidence for protein damage at environmental temperatures: seasonal changes in levels of ubiquitin conjugates and hsp70 in the intertidal mussel, *Mytilus trossulus*. J Exp Biol 198:1509–1518

Hofmann GE, Buckley BA, Airaksinen S, Keen J, Somero GN (2000) The Antarctic fish *Trematomus bernacchii* lacks heat-inducible heat shock protein synthesis. J Exp Biol 203:2331–2339

Hoyoux A, Blaise V, Collins T, D'Amico S, Gratia E, Huston AL, Marx J-C, Sonan G, Zeng Y, Feller G, Gerday C (2004) Extreme catalysts from low-temperature environments. J Biosci Bioeng 98:317–330

Iwama GK, Thomas PT, Forsyth RB, Vijayan MM (1998) Heat shock protein expression in fish. Rev Fish Biol Fish 8:35–56

Jaenicke R (1990) Protein structure and function at low temperature. Philos Trans R Soc Lond B Biol Sci 326:535–553

Johnston IA (2003) Muscle metabolism and growth in Antarctic fishes (suborder Notothenioidei): evolution in a cold environment. Comp Biochem Physiol B 136:701–713

Johnston IA, Calvo J, Guderley H, Fernandez D, Palmer L (1998) Latitudinal variation in the abundance and oxidative capacities of muscle mitochondria in perciform fishes. J Exp Biol 210:1–12

Kawall HG, Torres JJ, Sidell BD, Somero GN (2002) Metabolic cold adaptation in Antarctic fishes: evidence from enzymatic activities of the brain. Mar Biol 140:279–286

Lindquist S (1986) The heat shock response. Annu Rev Biochem 55:1151–1191

Marsh AG, Maxson RE, Manahan DT (2001) High macromolecular synthesis with low metabolic cost in Antarctic sea urchin embryos. Science 291:1950–1952

Marx J-C, Collins T, D'Amico S, Feller G, Gerday C (2007) Cold-adapted enzymes from marine Antarctic microorganisms. Mar Biotech 9:293–304

O'Grady SM, DeVries AL (1982) Osmotic and ionic regulation in polar fishes. J Exp Mar Biol Ecol 57:219–228

Place SP, Hofmann GE (2005) Constitutive expression of a stress-inducible heat shock protein gene, *hsp70*, in phylogenetically distant Antarctic fish. Polar Biol 28:261–267

Place SP, Zippay ML, Hofmann GE (2004) Constitutive roles for inducible genes: evidence for the alteration in expression of the inducible *hsp70* gene in Antarctic notothenioid fishes. Am J Physiol 287:R429–R436

Pörtner HO (2006) Climate-dependent evolution of Antarctic ectotherms: an integrative analysis. Deep Sea Res II 53:1071–1104

Rinehart JP, Hayward SAL, Elnitsky MA, Sandro LH, Lee RE, Denlinger DL (2006) Continuous up-regulation of heat shock proteins in larvae, but not adults, of a polar insect. Proc Natl Acad Sci USA 103:14223–14227

Robertson RF, el Haj AJ, Clarke A, Taylor EW (2001) The effects of temperature on metabolic rate and protein synthesis following a meal in the isopod *Glyptonotus antarcticus* Eights (1852). Polar Biol 24:677–686

Schubert U, Anton LC, Gibbs J, Norbury CC, Yewdell JW, Bennink JR (2000) Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. Nature 404:770–774

Sherman M, Goldberg AL (2001) Cellular defenses against unfolded proteins: a cell biologist thinks about neurodegenerative diseases. Neuron 29:15–32

Siddiqui KS, Cavicchioli R (2006) Cold-adapted enzymes. Annu Rev Biochem 75:403–433

Smith MAK, Haschemeyer AEV (1980) Protein metabolism and cold adaptation in Antarctic fishes. Physiol Zool 53:373–382

Somero GN (1995) Proteins and temperature. Annu Rev Physiol 57:43–68

Storch D, Pörtner HO (2003) The protein synthesis machinery operates at the same expense in eurythermal and cold stenothermal pectinids. Physiol Biochem Zool 76:28–40

Storch D, Heilmayer O, Hardewig I, Pörtner HO (2003) In vitro protein synthesis capacities in a cold stenothermal and a temperate eurythermal pectinid. J Comp Physiol B 173:611–620

Storch D, Lannig G, Pörtner HO (2005) Temperature-dependent protein synthesis capacities in Antarctic and temperate (North Sea) fish (Zoarcidae). J Exp Biol 208:2409–2420

van Breukelen F, Carey HV (2002) Ubiquitin conjugate dynamics in the gut and liver of hibernating ground squirrels. J Comp Physiol B 172:269–273

Velickovska V, Lloyd BP, Qureshi S, vanBreukelen F (2005) Proteolysis is depressed during torpor in hibernators at the level of the 20S core protease. J Comp Physiol B 175:329–335

Wells RMG, Ashby MD, Duncan SJ, MacDonald JA (1980) Comparative study of the erythrocytes and haemoglobins in notothenioid fishes from Antarctica. J Fish Biol 17:517–527

Whiteley NM, Taylor EW, el Haj AJ (1996) A comparison of the metabolic cost of protein synthesis in stenothermal and eurythermal isopod crustaceans. Am J Physiol 271:R1295–R1303

Wickner S, Maurizi MR, Gottesman S (1999) Posttranslational quality control: folding, refolding and degrading proteins. Science 286:1888–1893

Wing SS, Haas AL, Goldberg AL (1995) Increase in ubiquitin-protein conjugates concomitant with the increase in proteolysis in rat skeletal muscle during starvation and atrophy denervation. Biochem J 307:639–645