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ORIGINAL PAPER

Antarctic marine molluscs do have an HSP70 heat shock response

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Abstract The success of any organism depends not only on niche adaptation but also the ability to survive environmental perturbation from homeostasis, a situation generically described as stress. Although species-specific mechanisms to combat "stress" have been described, the production of heat shock proteins (HSPs), such as HSP70, is universally described across all taxa. Members of the HSP70 gene family comprising the constitutive (HSC70) and inducible (HSP70) members, plus GRP78 (glucose-regulated protein, 78 kDa), a related HSP70 family member, were cloned using degenerate polymerase chain reaction (PCR) from two evolutionary divergent Antarctic marine molluscs (*Laternula elliptica* and *Nacella concinna*), a bivalve and a gastropod, respectively. The expression of the HSP70 family members was surveyed via quantitative PCR after an acute 2-h heat shock experiment. Both species demonstrated significant up-regulation of HSP70 gene expression in response to increased temperatures. However, the temperature level at which these responses were induced varied with the species (+6–8°C for *L. elliptica* and +8–10°C for *N. concinna*) compared to their natural environmental temperature). *L. elliptica* also showed tissue-specific expression of the genes under study. Previous work on Antarctic fish has shown that they lack the classical heat shock response, with the inducible form of HSP70 being permanently expressed with an expression not further induced under higher temperature regimes. This study shows that this is not the case for other Antarctic animals, with the two molluscs showing an inducible heat shock response, at a level probably set during their temperate evolutionary past.

Keywords Antarctic · Heat shock protein · Stress · Temperature · Climate change

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Introduction

All organisms are adapted to life within a constrained environmental envelope, with consequential specialisations in ecology, physiology and biochemistry. Associated with these adaptations is a species-specific capacity to cope with environmental change. If an organism is taken outside of its "normal" environmental envelope by, for example, changing temperature, salinity or oxygen availability, the organism becomes vulnerable, a situation generically described as stress. In turn, this environmental challenge triggers a biochemical response, the aim of which is to counteract or mitigate any potential cell damage caused by the environmental insult and to enhance survival.

At the molecular level, the production of stress proteins (reviewed in Gross 2004), such as the heat shock proteins (HSPs), is regarded as a classical response. These are a family of highly conserved proteins that act as chaperones to stabilise and refold denatured proteins, preventing the formation of cytotoxic aggregates (Parsell and Lindquist 1993; Hartl 1996; Fink 1999). Numerous families of heat shock proteins have been identified, the naming of which is related to their weight in kilodaltons. The most studied of these family members are the 70-kD heat shock proteins (HSP70s), comprising constitutive (HSC70, heat shock cognate 70), stress-inducible (HSP70s, heat shock protein 70) and glucose-regulated forms (GRP78, glucose-regulated protein, 78 kD). Whilst their action has been described in response to a wide variety of

stresses, the classical activation of the inducible HSP70 genes is in response to elevated environmental temperatures and is tightly controlled by the heat shock transcription factor (HSF1; reviewed in Morimoto 1998).

The classic heat shock response involving a strong up-regulation of HSP70 production has been demonstrated in all organisms examined to date with the exception of *Hydra oligactis* (Bosch et al. 1988), an Antarctic ciliate *Euplotes focardii* (La Terza et al. 2001, 2004) and several species of Antarctic notothenioid fish (Place and Hofmann 2005). However, the fish case is complex. Three distantly related Antarctic species, *Trematomus bernacchii*, *Pagothenia borchgrevinki* and *Lycodichthys dearborni* permanently express the inducible form of HSP70 (Place and Hofmann 2005), but the Nototheniidae lack the ability to further up regulate this gene in response to elevated environmental temperatures (Place et al. 2004; Buckley et al. 2004). Given these data, the question arises as to whether the permanent expression of HSP70 and lack of a heat shock response in Antarctic Notothenioids is family specific and/or a consequence of adaptation to highly stable, cold Antarctic seawater temperatures and could therefore be a general phenomenon extending to other non-piscine Antarctic marine organisms.

Antarctic invertebrates are, in general, as stenothermal and, in some cases, more stenothermal than the endemic fish species (Somero and DeVries 1967; Peck and Conway 2000) with many species having survivable temperature envelopes between 5°C and 12°C above the minimum sea temperature of −1.86°C (Peck 2002). Even within this temperature range, animals start to lose critical biological functions such as swimming (scallops), righting responses (limpets) and reburying (clams; Peck et al. 2004), all of which are lost with temperature elevations of only 1–2°C above current summer maximum seawater temperatures (0–1.8°C). Many Antarctic invertebrates, including the groups mentioned above, die at temperatures below +10°C (Peck 1989; Peck et al. 2002; Pörtner et al. 2006). Shallow seawater temperatures along the west Antarctic Peninsula have risen in excess of 1°C over the last 50 years (Meredith and King 2005), whilst the IPCC (2001) Third Assessment Climate Model predicts a further 2°C increase in global seawater temperatures over the next 100 years, albeit with large regional variations and confidence intervals. In light of current and predicted seawater temperature increases, Antarctic stenotherms are, therefore, at considerable future risk of seasonal exposure to ambient water temperatures that exceed those known to result in the loss of critical biological functions (Peck et al. 2004).

In this study, members of the HSP70 gene family comprising the constitutive (HSC70) and inducible (HSP70) members, plus GRP78 (glucose-regulated protein, 78 kDa), a related HSP70 family member, were cloned using degenerate polymerase chain reaction (PCR) from two evolutionary divergent Antarctic marine molluscs [*Laternula elliptica* (a bivalve) and *Nacella concinna* (a gastropod)]. *L. elliptica* is a sediment burrowing mollusc, whereas *N. concinna* is a common inter-tidal species and can be found anywhere from mid-tide level (Walker 1972) to depths greater than 110-m depth (Powell 1951, 1973). The *N. concinna* used in this study were collected by divers at 8- to 10-m depths. Given these collection distributions, both species experience yearly water temperatures restricted between −1.86°C and +1°C. The expression of the HSP70 family members was assessed using quantitative PCR (Q-PCR) after an acute 2-h heat shock experiment. The data are discussed within the context of adaptation to life in an extreme and changing environment.

Methods

Animal sampling and experimental work

All animals used in experimental work were collected at Rothera Research Station, Adelaide Island, Antarctic Peninsula (67°34′07″ S, 68°07′30″ W), by scuba divers during the austral summer at depths of 10–15 m (*L. elliptica*) and 6 m (*N. concinna*). Seasonal water temperatures for this collection site are provided in Fig. 1. *Laternula elliptica* (bivalve) and *Nacella concinna* (gastropod) were collected and immediately returned to the laboratory where they were maintained in a through-flow aquarium under a simulated natural light/dark cycle. Predicted sunrise and sunset times (Poltips 3, Proudman Oceanographic Laboratory) were used in conjunction with a mechanical timer to control the lighting regime. During the time the animals were held in the aquarium, the water temperature was 0.75°C±0.0°C. All experimental work was

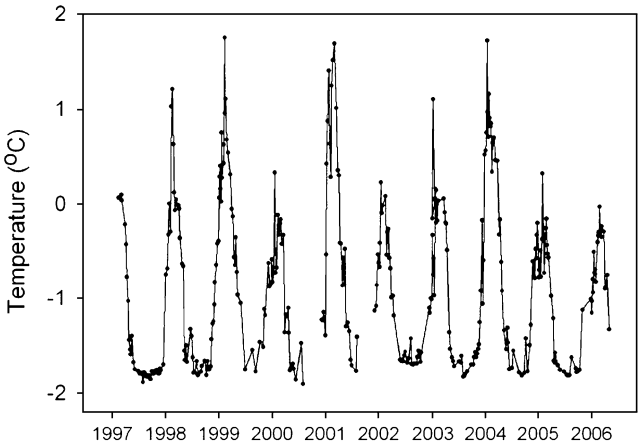


Fig. 1 Ten-year time course series of annual seawater temperature fluctuations around Rothera Research Station at 15 M (scuba operation) depth. Range of temperatures over this whole period varied from a minimum of −1.8°C to a maximum of +1.7°C. Data provided by Professor Andrew Clarke from the RaTS (Rothera Time course Series) Long-Term Monitoring Programme

carried out in January 2004. *L. elliptica* and *N. concinna* were not fed when held in the aquarium, but the former were observed filtering seawater in the tanks and would have obtained some food via this route, whereas the latter were observed grazing biofilms on the aquarium tanks. Animals were maintained for 5–7 days in the aquarium before the experimental work.

The two species were exposed to a thermal shock by immediate transfer to seawater maintained at a range of temperatures [1.33°C±0.07°C (control), 4.8°C±0.06°C, 6.0°C±0.03°C, 8.12°C±0.05°C, 10.16°C±0.07°C, 14.9°C± 0.0°C and 20.09°C±0.04°C (*N. concinna* only)] for 2 h. Groups of five animals of each species were transferred to the experimental tanks. After the 2-h thermal shock, the animals were weighed (±0.1 g), the shell length was measured (±0.1 mm), the animals were killed and the tissues were collected and placed either in RNALater (Ambion) or snap frozen in liquid nitrogen for subsequent analysis. Tissue samples were collected from the foot of *N. concinna*, and gill, foot muscle, digestive gland, gonad, mantle and siphon from *L. elliptica*.

Sample analysis

RNA extraction and isolation of HSP genes Total RNA was extracted from tissues using TRI reagent (Sigma) according to the manufacturer's instructions. One microgram of total RNA was DNAse treated using 0.4U DNase I (Ambion) in 10 mM DTT/100 mM $MgCl_2$ buffer and reverse transcribed using a first-strand synthesis kit (Promega). Degenerate primers for HSP70 were designed from a protein alignment of HSP70 genes from a variety of species (*H. sapiens* to molluscs). The primers (HSP70F, ATCATCGCYAACGAC CAGGGMRAC; HSP70R, GTTGTTGAAGTARGCDGG SACBGT) amplified a 500-bp fragment encompassing amino acids 30–125 (motifs used for primers: IIANDQGD and TVPAYFNN). PCR cycling conditions varied according to the organism: *L. elliptica*, 95°C, 5 min; 35 cycles of 95°C, 20 s; 55°C, 20 s; and 72°C for 40 s with a final elongation step of 72°C for 5 min. The same basic programme was kept for *N. concinna* but with the annealing temperature reduced to 45°C and the number of cycles increased to 40. Products were subcloned into p-GEMT-easy (Promega), transformed into *Escherichia coli* strain XL-2 Blue MRF' (Stratagene) and a minimum of 48 clones sequenced from each organism. Sequence data was assembled using the phred, Phrap and consed packages (Ewing et al. 1998; Gordon et al. 1998). Consensus sequences were database searched using WU-blast2 (WU-blastx; Altschul et al. 1997) against Uniprot (Boeckmann et al. 2003; Wu et al. 2006) to assign their HSP identity. The nucleotide sequences were aligned using Clustal W (Thompson et al. 1994) and specific primers designed to each different member of the HSP family for each organism, all with an annealing temperature of 60°C. Amplified fragment sizes varied between 119 and 142 bp. The specificity of each of the primers was checked by amplification and sequencing of the products. All HSP sequence fragments have been submitted to the European Molecular Biology Laboratory database with the accession numbers AM293594–AM293601 inclusive.

Isolation of β actin genes For comparative analysis to be made between the different HSP genes, a housekeeping sequence β actin was isolated from both organisms. Degenerate primers were designed from a ClustalW alignment of a number of β actin genes from *Takifugu* (Venkatesh et al. 1996), two Asteroidea and one Orthogastropoda (accession numbers P53484, p12716, p123717 and P17304, respectively; SeaactinF, ACCGACTACYTSAKKAA GATCCT; SeaactinR, GAVGCVAGGATGGAGCCRCC). The PCR conditions for *L. elliptica* were as follows: 95°C, 5 min; 35 cycles of 95°C, 20 s; 60°C, 20 s; and 72°C for 40 s with a final elongation step of 72°C for 5 min. The same basic programme used for *N. concinna* but with a lower annealing temperature of 45°C, and the number of cycles increased to 40. PCR products were sequenced, assembled and checked as described above for the HSP genes. In this instance, if multiple β actin fragments were amplified from the same organism, primers were designed to regions of identity between the different family members. Primers were designed to anneal at 60°C. Expression levels of β actin between different tissues and different treatment states were checked to ensure constant expression and reproducibility. Sequences of all primers are listed in Table 1.

Quantitative PCR HSP and actin sequences were amplified from each organism under each treatment condition using specific primers, Brilliant SYBR® Green QPCR Master Mix (Stratagene) and an MX3000P (Stratagene). PCR conditions were as follows: 95°C, 10 min; 40 cycles of 95°C, 30 s; 60°C, 1 min; and 72°C for 1 min with a final dissociation curve step as per manufacturer's recommendations. The plate setup for each Q-PCR experiment consisted of five control individuals and five experimental (treated) individuals; both sets were amplified with a specific HSP primer pair and an actin control primer set. All amplifications were reproduced in triplicate. Each primer set was checked to ensure that no primer dimers were produced during the course of the amplification reaction. RSq values and PCR efficiencies were checked over a fourfold 10× dilution series and the values calculated using the MxPro–MX3000P v 3.00 Build 311 Schema 74 software (Table 1). Primers producing low RSq values were discarded and new primers designed. Amplifications were analysed using the MxPro–MX3000P v 3.00 Build 311 Schema 74 software and Ct (dR) values exported into Excel. Relative expression ratios

Table 1 HSP and actin gene primer sets for *L. elliptica* and *N. concinna*

Organism	Primer set	Gene	Primer sequence		RSq	PCR efficiency (%)
Nacella concinna	1F2 and 1Rev2	HSP70A	Nco1F2	ATTCGATGACGAGACGGTTCA	0.968	134.90
			Nco1Rev2	AACGTCTTCAATTCGCTTTTGTA		
	3F and 3Rev	HSP70B	Nco3F	AGTTCACCGACGACACAGTAC	0.945	103.70
			Nco3Rev	TATTTTAGTCTCTGATTTGTACTC		
	7F and 7Rev2	GRP78	Nco7F	CTTGGGATGATAAATCTGTCCA	0.996	86.40
			Nco7Rev2	CTTTGTCAGAACCTTGTACATTA		
	9F and 9Rev	HSC70	Nco9F	AATTTGACGATGGACACGTTCAA	0.988	87.00
			Nco9Rev	GGTCTTTTGTTCACCCTTGTAG		
	ActinF and ActinR4	Actin	NcoActinF	GAGAAATCGTCCGAGACATCAA	0.983	95.00
			NcoActinRev4	CAGCAGATTCCATACCCAAGAA		
Laternula elliptica	2F and 2Rev	HSP70A	Lel2F	CTGTCTTGAGCGATGGTGGC	0.998	116.80
			Lel2Rev	TTTGTTACGGTCTTTCCTAAGTA		
	3F3 and 3Rev	HSC70	Lel3F3	CAATGACAACACTCGCCCCA	0.996	84.30
			Lel3Rev	TGTTGACAGTCTTTCCGAGGTA		
	4F and 4Rev	HSP70B	Lel4F	AAGCTTGTCAACCACGGCGG	0.975	107.20
			Lel4Rev	CCTTGACCCTTTGGCCAAGG		
	5F and 5Rev	GRP78	Lel5F	GGTCAAGAACAAGAACAACAAAC	1.000	94.90
			Lel5Rev	TGACGATTTTCTCTCCCAGGAA		
	Actin F and ActinRev	Actin	LelActinF	CGACGGTCAGGTCATCACCA	0.999	95.90
			LelActinRev	GACAGGACAGTGTTGGCGTA		

RSq and PCR efficiency values are included, as calculated using the Stratagene MxPro–MX3000P v. 3.00 Build 311 Schema 74 software.

of the HSP genes compared to the actin housekeeping genes between the control and the treated samples were derived using the Relative Expression Software Tool (REST; http://www.gene-quantification.info/; Pfaffl 2001; Pfaffl et al. 2002). This is an excel macro that incorporates both a mathematical model to calculate relative expression ratios on the basis of the PCR efficiency and crossing point derivation of the investigated samples and a two-sided pairwise fixed reallocation randomisation test. This test makes no assumptions about distribution (such as normality of distribution) and assumes that treatments were randomly allocated. The randomisation test repeatedly and randomly reallocates the observed values to the two groups and notes the apparent effect (expression ratio). The proportion of these effects, which are as great as that actually observed in the experiment, provides the p value of the test. Two thousand randomisations were used in the test (Pfaffl 2001; Pfaffl et al. 2002). These results were then followed by further statistical analysis (Minitab v 14) using a two-way analysis of variance (ANOVA) to test for the significance of an effect of either temperature or tissue.

Results

Four members of the HSP70 gene family were isolated from both *L. elliptica* and *N. concinna*. These were defined according to their sequence similarity scores after searching the sequence databases using WU-blastx. In each animal, these comprised two members of the inducible form (HSP70, designated HSP70A and HSP70B), one heat shock cognate HSC70 gene and one for glucose-regulated protein, 78kDa (GRP78; Table 2). Meaningful phylogenetic comparisons were not possible, as there are only two other sequences for mollusc GRP78 and HSC70 genes in the public databases.

Nacella concinna Initially to obtain a general idea of the relative expression levels of each of the HSP genes in *N. concinna*, the genes were assayed using standard PCR and gel electrophoresis in a set of control animals (Fig. 2). Both inducible forms of the HSP70 genes were present but at a relatively low level. In contrast, GRP78 was more strongly expressed by approximately twofold, but the constitutive HSC70 gene was very strongly expressed at a level similar to that of the β actin gene. Neither HSC70 nor GRP78 showed any significant change in gene expression levels assessed using Q-PCR after exposure to 10°C, 15°C or 20°C. Although both HSP70 genes showed no significant change in level at 10°C, there was massive up-regulation of both genes at 15°C and 20°C. HSP70A was up regulated by almost 2,000-fold at both temperatures, whereas HSP70B was up regulated by approximately 350-fold at 15°C, which increased to almost 750-fold at 20°C (Fig. 3). A two-way ANOVA on the combined dataset showed both a significant difference between genes ($F_{3,6}$=8.10, p=0.016) and an effect of temperature ($F_{2,6}$=5.38, p=0.046). Partitioning the

Table 2 Designation of HSP gene family member status based on BLAST match results from database sequence similarity searches

Organism	Primer set	Gene designation	Closest database match	Score	Percent identity	Probability
Nacella concinna	1F2 and 1Rev2	HSP70A	P08106: *Gallus gallus* (chicken)	448	81	$1.3e^{-40}$
	3F and 3Rev	HSP70B	Q86QM8: *Locusta migratoria* (Migratory locust)	557	81	$3.6e^{-52}$
	7F and 7Rev2	GRP78	Q75W49: *Crassostrea gigas* (Pacific oyster)	565	81	$5.2e^{-53}$
	9F and 9Rev	HSC70	Q9XZJ2: *Crassostrea gigas* (Pacific oyster)	559	81	$2.2e^{-52}$
Laternula elliptica	2F and 2Rev	HSP70A	Q2MJK5: *Haliotis discus hannai* (Abalone)	534	85	$9.9e^{-50}$
	3F3 and 3Rev	HSC70	Q76N60: *Paralichthys olivaceus* (Japanese flounder)	555	96	$5.9e^{-52}$
	4F and 4Rev	HSP70B	Q86MC3: *Balanus amphitite* (barnacle)	471	81	$4.7e^{-43}$
	5F and 5Rev	GRP78	Q75W49: *Crassostrea gigas* (Pacific oyster)	451	76	$9.1e^{-41}$

dataset (into HSP70 A and B; GRP78 and HSC70) and reanalysing shows that, with the inducible HSP70s, there is no effect of gene ($F_{1,2}$=0.81, p=0.462), but there is an effect of temperature ($F_{2,2}$=29.97, p=0.032), as evidenced by the massive up regulation of these genes; whereas with GRP78 and HSC70, there is no effect of either gene ($F_{1,2}$= 0.12, p=0.76) or temperature ($F_{2,2}$=0.70, p=0.590).

Laternula elliptica This mollusc was originally assayed for HSP expression across a range of five tissues (gill, digestive gland, mantle, foot and siphon) in control animals. Gonad tissue was not included in the heat shock evaluation because heat is one method of inducing spawning in a number of marine organisms, and as such, this would influence gene expression in this tissue. In *L. elliptica*, both inducible forms of the HSP70 gene were permanently expressed, with HSP70A expressed more strongly and uniformly than HSP70B. HSP70B showed tissue-specific expression levels with highest expression in mantle, followed by gut and very low levels in the foot, gill and siphon. GRP78 was fairly uniformly expressed at approximately two- to threefold higher levels than the HSP70A gene (Fig. 4). The gene designated as HSC70 showed a very low level of expression, which did not change appreciably after the heat shock treatment. Therefore, the data for this gene, whilst surveyed across all temperature ranges, and tissues have not been included in this analysis.

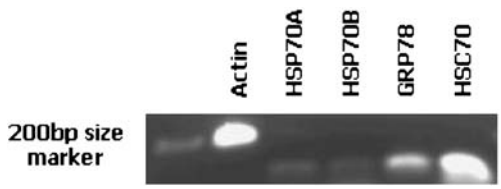


Fig. 2 Control expression levels of *Nacella concinna* actin and HSP70 genes performed using PCR on 1 µg of total RNA/ complementary DNA (cDNA)

Samples were sequentially analysed starting from the highest temperature of 15°C and then surveyed at lower temperatures if the genes showed up-regulation. Hence, some tissues were checked at the lower temperatures of 6°C and 4°C.

Gene	Temp	p-value	Relative Gene expression	Range	Gene Regulation
HSP70A	10	0.418	2.440	0.38-15.63	up
HSP70A	15	0.003	1822.320	166.16-19985.85	up
HSP70A	20	0.001	1809.950	164.74-19884.58	up
HSP70B	10	0.299	4.810	1.30-17.70	up
HSP70B	15	0.192	345.830	97.46-1227.10	up
HSP70B	20	0.007	752.660	337.94-1676.30	up
GRP78	10	0.931	1.540	0.09-26.16	up
GRP78	15	0.996	0.530	0.20-1.37	-1.847
GRP78	20	0.524	2.800	0.80-9.80	up
HSC70	10	0.298	0.140	0.02-0.77	-6.707
HSC70	15	0.236	2.450	1.36-4.40	up
HSC70	20	0.650	2.030	0.62-6.59	up

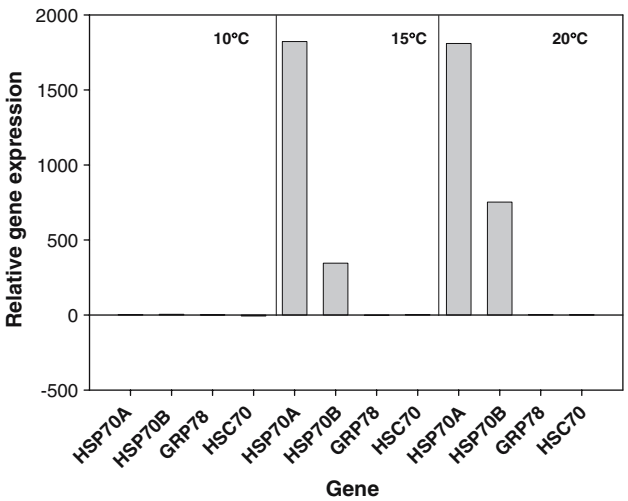


Fig. 3 Q-PCR results for *Nacella concinna* over three different temperature heat shocks. Relative expression ratios of the HSP genes from the control compared to the experimental animals are shown both in table format and graphically

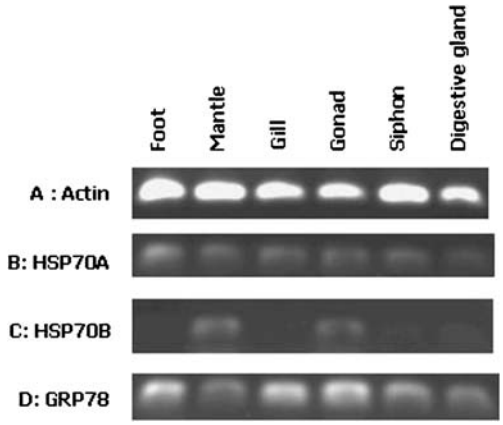
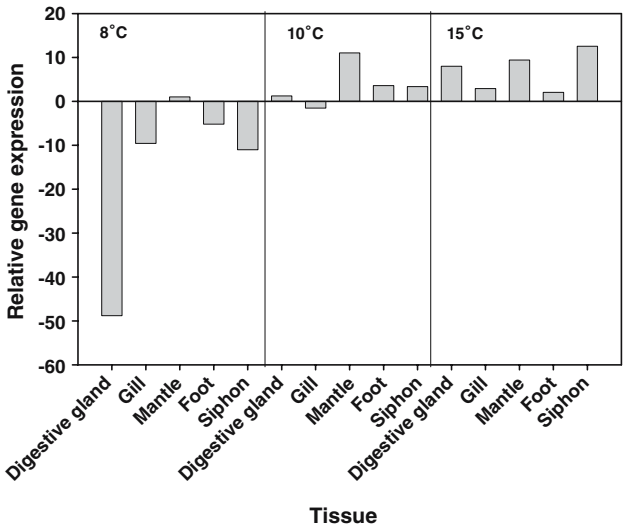


Fig. 4 Control expression levels of *Laternula elliptica* actin and HSP70 genes performed using PCR on 1 μg of total RNA/cDNA. *A* actin, *B* HSP70A, *C* HSP70B, *D* GRP78

All three genes (HSP70A, HSP70B and GRP78) under study appeared to show increased expression levels in response to temperature. The relative expression levels of HSP70A showed a significant response to temperature ($F_{2,8}$=25.30, p=<0.001) and an effect of tissue specificity at the 10% level ($F_{4,8}$=2.91, p=0.093). This gene showed up-regulation from 10°C. The maximum increase in relative expression of this gene was approximately 12× (Fig. 5). HSP70B exhibited higher maximal expression levels, up to 86× (Fig. 6). There was relatively uniform expression in all tissues at both 15°C and 10°C as evidenced by the ANOVA results ($F_{4,4}$=0.30, p=0.862), with a significant effect of temperature ($F_{1,4}$=23.97, p=0.008). The survey of tissues at 8°C showed significant relative expression in both foot and gill (according to the individual p values). Foot muscle was further surveyed at lower temperatures and showed no appreciable up-regulation (Fig. 6). When the whole 8°C dataset is combined with that from 10°C and 15°C and subjected to a two-way ANOVA, the seemingly tissue-specific nature of the response at this lower temperature masks the effects of both tissue and temperature ($F_{4,8}$= 0.81, p=0.550; $F_{2,8}$=2.34, p=0.158), respectively. Therefore, it is only possible to say that HSP70B is statistically significantly up regulated at 10°C. In the case of GRP78, there appeared to be differences in tissue-specific expression levels and a potentially higher uniform induction

Fig. 5 Q-PCR results for the *Laternula elliptica* HSP70A gene at 8°C (**a**), 10°C (**b**) and 15°C (**c**) in both tabular and graphical format

	Tissue	Temp	p-value	Relative Gene expression	Range	Gene Regulation
a	Digestive Gland	8	0.039	0.02	0.00005-7.43	-48.82 down regulated
	Gill	8	0.460	0.10	0.0007-15.18	-9.57 down regulated
	Mantle	8	0.963	1.02	0.02-3.94	up
	Foot	8	0.009	0.19	0.11-0.31	-5.19 down regulated
	Siphon	8	0.004	0.09	0.03-0.22	-11.016 down regulated
b	Digestive Gland	10	0.885	1.25	0.31-4.93	up
	Gill	10	0.866	0.64	0.08-5.01	-1.54 down regulated
	Mantle	10	0.234	11.05	4.78-25.50	up
	Foot	10	0.176	3.60	1.42-9.13	up
	Siphon	10	0.154	3.38	1.40-8.15	up
c	Digestive Gland	15	0.264	8.00	3.66-19.21	up
	Gill	15	0.221	2.91	0.90-9.81	up
	Mantle	15	0.007	9.42	3.37-26.33	up
	Foot	15	0.513	2.05	0.68-6.18	up
	Siphon	15	0.021	12.57	3.31-47.73	up



	Tissue	Temp	p-value	Relative Gene expression	Range	Gene Regulation
a	Foot	4	0.997	0.68	0.14-3.10	-1.46 down regulated
b	Gill	6	0.999	0.09	0.03-0.23	-10.61 down regulated
	Foot	6	0.962	1.90	0.44-8.16	up
c	Digestive Gland	8	0.030	5.62	3.49-9.04	up
	Gill	8	0.002	32.94	11.58-93.62	up
	Mantle	8	0.863	0.09	0.0005-16.98	-10.67 down regulated
	Foot	8	0.005	86.23	15.44-481.50	up
	Siphon	8	0.984	0.92	0.38-2.22	-1.08 down regulated
d	Digestive Gland	10	0.030	6.72	1.34-33.67	up
	Gill	10	0.203	2.48	0.53-11.48	up
	Mantle	10	0.239	6.46	0.47-87.75	up
	Foot	10	0.214	6.14	1.58-23.81	up
	Siphon	10	0.986	1.57	0.49-4.94	up
e	Digestive Gland	15	0.032	32.45	8.17-128.88	up
	Gill	15	0.001	41.28	9.63-176.97	up
	Mantle	15	0.001	31.17	10.78-90.09	up
	Foot	15	0.005	17.26	5.01-59.35	up
	Siphon	15	0.008	40.40	6.00-272.06	up

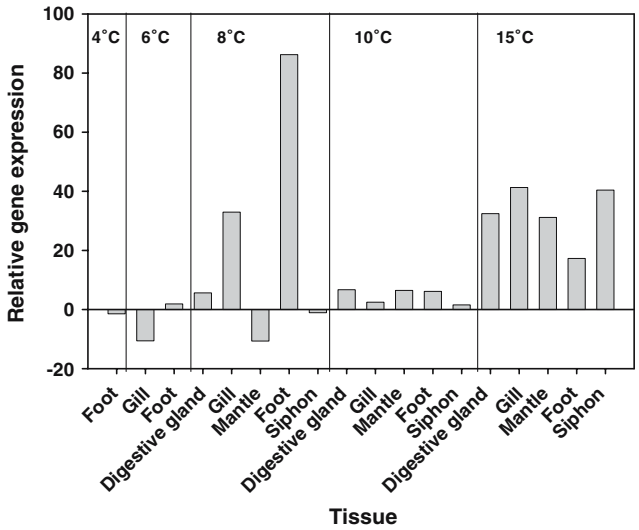


Fig. 6 Q-PCR results for the *Laternula elliptica* HSP70B gene at 4°C (**a**), 6°C (**b**), 8°C (**c**), 10°C (**d**) and 15°C (**e**) in both tabular and graphical format. Significant individual *p* values at 15°C are *shaded*

threshold of 15°C (Fig. 7). A two-way ANOVA test on the 8–15°C dataset produced no significant result for either tissue or temperature ($F_{4,8}$=0.68, p=0.626; $F_{2,8}$=2.28, p=0.165), respectively. Individual p values indicate that significant up-regulation of expression occurs in gill, mantle and siphon (p values of 0.48, 0.002 and 0.008, respectively) at 15°C; however, a two-way ANOVA test on the restricted 10–15°C dataset do not show an overall effect of either tissue or temperature ($F_{4,4}$=1.69, p=0.312; $F_{1,4}$=3.15, p=0.150). Foot tissue showed the highest expression at 8°C and so was surveyed at lower temperatures, but no up-regulation was indicated (Fig. 7).

Discussion

Both Antarctic marine molluscs in this study possess a quantifiable heat shock response, which varies in magnitude between the two species (maximum of 2,000-fold in *N. concinna* but only 40-fold in *L. elliptica*) and also in induction temperature. *N. concinna* exhibits a massive heat shock response at 15°C, whereas the up-regulation threshold for *L. elliptica* is statistically significant from 10°C depending on the gene.

Four genes, HSP70A, HSP70B, HSC70 and GRP78, were cloned from each of the two animals. The two inducible forms of HSP70 are very similar: The two *N. concinna* genes are 78.2% identical, whereas the two *L. elliptica* genes are 70.2% identical both at the DNA level. The most parsimonious explanation for which is propagation via gene duplication, either a gene-specific or a whole-genome event. Each of the HSP70 genes identified in each organism exhibits different control levels of gene expression compared to its paralogue in the same organism (in particular, HSP70B in *L. elliptica* is the subject of tissue-specific expression; for further discussion, see below). This is in line with the retention and evolution of duplicate genes via sub-functionalisation (Force et al. 1999), and maintenance of duplicates in this instance may reflect different tissue requirements to protect against low temperature or ice-nuclei insult.

	Tissue	Temp	p-value	Relative gene expression	Range	Gene Regulation
a	Foot	4	0.998	0.76	0.09-6.21	-1.31 down regulated
b	Foot	6	0.972	1.78	0.46-6.86	up
	Digestive Gland	8	0.597	0.73	0.27-1.96	-1.35 down regulated
	Gill	8	0.983	1.07	0.26-4.40	up
c	Mantle	8	0.726	1.95	0.26-14.43	up
	Foot	8	0.010	16.71	4.33-64.40	up
	Siphon	8	0.893	0.56	0.22-1.42	-1.75 down regulated
	Digestive Gland	10	0.993	1.01	0.29-3.40	up
	Gill	10	0.849	2.23	0.33-14.76	up
	Mantle	10	0.856	0.67	0.145-3.10	-1.488 down regulated
d	Foot	10	0.093	5.96	1.85-19.17	up
	Siphon	10	0.100	26.87	2.93-245.89	up
	Digestive Gland	15	0.048	6.90	1.85-25.65	up
	Gill	15	0.048	6.71	1.79-25.16	up
	Mantle	15	0.002	36.04	10.12-128.28	up
e	Foot	15	0.922	2.16	0.50-9.25	up
	Siphon	15	0.008	84.61	3.71-1927.05	up

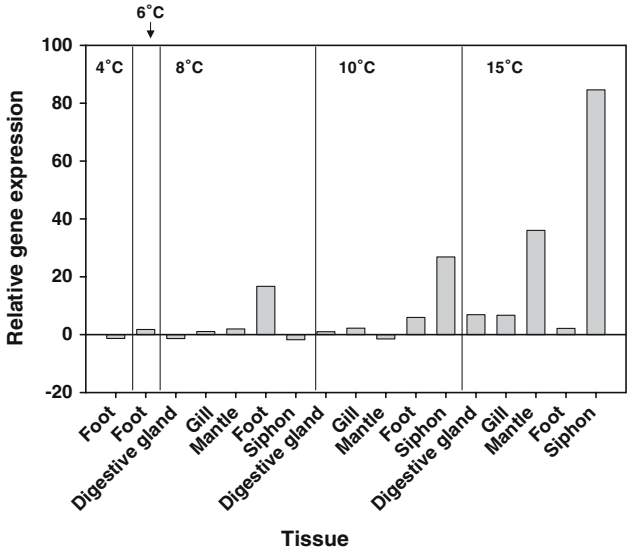


Fig. 7 Q-PCR results for the *Laternula elliptica* GRP78 gene at 4°C (**a**), 6°C (**b**), 8°C (**c**), 10°C (**d**) and 15°C (**e**) in both tabular and graphical format. Significant individual *p* values at 15°C are *shaded*

GRP78 is located in the endoplasmic reticulum and is a classic marker of the unfolded protein response with an HSP-like chaperon function (Sommer and Jarosch 2002), which in mammals, is not classically activated in response to temperature (Hendershot et al. 1994). However, evidence from both the platyfish and Japanese oyster indicate that, in lower vertebrates/invertebrates, it is up regulated in response to temperature (Yamashita et al. 2004; Yokoyama et al. 2006). Our experiments substantiate these findings.

In the experiments with *N. concinna*, only foot muscle was surveyed. *L. elliptica* is much larger, and several different tissues could be dissected and examined. The magnitude of response varied between tissues, with some tissues showing a statistically significant difference in expression levels of some genes compared to others (cf. gill, mantle and siphon with GRP78). Certainly, in the control animals, HSP70A showed fairly uniform expression levels across tissues, whereas HSP70B showed some indication of tissue specificity, with high expression in the mantle carried through to the 15°C experiments. HSP70B shows statistically significant individual results for gill, mantle and foot (and siphon at the 10% level) at 15°C, whereas GRP78 shows significant up-regulation in gill, mantle and siphon at 15°C. This is perhaps not surprising, as the foot, in particular, is the crucial organ for mobility in this animal, and gills present a large surface area in contact with the surrounding environment and are critical for respiration. The siphon is the only part of the animal, which is in direct contact with flowing seawater when buried and would be the first contact for increased water temperatures. The results for mantle are interesting, as this organ is largely responsible for secretion of the shell; however, it does enclose the critical respiratory chamber containing the gills and is also continuous with the siphon, and so maybe it too acts as a first response to increased seawater temperatures.

When examining the HSP response in these animals using Q-PCR, care has to be taken with regard to the change in relative gene expression as an absolute figure rather than looking at the data overall and extracting

general trends. This is because repeat sampling of animals before and after treatment was not possible. As a result, this meant that analysis of five "paired" results was, in fact, analysis of 10 different individuals; hence, the Q-PCR results were highly variable with large confidence intervals. Individual variation in gene expression is clearly high and may have been exacerbated by different sized animals being used in each set of experiments (for example, in the 10°C *N. concinna* cohort, weight varied from 1.3 to 7.1 g, and in the 10°C *L. elliptica* cohort, weight ranged from 23.7 to 75.8 g). Previous work examining physiological responses to increased water temperatures showed a correlation of response magnitude with size (Peck and Bailey in review; Peck et al. in review), and therefore, by inference, size may also affect gene expression levels or at least rate of change. This is clearly a factor that requires further investigation for future work but is impossible to eliminate completely, as it is not always possible to choose similar sized animals for the experiments when evaluating animals from extreme environments.

The two invertebrates studied demonstrated that they are capable of a heat shock response. This is in contrast to Antarctic Notothenioids, which show permanent expression of the inducible forms of the HSP70 genes and no heat shock response (Hofmann et al. 2000, Place et al. 2004; Place and Hofmann 2005). The two mollusc species in this study also show some permanent expression of the inducible forms of the HSP70 genes, albeit at a relatively low level. They also show permanent expression of the HSC70 genes; *N. concinna*, in particular, shows relatively high control levels of HSC70 similar to that of the actin gene. As the *N. concinna* foot was used for these experiments, actin would be expected to be one of the major genes expressed. The fact that the HSC70 gene is expressed at a similar level to actin suggests by inference that it is one of the most highly expressed genes in this animal. GRP78 is also constitutively expressed at high levels in this animal. As both HSC70 and GRP78 are expressed at such high-level constitutively, this may be the reason why they are not up regulated further in *N. concinna* in response to increased seawater temperatures, particularly when compared to the situation in *L. elliptica* where relative control levels of GRP78 are lower and HSC70 is poorly expressed.

Taking the data overall, the permanent expression of the inducible HSP70 genes, species-specific high expression of HSC70 (*N. concinna*) and permanent expression of GRP78 (*N. concinna* and *L. elliptica*) indicates that, as for Antarctic fish, chaperone proteins form an essential part of the adaptation of biochemical machinery of these Antarctic animals to low but stable temperatures. High constitutive levels of HSP gene family member expression may be a compensatory mechanism for coping with elevated protein damage at low temperatures analogous to the permanent expression of HSP70 in the Antarctic notothenioids. There is some evidence that protein degradation rates and protein carbonyl concentrations (as a measure of protein oxidation) appear comparatively higher in invertebrates at polar water temperatures than in species living at warmer temperatures (Fraser et al. 2002; Philipp et al. 2005). Other studies have also shown elevated levels of ubiquitin-conjugated proteins in polar fishes, a likely indication of increased levels of denatured proteins at polar water temperatures (Place et al. 2004). Taken together, this evidence suggests that transcribing, translating and folding proteins at polar water temperatures is problematic. Indeed, cold denaturation of proteins has previously been documented (Privalov 1990) and exposure of endotherm cells and ectotherms to cold shock can induce HSP70 expression (Ali et al. 2003; Laios et al. 1997).

Whilst both species show constitutive expression of HSP gene family members and up-regulation of HSP70 genes, there is a clear difference in the response between *N. concinna* and *L. elliptica.* This may be phylogenetically constrained but impossible to determine given the limited species sampling. Certainly, the level may be pre-determined according to the lifestyle of the organism. *L. elliptica* is a sediment-burrowing mollusc that experiences a seasonal sea temperature range of between −1.86°C to +1.86°C at Rothera Point (Peck et al. 2006; Fig. 1). This species has also been shown to be one of the most stenothermal Antarctic marine species studied to date with an effective temperature tolerance between −2°C to +2.5°C, an upper lethal temperature of +7.5°C and a critical temperature window of 5–7°C, above which anaerobiosis starts (Peck et al. 2002, 2004; S. Morley, personal communication). In contrast, *N. concinna* is a common inter-tidal species and can be found anywhere from mid-tide level (Walker 1972) to depths greater than 110-m depth (Powell 1951, 1973). The *N. concinna* used in this study were collected by divers at 8- to 10-m depths, where again, the yearly water temperature range only varies between −1.86°C and +1°C. Although data collected during the austral summer at South Cove, Rothera Point, on inter-tidal limpets demonstrated that, on a sunny day, the foot temperature of intertidal *N. concinna* exceeded 6.3°C, whereas the maximum shell temperatures reached 7.2°C (C. Waller, personal communication). According to the current study, these foot temperatures are still considerably lower than that required to induce HSP up-regulation (15°C). When examining the threshold for HSP induction in temperate marine organisms, a value of +8°C to +10°C over habitat temperature is common (Tomanek and Somero 1999; Buckley et al. 2001) and, therefore, the threshold at which HSPs are induced in these two molluscs is similar to the temperature rise required for the normal induction temperature for temperate species (Hofmann 2005). There are no accurate fossil records for *N. concinna* in the Antarctic (A.

Crame, personal communication), but fossils of *L. elliptica* have been described from the late Pliocene (5 Ma; Soot-Ryen 1952; Jonkers 1999) Hence, these founder animals may have an HSP regulation that evolved in a warmer and more variable climate.

Given that the HSP response in these molluscs is induced at typical temperate habitat levels, the question remains as to why the level at which the thermostat has been set has not been reduced in line with the very narrow temperature range that these animals currently experience in their natural environment. Ultimately, there is an energetic cost associated with the production of heat shock proteins, and overproduction may be cytotoxic (Feder and Hofmann 1999; Sorensen et al. 2003). As both *N. concinna* and *L. elliptica* constitutively express members of the HSP70 family (HSC70 and GRP78: *N. concinna* and GRP78 *L. elliptica*) and are permanently investing heavily in their production, they may not have the spare energetic capacity for significant up-regulation without significant deleterious effects on other cellular processes.

A second explanation may be that the constitutive requirement for the HSP family to mitigate problems of protein conformity at low temperature decrease with small to moderate elevations in temperature, such as those below 10°C. This could either reduce the expression levels somewhat of the relevant genes or balance any increase in protein unfolding with rising temperature. Eventually, the insult from the temperature shock would overwhelm the balance, and a marked increase in HSP gene expression would result similar to that observed. We currently do not have data to differentiate the competing hypotheses.

In summary, these results show that, in contrast to Antarctic Notothenioids, two Antarctic molluscs exhibit the classical biochemical-based heat shock response to elevated environmental temperatures. The level and magnitude of the response varies with the species. The level at which this response is activated, under experimental conditions, indicates that the control of this function is probably a relic from temperate ancestors and, given the data presented here, probably would not be activated under increased seawater temperatures associated with global warming predictions. Therefore, genetic factors other than HSP production might be expected to play a more important role in the adaptation of these animals to life in higher temperatures should they survive. A more comprehensive investigation into the complex transcriptional changes that take place in these animals associated with increased environmental temperatures is currently underway in our laboratory.

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