Low heat shock thresholds in wild Antarctic intertidal limpets (*Nacella concinna*).

[1]Melody S. Clark, Paul Geissler, Catherine Waller, Keiron P.P. Fraser, David K. A. Barnes; Lloyd S. Peck

British Antarctic Survey, Natural Environment Research Council, High Cross, Madingley Road, Cambridge, CB3 0ET, UK

[1]Author for correspondence: Melody S. Clark, British Antarctic Survey, Natural Environment Research Council, High Cross, Madingley Road, Cambridge, CB3 0ET, UK. Email: mscl@bas.ac.uk

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Summary

Heat shock proteins are a family of genes classically used to measure levels of organism stress. We have previously identified two HSP70 genes (HSP70A and HSP70B) in subtidal populations of the Antarctic limpet (*Nacella concinna*). These genes are up regulated in response to increased seawater temperatures of 15ºC or more during acute heat shock experiments, temperatures, which have very little basis when considering the current Antarctic ecology of these animals. Therefore the question was posed as to whether these animals could express HSP70 genes when subjected to more complex environmental conditions, such as those that occur in the intertidal. Intertidal limpets were collected on three occasions in different weather conditions at South Cove, Rothera Point over a complete tidal cycle and the expression levels of the HSP70 genes measured. Both genes showed relative up regulation of gene expression over the period of the tidal cycle. The average foot temperature of these animals was 3.3°C, far below that of the acute heat shock experiments. These experiments demonstrate that the temperature and expression levels of HSP production in wild animals cannot be accurately extrapolated from experimentally induced treatments, especially when considering the complexity of stressors in the natural environment. However, experimental manipulation can provide molecular markers for identifying stress in Antarctic molluscs, provided it is accompanied by environmental validation, as demonstrated here.

Introduction

To successfully colonise and reproduce in a particular environment, any organism has to be responsive to, and able to adapt to, changes in their physical surroundings. This is particularly crucial for marine intertidal invertebrates as they regularly experience conditions that for most species would constitute acute, and potentially lethal, stress (for Antarctic intertidal stresses see Waller et al 2006). As part of their daily routine, they have to cope with periodic tidal emersion and the consequential changes in temperature, desiccation, humidity and ultraviolet irradiation. How animals cope with regular acute stresses has always been of interest to biologists. In particular thermal biologists/ecologists are interested in how tolerance of temperature varies between species and the effect this has on setting species range boundaries and how species distribution patterns might be affected by climate change (Reviewed in Somero 2002 and Hofmann 2005).

A key tool in the investigation of stress tolerance has been the characterisation and monitoring of heat shock proteins. These are a family of highly conserved proteins, which act as chaperones to stabilise and refold denatured proteins, preventing the formation of cytotoxic aggregates (Parsell & Lindquist 1993, Hartl 1996, Fink 1999, Gross 2004). Numerous families of heat shock proteins have been identified, the naming of which is related to their weight in kiloDaltons. One such is the HSP70 (heat shock protein 70) family, which although up-regulated under different stress conditions is

classically associated with transcriptional up regulation in response to elevated environmental temperatures in most organisms studied to date.

The induction and expression of these HSP70 genes is highly plastic. Levels of induction are influenced by thermal history such as seasonal temperature cycling, vertical zonation and biogeography (reviewed in Somero 2002; Hofmann 2005). Certainly prior exposure to slightly elevated temperatures (such as summer verses winter water temperatures) increases the temperature at which the HSP70 genes are induced (Buckley et al 2001, Tomanek 2002). Also the height of the vertical zonation of animals in the intertidal region does influence their heat shock thresholds (Halpin et al 2002, Stillman 2002, Tomanek 2002). The animals in the studies described above are eurythermal. However this work has lead to great interest in the response of Antarctic marine organisms, which are highly stenothermal (Somero & DeVries 1967, Peck & Conway 2000, Peck 2002) and lose critical biological functions with temperature elevations of only 1-2°C above current summer maximum seawater temperatures (0-1.8°C) (Peck et al 2004).

The first studies of heat shock responses in Antarctic marine ectotherms showed that both microorganisms and fish did not increase HSP70 expression when warmed (Carratù et al 1998, Hofmann et al 2000). However, recent data (Clark et al, in press) on the heat shock response of Antarctic marine molluscs has shown that the classical heat shock response is initiated in the Antarctic limpet (*Nacella concinna*) at 15ºC and the Antarctic clam (*Laternula*

elliptica) at 8ºC. Given that these are temperatures in excess of that which is currently experienced by these animals (Waller et al 2006), the obvious question to ask is whether these animals express HSP70 when subjected to longer, lower levels of elevated temperatures in the natural environment or in response to multiple environmental insults? i.e. is there an environmental context to this response?

N. concinna is an ideal candidate species with which to pose these questions. It is a common Antarctic intertidal species and therefore regularly experiences periods of acute stress when it is uncovered at low tide. Intertidal limpets were collected on both sunny and cloudy days during the austral summer at South Cove, Rothera Point from the high water level over a complete tidal cycle. For comparative seasonal data, samples were also collected from the same site at the end of spring. The temperatures of the air, water, limpet foot and shell were logged and muscle tissue assayed for the expression of previously characterized inducible HSP70 genes. The objective of this study was to take HSP70 genes, which are known to be up regulated in response to temperature with a 15°C threshold temperature in laboratory experiments and identify their threshold limits in the field.

Materials and Methods

Animal sampling and experimental work

Nacella conncina were collected from South Cove, Rothera Research Station, Adelaide Island (67 34 11S, 068 07 88W) during the austral summer (January 2004) and the late spring, whilst there was still snow cover (November 2004). Each sample batch consisted of 5 animals. Control samples were collected using SCUBA from subtidal populations (6M depth). Experimental (intertidal) samples were collected from the mean mid-tide water level over a complete tidal cycle, and so subsequent batches experienced progressively longer exposure to air temperatures (5 collections were made for the sunny day, whilst 6 collections were made for each of the cloudy and spring days). Hence batch one were just exposed to air, batch two had one hours exposure and subsequent batches at hourly intervals until they were re-immersed (see Supplemental Tables 2-4 for full details). Only one set of spring samples was collected, but two batches of summer samples were collected under sunny and cloudy conditions on separate occasions; the air and water temperatures were recorded (±0.1^{o}C) at each sampling point. Limpet shell and foot temperatures were recorded to an accuracy of ±0.1^{o}C *in situ* using a handheld non-contact infrared thermometer (Supplemental Tables 1, 2, 3 and 4). The shell temperature of the limpet was recorded prior to the animal being gently pried from the rock. After removal animals were carefully placed in the shade and the foot temperature, shell length, width and height and

recorded using vernier calipers (±0.1mm). The shells were removed and a small (ca 200mg) sample of the foot muscle tissue placed in RNA later. These samples were stored at 4°C overnight then at –80°C until required.

Sample Analysis

RNA extraction: Total RNA was extracted from *N. concinna* whole foot muscle using TRI Reagent (Sigma) according to the manufacturer's instructions. 1µg of total RNA was DNAse treated using 0.4U DNase I (Ambion) in 10mM DTT/100mM $MgCl_2$ buffer and reverse transcribed using a first strand synthesis kit (Promega).

Q PCR: Species-specific HSP and actin sequences had been previously characterised (Clark et al, in press) and were amplified using the following primer sets: HSP70A: Nco1F: ATTCGATGACGAGACGGTTCA and Nco1Rev2P: AACGTCTTCAATTCGCTTTTGTA. HSP70B: Nco3F: AGTTCACCGACGACACAGTAC; Nco3Rev: TATTTTAGTCTCTGATTTGTACTC. Actin primers: NcoActinF: GAGAAATCGTCCGAGACATCAA; NcoActinRev4: CAGCAGATTCCATACCCAAGAA using Brilliant SYBR® Green QPCR Master Mix (Stratagene) with Sure Start® Taq DNA polymerase and an MX3000P (Stratagene). PCR conditions were as follows: 95°C 10 minutes, 40 cycles of 95°C 30 seconds, 60°C 1 minute and 72°C for 1 minute with a final dissociation curve step as per manufacturers recommendations. The plate set-up for each Q-PCR experiment consisted of 5 control individuals and 5 "treated" individuals amplified with a specific HSP primer set (designated Expt

1) and an actin control primer set (designated Normaliser). Each HSP and actin amplification was 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 four fold 10x dilution series and the values calculated using the MxPro - MX3000P v 3.00 Build 311 Schema 74 software (for RSq and efficiency details see: Clark et al, in press). 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 (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 Pair Wise 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. 2000 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 2-way ANOVA to test for the significance of an effect of either gene or

batch number. Overall results for each primer pair under each sampling regime were subjected to a 2-sample t-test to assign significance of results from random variation.

Results

Both heat shock protein genes (HSP70A and HSP70B) were induced under all intertidal conditions surveyed. A two way ANOVA of the 3 combined datasets showed an effect of batch number (at the 10% level) ($F_{4,4} = 2.48$, $p=0.77$), but no effect of gene ($F_{1,4} = 1.54$, $p = 0.229$). Analysing the datasets individually using a 2 way ANOVA showed no effect of either gene or batch number for the sunny day samples ($F_{1,4} = 3.01$, $p = 0.158$; $F_{4,4} = 1.03$, $p = 0.49$, respectively), although the individual p values for batches 1 and 2 for HSP70A were significant at the 10% level (Figure 1). The cloudy day samples showed no effect of either gene or batch number ($F_{1,4} = 6.19$, $p = 0.68$; $F_{4,4} = 2.56$, $p = 0.193$, respectively), but again provided individually significant results with batch 1 for HSP70A significant at the 5% level and levels of HSP70B significant at the 5% level (batch 4) and 10% level (batches 3 and 6) (Figure 2). The spring day samples showed no effect of either gene ($F_{1,4} = 2.55$, $p = 0.185$), but showed an effect of batch at the 10% level ($F_{4,4} = 4.62$, $p = 0.084$). An individually significant result at the 5% level was shown for HSP70B, batch 5 only (Figure 3). Within the three groups of samples relative gene expression levels varied from 27.70 – 0.41 for HSP70A and 14.22 – 3.90 for HSP70B, with the highest relative expression levels for HSP70A being

more apparent at the early tidal cycle batches where the animals are just becoming uncovered.

The average foot temperature of the sunny day animals was 3.3°C with an SD of 1.07 and a range of 4.6 (data from Supplemental Table 2). The average foot temperature of the animals on a cloudy day was 3.8°C with an SD of 1.26 and a range of 5.5 (data from Supplemental Table3). The spring samples, whilst overall having a lower mean foot temperature of 2.1°C, showed a much wider temperature range of 9.4°C, which is reflected in the higher SD of 2.0 when compared to the other two samplings (data from Supplemental Table 4). In this sampling set, it was noted that some of the animals that were taken were in the sun and therefore were exposed to higher levels of solar radiation.

Discussion

Previous experiments (Clark et al, in press) have indicated that *N. concinna* has a 15°C induction threshold for the HSP70A and HSP70B genes. The data presented here shows that in the natural intertidal environment these genes are up regulated when the foot temperatures of the animals are only 3°C. Heat shock proteins were named initially because of their role in cellular protection from heat stress but are, in fact, generalised stress proteins and are activated under several different conditions e.g. hypoxia, cadmium etc (reviewed in Sorensen et al 2003). Intertidal animals, in particular, have many

different environmental conditions to accommodate: periodic tidal emersion, changes in temperature, desiccation, oxygen availability, humidity and ultraviolet irradiation (Menge & Branch 2001). In Antarctica these stresses are enhanced by physical disturbance from ice, low temperature freezing and extreme seasonality of resource availability (Peck et al 2006). The overall stress load on intertidal limpets is, therefore great and it may be that the combined stress is equivalent to the higher temperatures needed in the laboratory to elicit a heat shock response (c.f. Halpin et al 2002, Todgham et al 2006).

Overall there is a statistically significant effect (at the 10% level) of batch indicating that tidal emersion and immersion play a role in the induction of heat shock genes in these animals. Comparing the individual p values and the ANOVA analyses, there appears to be a difference in the relative expression levels of HSP70A compared to HSP70B over the period of the tidal cycle, with HSP70A most highly expressed when the animals are first uncovered and significant results for HSP70B present in the latter half of the tidal cycle (when the animals are becoming covered again). This difference in expression pattern between paralogous genes is not uncommon. The most parsimonious explanation for the presence of two closely related HSP70 genes is a gene duplication event and maintenance of such duplicates is invariably by a process of sub-functionalisation (Force et al 1999), with each gene performing a slightly different, but vital cellular function. Work is currently underway in our laboratory to identify the triggers of HSP70 expression in

these animals, as factors other than heat are clearly involved given the HSP threshold induction temperature of 15°C in Clark et al (in press).

The statistical significance of the results under the three different environmental conditions is confounded by high individual genetic variability (discussed in greater detail in Clark et al, in press). Hence care has to be taken with the Q-PCR results and regarding the relative differences in gene expression as an absolute figure. In reality, the data has to be examined more globally and general trends extracted. Virtually all the different batches of animal collections show up-regulation of both HSP70A and HSP70B expression. If this was a random event, 50% up-regulation and 50% down-regulation would be expected. These results are highly significant as shown by the results of the 2-sample t-tests. Therefore the environmentally sampled animals do show significant up-regulation of both HSP70 genes compared to the subtidal control animals, indicating higher endogenous levels of chronic stress. Subtidal animals were chosen as the controls as they inhabit a stable environment and were the animals shown to have an acute HSP threshold temperature of 15°C. The tides at Rothera are normally limited to 0.8M and therefore all animals in this zone will experience a wider range of temperatures even when covered by water.

For decades it was considered that the Antarctic intertidal zone did not have permanent residents and species e.g. *N. concinna* were merely transient. Recent intensive sampling has shown that in fact many diverse species occur

there and that some, again such as *N. concinna*, survive below the icefoot covering the intertidal in winter (Waller et al 2006). Although the long term monitoring of air temperatures at Rothera shows variation between +5°C and -25°C, and seawater at 15m depth ranging from +1.8°C to –1.8°C (Figure 4A and B), microhabitat temperatures e.g. rock surfaces can differ considerably from this and the animals will be affected by zonation (figure 4C). Few marine invertebrates are capable of colonising the high intertidal zone and surviving there (Waller et al 2006), so *N. concinna* probably experiences amongst the widest temperature ranges of any marine Antarctic endemic species (Barnes et al 2006). This is true for both subtidal and intertidal *N. concinna* due to its wide geographic and tidal range (Barnes et al 2006). Therefore despite being an Antarctic endemic, the model organism we studied is in some ways extreme.

We have now shown that two very distantly related Antarctic molluscs (*N. concinna* and *L. elliptica*) have the ability to up regulate HSPs (Clark et al, in press). We have extended these laboratory-based studies with an investigation of HSP expression in wild *N. concinna.* Which shows that the temperature and expression levels of HSP production in wild animals cannot be accurately predicted using experimentally induced acute heat shock treatments (c.f. Lund et al 2002), especially in this case when considering the much more complex stresses of the intertidal zone. However, the controlled laboratory based physiological experiments were essential for the cloning of the genes and defining the nature of the heat shock response, particularly

against a high background of inter-individual genetic variation. Our work on *N. concinna* has provided us with a methodology for identifying molecular stress markers in Antarctic molluscs and their validation in environmental monitoring studies. These data take advantage of the intertidal ecosystem and demonstrate for the first time, the effective combination of field ecology and physiology in Polar research.

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Table Legends

All should be made available in supplemental on-line format, not in the paper text.

Table 1: Details of control animals collected by SCUBA divers from 6M depth, South Cove: 17/01/2004.

Table 2: Environmental and morphometric details of intertidal *N. concinna* collected on a summer "sunny" day: 11/01/2004.

Table 3: Environmental and morphometric details of intertidal *N. concinna* collected on a summer "cloudy" day: 07/01/2004.

Table 4: Environmental and morphometric details of intertidal *N. concinna* collected during November (spring): 15/11/2004.

Figure Legends

Figure 1: Q-PCR results for *Nacella concinna* HSP70A and HSP70B genes from animals collected on a sunny day in the austral summer. Relative expression ratios of the HSP genes from control compared to experimental animals are shown both in table format and graphically. Significant individual

p values (10% level) with the different batches are shaded. 2-sample t-test of HSP70A expression levels in environmental samples compared to controls gave a P-Value of 0.000. 2-sample t-test of HSP70B expression levels in environmental samples compared to controls gave a P-Value of 0.000.

Figure 2: Q-PCR results for *Nacella concinna* HSP70A and HSP70B genes from animals collected on a cloudy day in the austral summer. Relative expression ratios of the HSP genes from control compared to experimental animals are shown both in table format and graphically. Significant individual p values (5% and 10% level) with the different batches are shaded. 2-sample t-test of HSP70A expression levels in environmental samples compared to controls gave a P-Value of 0.062. 2-sample t-test of HSP70B expression levels in environmental samples compared to controls gave a P-Value of 0.000.

Figure 3: Q-PCR results for *Nacella concinna* HSP70A and HSP70B genes from animals collected on a late day in the austral summer. Relative expression ratios of the HSP genes from control compared to experimental animals are shown both in table format and graphically. Significant individual p values (5% and 10% level) with the different batches are shaded. 2-sample t-test of HSP70A expression levels in environmental samples compared to controls gave a P-Value of 0.011. 2-sample t-test of HSP70B expression levels in environmental samples compared to controls gave a P-Value of 0.000.

Figure 4: Mean air (A), seawater (B) and intertidal temperatures (C) recorded at Rothera Research Station. Air temperature data was provided by the British Antarctic Meteorological Department, seawater temperatures (at 15M depth) were taken from the Rothera oceanographic Time Series (RaTS) and intertidal data taken from C Waller PhD thesis, 2007.

Gene	Batch	p value	Relative Gene expression	Range	Gene Regulation
HSP70A	1	0.080	18.28	4.88-68.14	up
HSP70A	2	0.059	27.70	1.54-497.20	up
HSP70A	3	0.844	5.50	1.11-27.11	up
HSP70A	4	0.944	7.44	2.54-159.30	up
HSP70A	5	0.072	7.01	0.77-63.69	up
HSP70B	1	0.202	8.64	2.59-28.84	up
HSP70B	2	0.491	4.20	1.01-17.51	up
HSP70B	3	0.966	5.02	1.93-13.07	up
HSP70B	4	0.915	9.30	2.78-31.11	up
HSP70B	5	0.857	3.74	0.57-24.18	up

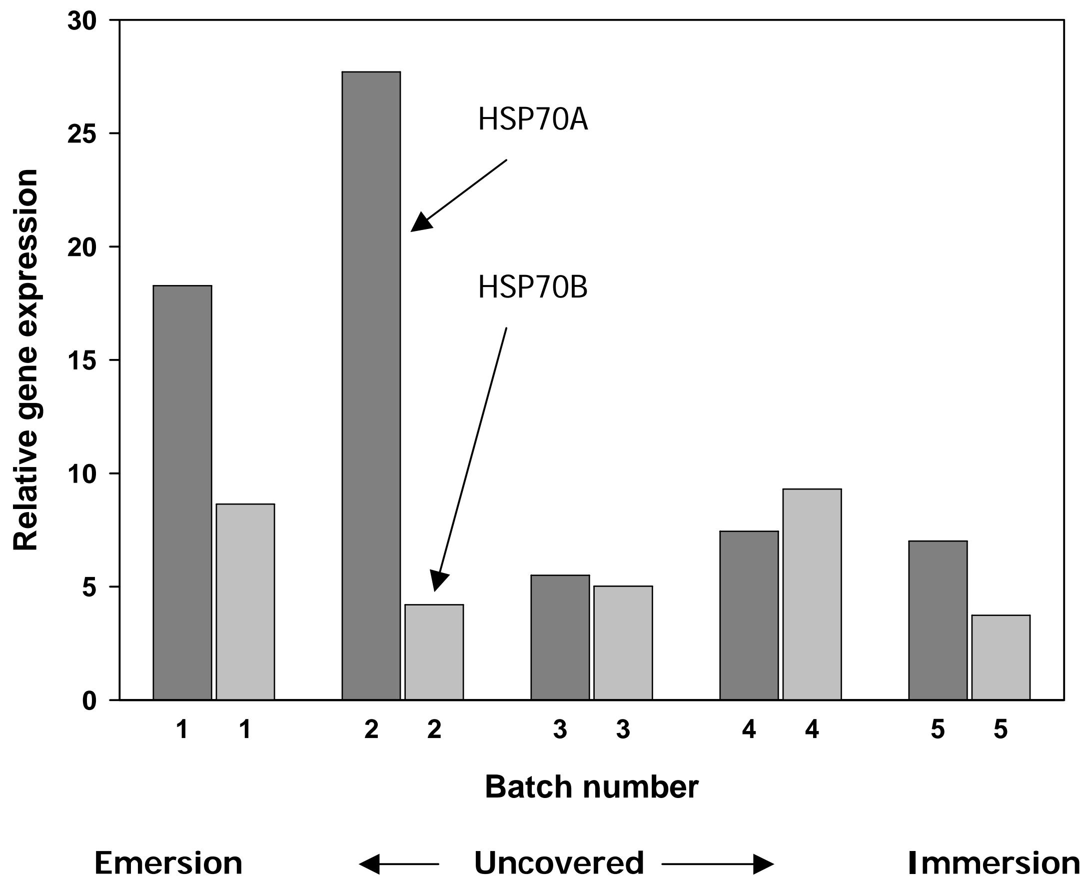
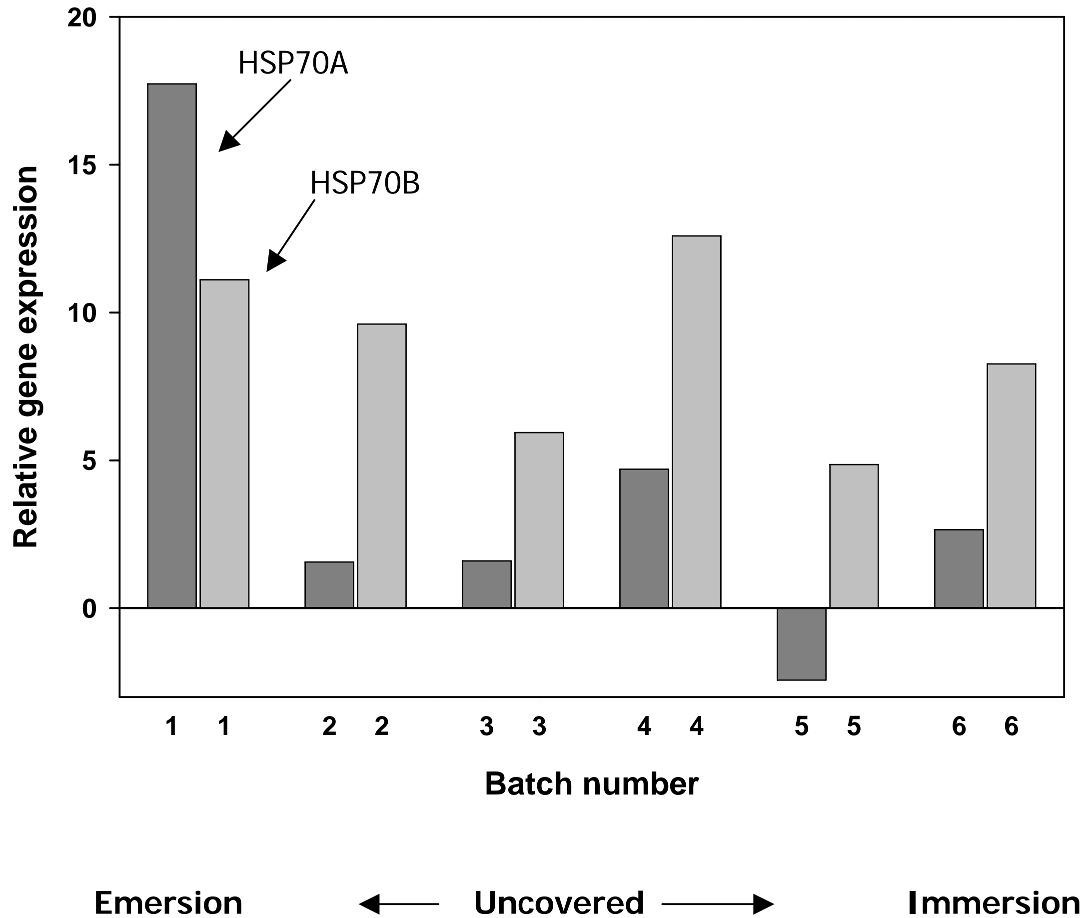


Figure 1

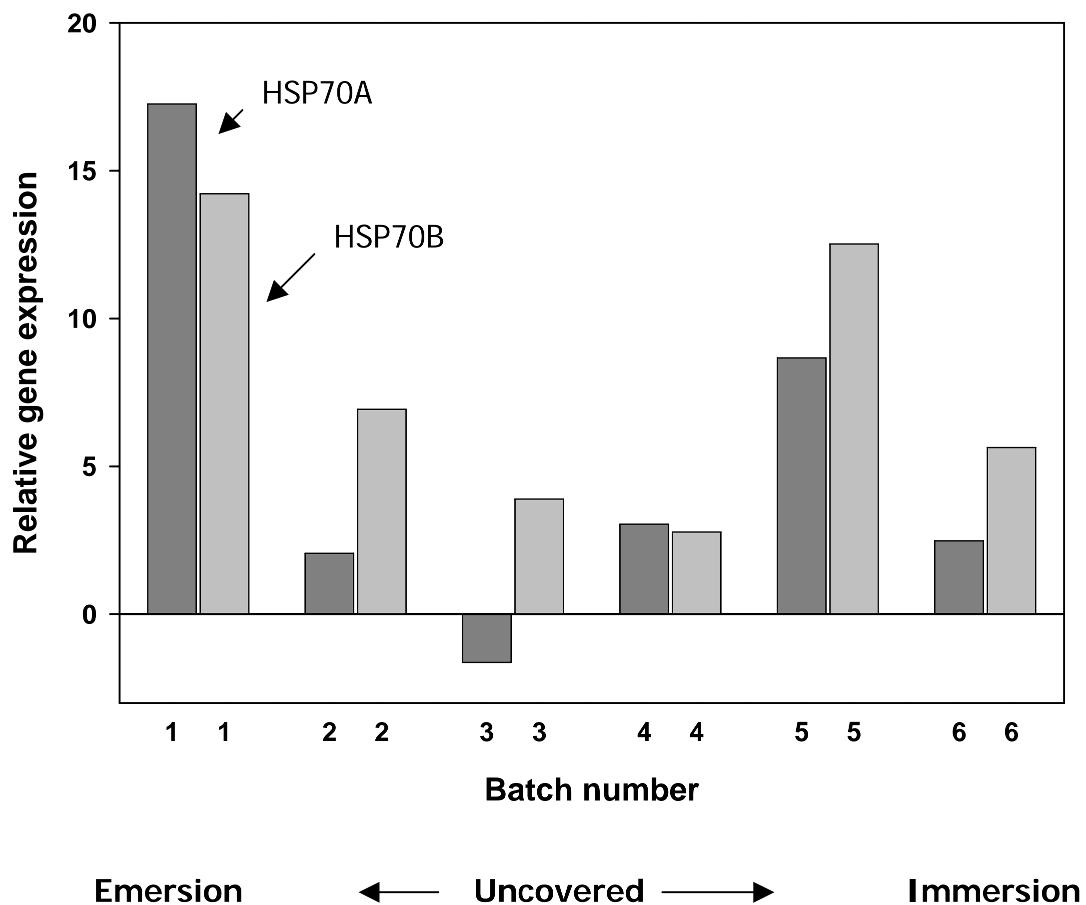
Gene	Batch	p value	Relative gene expression	Range	Gene Regulation
HSP70A	1	0.017	17.73	1.16-207.31	up
HSP70A	2	0.722	1.56	0.11-21.86	up
HSP70A	3	0.575	1.60	0.05-43.68	up
HSP70A	4	0.586	4.70	0.72-30.51	up
HSP70A	5	0.995	0.41	0.03-5.33	-2.43 down regulated
HSP70A	6	0.536	2.66	0.27-25.93	up
HSP70B	1	0.147	11.11	5.26-23.46	up
HSP70B	2	0.409	9.61	2.92-31.61	up
HSP70B	3	0.070	5.94	1.78-19.79	up
HSP70B	4	0.016	12.59	3.34-47.38	up
HSP70B	5	0.249	4.86	1.07-22.11	up
HSP70B	6	0.022	8.26	1.53-44.52	up



Emersion ← Uncovered → Immersion

Figure 2

Gene	Batch	p value	Relative gene expression	Range	Gene Regulation
HSP70A	1	0.187	17.25	0.45-658.75	up
HSP70A	2	0.609	2.06	0.15-27.02	up
HSP70A	3	0.710	0.61	0.06-5.43	-1.63 down regulated
HSP70A	4	0.220	3.05	0.41-22.62	up
HSP70A	5	0.189	8.67	0.26-288.67	up
HSP70A	6	0.788	2.48	0.21-28.68	up
HSP70B	1	0.236	14.22	1.12-179.87	up
HSP70B	2	0.163	6.93	1.30-36.95	up
HSP70B	3	0.212	3.90	0.82-18.34	up
HSP70B	4	0.341	2.78	0.89-8.66	up
HSP70B	5	0.024	12.52	1.33-117.14	up
HSP70B	6	0.255	5.64	0.77-41.37	up



Emersion ← **Uncovered** → **Immersion**

Figure 3

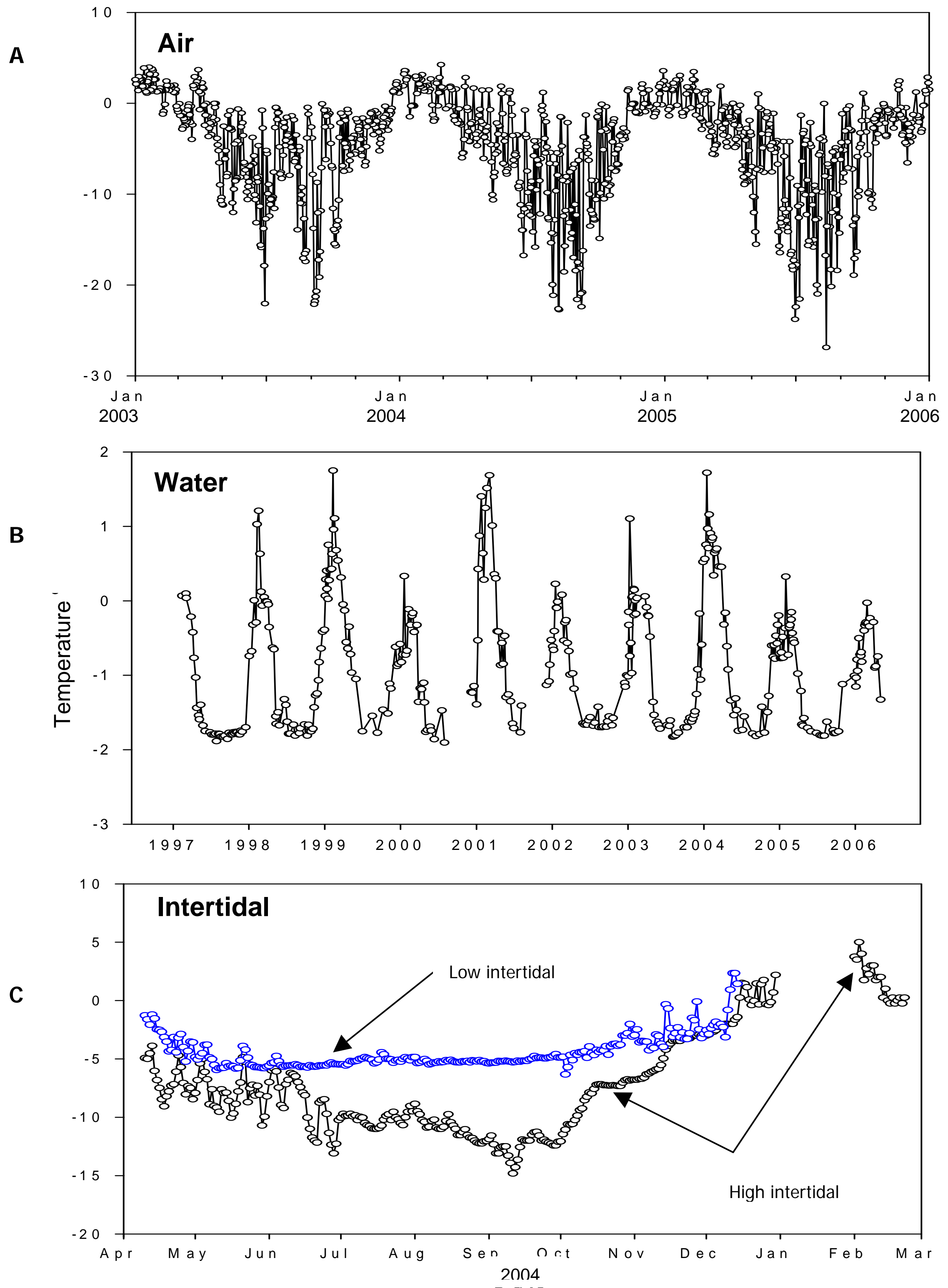


Figure 4

Air temp: N/A		Water temp: 1.5°C				
Limpet No.	**foot temp**	**shell temp**	**l/mm**	**w**	**h**	**time**
C1	1.5	3.2	27.7	19.0	8.9	11.14
C2	2.7	2.7	21.4	14.4	6.2	11.18
C3	2.7	2.7	22.4	15.0	6.2	11.20
C4	2.0	2.2	26.8	18.3	8.1	11.23
C5	3.0	3.0	21.7	14.4	6.3	11.26

Table 1_Supplemental on-line

Batch 1: subtidal, just covered						
Air temp: 9.2°C		Water temp: 2.5°C				
Limpet No.	**foot temp**	**shell temp**	**l/mm**	**w**	**h**	**time**
B1	3.7	3.7	21.2	15.4	6.2	15.16
B2	1.9	3.9	22.7	15.8	7.1	15.20
B3	5.2	3.3	21.8	15.4	6.9	15.27
B4	3.5	3.8	23.4	16.1	6.3	15.31
B5	3.5	3.7	25.8	17.5	7.1	15.39
Batch 2: partially exposed						
Air temp: 10°C		Water temp: 2.2°C				
Limpet No.	**foot temp**	**shell temp**	**l/mm**	**w**	**h**	**time**
B6	2.3	2.4	26.5	19.8	7.9	16.17
B7	1.7	3.3	30.9	21.5	12.1	16.20
B8	2.9	3.3	24.1	16.3	7.5	16.24
B9	2.0	3.0	23.5	16.4	7.2	16.28
B10	2.3	3.4	28.8	20.4	10.2	16.31
Batch 3: exposed, occasional wave breaking over them						
Air temp: 8°C		Water temp: 3°C				
Limpet No.	**foot temp**	**shell temp**	**l/mm**	**w**	**h**	**time**
B11	3.7	3.9	22.2	19.1	6.1	17.18
B12	6.3	7.2	30.7	17.1	10.3	17.21
B13	5.2	6.3	20.6	18.8	5.1	17.23
B14	2.6	5.2	25.1	17.0	7.2	17.28
B15	3.7	5.1	26.1	22.0	8.4	17.29
Batch 4: mostly covered, some exposed						
Air temp: 12.3°C		Water temp: 3.1°C				
Limpet No.	**foot temp**	**shell temp**	**l/mm**	**w**	**h**	**time**
B16	3.2	3.5	28.7	19.6	10.5	18.18
B17	3.8	4.6	29.2	21.2	9.7	18.21
B18	3.6	4.2	23.7	16.1	6.6	18.24
B19	4.0	4.6	23.9	15.9	6.9	18.27
B20	3.4	3.1	31.7	23.1	11.3	18.29
Batch 5: covered						
Air temp: 8.4°C		Water temp: 2.3°C				
Limpet No.	**foot temp**	**shell temp**	**l/mm**	**w**	**h**	**time**
B21	3.5	5.9	28.4	20.0	8.8	19.20
B22	3.4	6.6	21.3	14.2	6.2	19.23
B23	3.4	6.1	25.8	17.9	7.6	19.27
B24	2.4	6.6	24.9	15.6	6.3	19.30
B25	2.6	5.9	20.0	14.2	6.1	19.33

Table 2_supplemental on-line

Batch 1: subtidal, just covered						
Air temp: 6.1°C		Water temp:1.5°C				
Limpet No.	**foot temp**	**shell temp**	**l/mm**	**w**	**h**	**time**
A1	3.4	2.9	29.9	21.2	9.2	12.30
A2	1.4	2.4	31.8	22.2	10.9	12.35
A3	4.6	3.3	25.2	21.2	7.4	12.39
A4	3.3	2.0	25.1	25.1	8.1	12.43
A5	4.0	2.7	X	X	X	12.47
Batch 2: exposed						
Air temp: 9.1°C		Water temp: 1.7°C				
Limpet No.	**foot temp**	**shell temp**	**l/mm**	**w**	**h**	**time**
A6	4.3	6.0	27.1	18.9	8.9	13.35
A7	3.9	4.4	27.4	19.1	8.2	13.42
A8	3.1	6.3	28.0	20.1	9.0	13.46
A9	2.8	4.0	20.4	14.1	6.1	13.50
A10	2.8	3.7	23.2	16.1	8.1	13.55
Batch 3: exposed						
Air temp: 7.2°C		Water temp: 1.7°C				
Limpet No.	**foot temp**	**shell temp**	**l/mm**	**w**	**h**	**time**
A11	4.6	8.2	27.2	19.1	8.5	14.39
A12	3.5	5.7	24.4	17.1	6.8	14.42
A13	3.2	5.2	27.0	18.8	8.1	14.46
A14	2.0	4.1	25.5	17.0	7.8	14.51
A15	3.5	5.1	30.0	22.0	9.2	14.55
Batch 4: exposed						
Air temp: 9.1°C		Water temp: 2°C				
Limpet No.	**foot temp**	**shell temp**	**l/mm**	**w**	**h**	**time**
A16	5.6	6.7	25.1	17.0	7.2	15.37
A17	5.4	7.0	26.3	18.8	6.8	15.43
A18	5.6	7.3	22.6	15.0	6.4	15.46
A19	6.4	8.1	22.9	15.2	7.6	15.50
A20	6.9	8.6	26.6	18.5	8.8	15.55
Batch 5: occasionally covered						
Air temp: not recorded		Water temp: not recorded				
Limpet No.	**foot temp**	**shell temp**	**l/mm**	**w**	**h**	**time**
A21	5.1	7.0	24.6	15.8	7.4	16.33
A22	3.3	5.1	26.7	17.6	8.4	16.36
A23	3.9	5.4	21.3	15.2	7.2	16.40
A24	3.9	5.5	22.4	16.0	7.5	16.44
A25	3.0	3.9	24.2	16.2	8.3	16.48
Batch 6: just covered						
Air temp: 10°C		Water temp: 1.7°C				
Limpet No.	**foot temp**	**shell temp**	**l/mm**	**w**	**h**	**time**
A26	3.9	2.8	24.4	16.2	6.8	17.34
A27	2.7	3.6	23.1	15.5	6.8	17.37
A28	4.7	1.5	27.3	19.0	7.5	17.41
A29	3.3	2.9	23.8	16.1	6.8	17.45
A30	2.3	2.7	25.2	17.1	7.6	17.49

Table 3_supplemental on line

Batch 1: just uncovered						
Air temp: 3.3°C		Water temp:1.6°C				
Limpet No.	**foot temp**	**shell temp**	**l/mm**	**w**	**h**	**time**
WA1	0.2	0.6	26.0	17.0	7.0	13.45
WA2	0.2	0.5	25.0	17.0	7.0	13.47
WA3	0.3	0.5	21.0	15.0	7.0	13.49
WA4	-0.2	0.6	32.0	23.0	12.0	13.51
WA5	-0.6	0.5	32.0	22.0	12.0	13.53
Batch 2: uncovered						
Air temp: 4.3°C		Water temp: 3.6°C in rock pool, 1.5°C open to sea				
Limpet No.	**foot temp**	**shell temp**	**l/mm**	**w**	**h**	**time**
WB1	2.1	1.6	31.0	22.0	19.0	15.10
WB2	1.4	1.6	28.0	19.5	9.0	15.12
WB3	1.5	1.6	25.0	18.0	8.0	15.15
WB4	2.8	1.8	24.0	17.0	7.0	15.18
WB5	2.7	2.2	24.0	17.0	8.0	15.20
Batch 3: uncovered, 4 and 5 in sun						
Air temp: 2.9°C		Water temp: 6.2°C in rock pool, 1.7°C open to sea				
Limpet No.	**foot temp**	**shell temp**	**l/mm**	**w**	**h**	**time**
WC1	2.4	2.7	32.0	23.0	12.0	16.28
WC2	1.7	2.3	34.0	25.0	12.0	16.29
WC3	1.3	1.4	31.0	22.0	10.0	16.30
WC4	5.2	6.9	30.0	22.0	11.0	16.31
WC5	3.9	6.4	28.0	18.0	8.0	16.32
Batch 4: uncovered, 4 and 5 in sun						
Air temp: 2.3°C		Water temp: 5.3°C in rock pool, 2.2°C open to sea				
Limpet No.	**foot temp**	**shell temp**	**l/mm**	**w**	**h**	**time**
WD1	1.3	1.9	35.0	25.0	13.0	17.44
WD2	1.5	1.4	30.0	21.0	10.0	17.45
WD3	0.8	0.2	30.0	21.0	10.0	17.47
WD4	4.7	5.3	25.0	12.0	9.0	17.48
WD5	8.8	11.2	28.0	19.0	8.0	17.50
Batch 5: being lapped by encroaching tide						
Air temp: 1.0°C		Water temp: 2.6°C in rock pool, 1.5°C open to sea				
Limpet No.	**foot temp**	**shell temp**	**l/mm**	**w**	**h**	**time**
WE1	5.4	7.4	31.0	21.0	9.0	18.21
WE2	1.0	1.7	30.0	22.0	10.0	18.23
WE3	0.4	0.0	29.5	21.0	9.0	18.24
WE4	3.6	3.8	26.0	18.0	11.0	18.25
WE5	4.0	3.6	28.0	20.0	10.0	18.26
Batch 6: covered						
Air temp: 1.9°C		Water temp: 1.5°C				
Limpet No.	**foot temp**	**shell temp**	**l/mm**	**w**	**h**	**time**
WF1	1.6	1.4	27.0	19.5	8.0	19.17
WF2	1.5	1.4	31.0	22.0	8.0	19.19
WF3	1.2	1.3	23.5	16.0	7.0	19.20
WF4	1.3	1.2	26.0	17.0	7.0	19.21
WF5	1.3	1.2	26.0	17.0	8.0	19.23

Table 4_supplemental on-line