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RESEARCH ARTICLE

The effect of temperature adaptation on the ubiquitin–proteasome pathway in notothenioid fishes

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ABSTRACT

There is an accumulating body of evidence suggesting that the sub-zero Antarctic marine environment places physiological constraints on protein homeostasis. Levels of ubiquitin (Ub)-conjugated proteins, 20S proteasome activity and mRNA expression of many proteins involved in both the Ub tagging of damaged proteins as well as the different complexes of the 26S proteasome were measured to examine whether there is thermal compensation of the Ub–proteasome pathway in Antarctic fishes to better understand the efficiency of the protein degradation machinery in polar species. Both Antarctic (*Trematomus bernacchii*, *Pagothenia borchgrevinki*) and non-Antarctic (*Notothenia angustata*, *Bovichtus variegatus*) notothenioids were included in this study to investigate the mechanisms of cold adaptation of this pathway in polar species. Overall, there were significant differences in the levels of Ub-conjugated proteins between the Antarctic notothenioids and *B. variegatus*, with *N. angustata* possessing levels very similar to those of the Antarctic fishes. Proteasome activity in the gills of Antarctic fishes demonstrated a high degree of temperature compensation such that activity levels were similar to activities measured in their temperate relatives at ecologically relevant temperatures. A similar level of thermal compensation of proteasome activity was not present in the liver of two Antarctic fishes. Higher gill proteasome activity is likely due in part to higher cellular levels of proteins involved in the Ub–proteasome pathway, as evidenced by high mRNA expression of relevant genes. Reduced activity of the Ub–proteasome pathway does not appear to be the mechanism responsible for elevated levels of denatured proteins in Antarctic fishes, at least in the gills.

KEY WORDS: Antarctic fish, Protein homeostasis, Ub–proteasome pathway, Cold adaptation, Notothenioid

INTRODUCTION

Antarctic marine ectotherms have evolved a number of physiological and biochemical adaptations to counteract the temperature constraints of inhabiting a sub-zero environment (for review, see Coppes Petricorena and Somero, 2007; Pörtner et al., 2007). Some of the most well studied of these adaptations include antifreeze glycoproteins that inhibit the growth of ice crystals (DeVries, 1983), enzymes that are cold-adapted to support metabolism at low temperatures (Crockett and Sidell, 1990; Somero, 1995; Fields and Somero, 1998) and mitochondrial proliferation to compensate for the effects of low temperature on aerobic metabolism (Johnston, 1989; O'Brien et al., 2003; Guderley, 2004). Recently, there has been an accumulating body of literature to suggest that protein homeostasis – the maintenance of a functional protein pool – has been highly impacted by evolution under these cold and stable conditions.

Maintaining protein homeostasis is a fundamental physiological process, reflecting a dynamic balance in synthetic and degradation processes. There are numerous lines of evidence to suggest temperature compensation of protein synthesis in Antarctic invertebrates (Whiteley et al., 1996; Marsh et al., 2001; Robertson et al., 2001; Fraser et al., 2002) and fish (Storch et al., 2005). In zoarcid fishes, it has been demonstrated that Antarctic eelpouts (*Pachycara brachycephalum*) maintain higher protein synthesis capacities than their temperate relatives through reductions in activation energies of protein synthesis and increases in RNA translational capacity (mg protein synthesized μg^{-1} RNA day^{-1}) to counterbalance the decrease in reaction rates by low temperature (Storch et al., 2005). Studies examining the energetic cost of protein synthesis in invertebrates have been more variable in their conclusions; however, it appears that invertebrates maintain elevated RNA levels, possibly as a mechanism to offset the low RNA translational efficiency in these species (Smith and Haschemeyer, 1980; Whiteley et al., 1996; Marsh et al., 2001). How millions of years living at subzero temperatures has affected protein turnover and degradation is less well understood. Previous research in a variety of Antarctic species has documented that the inducible isoform of the 70-kDa heat shock protein (Hsp70), a classic molecular chaperone targeting denatured and damaged proteins, is expressed highly under basal conditions and therefore has been recruited for a constitutive role in species that inhabit stable sub-zero conditions (Hofmann et al., 2000; La Terza et al., 2001; Buckley et al., 2004; Place et al., 2004; Rinehart et al., 2006). In concordance with higher levels of molecular chaperones, higher levels of ubiquitin (Ub)-tagged proteins, a measure of damaged, non-native proteins targeted for protein breakdown, have been observed in Antarctic fishes compared with their close temperate relatives (Place and Hofmann, 2005; Todgham et al., 2007). Taken together, the sub-zero Antarctic marine environment appears to place physiological constraints on maintaining proteins in their native state.

If damaged or misfolded proteins accumulate in the cell, these non-native proteins become cytotoxic and interfere with proper functioning of the cell (Sherman and Goldberg, 2001). Molecular chaperones, such as Hsps, are involved in remodelling damaged proteins back to their native state; however, if these proteins are beyond repair, they will be degraded by proteases, primarily by the 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;

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Goldberg, 2003). The Ub–proteasome pathway is a highly regulated and temporally controlled process that plays a very important role in a broad array of cellular processes and specifically targets numerous substrates (e.g. regulators of cell cycle, components of signal transduction pathways, enzymes involved in metabolism and damaged proteins) (Glickman and Ciechanover, 2002). Mechanistically, it is unclear whether the elevated levels of Ub conjugates measured in Antarctic fishes (Place and Hofmann, 2005; Todgham et al., 2007) are the direct denaturation of proteins at sub-zero temperatures in an oxygen-rich environment or a more cellular mechanism where the process of protein homeostasis (e.g. protein folding or protein breakdown) is affected because of limited cold adaptation of the machinery. However, it is becoming apparent that one of the costs associated with life in the cold is defending the integrity of the cellular protein pool.

The objective of the present study was to examine whether there is thermal compensation of the Ub–proteasome pathway in Antarctic fishes to better understand the efficiency of the protein breakdown machinery in polar species. Studies of temperature compensation of enzymes in Antarctic species have demonstrated that polar organisms maintain higher enzyme activities than would be predicted when rates from temperate or tropical species are extrapolated down to subzero temperatures (Kawall et al., 2002; Fields and Houseman, 2004; Galarza-Muñox et al., 2011). To offset the effects of low temperature on reaction rates, we would predict some degree of thermal compensation of the activity of the 26S proteasome in Antarctic species; however, this has yet to be tested in comparison to temperate relatives. The Antarctic fish fauna is dominated by the perciform suborder Notothenioidei (Eastman, 1993; Clarke and Johnston, 1996). Although this group of fishes is largely endemic to the Antarctic region and members have evolved under stable sub-zero conditions for millions of years, there are several non-Antarctic notothenioid species that inhabit the southern coastal waters of New Zealand, Australia and South America, which rarely experience temperatures below 5°C. Notothenioids as a group offer a unique comparative study system to investigate the mechanisms of cold adaptation in polar species and provide us with an opportunity to separate the effects due to environment from those due to phylogeny and better understand thermal compensation of particular physiological processes, such as protein homeostasis.

MATERIALS AND METHODS

Fish collection

Adult specimens of two Antarctic notothenioids [*Trematomus bernacchii* Boulenger 1902 and *Pagothenia borchgrevinki* (Boulenger 1902)] were collected in McMurdo Sound, Antarctica (77°53′S, 166°40′E), during October and November 2006 (n=10 for both species, sex not determined). Benthic nearshore specimens of *T. bernacchii* 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. Fish were euthanized immediately following capture. Specimens of two New Zealand notothenioids [*Notothenia angustata* Hutton 1875 (n=6, mass: 905.7±123.5 g, standard length: 338.5±17.1 mm, mean±s.e.m.) and *Bovichtus variegatus* Richardson 1846 (n=7, mass: 45.8±11.0 g, standard length: 124.1±8.7 mm)] 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* was collected using hand nets and the black cod *N. angustata*, 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 euthanized.

This research was conducted in accordance with US federal animal welfare laws via approval and oversight by the University of California, Santa Barbara Institutional Animal Care and Use Committee (IACUC) (protocol no. 634). Specimens were collected in compliance with the US regulations governing collection of Antarctic organisms, the Antarctic Conservation Act of 1978 (Public Law 95-541) and the Antarctic Marine Living Resources Convention Act of 1984 (Public Law 98-623) and complied with the current laws in New Zealand.

Tissue sampling

Fish were netted and rapidly anaesthetized with a high dose of MS-222 (0.2 g MS-222 l^{-1} water), and following onset of anaesthesia, the spinal cord was severed. Liver and gills were then rapidly excised, snap-frozen in liquid nitrogen and stored at −80°C until further analysis. Liver and gills were chosen as these tissues are both metabolically active, with the gills being directly in contact with the outside environment (i.e. potentially in contact with brash and anchor ice in Antarctic fishes) and one of the most active tissues with respect to protein turnover (Lyndon and Houlihan, 1998).

Dot blot analysis

Levels of Ub-conjugated protein were measured using immunochemical analysis of gill and liver using methods outlined in Todgham et al. (2007) in winter 2007. Equal amounts of total protein (0.5 µg) from each sample were blotted onto pre-wetted nitrocellulose membrane (0.2 µm pore size, Bio-Rad Laboratories, Hercules, CA, USA) in triplicate by gravity filtration using a BioDot dot blotter (Bio-Rad Laboratories). Values were standardized using dot intensity values from an individual sample of *T. bernacchii* liver homogenate that was run in triplicate concurrently on each gel (referred to as ‘standard’ in figures).

Proteasome activity

Proteasome activity was measured in winter 2007 on the same individuals as Ub-conjugated proteins but using a different section of tissue. Frozen tissue samples were homogenized with a Dounce homogenizer (Kontes, Vineland, NJ, USA) in SDS-free homogenization buffer (50 mmol l^{-1} Tris, 0.1 mmol l^{-1} EDTA, 1.0 mmol l^{-1} β-mercaptoethanol, pH 7.4), at a ratio of 200 mg tissue to 1 ml of buffer. Homogenates were then centrifuged at 20,000 ***g*** for 1 h at 4°C. 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 divided into aliquots and stored at −80°C until proteasome activity was measured within 48 h. We assumed no loss of activity with short-term storage at −80°C.

Biochemical studies of the 20S proteasome with fluorogenic peptide substrates have led to the description of three distinct proteolytic components within the inner rings of the core particle, designated as: (1) chymotrypsin-like activities, which cleaves after large hydrophobic residues, (2) trypsin-like activities, which cleaves after basic residues, and (3) peptidylglutamylpeptide hydrolyzing activities, which cleaves after acidic residues (Coux et al., 1996). To measure the chymotrypsin-like peptidase activity of the 20S proteasome, the fluorogenic peptide substrate succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (Suc-LLVY-AMC, 10 mmol l^{-1} stock in DMSO) (Boston Biochem, Cambridge, MA, USA) was

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used following a protocol modified from Velickovska et al. (2005) for a microplate. During setup, all reagents and labware were pre-chilled on ice to avoid inconsistencies in reaction rates due to variations in temperature. To begin, frozen protein homogenates were thawed on ice for 1 h. These samples were then diluted with SDS-free homogenization buffer to a standard protein concentration of 2.5 µg µl^{-1}. Five micrograms of diluted homogenate was loaded onto a 96-well opaque black plate, in replicates of eight, and incubated with 100 µl of reaction buffer (40 µmol l^{-1} Suc-LLVY-AMC, 100 mmol l^{-1} Tris, pH 7.4). To determine background fluorescence, stop solution (6 mol l^{-1} guanidine hydrochloride) was added to three of the eight replicates immediately after reaction buffer was added. The plate was then moved to a plate chiller, preset to the desired temperature, where the reaction was allowed to continue for 30 min. Proteasome activity was assessed at 0 and 10°C. Stop solution was added after the 30 min incubation to terminate the reaction. Fluorescence, as a measure of the cleaving of the fluorogenic product AMC from the Suc-LLVY, was determined by a Victor$_3$ Multilabel Plate Counter (Perkin Elmer, Waltham, MA, USA) with 380 nm excitation and 535 nm emission filters. The 20S proteasome activity is presented as arbitrary fluorescent units (AFU) minus background fluorescence.

Q_{10} calculations

To compare the temperature sensitivity of proteasome activity in Antarctic and New Zealand notothenioids, Q_{10} values were calculated for the 0–10°C interval in assay temperature using the following equation:

$$Q_{10} = (R_1/R_2)^{(10/T_2-T_1)}, \quad (1)$$

where R_1 represents proteasome activity when assayed at 0°C (T_1) and R_2 represents proteasome activity when assayed at 10°C (T_2). From the Q_{10} values calculated for the Antarctic notothenioids, proteasome activity of the gills and liver of these fish at their native temperature of −1.9°C was estimated.

RNA extraction and reverse transcription

Total RNA was extracted from gill tissues using the guanidine isothiocyanate method outlined by Chomczynski and Sacchi (1987) using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) in spring 2007. Following extraction, RNA was processed through an additional clean-up step to remove small-sized RNA degradation products. Dried RNA pellets were resuspended in 0.1 ml of nuclease-free water. Following resuspension, 0.3 ml of 6 mol l^{-1} guanidine hydrochloride and 0.2 ml of 100% ethanol (EtOH) were added and the entire volume was loaded onto a spin column (Ambion, Austin, TX, USA) and centrifuged for 1 min at 12,000 ***g*** at room temperature. Flow-through was discarded and filters were washed twice with 0.2 ml of 80% EtOH. RNA was eluted off the filters with 0.2 ml of nuclease-free water. To precipitate RNA, 0.1 vol of 3 mol l^{-1} sodium acetate (pH 5.0) and 2.5 vol of 100% EtOH was added to the eluted RNA, the contents were mixed by vortexing and then incubated for 1 h at −80°C. After this period, tubes were spun at 12,000 ***g*** for 20 min at room temperature. Pellets were rinsed twice with 80% EtOH and resuspended in 50 µl of nuclease-free water. RNA was quantified spectrophotometrically and electrophoresed on a 1.5% w/v agarose gel to verify RNA integrity. RNA was stored at −80°C. First-strand cDNA was synthesized from 1 µg total RNA using oligo(dT$_{18}$) primer and Improm-II reverse transcriptase following the manufacturer's instructions (Promega, Madison, WI, USA).

Isolation and sequencing of candidate genes from the Ub–proteasome pathway

In order to characterize the transcript dynamics of the Ub–proteasome pathway in notothenioid fishes, a number of candidate genes were chosen from the two main components of this pathway: ubiquitination of the substrate protein and the 26S proteasome complex.

Ubiquitination

The conjugation of Ub to the substrate protein targeted for degradation by the 26S proteasome requires the step-wise processing of the protein by Ub-activating (E1), Ub-conjugating (E2) and Ub-ligating (E3) enzymes. Polyubiquitin was chosen as a candidate gene because of its central role in the tagging of proteins bound for degradation by the 26S proteasome (Hershko and Ciechanover, 1998; Pickart and Eddins, 2004). The Ub-conjugating enzyme, E2D1 (UbE2D1), was chosen as a candidate gene for its specific role in mediating the rapid degradation of short-lived and abnormal cytosolic proteins, including damaged nascent proteins (Seufert and Jentsch, 1990; Chuang and Madura, 2005). The specific E3 ligase with co-chaperone activity, the carboxy terminus of the Hsp70-interacting protein (CHIP, also known as STIP1 homology and U-box containing protein 1 STUB1), was chosen as a candidate gene because of its dual role in preferentially ubiquitinating damaged proteins that are bound to chaperones, such as Hsp70 and Hsp90, as well as tagging Hsp70 for degradation by the proteasome (Connell et al., 2001; Qian et al., 2006).

26S proteasome complex

The 26S proteasome is a large protein complex consisting of a central 20S core particle that carries the catalytic activity of the proteasome and one or two 19S regulatory particles that are responsible for substrate recognition, unfolding and transport into the core particle (Voges et al., 1999). The cylindrical 20S core particle is composed of four stacked heptameric rings. The two outer rings are made up of α-subunits and are important in controlling the movement of proteins in and out of the proteasome. The two inner rings, composed of β-subunits, contain the proteolytic subunits of the core particle. The 20S proteasome subunit alpha type 2 (*PSMA2*) and proteasome subunit beta type 7 (*PSMB7*) were chosen as candidate genes representing an α- and β-subunit, respectively. The 19S regulatory cap complex contains six subunits with ATPase activity, which is thought to be important in the substrate unfolding necessary for translocation into the 20S core particle (Benaroudj et al., 2003). The candidate gene, 26S proteasome subunit ATPase 1 (*PSMC1*), was chosen as a representative ATPase subunit in the 19S regulatory particle.

Housekeeping genes

β-Actin and *elongation factor 1 alpha* (*EF1α*) were sequenced and evaluated as potential housekeeping genes. All primers were designed with the assistance of Primer3 software (Rozen and Skaletsky, 2000, http://fokker.wi.mit.edu/primer3/input-040.htm) and partial sequences for each gene of interest are reported in Table S1.

PCRs were carried out in a DNA Engine Dyad Peltier Thermal Cycler (Bio-Rad Laboratories) using 1.25 U Taq DNA polymerase (New England Biolabs, Ipswich, MA, USA) and isolated gill cDNA. PCR products were electrophoresed on 1.5% agarose gels containing ethidium bromide and bands of appropriate size were extracted from the gels using the QIAEX II gel extraction kit (Qiagen, Valencia, CA, USA). The extracted PCR product was ligated into a T-vector (pGEM-T easy; Promega), transformed into

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heat shock competent *Escherichia coli* (strain JM109; Promega) and colonies were grown on ampicillin LB-agar plates. Several colonies containing the ligated PCR product were selected and grown overnight in LB bacterial growth medium. Plasmids were isolated from the liquid culture using GenElute Plasmid Miniprep kit (Sigma-Aldrich, St Louis, MO, USA) and sequenced on a CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA).

Quantitative real-time PCR analysis of gene expression

The relative levels of mRNA for the *Ub*, *UbE2D1*, *CHIP*, *PSMA2*, *PSMB7*, *PSMC1* and *β-Actin* genes from gill tissue were determined using quantitative real-time PCR on an iCycler Thermal Cycler (Bio-Rad). Gene-specific primers were designed using Primer Express software (version 2.0.0; Applied Biosystems, Foster City, CA, USA) and are reported in Table S2. Quantitative real-time PCR reactions were performed with 1 µl cDNA, 4 pmol of each primer and 2× SYBR Green Master Mix (Bio-Rad) to a total volume of 20 µl. All qRT-PCR reactions were run as follows: 1 cycle of 94°C for 3 min, 40 cycles of 94°C for 20 s, 55°C for 20 s, 1 cycle of 94°C for 1 min, and 1 cycle of 55°C for 1 min. At the end of each PCR reaction, PCR products were subjected to a melt curve analysis to confirm the presence of a single amplicon.

Statistical analyses

Because of the unbalanced factorial design of the data set, a split-plot analysis using residual maximum likelihood (REML) was used to determine significant (P<0.05) differences in Ub-conjugated protein levels between species and tissue types and in proteasome activity between species, tissue types and assay temperature (Patterson and Thompson, 1971). All data were log transformed to satisfy the assumption of homogeneity of variance. Adjusted means (for the unbalanced design) were compared using Tukey's HSD test. Results for Ub-conjugated protein and proteasome activity are reported as means±s.e.m. on the original scale.

The expression of two housekeeping genes (HKG; *β-Actin* and *EF1α*) was evaluated as potential housekeeping genes in field-collected fish of all four species (n=8 fish per species) using the Excel-based program BestKeeper (Pfaffl et al., 2004). BestKeeper determines the most stable HKGs through repeated pairwise correlation analyses of threshold cycle (C_t) values from HKGs and candidate genes. *β-Actin* was selected as the HKG for the current analyses as it had the lowest standard deviation (0.64), demonstrating the consistent expression of *β-Actin* across species, and a high correlation coefficient (r=0.959) using the BestKeeper Index (Pfaffl et al., 2004). To quantify *Ub*, *UbE2D1*, *CHIP*, *PSMA2*, *PSMB7* and *PSMC1* mRNA expression, one control cDNA sample was used to develop a standard curve for all primer sets relating C_t to cDNA amount, and this standard curve was run on each plate. All results were expressed relative to these standard curves and mRNA values were normalized relative to *β-Actin*. One-way ANOVA was used to determine significant (P<0.05) differences in mRNA levels of a particular gene between fish species. Means were compared using the *post hoc* Tukey's HSD multiple comparison test (P<0.05). All data were tested for normality (Shapiro–Wilk test) and homogeneity of variance (Levene's median test). In cases where these assumptions were not met, values were square root transformed and the statistical analysis was repeated. Results for *Ub*, *UbE2D1*, *CHIP*, *PSMA2*, *PSMB7* and *PSMC1* mRNA expression are reported as means±s.e.m. on the original scale. All statistical analyses were carried out using JMP (SAS Institute, Cary, NC, USA).

RESULTS

Ubiquitin-conjugated protein levels

Ub can tag many size classes of proteins for degradation via the Ub–proteasome pathway. For quantitative purposes, Ub-conjugated proteins were analyzed using dot blot analysis. Overall, there were significant differences in the levels of Ub-conjugated proteins between the Antarctic and New Zealand notothenioids (Fig. 1). Results of the two-factor factorial, split-plot analysis using REML showed that there was a significant effect of fish species (P<0.0001), with the New Zealand tidepool thornfish, *B. variegatus*, having significantly lower levels of Ub conjugates than the Antarctic notothenioids *T. bernacchii* and *P. borchgrevinki* as well as the New Zealand black cod, *N. angustata*, which did not differ significantly from one another. There was no significant effect of tissue type (P=0.3403), indicating that there was no significant intraspecific difference between gill and liver levels of Ub conjugates in any of the four notothenioid species. Finally, there was no significant interaction between fish species and tissue type (P=0.1441).

Proteasome activity

Results of the three-factor factorial, split-plot analysis using REML showed that there was a significant effect of fish species

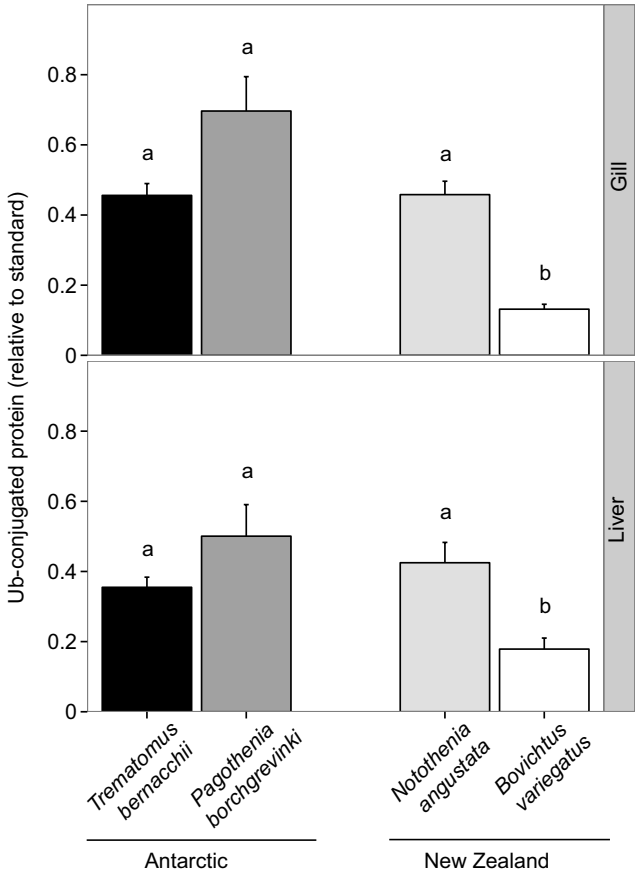

Fig. 1. Ubiquitin (Ub)-conjugated protein levels in the gill and liver tissues collected from wild-caught fish. Specimens of *Trematomus bernacchii* and *Pagothenia borchgrevinki* were collected in McMurdo Sound, Antarctica (n=10 for both species). Specimens of *Notothenia angustata* (n=6) and *Bovichtus variegatus* (n=7) were collected on the Otago Peninsula of the South Island, New Zealand. Ub-conjugated protein levels are shown as relative values based on dot intensities standardized against an individual sample of *T. bernacchii* liver homogenate (means±s.e.m.). Different letters denote significant differences between fish species within a specific tissue type (P<0.05).

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(P<0.0001), with *T. bernacchii* and *P. borchgrevinki* having the highest 20S proteasome activity, followed by *N. angustata*, which had significantly lower proteasome activity than the two Antarctic notothenioids but a significantly higher activity than *B. variegatus* (Fig. 2). Overall, there was a significant effect of tissue type (P=0.0003) on proteasome activity, with activity in the gill tissue being significantly higher than levels measured in the liver tissue. Finally, proteasome activity assayed at 0°C was significantly lower than activity assayed at 10°C (P<0.0001).

The split-plot analysis revealed that there was a significant interaction between fish species and tissue type (P<0.0001), with *T. bernacchii* and *P. borchgrevinki* having higher proteasome activity in the gills than in the liver, whereas in *B. variegatus*, proteasome activity was higher in the liver than in the gills. Tissue proteasome activity did not differ between liver and gills in *N. angustata*, which is intermediary between what was shown in the Antarctic notothenioids and the intertidal New Zealand notothenioid. There was also an interaction between tissue type and assay temperature in regards to proteasome activity (P=0.0125), which demonstrated that 20S proteasome activity was significantly different in gill and liver tissue when proteasome activity was assayed at 0°C, but there was no significant difference between gill and liver proteasome activity when assayed at 10°C. Finally, there was no significant interaction between fish species and assay temperature (P=0.7442), nor was there an interaction between fish species, tissue type and assay temperature (P=0.3659).

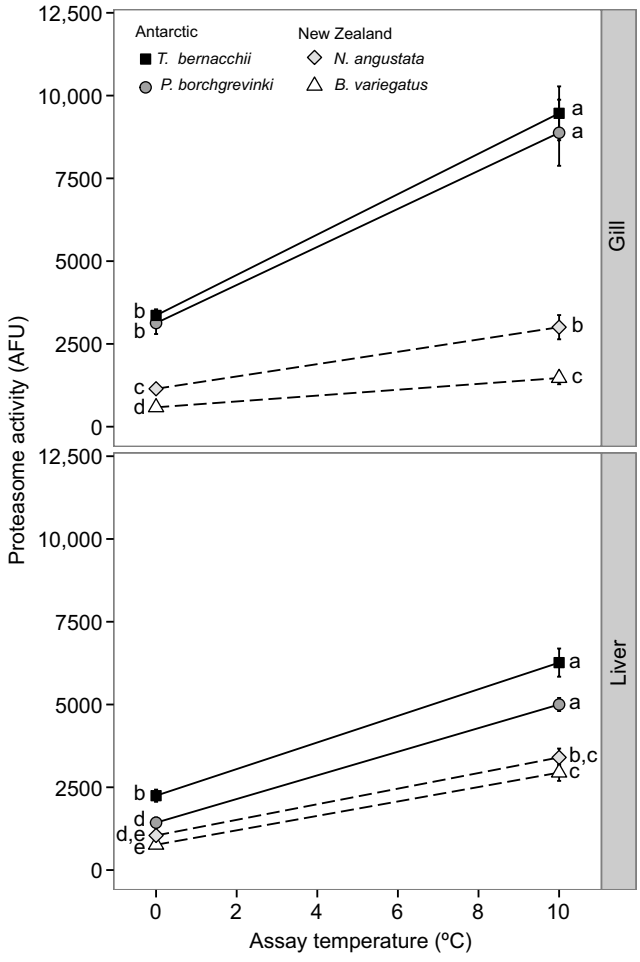

Fig. 2. Proteasome activity in the gill and liver tissues collected from wild-caught fish. Proteasome activity was assessed at 0 and 10°C and is presented as arbitrary fluorescent units (AFU) minus background fluorescence (means±s.e.m.). Different letters denote significant differences between fish species at a particular assay temperature and between assay temperatures within a specific tissue type (P<0.05).

Table 1. Estimated Q_{10} values for proteasome activity in the gill and liver of field-collected Antarctic and New Zealand notothenioids

	Q_{10} estimation (0–10°C)	
Species	Gill	Liver
Antarctic		
Trematomus bernacchii	2.80±0.33 (6)^{b,c}	2.81±0.30 (6)^{b,c}
Pagothenia borchgrevinki	2.85±0.35 (6)^{b,c}	3.60±0.61 (6)^{a,b}
New Zealand		
Notothenia angustata	2.64±0.37 (6)^{c}	3.31±0.65 (6)^{a,b,c}
Bovichtus variegatus	2.51±0.51 (6)^{c}	3.86±0.41 (6)^{a}

Values are means±s.d. (number of individuals). Different letters denote significant differences in Q_{10} values

Analysis of proteasome activity data: Q_{10} values and thermal compensation

To estimate the temperature sensitivity of proteasome activity in the gills and liver of field-collected fishes, Q_{10} values were calculated from the proteasome activities assayed at 0 and 10°C (Table 1). Two-factor factorial, split-plot analysis using REML indicated that there was no significant difference in the Q_{10} values of the four fishes when tissue data were combined (P=0.1511). There was a significant effect of tissue type (P<0.0001), with Q_{10} values in the liver being significantly elevated over values calculated in the gills. Statistical analysis revealed that there was a significant interaction between species and tissue type (P=0.0122), indicating that the relationship between Q_{10} values in the gills and liver of the four fish species were different. In *T. bernacchii*, Q_{10} values in the gills and liver did not differ significantly from one another. In *P. borchgrevinki* and *N. angustata*, Q_{10} values in the gills were lower, although not significantly, than the Q_{10} values calculated for the liver. Finally, in *B. variegatus*, the Q_{10} value for proteasome activity in the gills was significantly lower than the Q_{10} value in the liver.

Q_{10} values were used to estimate the proteasome activity of Antarctic fishes at their native environmental temperature (−1.9°C) in Antarctic waters. These activity values were then compared with the proteasome activities measured at 10°C in the New Zealand species, an average temperature experienced in their native New Zealand waters (Table 2). Within the gills, there was a significant effect of species (P=0.0031) on proteasome activity of the four fish at native environmental temperatures. Proteasome activity in the gills of *N. angustata*, *T. bernacchii* and *P. borchgrevinki* did not

Table 2. Estimated proteasome activity in the gill and liver of field-collected Antarctic and New Zealand notothenioids (n=6 for all species) at their native environmental temperatures (−1.9°C for Antarctic species, 10°C for New Zealand species)

	Proteasome activity (AFU)	
Species	Gill	Liver
Antarctic (−1.9°C)		
Trematomus bernacchii	2186±230^{a,b}	1437±151^{b}
Pagothenia borchgrevinki	1959±206^{a,b}	672±77^{c}
New Zealand (10°C)		
Notothenia angustata	2893±304^{a}	3361±353^{a}
Bovichtus variegatus	1408±148^{b}	2864±301^{a}

Values are means±s.e.m. Different letters denote significant differences in proteasome activity within a particular tissue type.

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differ significantly from one another; however, all three fish had higher branchial proteasome activity than *B. variegatus*, with significant differences between *B. variegatus* and *N. angustata*. Within the liver, there was also a significant difference in proteasome activity between the fish species (P<0.0001). In contrast to what was seen in the gills, hepatic proteasome activity was significantly higher in the New Zealand species.

Gene expression

To quantify gene expression of the Ub–proteasome pathway, candidate genes were examined from the two major components of this pathway: the conjugation of Ub to the substrate protein targeted for degradation by the proteasome (*Ub*, *UbE2D1* and *CHIP*) and the 26S proteasome complex itself (*PSMA2*, *PSMB7* and *PSMC1*). Results from the one-way ANOVA on candidate gene expression in the gills of field-collected *T. bernacchii*, *P. borchgrevinki*, *N. angustata* and *B. variegatus* revealed that there were significant differences between species in *Ub* ($F_{3,29}$=291.84, P<0.0001), *UbE2D1* ($F_{3,29}$=72.23, P<0.0001), *CHIP* ($F_{3,29}$=452.64, P<0.0001), *PSMA2* ($F_{3,29}$=32.50, P<0.0001), *PSMB7* ($F_{3,29}$=161.03, P<0.0001) and *PSMC1* ($F_{3,29}$=223.37, P<0.0001) mRNA levels (Figs 3, 4). *Ub* mRNA levels in field-acclimatized notothenioids showed that the highest transcript levels were measured in the New Zealand black cod, *N. angustata* (Fig. 3). *Ub* gene expression was 2.5-fold higher in *N. angustata* when compared with the Antarctic notothenioids *T. bernacchii* and *P. borchgrevinki*, which did not differ significantly from one another. The lowest levels of *Ub* transcript were measured in the New Zealand thornfish, *B. variegatus*. When comparing transcript levels of the Ub-conjugating enzyme *UbE2D1*, which has a specific role in mediating the rapid degradation of short-lived and abnormal cytosolic proteins (Seufert and Jentsch, 1990; Chuang and Madura, 2005), *P. borchgrevinki* and *N. angustata* had significantly higher expression than *T. bernacchii* and all three fish had significantly higher *UbE2D1* mRNA levels than *B. variegatus* (Fig. 3). Transcript levels of the E3 ligase *CHIP*, which is responsible for

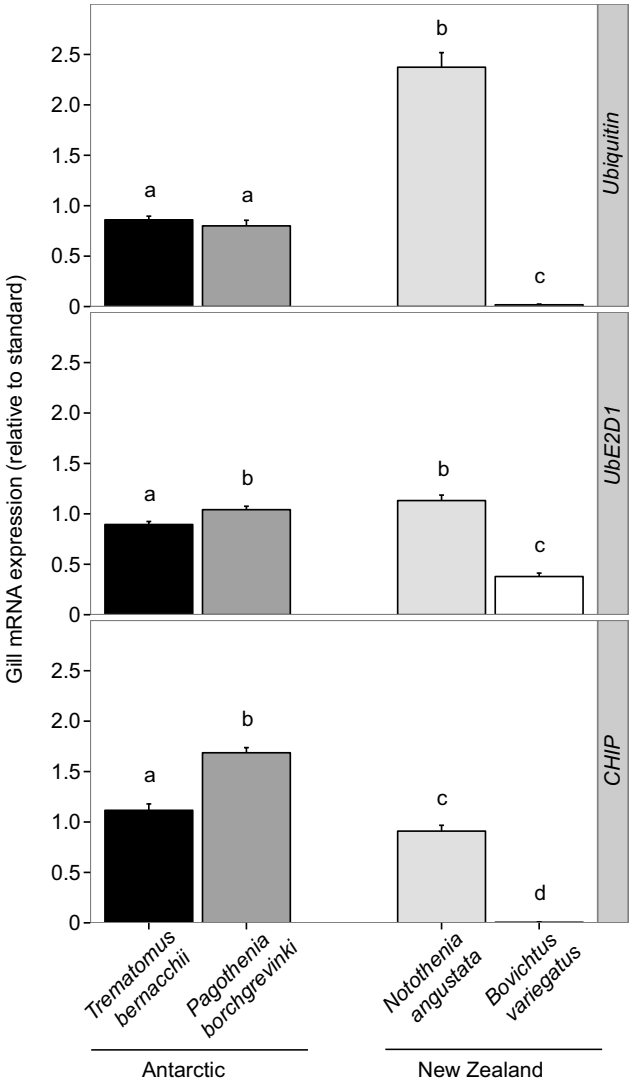

Fig. 3. Branchial mRNA levels of genes involved in the Ub tagging of proteins for breakdown by the 26S proteasome from wild-caught fish. *Ubiquitin*, *Ub2ED1* and *CHIP* mRNA levels are normalized to a control gene, *β-Actin* (means±s.e.m.). Different letters denote significant differences in mRNA levels between fish species for a particular gene (P<0.05).

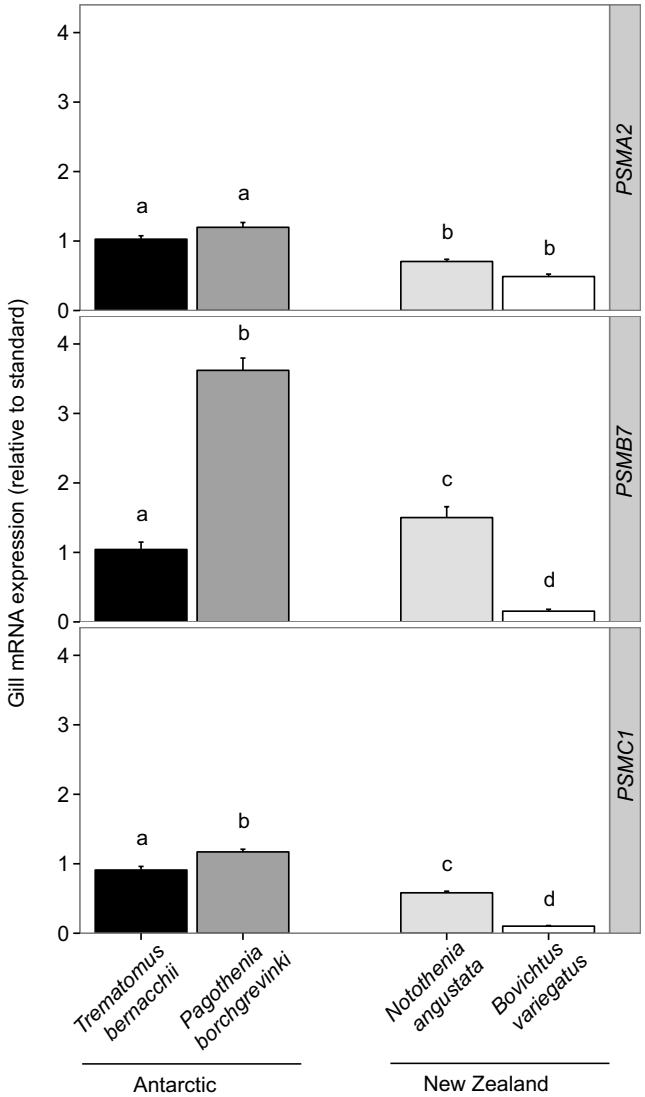

Fig. 4. Branchial mRNA levels of genes involved in the 26S proteasome complex from wild caught fish. *PSMA2* mRNA, *PSMB7* mRNA and *PSMC1* mRNA levels are normalized to a control gene, *β-Actin* (mean±s.e.m.). Different letters denote significant differences in mRNA levels between fish species for a particular gene (P<0.05).

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the ubiquitination of proteins bound to molecular chaperones as well as the ubiquitination of molecular chaperones targeted for degradation by the proteasome (Connell et al., 2001; Qian et al., 2006), differed significantly between all four notothenioid fishes. *Pagothenia borchgrevinki* had the highest *CHIP* mRNA levels, followed by *T. bernacchii*, followed by *N. angustata*, with *B. variegatus* having the lowest transcript levels of *CHIP* (Fig. 3). The Antarctic notothenioids *T. bernacchii* and *P. borchgrevinki* had significantly higher expression levels of the proteasome α-subunit *PSMA2* compared with their New Zealand counterparts *N. angustata* and *B. variegatus*, which did not differ significantly from one another (Fig. 4). The expression of the proteasome subunits *PSMB7* and *PSMC1* were similar for these two genes, with all four notothenioids having significantly different transcript levels from one another. The gills of *P. borchgrevinki* had the highest levels of *PSMB7* expression compared with the other notothenioids, followed by *N. angustata* and *T. bernacchii*, and *B. variegatus* had the lowest levels of *PSMB7* mRNA expression. Similarly, field-collected *P. borchgrevinki* and *B. variegatus* had the highest and lowest transcript levels, respectively, of *PSMC1*; however, with this candidate gene, the second highest levels were measured in *T. bernacchii*, followed by *N. angustata* (Fig. 4).

DISCUSSION

The main objective of this research was to examine mechanisms of protein degradation in Antarctic notothenioid fishes and to determine whether thermal compensation of the Ub–proteasome pathway might exist in the representative study species. We also adopted a comparative approach, noting that comparison of proteasome activities of these two pairs of closely related but differently thermally adapted species might increase our understanding of thermal adaptation that has occurred with cold adaptation of Antarctic fish species with regard to the maintenance of protein homeostasis. Overall, our results suggest that proteasome activity in the gills of Antarctic fish species have undergone a high degree of cold adaptation such that activity levels were similar to activities measured in their temperate relatives at ecologically relevant temperatures. A similar level of thermal compensation has not occurred in the proteasome activity of the liver of Antarctic fishes, although partial temperature compensation is evident. Furthermore, basal mRNA expression in the gills of three proteins involved in the Ub tagging of damaged proteins as well as three proteins involved in the different complexes of the 26S proteasome are higher in the Antarctic species compared with the New Zealand *B. variegatus*. Therefore, higher gill proteasome activity is likely due in part to higher cellular levels of proteins involved in the Ub–proteasome pathway. Lastly, *N. angustata*, a New Zealand temperate relative with ancestors believed to have once lived in Antarctic waters, shows evidence at the level of Ub-conjugated proteins, proteasome activity and mRNA expression of Ub–proteasome pathway genes of an intermediary biochemical phenotype with respect to protein catabolism machinery between the Antarctic fishes and the other New Zealand temperate relative, *B. variegatus*.

Temperature compensation of the Ub–proteasome pathway

20S proteasome activity, as reflected in the capacity to degrade a synthetic peptide, is temperature compensated in the gills but not the liver of *T. bernacchii* and *P. borkgrevinki*. Proteasome activity in the gills of Antarctic fishes at 0°C is similar to that of *N. angustata* at 10°C and significantly elevated above that of *B. variegatus* at 10°C (Fig. 2). When proteasome activity was extrapolated down to −1.9°C (Table 2), activity was not significantly different from either species, with activity being higher in *N. angustata* and lower in *B. variegatus* when compared with the Antarctic notothenioids. Temperature compensation and cold adaptation of proteins with catalytic functions in Antarctic species has been an area of research that has received significant attention over the last few decades in fishes (Crockett and Sidell, 1990; Fields and Somero, 1998; Kawall et al., 2002; Fields and Houseman, 2004) and invertebrates (Galarza-Muñoz et al., 2011). These studies have focused on the structural and physiological adjustments that provide proteins with the flexibility to compensate for the reduction in chemical reaction rates that is due to sub-zero temperatures while preserving the stability necessary to maintain function (Somero, 1995). Enzymes involved in aerobic metabolism and β-oxidation of lipid fuels are cold adapted in Antarctic fish, as is lactate dehydrogenase (Crockett and Sidell, 1990; Fields and Somero, 1998; Kawall et al., 2002). Much of our understanding of cold adaptation of proteins comes from single-protein studies (e.g. enzymes or active ion transporters), with less attention to protein complexes. Protein synthesis machineries and capacities in polar zoarcids are cold-adapted through increased RNA translational capacity (Storch et al., 2005). Results from the present study demonstrate that protein degradation machinery in the gills also shows partial temperature compensation and likely cold adaptation.

Protein synthesis and degradation are energetically costly (Houlihan, 1991); therefore, in Antarctic fishes, where oxygen consumption rates and thus energy supply are low, there is a potential energetic trade-off in maintaining protein homeostasis and managing the accumulation of damaged proteins. Why liver proteasome activity does not show the same level of temperature compensation as gill proteasome activity requires further investigation, but may be due to differences in how energy is allocated in a particular tissue. Tissue-specific differences may be due to lower protein turnover rates in liver compared with gill tissue, a tissue known to be highly active with respect to protein turnover (Lyndon and Houlihan, 1998), and therefore a decreased reliance on protein degradation machinery in the liver. Alternatively, our selected Antarctic fishes species live in environments where their gills may be in direct contact with brash ice (*P. borchgrevinki*) or anchor ice (*T. bernacchii*) and therefore have an increased potential for gill tissue damage and a greater demand on maintaining protein integrity. In a previous study, gill Ub-conjugated protein levels were significantly higher in the gills of *T. bernacchii* and *Lycodichthys dearborni* compared with the liver (Todgham et al., 2007); however, in the present study, levels of Ub-conjugated proteins were only slightly higher (Fig. 1, non-significant) in the gills. The reason why liver proteasome activity is not temperature compensated to the same degree as in the gills remains conjectural and requires additional study.

The mechanistic basis for higher proteasome activity in the gills of Antarctic fishes compared with their temperate New Zealand relatives could be due to higher concentrations or cellular levels of proteasomes, more efficient proteasomes (i.e. higher catalytic efficiency) or a combination of the two. Gene expression analysis of proteins involved in the tagging of Ub to denatured proteins (*Ub*), the shuttling of these proteins to the proteasome (*UbE2D1*, *CHIP*) and the different regulatory and catalytic complexes of the proteasome itself (*PSMA2*, *PSMB7*, *PSMC1*) provide direct evidence of higher basal levels of mRNA transcripts in Antarctic fishes (Figs 3, 4) compared with *B. variegatus* (the intermediary nature of *N. angustata* is discussed below), suggesting higher cellular levels of proteasomes. A transcriptome comparison of the Antarctic toothfish *Dissostichus mawsoni* and five temperate and

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tropical teleosts demonstrated that Antarctic toothfish have significant upregulation of genes involved in the Ub–proteasome pathway (e.g. Ub, Ub-conjugating enzymes E2s and proteasome α subunits) as well as extracellular proteases such as Cathespin Z, suggesting a greater demand for these functions in the sub-zero Antarctic polar environment (Chen et al., 2008). Pepsins, a family of aspartic proteinases important in digestive functions, have been isolated and characterized from *T. bernacchii* (Brier et al., 2007). Results suggest that Antarctic pepsins are not cold-adapted, having specific activities that were similar to or lower than pig pepsin. The authors speculated that *T. bernacchii* likely increase production of pepsin to compensate for the reduced kinetic efficiency at low temperature (Brier et al., 2007). Cold acclimation of juvenile spotted wolfish, *Anarhichas minor*, to 4°C resulted in increased 20S proteasome activity (Lamarre et al., 2009) and cold acclimation has been shown to increase expression of genes involved in the Ub–proteasome pathway in the common carp, *Cyprinus carpio* (Gracey et al., 2004), the annual killifish, *Austrofundulus limnaeus* (Podrabsky and Somero, 2004), and the zebrafish, *Danio rerio* (Chou et al., 2008). Taken together, both acclimation and adaptation to low temperatures results in higher proteasome activities with evidence of higher cellular concentrations; however, additional research is needed to determine the catalytic efficiency of the 20S proteasome of Antarctic fishes and whether catalytic rate constants (k_{cat}) and Michaelis–Menten constants (K_m) are modified similarly to what has been documented for lactate dehydrogenase (A_4-LDH; Fields and Somero, 1998). Furthermore, body size is known to influence enzyme activity (Somero and Childress, 1980) and protein turnover (Houlihan, 1991), and growth rate has been shown to influence proteasome activity in the white muscle of wolffish (Lamarre et al., 2010). Additional research is needed to understand the effects of body size on proteasome activity of notothenioid fishes.

Thermal sensitivity of 20S proteasome activity of Antarctic and New Zealand notothenioids do not differ substantially in gill (range of Q_{10} values: 2.51–2.85; Table 1) and liver tissues (range of Q_{10} values: 2.81–3.86; Table 1), with no significant differences between tissues, except for *B. variegatus*. The estimated Q_{10} values of proteasome activity in notothenioid fishes at their environmental temperatures (Table 2) are greater than those reported in the liver of juvenile spotted wolffish (Q_{10}=1.3, Lamarre et al., 2009) but within the range reported for hibernating ground squirrels as temperature is reduced (range of Q_{10} values estimated from Fig. 2 as temperature decreased from 30 to 10°C: 2.0–2.7; Velickovska et al., 2005).

Potential mechanisms underlying elevated levels of damaged proteins in Antarctic fishes

Higher proteasome activity along with other indices of protein homeostasis confirm that persisting at sub-zero temperatures places fundamental constraints on maintaining protein integrity in Antarctic notothenioids. Three potential mechanisms that may underlie the constraints on protein homeostasis are: (1) proteins are temperature sensitive and denatured by cold, (2) protein folding efficiency is compromised thermodynamically at cold temperatures, resulting in improperly folded proteins requiring degradation and (3) a factor other than cold temperature, such as oxidative stress, is denaturing the proteins or preventing them from folding properly in the first place. It is unlikely that the extremely cold temperatures of Antarctic waters denature proteins given the long evolutionary time frame (11–14 million years) that notothenioid fishes have had to adapt to subzero temperatures and undergo appropriate modifications to maintain stability under natural conditions. There are numerous studies that have investigated the modifications of cold-adapted proteins that allow them to function at subzero temperatures (Jaenicke, 1990; Hoyoux et al., 2004; Siddiqui and Cavicchioli, 2006). While cold denaturation of proteins has been demonstrated *in vitro* by sub-zero temperatures, these studies were not conducted in a physiological system that accurately represents the conditions experienced by a protein in a cell. Although the capacity of Antarctic fishes to properly fold newly synthesized proteins has not been characterized, elevated Ub-conjugated proteins and activity of the 20S proteasome may reflect inefficiencies in protein folding associated with low 'operating temperatures' and accumulations of misfolded proteins. There is inherent inefficiency in protein folding such that approximately 30% of newly synthesized proteins are degraded within 10 min of synthesis by the proteasome (Schubert et al., 2000). The sub-zero temperatures of polar environments may exacerbate this non-productive folding such that a cost of living for cold-adapted ectotherms is committing more effort to maintaining protein homeostasis. This hypothesis requires further investigation.

Oxidative stress may place constraints on protein homeostasis in Antarctic fishes. Increased mitochondrial abundance, lipid polyunsaturation and lipid deposits all favour increased oxidative capacity at cold temperatures in Antarctic fishes; however, these cold adaptive strategies come at a cost of increased formation of reactive oxygen species (ROS) and increased lipid peroxidation (for review, see Guderley, 2004). The mitochondrial respiratory chain produces the majority of ROS in a cell. Approximately 0.15% of oxygen that is consumed is converted to ROS (St-Pierre et al., 2002) and similar rates of ROS production were documented in two Antarctic fishes (Mueller et al., 2011). Mitochondrial proliferation in Antarctic fishes is not accompanied by compensation of maximal capacities of oxidative phosphorylation, owing to reduced cristae density, likely as a mechanism to limit ROS production by limiting mitochondrial activity while maintaining enhanced oxygen distribution (Johnston et al., 1998; Sidell, 1998). Nonetheless, polar species have elevated levels of antioxidant protection to mitigate oxidative damage from ROS production (Abele and Puntarulo, 2004; Regoli et al., 2005; Benedetti et al., 2010) and show elevated levels of antioxidant defense genes when compared with temperate and tropical teleosts (Chen et al., 2008). It is possible that the antioxidant defense mechanisms in place are not sufficient to fully protect cellular protein integrity and the Ub–proteasome system has an important role in ensuring oxidatively damaged proteins are quickly degraded. The Ub–proteasome pathway has a key role in the degradation of oxidized proteins (Grune et al., 2003; Poppek and Grune, 2006). Lamarre and colleagues (2009) suggest that wolffish maintain high 20S proteasome activity following cold acclimation as a mechanism to remove proteins damaged by oxidative stress. Oxidative stress itself has been shown to inhibit proteasome degradation (Ullrich and Grune, 2001), but whether this is a factor in the accumulation of Ub-conjugated proteins tagged for breakdown by the proteasome in Antarctic fishes requires further investigation.

Evidence of cold adaptation of the Ub–proteasome pathway from *Notothenia angustata*

The intermediary nature of levels of Ub-conjugated proteins (also seen in Todgham et al., 2007), 20S proteasome activity in the gill, and gill mRNA expression of genes involved in the Ub–proteasome pathway in *N. angustata* provides additional evidence for cold adaptation of the protein breakdown machinery in Antarctic notothenioids. It is widely believed that the ancestors of *N.*

angustata inhabited the sub-zero Antarctic environment for millions of years (Cheng et al., 2003) before being displaced to New Zealand with the temporary northward movement of the Antarctic Polar Front. Therefore, this 'Antarctic escapee' likely shares many of the cold-adapted physiological traits of other Antarctic notothenioids. *Notothenia angustata* resemble their Antarctic relatives in that they possess functional antifreeze genes despite living in a temperate marine environment that rarely falls below 5°C (Cheng et al., 2003), the conserved sequence identity and diversity of haemoglobins (D'Avino and di Prisco, 1997) and a kidney that is intermediate in structure between Antarctic and non-Antarctic notothenioids (Eastman, 1993). *Notothenia angustata* are nearshore benthic species that inhabit a relatively stable temperate marine environment compared with the more thermally variable intertidal habitat in which *B. variegatus* reside, where you might expect a greater likelihood of protein denaturation. Given that *N. angustata* does not experience sub-zero temperatures in its present-day marine environment, the intermediary nature of the Ub–proteasome pathway of *N. angustata* provides additional evidence of an ancestral Antarctic existence as well as an evolutionarily conserved intrinsic mechanism of cold adaptation of protein homeostasis machinery.

Concluding remarks

In summary, Antarctic notothenioids living at sub-zero temperatures exhibit high levels of Ub-conjugated proteins and significant or partial temperature compensation of 20S proteasome activity in the gills and liver, respectively. Expression of genes involved in the Ub–proteasome pathway suggest that temperature compensation is in part likely due to higher concentrations of proteasomes in the cell, but additional research on the catalytic efficiency of the 20S proteasome of Antarctic fishes is warranted. Overall, these results provide further evidence that cold adaptation of Antarctic fishes to the sub-zero polar marine environment likely occurred with some trade-offs to protein homeostasis and the maintenance of a functional protein pool. Future studies are warranted to understand the origin of the elevated levels of denatured proteins, with a particular focus on the efficiency of protein folding *in vivo* at sub-zero temperatures and the sensitivity of proteins to oxidative stress.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

A.E.T and G.E.H. designed the experiment, A.E.T performed the experiments and A.E.T and T.A.C. analyzed the data. A.E.T wrote the manuscript with editorial comments from G.E.H.

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Data availability

Sequences were deposited into GenBank (accession numbers: GQ229124–GQ229147).

Supplementary information

Supplementary information available online at
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Table S1. Gene-specific degenerate primers used to amplify and clone candidate genes involved in the ubiquitin-proteasome pathway of Antarctic and New Zealand notothenioids. Partial *Ub* sequences were obtained using primers outlined in Okubo et al. (2002). Partial sequences for *UbE2D1* were obtained using primers designed from conserved regions of *Danio rerio* (Accession No. NM_199664), *Xenopus laevis* (Accession No. BC076728) and *Canis familiaris* (Accession No. XM_845637). Partial sequences for *CHIP* were obtained using primers determined from conserved regions of *D. rerio* (Acession No. NM_199674) and *Tetraodon nigroviridis* (Accession No. CR691698). Partial sequences for *PSMA2* were obtained using primers determined from conserved regions of *D. rerio* (Accession No. BC059539) and *Carassius auratus* (Accession No. AB013342). Partial sequence for *PSMB7* was obtained using primers determined from conserved regions of *D. rerio* (Accession No. NM_001045564), *Gasterosteus aculeatus* (Accession No. BT028121) and *T. nigroviridis* (Accession No. CR718794). Partial sequences for *PSMC1* were obtained using primers designed from conserved regions of *D. rerio* (Accession No. NM_200033), *G. aculeatus* (Accession No. BT027408) and *T. nigroviridis* (Accession No. CR650415). Partial sequences for *β-Actin* were obtained using primers determined from conserved regions of *Oryzias latipes* (Accession No. S74868), *D. rerio* (Accession No. AF057040), *Fundulus heteroclitus* (Accession No. AF397164) and *Pagrus major* (Accession No. AY190686). Partial sequences for *EF1α* were obtained using primers outlined in Todgham et al. (2006).

Candidate gene	**Primer sequences**
Ub	Forward: 5'-CTG GAA GAT GGG CGA ACC C-3' Reverse: 5'-CTG CTT GCC AGC AAA GAT CAA CC-3'
UbE2D1	Forward: 5'-TTR TTY CAC TGG CAA GCA AC-3' Reverse: 5'-TTC TGR GTC CAT TCT CTT GC-3'
CHIP	Forward: 5'-GSA TCM RCC ARG AGA RCG AG-3' Reverse: 5'-ATR AAD GCR TCR ATM ACC TC-3'
PSMA2	Forward: 5'-GTT GTG CTG GCA ACW GAG AA-3' Reverse: 5'-GCG AAA YCC TGC TTC ATT AC-3'
PSMB7	Forward: 5'-TCC AAC CTG GAG CTK CAY KC-3' Reverse: 5'-GST YTC CTC CAC CAY MTC CA-3'
PSMC1	Forward: 5'-CCC ATG TCT GTS GGM ACT CT-3' Reverse: 5'-GAT CTC VCG CTC WCC KCC WG-3'
β-Actin	Forward: 5'-CAA CGG MTC YGG TAT GTG CAA AG-3' Reverse: 5'-TGG CRT GGG GMA GRG CRT ARC C-3'
EF1α	Forward: 5'-GAA GGA AGC HGC TGA GAT GG-3' Reverse: 5'-CGG TCT GCC TCA TGT CAC GC-3'

Table S2. Gene-specific quantitative RT-PCR primers used to amplify candidate genes involved in the ubiquitin-proteasome pathway of Antarctic and New Zealand notothenioids.

Candidate gene	Primer sequences
Ub	Forward 5'-GGA AAG ACC ATC ACC CTA GAG GTA-3' Reverse 5'-TGG ATC TTG GCC TTC ACG TT-3'
UbE2D1	Forward 5'-GGA CCG AAT GAC AGC CCT TA-3' Reverse 5'-TCA GTG GGA AAA TGG ACA GAA A-3'
CHIP	Forward 5'-CTT ATG ATC GCA AGG ACA TTG AAG-3' Reverse 5'-GGT GAC CGG GTC GAA GTG-3'
PSMA2	Forward 5'-CGA CGA GCC CGG AAG TT-3' Reverse 5'-GGG ATG GGC TCC TGG TAA A-3'
PSMB7	Forward 5'-CAC GAG GAG GCC AAC AAG AA-3' Reverse 5'-CAC GCC TGT GGT TCC TCT CT-3'
PSMC1	Forward 5'-TGA TCC CCT GGT GAC AGT GA-3' Reverse 5'-CGA TGT CAG CGT AGG TTT CTT G-3'
β-Actin	Forward 5'-AGT ACC CCA TTG AGC ACG GTA TT-3' Reverse 5'-AAG GTG TGA TGC CAG ATC TTC TC-3'
EF1α	Forward 5'-TGA CTG CGC TGT GCT GAT C-3' Reverse 5'-CTT GGA GAT ACC GGC CTC AA-3'

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