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ORIGINAL PAPER

Triggers of the HSP70 stress response: environmental responses and laboratory manipulation in an Antarctic marine invertebrate (*Nacella concinna*)

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Abstract The Antarctic limpet, *Nacella concinna*, exhibits the classical heat shock response, with up-regulation of duplicated forms of the inducible heat shock protein 70 (HSP70) gene in response to experimental manipulation of seawater temperatures. However, this response only occurs in the laboratory at temperatures well in excess of any experienced in the field. Subsequent environmental sampling of inter-tidal animals also showed up-regulation of these genes, but at temperature thresholds much lower than those required to elicit a response in the laboratory. It was hypothesised that this was a reflection of the complexity of the stresses encountered in the inter-tidal region. Here, we describe a further series of experiments comprising both laboratory manipulation and environmental sampling of *N. concinna*. We investigate the expression of HSP70 gene family members (HSP70A, HSP70B, GRP78 and HSC70) in response to a further suite of environmental stressors: seasonal and experimental cold, freshwater, desiccation, chronic heat and periodic emersion. Lowered temperatures (−1.9°C and −1.6°C), generally produced a down-regulation of all HSP70 family members, with some up-regulation of HSC70 when emerging from the winter period and increasing sea temperatures. There was no significant response to freshwater immersion. In response to acute and chronic heat treatments plus simulated tidal cycles, the data showed a clear pattern. HSP70A showed a strong but very short-term response to heat whilst the duplicated HSP70B also showed heat to be a trigger, but had a more sustained response to complex stresses. GRP78 expression indicates that it was acting as a generalised stress response under the experimental conditions described here. HSC70 was the major chaperone invoked in response to long-term stresses of varying types. These results provide intriguing clues not only to the complexity of HSP70 gene expression in response to environmental change but also insights into the stress response of a non-model species.

Keywords Environmental change · Chronic stress · Acute stress · Inter-tidal · Macrophysiology

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Introduction

Understanding environmental stress responses in animals is becoming increasingly important in our changing world, as we seek to identify what factors set species range boundaries and how they will be affected under climate change scenarios. Of necessity, such an understanding will have to comprise not only a mix of environmental sampling and laboratory experimentation but also an understanding of the links between and limitations of the two different approaches (Barnes and Peck 2008). A priori of this work is the requirement to develop molecular biomarkers to enable us to determine when a species comes under threat and the extent of such (Dupont et al. 2007). Given that environmentally relevant species are almost universally genome data poor, the primary targets to date have been the heat shock protein 70 (HSP70) gene family (Hofmann 2005; Clark et al. 2008a). These are a family of highly conserved proteins, all with an approximate molecular weight of 70 kDa, which act as chaperones to stabilise and re-fold denatured proteins, preventing the formation of deleterious cytotoxic aggregates (Bucciantini et al. 2002). The combination of the fact that these genes are highly conserved

(which facilitates de novo cloning) with the classical activation of certain family members with temperature (the inducible form of HSP70) has promoted their use in environmental monitoring (cf. Hofmann 2005).

There has been a considerable recent interest in the expression of these proteins, particularly in relation to Antarctic species and the induction of the classical heat shock response (Clark and Peck 2009). These organisms live within a very restricted temperature regime, ranging from −1.86°C in winter to a summer maximum of −0.5°C in McMurdo Sound, Ross Sea and +1°C along the Antarctic Peninsula (Barnes et al. 2006). Both the Antarctic fish and shallow water invertebrates are very stenothermal (Somero and DeVries 1967; Peck and Conway 2000; Peck et al. 2009, under review), with many species having survivable temperature envelopes between 5°C and 12°C above the minimum sea temperature of −1.86°C (Peck 2002; Robinson and Davison 2008) and loss of critical biological functions at temperatures much below this (Peck et al 2004). In contrast to animals elsewhere in the world, when a range of these species were examined for a classical heat shock response, i.e. elevation of expression of the inducible form of HSP70 (genes and/or proteins), the majority did not exhibit such a response (Clark and Peck 2009). Intriguingly, two species, the limpet *Nacella concinna* and the clam *Laternula elliptica* do exhibit the classical response (Clark et al. 2008a), but at temperatures far in excess of any that they experience in the natural environment or would experience even under the more extreme global warming scenarios (IPCC 2007).

The immediate consequence of these studies was to question the suitability of the HSP70 gene family as "stress" biomarkers in Antarctic species. However, further in-depth analysis on the limpet highlighted marked differences between laboratory experimentation and environmental sampling. Laboratory experiments showed that out of four HSP70 family members cloned, only two genes with sequence similarity to inducible HSP70s, HSP70A and HSP70B were induced in response to water temperatures in excess of 15°C (Clark et al. 2008a). The signal for this response was very clear, with no increased expression at 10°C compared to control animals and a dramatic increase to almost 2,000-fold for HSP70A and 350-fold for HSP70B at +15°C (Clark et al. 2008a). Since these were the only two of the four genes tested that showed a response, these were then tested in an environmental context by sampling limpets that occurred in the inter-tidal zone. Surprisingly, both genes showed up-regulation during the period of a tidal cycle, despite temperatures being below 3°C throughout (Clark et al. 2008b). This latter result provided tantalising clues as to the complex nature of the HSP70 gene family expression and regulation in Antarctic species.

Much work has been conducted on the limpet *N. concinna* in many different fields ranging from growth kinetics (Peck et al. 1996; Clarke et al. 2004), colonisation processes (Bowden et al. 2006), metabolic responses to feeding (Peck and Veal 2001), fertilisation kinetics (Powell et al. 2001), nitrogen excretion (Clarke et al. 1994), protein metabolism (Fraser et al. 2007) and antioxidant production (Malanga et al. 2005) to population genetics (Hoffman et al., in review) and thermal sensitivity studies (Peck et al. 2004; Clark et al. 2008a, b). Given this comprehensive knowledge and the fledgling molecular work on this species, *N. concinna* is probably the best candidate so far for an Antarctic model organism. It also has an extensive Antarctic circumpolar distribution (Powell 1951) occurring anywhere from the inter-tidal (Walker 1972) to depths greater than 110 m (Powell 1951) and has closely related temperate congeners (Yoon and Kim 2007). Certainly, given the data described here, it is an ideal candidate for studying the complexity of the HSP70 stress response in a marine organism and for making links between laboratory experiments and environmental observations. Given the occurrence of *N. concinna* in the inter-tidal region, we expanded the stressors under study from heat to:

- Freshwater: to simulate freshwater runoff in the inter-tidal
- Cold: inter-tidal limpets migrate below water level in winter to escape ice (Walker 1972), but then permanently encounter sea temperatures below zero or if exposed to air, the temperatures can be −20°C
- Dry heat: to simulate desiccation effects and the relative effect of temperature
- Chronic stress: longer-term effects of increased temperature albeit at known survival levels
- Tidal simulation: to study the effects of periodic repeated stresses

The results are discussed in relation to the complexity of the inter-tidal environment and the stress response, in particular, the use of the HSP70 gene family as biomarkers to describe responses in a changing world.

Materials and methods

Animal sampling and experimental work

N. concinna were collected by SCUBA divers from South Cove, Rothera Research Station, Adelaide Island (67°34.25′ S, 68°08.00′ W) in the late winter (June, July, September 2007; environmental samples) and also during the austral summer (January 2008; experimental and control samples). Each sample batch consisted of five to ten animals. All animals were taken from 6 m depth. The winter animals were immediately sampled and the tissues flash frozen in liquid nitrogen. The summer collected animals were maintained in a through-flow aquarium at Rothera under a

simulated natural light/dark cycle. During the time the animals were held in the aquarium, the water temperature was +0.80±0.01°C. Batches of summer collected animals were subjected to the following treatments (without acclimation, unless stated):

- Cold (seawater at −1.6±0.1°C for periods of 2 and 6 h)
- Freshwater (+0.8°C for 1 h)
- In air in either a refrigerator or incubator (+2°C and +15°C, respectively, for 2 h)
- Chronic heat exposure (seawater in an experimental tank initially at 0°C and the temperature raised to +2°C over 2 days and then maintained at +2°C, with animals sampled at 6 and 30 days)
- Simulated tidal cycle (maintained in a re-circulating aquarium system at Cambridge (water temperature 0°C) and emersed for two periods of 2 h each day between 0800–1000 and 2000–2200 hours, with air temperature of the aquarium at 0°C). The animals were sampled during the final tidal simulation after 6 days of continual treatment

Animals maintained in the aquarium for the short-term challenges and those held in experimental tanks for the chronic exposure were not actively fed, but grazed on the biofilm on the side of the aquarium tanks. All animal procedures were carried out according to current UK guidelines.

Sample analysis

RNA extraction Total RNA was extracted from *N. concinna* whole foot muscle using TRI Reagent (Sigma) according to the manufacturer's instructions. One microgram of total RNA was DNAse treated using 0.4 U DNase I (Ambion) in 10 mM dithiothreitol/100 mM $MgCl_2$ buffer and reverse transcribed using a first strand synthesis kit (Promega).

Q-PCR *Nacella*-specific primers HSP70AF: ATTCGATGACGAGACGGTTCA, HSP70ARev: AACGTCTTCAATTCGCTTTTGTA; HSP70BF: AGTTCACCGACGACACAGTAC, HSP70Rev: TATTTTAGTCTCTGATTTGTACTC; HSC70F: AATTTGACGATGGACACGTTCAA, HSC70Rev: GGTCTTTTGTTCACCCTTGTAG; GRP78F: CTTGGGATGATAAATCTGTCCA, GRP78Rev: CTTTGTCAGAACCTTGTACATTA; and ActinF: GAGAAATCGTCCGAGACATCAA, ActinRev: CAGCAGATTCCATACCCAAGAA were used for the quantitative polymerase chain reaction (Q-PCR) as previously detailed (Clark et al. 2008a). The four HSP genes and the actin housekeeping sequences were amplified using SensiMix Plus SYBR® Green QPCR Master Mix (Quantace) with heat-activated Taq DNA polymerase and an MX3000P (Stratagene) using conditions as described in Clark et al. (2008a). 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 of the HSP genes compared to the actin housekeeping genes between the control and treated samples were derived using the Relative Expression Software Tool (http://www.gene-quantification.info/; Pfaffl et al 2002; Clark et al. 2008a). 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 gene or experimental condition.

Results

Cold stresses

Cold was the first stress tested and the results comprised both seasonal environmental sampling and experimental cold manipulation. Animals were sampled from below the sea ice at around 6 m depth in midwinter (June) and in July and September, as winter emerged into early spring. The results from June show down-regulation of all genes with the exception of HSC70 (the expression of which barely changes compared to control animals sampled in the summer; Fig. 1). The July results are all generally more negative, a trend which is reversed in September, when the sea temperature increases. Only HSP70A remains negatively expressed compared to results in controls, with the other genes being more positively expressed, particularly HSC70 (Fig. 1). Whilst the Q-PCR analyses showed a trend in expression levels as the winter progressed, two-way ANOVA analysis showed no significant effect of either the gene under study or the month of the year (gene: df=3, F=1.75, P=0.257; month: df=2, F=2.27, P=0.184). When summer animals were treated at −1.6°C for 2 h, the expression levels of all the genes remained similar to those in summer control animals. A longer cold treatment (6 h) produced a uniform decrease in HSP gene expression, particularly of HSP70A, where levels of expression were significantly reduced to −11.9-fold compared to controls (P=0.038) (Table 1).

Freshwater stress

Freshwater runoff is potentially a problem in the Antarctic inter-tidal zone (Davenport 2001) and so the second stress tested was by immersing the animals in freshwater for 1 h at 0°C. This produced very little change in gene expression for either of the inducible HSP70 genes and GRP78; however, HSC70 again showed a more pronounced increase 4.1-fold compared to controls (Table 2).

Gene	P value	Relative gene expression	Range
June -1.9ºC			
HSP70A	0.926	0.786	0.003-193.689
HSP70B	0.830	0.517	0.013-20.186
GRP78	0.772	0.530	0.026-10.595
HSC70	0.927	1.147	0.150-8.746
July -1.9ºC			
HSP70A	0.472	0.123	0.030-0.487
HSP70B	0.748	0.325	0.131-0.803
GRP78	0.891	0.641	0.259-1.586
HSC70	0.689	0.533	0.159-1.783
Sept -1.6ºC			
HSP70A	0.154	0.300	0.139-0.647
HSP70B	0.770	1.300	0.700-2.414
GRP78	0.971	1.044	0.566-1.925
HSC70	0.152	4.076	1.680-9.888

Fig. 1 Graphical description of the expression levels of the four HSP70 gene family members from the winter environmental sampling over a period of 4 months with seawater temperatures ranging from −1.9°C to −1.6°C. Animals were sampled by divers from 6 m depth. Ranges of gene expression and *P* values are given for each sample of five animals taken at each time point

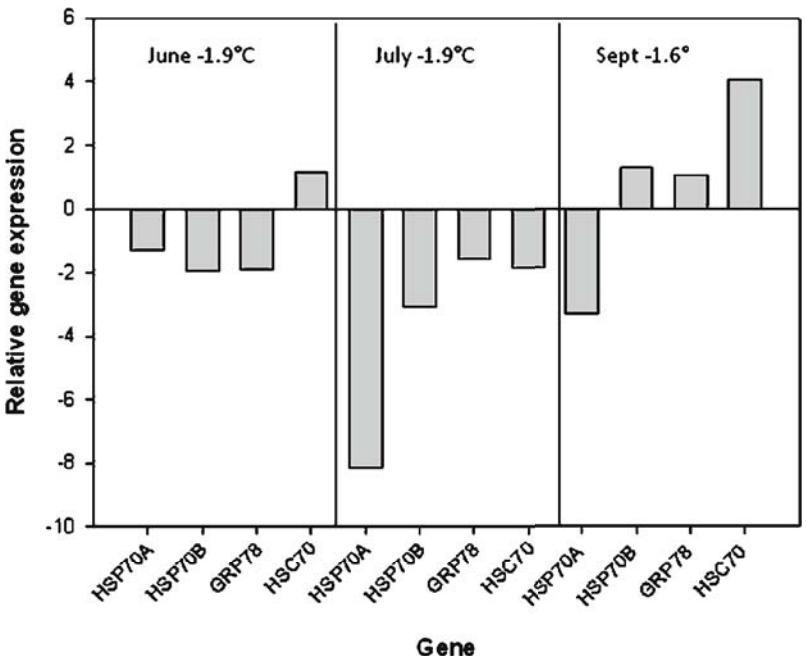

Table 1 Relative gene expression levels, ranges and *P* values of the four HSP70 gene family members in response to limpet experimental immersion in cold water at −1.6°C

Gene	*P* value	Relative gene expression	Range	Gene regulation
−1.6°C, 2 h				
HSP70A	1.000	1.000	0.200–4.992	+1.000 up
HSP70B	0.941	1.328	0.720–2.448	+1.328 up
GRP78	0.997	1.206	0.759–1.916	+1.206 up
HSC70	0.999	2.146	0.945–4.874	+2.146 up
−1.6°C, 6 h				
HSP70A	0.038	0.084	0.018–0.389	−11.935 down
HSP70B	0.258	0.445	0.283–0.679	−2.247 down
GRP78	0.409	0.614	0.309–1.215	−1.630 down
HSC70	0.107	0.487	0.238–0.995	−2.053 down

Animals were sampled after 2 and 6 h

Table 2 Relative gene expression levels, ranges and *P* values of the four HSP70 gene family members in response to limpet experimental immersion in freshwater at 0°C for 1 h

Gene	*P* value	Relative gene expression	Range	Gene regulation
HSP70A	0.988	1.237	0.220–6.926	+1.237 up
HSP70B	0.990	1.117	0.498–2.502	+1.117 up
GRP78	0.996	1.520	0.547–4.063	+1.520 up
HSC70	0.987	4.142	1.849–9.689	+4.142 up

Heat/desiccation stresses

Previously, animals had been heat stressed in seawater (Clark et al. 2008a). To study the potential effects of heat combined with desiccation, dry heat at +2°C and +15°C was used. The results of the +2°C treatment showed very little change in the expression of HSP70A and GRP78, with higher expression levels produced by HSC70 (4.0-fold) and HSP70B (6.2-fold; Fig. 2). The results of treatment at +15°C showed a significant increase in the expression of both HSP70A (134.2-fold, P=0.038) and HSP70B (63.2-fold, P=0.032; Fig. 2). Although certain treatments showed a significant increase in gene expression, overall, because of the wide variation in expression levels between individuals and the limited sample size, the combined results showed no significant effect of either gene or temperature (gene: df=3, F=0.99, P=0.504; temperature: df=1, F=2.31, P=0.504). The previously reported experiments also only reported responses to short-term heat stress (2 h; Clark et al. 2008a). The animals in this series of experiments were also subjected to longer-term heat stress of +2°C and sampled after 6 and 30 days. Up-regulation of all four genes was shown, but only significantly for HSP70A and HSP70B (46.9- and 68.2-fold, P=0.022 and P=0.011, respectively; Fig. 2), but the situation changed with a longer chronic heat treatment. At 30 days, the level of HSP70B expression was still very high (67.8-fold) compared to controls and similar to that at 6 days. However, the elevated expression levels of HSP70A at 6 days were reduced fourfold by 30 days. GRP78 had increased slightly to 13.9-fold at day 30, but levels of HSC70 had massively increased to 126.4-fold at 30 days compared to control animals (Fig. 2). Two-way ANOVA analysis of the chronic stress results showed similar results to that of acute dry heat, with no significant effect of either gene or time (gene: df=3, F=0.73, P=0.600; time: df=1, F=0.48, P=0.539).

Tidal cycle

The final experiment was an attempt to mimic a tidal cycle via periodic emersion twice daily, continually for 6 days. Animals were sampled during the cycle from immersion in water at 0°C, through exposure to air for different times (maximally 2 h) and then re-immersion. Some rhythmicity of expression was starting to appear after 6 days with both the expression levels of HSP70B and GRP78 peaking at +1 to +2 h of exposure to air and reducing as the animals were immersed for a further 12 h (Fig. 3). HSP70A showed very little change in expression (−1.3- to a maximum of +2.1-fold) over the whole cycle (Fig. 3). In contrast, HSC70 expression levels indicated a baseline expression level of around 50-fold compared to control animals with an increase to approximately 70-fold during exposure to air and a subsequent further increase to 123.6-fold when the animals had been immersed for an hour (Fig. 3). By extrapolation, this expression level would then decrease to around 50-fold (compared to that of controls) over 12 h before the commencement of the next emersion event. Elevation of HSC70 expression levels to 50-fold was significant at the 10% level (P=0.063), whilst expression to circa 70-fold was significant (P=0.001–0.034), but that of 123.6-fold was not (P=0.183). This was confirmed by two-way ANOVA analysis analysing the effect of gene and tidal period. There was a clear effect of the gene under study (df=3, F=37.22, P<0.000), but no significant effect of the tidal period (df=4, F=1.40, P=0.293).

Discussion

These experiments and environmental observations clearly demonstrate the complexity of the HSP70 stress response in *N. concinna*. Whilst this animal occurs over a wide range of depths (from greater than 110 m to the inter-tidal zone; Powell 1951; Walker 1972), the greatest variety of stressors occur in the inter-tidal including periodic tidal emersion, changes in temperature, desiccation, oxygen availability, humidity and ultraviolet irradiation (Menge and Branch 2001). The inter-tidal in Antarctica is one of the most difficult for life to establish because alongside problems of rapid temperature change, immersion and emersion from seawater and freshwater runoff, organisms living there also have to survive regular ice impacts, winter encasement in ice and extreme seasonality of resource availability (Peck et al 2006). Two different *N. concinna* morphotypes have been described (an inter-tidal "polaris" form and a sub-tidal "concinna" form) (Powell 1951). However, recent work has shown that these morphotypes are from a single homogeneous population and observed morphological and physiological

Gene	P value	Relative gene expression	Range	Gene regulation
Dry heat stress				
+2ºC				
HSP70A	0.951	2.320	0.509-10.560	+2.320 up
HSP70B	0.900	6.271	2.390-16.452	+6.271 up
GRP78	0.997	2.297	0.830-6.350	+2.297 up
HSC70	0.992	4.070	1.553-10.663	+4.070 up
+15ºC				
HSP70A	0.038	134.176	49.507-363.649	+134.176 up
HSP70B	0.032	63.225	25.865-154.596	+63.225 up
GRP78	0.998	3.400	1.320-8.753	+3.400 up
HSC70	0.997	3.686	1.423-9.541	+3.686 up
Chronic heat stress				
+2ºC, 6 days				
HSP70A	0.022	46.969	1.993-1107.654	+46.969 up
HSP70B	0.011	68.292	8.423-553.355	+68.292 up
GRP78	0.151	3.826	1.777-8.231	+3.826 up
HSC70	0.082	5.486	2.261-13.303	+5.486 up
+2ºC, 30 days				
HSP70A	0.982	10.375	0.375-287.148	+10.375 up
HSP70B	0.929	67.796	6.903-665.807	+67.796 up
GRP78	0.995	13.972	7.134-27.357	+13.972 up
HSC70	0.900	126.407	31.563-506.234	+126.407 up

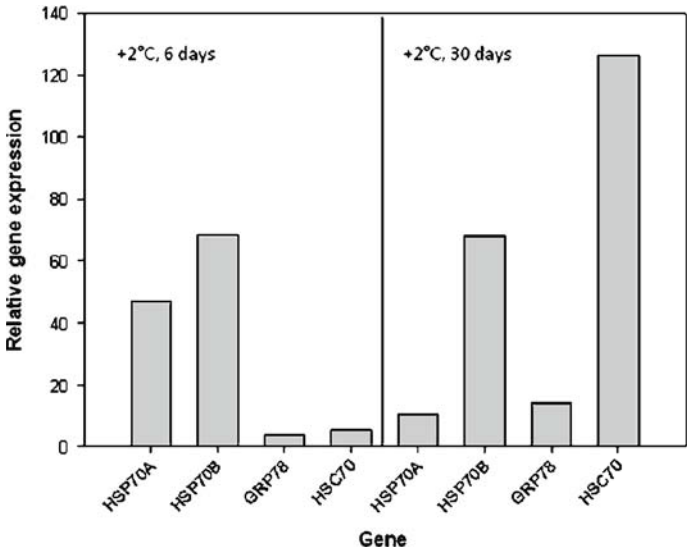

Fig. 2 Graphical description of the expression levels of the four HSP70 gene family members from the chronic heat exposure at +2°C. Animals were sampled after 6 and 30 days. Ranges of gene expression and *P* values are given for each sample of five animals taken at each time point. Data are also shown for the dry heat stress experiment with 2-h exposures at +2°C and +15°C

differences a result of phenotypic plasticity (Hoffman et al., in review). This is in contrast to many previous studies on the HSP70 response in inter-tidal animals, where one of the overriding interests is what sets the vertical zonation of the different species and the species under study are distinctly stratified in their habitat boundaries (reviewed in Hofmann 2005). Hence, there is added value in studying *N. concinna* as a wide variety of environmental sampling is possible alongside experimental manipulation with the results relevant across the whole species consisting of a homogeneous population. *N. concinna* is also one of the few macro-invertebrates to widely colonise the inter-tidal in Antarctica and thus become exposed to the wide variety of stressors (Waller et al. 2006a).

Cold stress

Protein folding is less efficient at cold temperatures (Privalov 1990) and HSP70 expression has been shown in response to cold shock in endotherm cells and ectotherms (Ali et al. 2003; Laios et al. 1997). As a consequence, the observation that Antarctic marine species exhibit enhanced expression of chaperone proteins to facilitate this requirement, either in the form of permanent expression of the inducible form of HSP70 or the permanently enhanced expression of other HSP70 family members (reviewed in Clark and Peck 2009), is explained as one of the more general adaptations to life in Antarctic waters required to protect proteins from low temperature damage. Much of the

Gene	P value	Relative gene expression	Range
Minus 1 hour (under water)			
HSP70A	0.962	0.766	0.028-20.287
HSP70B	0.596	8.125	0.683-96.544
GRP78	0.637	10.170	1.733-59.680
HSC70	0.063	52.210	9.234-295-302
Zero (10 mins exposed to air)			
HSP70A	0.987	1.406	0.040-48.422
HSP70B	0.808	12.277	1.620-92.277
GRP78	0.603	13.177	6.430-27.004
HSC70	0.034	75.604	24.337-234.862
Plus 1 hour (exposed to air for 1 hour)			
HSP70A	0.967	2.068	0.092-46.062
HSP70B	0.443	17.969	2.205-146.349
GRP78	0.339	22.746	11.178-46.293
HSC70	0.001	78.663	31.976-193.446
Plus 2 hours (exposed to air for 2 hours plus 10 mins under water)			
HSP70A	0.967	1.904	0.054-68.717
HSP70B	0.720	19.604	2.491-154.161
GRP78	0.694	18.077	8.166-40.044
HSC70	0.015	71.050	26.682-189.047
Plus 3 hours (exposed to air for 2 hours plus 1 hour under water)			
HSP70A	0.995	2.298	0.051-102.514
HSP70B	0.951	13.838	0.755-253.661
GRP78	0.872	16.006	5.501-46.572
HSC70	0.183	123.630	33.115-461.277

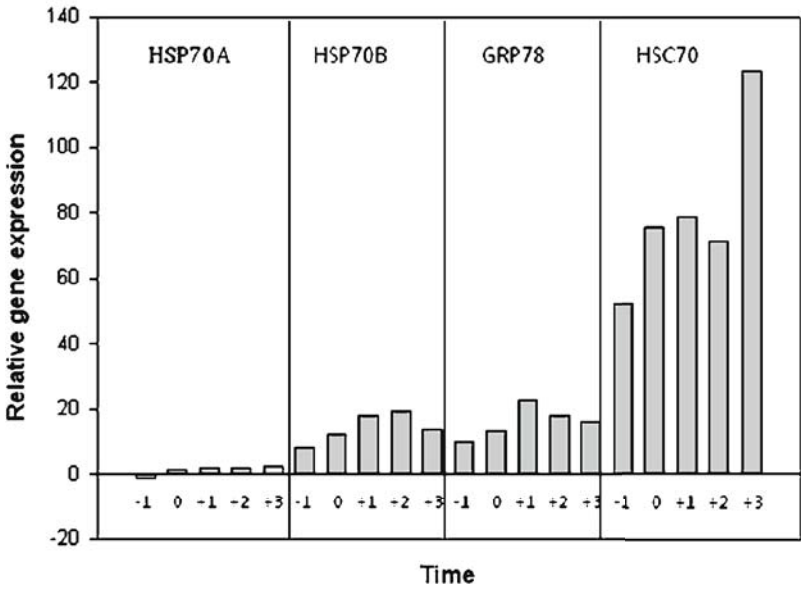

Fig. 3 Graphical description of the expression levels of the four HSP70 gene family members from the tidal simulation experiments with sampling taking place on day6. Key for time on the *x*-axis: *−1* limpets immersed, *0* limpet exposed to air for 10 min, *+1* limpets exposed to air for 1 h, *+2* limpets exposed to air for 2 h plus 10 min under water, *+3* limpets exposed to air for 2 h and re-immersed for 1 h. Table data are grouped according to time (ranges of gene expression and *P* values are given for each sample of five animals) with the graphical description shown grouped via gene, to better depict the tidal rhythm of expression patterns

work has been carried out on notothenioid fish species which inhabit a relatively stable environment with a temperature below zero (Place et al. 2004; Clark et al. 2008c). However, there was interest in the effect of cold on shallow nearshore marine and inter-tidal animals, as these inhabit a much more variable environment from positive summer temperatures down to −1.86°C in winter and potential encasement in ice (Waller et al. 2006a). Previous studies have shown that inter-tidal animals exhibit a variety of cold tolerances (Waller et al. 2006b) and the question arose as to whether the below zero winter temperatures induced further HSP70 expression as a protective mechanism. All the environmental samplings and experimental manipulations showed a reduction in HSP70 expression

(Table 1; Fig. 1). Whilst the animals in these experiments/samplings were subjected to temperatures lower than those of the control animals (at 0°C) and therefore presumably protein folding was less efficient, this reduction in expression has to be viewed in the context of whole animal physiology and energetics. Producing HSPs is expensive and there are costs involved at both the cellular and whole animal physiology level (reviewed in Sorensen and Loeschcke 2006). However, during winter, other factors are important and may take overriding precedence. The Antarctic environment is highly seasonal with regard to food supply (Clarke 1988) with phytoplankton blooms occurring for brief periods in the water column in summer (Clarke and Leakey 1996). Whilst *N. concinna* feeds all year round, exhibiting a low degree of metabolic seasonality (Fraser et al. 2002a), protein synthesis (and by extrapolation, gene expression) is reduced in winter (Fraser et al. 2002b). There are also additional potential requirements, such as mucus production to guard against the effects of the cold (Hargens and Shabica 1973). This protects the animal against ice nucleators, providing a layer of insulation. Energy budget calculations estimate that mucus production accounts for 12% of the total energy budget in summer acclimated animals (Peck et al. 1993), but it may be higher in winter alongside the increased requirement. There may also be, as yet, undiscovered functions of the HSP70 proteins, which only become apparent during down-regulation. For example, a closely related family member (HSP90) has been shown to buffer morphological variation in *Drosophila* and expression of cryptic variants (which may have fitness advantages) only become apparent when this HSP90 buffering is compromised and gene expression levels lowered during stressful conditions (Rutherford and Lindquist 1998).

Freshwater stress

This experiment was designed to mirror the potential for freshwater runoff from melt ice into the inter-tidal region. Davenport (2001) had previously shown that *N. concinna* does not tolerate freshwater; hence, this treatment was expected to induce a stress response. However, no significant increase in the HSP70 gene family expression was observed. Davenport's experiments showed that the reaction of *N. concinna* to freshwater was not to "clamp down" and try to isolate itself from the freshwater, but to detach from the rock substratum. On steep shores, these limpets would fall either into a more saline rock pool or the saline sub-tidal, but the main response mechanism seems to be simple avoidance and rapid recovery (Davenport 2001). Hence, there is no imperative for this species to invoke a cellular stress response, when it has already developed an alternative survival strategy. This inability to tolerate freshwater does therefore restrict its inter-tidal distribution to areas that only experience limited freshwater inundation (Davenport 2001).

Combined desiccation and heat stress

Previous experiments showed a very strong reaction of both HSP70A and HSP70B to elevated temperatures, with +15°C as a threshold (Clark et al. 2008a). These experiments used immersion in warm seawater, where heat conductance would be efficient. Not surprisingly therefore, the result for dry heat at +15°C was considerably muted by comparison, but still significant under the experimental conditions used here. Interestingly, +2°C showed some elevation of all family members, particularly HSP70B, and given the relatively short exposure time (2 h) and the inefficiency of heat conductance in air, this could be either a desiccation or oxygen deficiency effect rather than one of heat. These experiments were conducted in cabinets and so there was no effect of air turbulence, which would effectively increase any desiccation effects.

Chronic stress

The vast majority of molecular heat stress experiments in Antarctic invertebrates have been carried out for short periods and represent acute stress (Clark and Peck 2009). Whilst this may be appropriate for determining which genes to study, environmental change will induce much lower level chronic stresses, especially with predictions of changes to seasonality in Antarctica (Peck et al. 2006). Therefore, it was decided to subject *N. concinna* to a longer-term heat stress at +2°C, a temperature at which longer-term survival is possible, but the animal is reaching a threshold for maintenance of biological function (Peck et al. 2004). There was an interesting pattern to the relative expression of the four different gene family members over the month time course. There was initial up-regulation of both HSP70A and HSP70B. Given previous temperature experiments (described above), this is not surprising as these two genes have a clear temperature trigger. Over the course of a month, the relative expression levels changed, with that of HSP70A dramatically reduced from 46.9-fold down to 10.3-fold, but that of HSP70B remained constant. GRP78 increased slightly from 3.8- to 13.9-fold, whilst HSC70 expression levels rose significantly from 5.4- to 126.4-fold.

The changes in expression of the inducible forms in these experiments provide an interesting comparison to the environmental sampling over the period of a tidal cycle (Clark et al. 2008b). In each case, HSP70A expression shows a rapid but short-lived response. The overall expression levels of HSP70B are lower than those of

HSP70A under thermal stress, but the response is prolonged. It is possible that the expression levels and control of the HSP70B gene is due to a "preparative defence" mechanism to protect the cells against unpredictable stresses, a continual danger of life in the inter-tidal region (Dong et al. 2008). This represents a compromise between the energetics of maintaining a standing stock of HSP transcripts and that required to rapidly induce the genes under stress (Dong et al. 2008). Since the inducible form of HSP70 is present in the form of duplicated genes in *N. concinna*, this difference in response is both a flexible mechanism for dealing with a variety of environmental insults and logical when viewed in the context of the hypothesis of maintenance of duplicated genes by sub-functionalisation (Force et al. 1999). HSC70 is clearly the major HSP70 family member involved in response to chronic heat stress, the context of which is discussed below.

Tidal simulation experiments

The result of these experiments in many ways mirrored those of the chronic heat stress (Fig. 3). There was very little elevation in the levels of HSP70A, but the temperatures of the limpet foot varied only slightly as the animals were moved from water at 0°C to air at 0°C. The expression levels of both HSP70B and GRP78 showed some elevation compared to controls (between eight- to 22-fold) and some appearance of a cyclical expression pattern related to emersion. HSC70 again showed strong elevation in levels of expression with a clear baseline of 50-fold compared to control animals and increasing levels as the tidal cycle progressed. Differences in the patterns of expression of HSP70 and HSC70 with regard to the type of response (on/off as opposed to sustained) have previously been documented with respect to the inter-tidal environment (Todgham et al. 2006). Given the previous results and those demonstrated in this study, the major trigger for HSC70 expression is chronic exposure.

Overall, the periodicity of the expression patterns of all HSP70 family genes over a simulated tidal cycle is not as clear as those of the environmental sampling in Clark et al. (2008b), but the simulation described here was only carried out for a week and the limpets were taken from a stable environment. A more enhanced periodicity may well emerge over a longer time period and may be accompanied by a change in the relative expression levels of the genes, as the physiology of the animal accommodated a cyclical environmental challenge. However, the temperature cycling associated with the inter-tidal region is complex with other extrinsic factors acting in concert with temperature (cf. Todgham et al. 2006). In Antarctica, these include unpredictable contact with ice as well as wave surge, air exposure effects and very rapid temperature change. Therefore, the laboratory experiments would not be expected to exactly mirror the changes in gene expression associated with environmental sampling, but they do allow the dissection of some of the main inducing agents (Todgham et al. 2006).

HSP70 gene family expression

As an increasing number of organisms are studied in relation to their stress response, the complexity of the role of HSP70 gene family members is becoming apparent. Relative expression levels of HSP70 gene family members can vary according to bio-geographical location and demonstrate a plasticity of gene regulation within populations of the same species (Fangue et al. 2006; Place et al. 2008). Much of the research on the interaction between HSP70 genes and environmental stress involves non-model organisms and frequently uses gene fragments as assays (cf. Fangue et al. 2006). Without full genome information, it is impossible to categorically identify all members of a gene family and novel species-specific variants may always be discovered (cf. Leignel et al. 2007). However, it is becoming clear that each member of the HSP70 gene family may act differently depending on the species. For example, the enhanced expression of GRP78 has been described in response to heat in diverse temperate species such as the Atlantic salmon *Salmo salar* (Lund et al. 2002), the oyster *Crassostrea gigas* (Yokoyama et al. 2006) and the crustacean *Fenneropenaeus chinensis* (Luan et al. 2009). However, this mechanism is absent in mammalian and rainbow trout *Oncorhynchus mykiss* cell lines (Kozutsumi et al. 1989; Ojima et al. 2005) and the Antarctic invertebrates *Odontaster validus* and *Paraceradochus gibber* (Clark et al. 2008d). There is now an increasing number of examples of both HSP70 and HSC70 being induced in response to heat in marine species (reviewed in Place et al. 2008). The inducible HSP70 is often described as one of the major responses to acute heat stress even when 1,000s of genes are surveyed in microarrays (cf. Gracey et al. 2008); however, the constitutive HSC70 has been designated the main heat-dependant HSP70 family member in the Pacific white shrimp (*Litopenaeus vannamei*; Wu et al. 2008). This latter example may be species specific as there is increasing evidence that although HSC70 may upregulated by acute heat stress, it is often more likely to correlate with longer-term chronic stress such as that described here, in fluctuating temperature regimes (Podrabsky and Somero 2004; Todgham et al. 2006) and under environmentally diverse bio-geographical distributions (Fangue et al. 2006; Place et al. 2008). The great flexibility of the HSP70 gene complex means that if it is to be used as an environmental biomarker, clearly as many family members as possible should be surveyed under a number of different

conditions over both acute and chronic timescales. This requirement is not specific to the HSP70 family and any potential molecular biomarker needs to be rigorously tested both in the laboratory and under field conditions.

Conclusions

This study adds further data to the limited comparisons of marine gene expression studies using both laboratory and environmental sampling (Roberts et al. 1997; Halpin et al. 2004; Todgham et al. 2006; Clark et al. 2008b). These combined experiments are essential if we are to understand the complex nature of chaperone regulation. Whilst these molecules were originally described in response to stress (reviewed in Parsell and Lindquist 1993), they have since been realised to have pluripotent roles (Korcsmáros et al. 2007; Quinones et al. 2008) and are involved in diverse functions such as intracellular trafficking and neuronal protection (Goldfarb et al. 2006; Brown 2007). The growing interest in these proteins in the medical field is in their potential to protect against disease (cf. Brown 2007), but this work may provide clues to research in the environmental field. The expression of the type of chaperone molecule activated in response to a certain environmental trigger may reflect the most immediate target for damage and not purely be a generalised response to build up of cytotoxic aggregates. There has been some discussion as to whether HSP70 genes are effective environmental biomarkers (Iwama et al. 2004), particularly in Antarctica where all except one example of the absence of the classical heat shock response reside (Clark and Peck 2009). However, the growing realisation of the complexity of HSP70 gene expression will ensure that these molecules are studied in the future. It will be important to study these genes in both a transcriptome and macro-ecophysiological context to dissect out their potential multifunctional roles in environmental protection and the role they may play in the cellular defences against climate change effects.

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