Lack of an HSP70 heat shock response in two Antarctic marine invertebrates

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Abstract

Members of the HSP70 gene family comprising the inducible (HSP70) genes and GRP78 (Glucose-regulated protein 78kDa) were identified in an Antarctic sea star (*Odontaster validus*) and an Antarctic gammarid (*Paraceradocus gibber*). These genes were surveyed for expression levels via Q-PCR after an acute two-hour heat shock experiment in both animals and a time course assay in *O. validus*. No significant up-regulation was detected for any of the genes in either of the animals during the acute heat shock. The time course experiment in *O. validus* produced slightly different results with an initial down regulation in these genes at 2°C, but no significant up-regulation of the genes either at 2°C or 6°C. Therefore the classical heat shock response is absent in both species. The data is discussed in the context of the organisms' thermal tolerance and the applicability of HSP70 to monitor thermal stress in Antarctic marine organisms.

Keywords: Antarctic, Climate change, biomarker, stress, heat shock proteins

Introduction

The production of heat shock proteins is one of the classical cellular responses of all organisms to environmental insult. These highly conserved proteins act as chaperones to stabilise and refold denatured proteins, preventing the formation of cytotoxic aggregates, maintaining the integrity and functioning of the cell until conditions improve (Parsell and Lindquist, 1993, Hartl, 1996, Fink, 1999). The most studied of these chaperone molecules are the 70kD heat shock protein family (HSP70s) comprising constitutive forms (HSC70: heat shock cognate 70) and stress inducible forms (HSP70s: heat shock protein 70) (Ritossa, 1962; Morimoto et al, 1998). Their action has been described in response to a wide variety of stresses, however the classical activation of this family is in response to elevated environmental temperatures (cf. Lund et al, 2002; Tomanek and Sanford, 2003). Indeed they are often suggested as primary ecological biomarkers (Feder and Hofmann, 1999; Sorensen et al, 2003).

One of the regions of most rapid climate change on Earth is the Antarctic Peninsula, where shallow seawater temperatures along the west Antarctic Peninsula have risen in excess of 1°C over the last 50 years (Meredith and King, 2005). This marine environment is also the habitat of some of the World's most stenothermal species described to date (Peck and Conway, 2000). Although Antarctic invertebrates have survivable temperature envelopes between 5°C and 12°C above the minimum sea temperature of -

2°C (Peck 2002), they start to lose critical biological functions at temperatures 2-3°C higher than current summer maxima (Peck et al, 2004). The question posed is how do we monitor the effects of heat stress in such organisms and are heat shock proteins the most appropriate biomarker for environmental stress in the Antarctic?

The latter is particularly pertinent, given that with the exception of *Hydra oligactis* (Bosch et al, 1988), the only species that have been shown to lack the classical heat shock response are an Antarctic ciliate (La Terza et al 2001, 2004) and two Antarctic notothenioid fish (Carpenter and Hofmann, 2000; Hofmann et al, 2000; Place and Hofmann, 2005; Clark et al, 2007). In addition two Antarctic molluscs *Laternula elliptica* (bivalve) and *Nacella concinna* (gastropod) have a demonstrable heat shock response, but at threshold levels (10-15°C) that are incompatible with moderating any deleterious effects from elevated Polar sea water temperatures (Clark et al, 2008a). The example of *N. concinna* is slightly more complex, in that it occurs inter-tidally and HSP70 genes are induced in response to emersion, a situation with a more complex suite of stressors than purely temperature (Clark et al, 2008b). In a continuing survey into the HSP70 response and thermal stress in Antarctic marine species, we expanded the phylogenetic range of species to include a gamarid crustacean, *Paraceradocus gibber* and an echinoderm, *Odontaster validus*, two sub-tidal species that seasonally experience a temperature window restricted to between –1.86°C and +1°C (from the Rothera Time Series long term data set, provided by Professor A. Clarke).

P. gibber is the largest Antarctic amphipod. It lives under stones in burrows in the sediment and is predominantly a detrital feeder (Coleman, 1989). *O. validus* is ubiquitous and abundant in Antarctic waters with a circum-polar distribution (McClintock et al, 1988). Although it is known as a predatory scavenger, it also gets much of its energy from detrital feeding (Pearse, 1965). So these are both species that are positioned towards the top of the Antarctic marine food web and have different lifestyles to those Antarctic species already studied for their HSP response.

In this study members of the HSP70 gene family comprising inducible (HSP70) members and GRP78, a related HSP70 family member were cloned using degenerate PCR from *P. gibber* and *O. validus*. The expression of these genes was surveyed via Q-PCR after an acute heat shock experiment in both organisms, and an additional time course experiment in *O. validus*. The data is discussed in the context of the organisms' evolutionary adaptation to life in an extreme environment, thermal tolerance and the applicability of HSP70 to monitor thermal stress in Antarctic marine organisms.

Methods

Animal sampling and experimental work

All *Odontaster validus* (sea star) and *Paracerodocus gibber* (amphipod) used in experimental work were collected at Rothera Research Station, Adelaide Island, Antarctic Peninsula (67° ″07 ′34 S, 68° ″30 ′07 W) by SCUBA divers during the austral summer and returned to the British Antarctic Survey's aquarium in Cambridge, UK. The animals were maintained throughout in a re-circulating seawater aquarium at a mean water temperature of -0.7°C and a 12:12 light:dark cycle.

O. validus and *P. gibber* were exposed to 10^0C (9.96±0.05°C) and 15^0C (14.85±0.05°C) for 2h (n=5/species). Animals were directly transferred from -0.7°C to the elevated temperatures without an acclimation period. Separate control groups (n=5/species) of *O. validus* and *P. gibber* were also sampled from the aquarium. *O. validus* was also subjected to 2 time course experiments at 2^0C (2.0±0.05°C) and 6^0C (6.0±0.02°C) for periods of 2, 6, 12, 24, 48 and 168 hours each (n=4 at each time period).

Sample Analysis

RNA extraction and isolation of Heat Shock Protein (HSP) genes: Total RNA was extracted from whole body *(P. gibber*) and gonad (*O. validus*) 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). Degenerate primers for HSP70 were designed from a protein alignment of HSP70 genes from a variety of species as described in Clark et al (2008a) (Table 1). PCR cycling conditions were as follows: 95°C 5 minutes, 35 cycles of 95°C 20 seconds, 45°C 20 seconds and 72°C for 40 seconds with a final elongation step of 72°C for 5 minutes with 40 cycles. Products were subcloned into p-GEMT-easy (Promega), transformed into E.coli strain XL-2 Blue MRF' (Stratagene) and a minimum of 48 clones sequenced from each species. 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 species (Table 1), all with an annealing temperature of 60°C. Amplified fragment sizes varied between 86bp and 146bp. The specificity of each of the primers was checked, by amplification and sequencing of the products.

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 species. Degenerate primers were designed from a ClustalW alignment of a number of β actin genes as described in Clark et al (2008a) (Table 1). The PCR conditions were as follows: 95°C 5 minutes, 40 cycles of 95°C 20 seconds, 45°C 20 seconds and 72°C for 40 seconds with a final elongation step of 72°C for 5 minutes. PCR products were sequenced, assembled and checked as described above for the HSP genes. Where 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.

The HSP sequences described in this paper have been submitted to the EMBL database with accession numbers AM408048 (*O. validus*, HSP70), AM408049 (*O. validus*, GRP78), AM408050 (*P. gibber*, HSP70A), AM408051 (*P. gibber*, HSP70B), AM408052 (*P. gibber*, HSP70C) and AM408053 (*P. gibber*, HSC70)

Q 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 Q-PCR machine (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 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 four fold 10x dilution series and the values calculated using the MxPro - MX3000P v 3.00 Build 311 Schema 74 software (Table 2). 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 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 Fisher's test for combining probabilities (Fisher, 1954) and 2-way ANOVA tests were run on the time course dataset to identify any effect of either gene, temperature or time.

Results

Four different HSP gene family members were cloned from both *P. gibber* and *O. validus*. These were defined according to their sequence similarity scores after searching the sequence databases using WU-blastx and comprised three inducible HSP70's and GRP78 (*P. gibber*) and one inducible HSP70 and GRP78 (*O.validus*) (Table 1). Specific primers for Q-PCR were successfully designed to all clones (Table 2). A further two clones were isolated from *O. validus* for another inducible form of HSP70 and also HSC70, however in spite of repeatedly testing different primers for these clones (30 potential primer combinations for each) none worked effectively either in terms of amplification, reproducibility or producing a linear relationship in a dilution series and they were excluded from the analysis. To obtain a crude estimate of the relative expression levels of each of the HSP genes in each organism the genes were assayed using PCR and gel electrophoresis with ethidium bromide staining in a set of control animals (data not shown). Actin expression, as expected, produced a strong signal in each species, but there was no discernable expression of the HSP family members either in *P. gibber* or *O. validus*.

The acute heat shock experiments at 10°C and 15°C showed no significant up regulation of HSP genes in either species (Figure 1). Application of Fisher's method for combining probabilities on each of the two species datasets is consistent with no detectable effect of temperature on HSP gene expression (for values see figure legend).

In the *O. validus* time course experiment, at 2°C both GRP78 and HSP70 were down regulated. With both genes there was a trend showing an initial decrease in relative expression levels (-40 fold with GRP78 and –6.9 fold with HSP70), which over the period of the time course gradually returned to levels approaching those of the controls (Figure 2). The 6°C time course experiment showed a different pattern with a non-significant level of up-regulation (cf. individual p values) in relative gene expression level for both genes. 2-way ANOVA testing on the combined 2°C and 6°C dataset identified an effect of both temperature and time (temperature: DF = 1, F = 63.84, p = 0.000; time: DF = 5, F = 4.34, p = 0.017), but no effect of the gene when tested against the variables of time and temperature (data not shown).

Discussion

Neither *P. gibber* nor *O. validus* showed any significant up-regulation of heat shock protein activity in the acute temperature challenges above the level of

individual variation/experimental noise for any of the family members (Figure 1). Whilst these are temperatures far in excess of those the animals would experience in the Antarctic marine environment, previous laboratory based experiments on *L. elliptica* and sub-tidal *N. concinna* have demonstrated the classical heat shock response at 10°C (*L. elliptica*) and 15°C (*N. concinna*) (Clark et al, 2008a). Hence there are Antarctic marine organisms that still possess the ability to up-regulate their HSP genes. Whether these genes are utilised in their natural environment, or the threshold level for induction is a remnant of a temperate ancestor remains to be determined and may become more apparent as a greater number of species are surveyed. One such example of related work on inter-tidal *N. concinna* has demonstrated the up-regulation of inducible forms of HSP70 (albeit to much lower levels than the laboratory experiments) in response to emersion (Clark et al, 2008b). Clearly the stresses imposed on inter-tidal animals are more complex, but this data does show that HSP70 genes, at least in *N. concinna* are induced by stresses in the natural environment and not merely a vestigial attribute from a phylogenetic history. That may be the situation with *P. gibber* and *O. validus* in that they have maintained a suite of HSP70 genes, but they may be induced by different cellular perturbations. Although only one inducible form of HSP70 was surveyed in *O. validus* (sequence data indicated that other forms were present (Clark, unpublished)), results from the bivalve, *L. elliptica* and *N. concinna*, limpets, show that heat stress reproducibly induced all forms of HSP70 in the latter species (Clark et al, 2008a). Therefore results from a single inducible HSP70, in this experimental context, are potentially indicative

of the generalised expression of multiple isoforms, where they exist. However validation will always required to define subtle differences in control between paralogous genes.

In the other main example of lack of a classical heat shock response, the Antarctic Notothenioids, have maintained HSP70 genes, but their expression mode has been altered from inducible to constitutive (Carpenter and Hofmann, 2000; Hofmann et al, 2000; Place et al, 2004; Place and Hofmann, 2005; Clark et al, 2007), unless like the limpets, these chaperones are induced by other environmental stresses. A PCR-based survey of the HSP70 genes in control animals of *P. gibber* and *O. validus* did not show constitutive expression of any of the genes under study. This is in contrast to the work on the Antarctic Notothenioids and the molluscs, where there is considerable constitutive expression of HSP70, HSC70 and GRP78 (Place and Hofmann, 2005; Clark et al, 2007, 2008a). This is thought to be due to an enhanced requirement for chaperone proteins to help with the problems of protein folding at low temperatures (Place et al, 2004). This requirement would not necessarily appear to be present in all species, but again requires further investigation.

The results of the *O. validus* time course experiment are more complex (Figure 2). Again, the individual p values show no significance at the 95% Confidence Interval. Although there would appear to be a trend in the 2°C experiment for an initial down-regulation of both genes, followed by a gradual

return to "normal" levels over the period of the week. This trend is validated by the ANOVA analysis, showing a combined effect of time and temperature. The shape of the 2°C graphs mirror the results of a 6°C 48 hour time course experiment in *Harpagifer antarcticus* for both HSP70 gene family members and the warm acclimated protein (WAP65) (Clark et al, 2007; Clark and Burns, 2007). It has been proposed that initial down-regulation of certain genes is due to the initial "shock" response followed by a return to equilibrium as these animals acclimate. Certainly Antarctic fish have been acclimated to +4°C (Carpenter and Hofmann, 2000; Lowe and Davidson, 2005; Jin and deVries, 2006, Podrabsky and Somero, 2006) and *H. antarcticus* has spent several months at 3°C in the Rothera aquarium (Clark, unpub.). *O. validus* has also been successfully acclimated to +6°C (Peck et al, 2008) and experiments on larvae indicate a certain level of temperature tolerance (Stanwell-Smith and Peck, 1998; Peck and Prothero-Thomas, 2002), indicating that the fish and the sea star are some of the more robust Antarctic stenotherms (Peck et al, submitted). Why the shape of the 2°C graphs are not mirrored in the 6°C experiments (with a lack of initial down regulation) is unknown. Even with this experiment, the longer timescale and higher temperature does not invoke significant HSP expression, but given the data of Peck et al (submitted) where oxygen consumption and feeding in *O. validus* are not affected by 25 days at 6°C, this is perhaps not surprising.

These two species (*P. gibber* and *O. validus*) expand the previous surveys into the heat shock response of Antarctic marine organisms. The more

species surveyed, the more complex the picture. There is now data on several fish species (Place and Hofmann, 2005; Clark et al, 2007), a filter feeding bivalve mollusc and a grazing gastropod mollusc (Clark et al, 2008a). We have now added to this with data for a detritivore and a carnivore/detritivore. This encompasses a wide phylogenetic range, different feeding guilds and variety in lifestyle. Not all species show permanent expression of inducible HSPs, not all species show the classical heat shock response and in at least one case, multiple stressors can invoke HSPs at temperatures lower than that tested in the laboratory. This combined data shows that it will not be a simple task to derive generalisations about the ability of Antarctic marine organisms to invoke the classic cellular first aid kit of HSP70 genes in response to elevated sea water temperatures or indeed the utility of HSPs as a biomarker for stress in Antarctic waters. The results of the gene expression level surveys also demonstrate that for a molecular biomarker (Antarctic or otherwise) to be effective, the change in gene expression has to be large. The one-off sampling regime for "before" and "after" effects and the high inter-individual variability of non-interbred, non-model organisms produces wide 95% Confidence Intervals with Q-PCR experiments (discussed in more detail in Clark et al, 2007). Therefore biomarker gene expression levels must show a strong signal above background variation in order for valid conclusions to be drawn about perturbation effects. This provides problems when evaluating field populations of non-model organisms when gene expression changes are moderate or small. In conclusion, the data presented here show that the HSP response in the Antarctic has to be investigated on a species-specific basis.

Wider surveys are required to elucidate the most common response patterns, along with investigation of the potential for different types of environmental perturbation to invoke HSP expression both in the laboratory and wild populations. Also given these limitations, a wider range of genes should be investigated and EST libraries are currently in production within our laboratory for a number of Antarctic invertebrates with the aim of targeting genes involved in the reaction to heat stress.

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Table and Figure Legends

Table 1:

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

Table 2:

Degenerate primers used to clone specific HSP and actin genes in *P. gibber* and *O. validus.* Q-PCR specific primer sets for HSP family members and actin. RSq and PCR efficiency values are included for Q-PCR reactions, as calculated using the Stratagene MxPro - MX3000P v 3.00 Build 311 Schema 74 software.

Figure 1:

Table shows Q-PCR results for *P. gibber* and *O. validus* after an acute 10°C and 15°C temperature heat shock. Absolute fold change in gene expression level is given, alongside REST calculated change in gene expression levels. Graph depicts tabulated results. Fisher's method for combining probabilities: Chi squared test on *P. gibber* dataset: F = 9.727, Df = 16, p = 0.119; Chi squared test on *O. validus* dataset: F = 4.458, Df = 8, p = 0.186.

Figure 2:

Table shows Q-PCR results for *O. validus* after a 2°C and a 6°C heat shock time course experiment. Absolute fold change in gene expression level is

given, alongside REST calculated change in gene expression levels. Graph depicts tabulated results.

Organism	Primer Set	Gene Designation	Closest database match	Score	% identity	Probability
Paraceradocus gibber	Pgi1F Pgi1Rev	HSP70A	Q6L6T3: HSP70 *Antheraea yamamai* (Japanese oak silk moth)	435	69	$4.2e^{-39}$
	Pgi2F Pgi2Rev2	HSP70B	P08106: HSP70 *Gallus gallus* (chicken)	476	74	$1.4e^{-43}$
	Pgi9F Pgi9Rev	HSP70C	Q517H8: HSP70b *Drosophila melanogaster* (fruit fly)	396	65	$9.3e^{-35}$
	Pgi11F Pgi11Rev	GRP78	HSC70 *Bombyx mori* (silk moth)	476	80	$1.4e^{-43}$
Odontaster validus	Ova2F2 Ova2Rev2	GRP78	Q9O593: GRP78 *Gallus gallus* (chicken)	487	78	$9.8e^{-45}$
	Ova9F Ova9Rev	HSP70	Q6S4R6: *Macrobrachium rosenbergii* (giant freshwater prawn)	479	86	$6.9e^{-44}$

Table 1

Organism	Gene	Primer Sequence		RSq	PCR efficiency
	Degenerate for HSP70	HSP70F	ATCATCGCYAACGACCAGGGMRAC	N/A	N/A
		HSP70Rev	GTTGTTGAAGTARGCDGGSACBGT		
	Degenerate for actin	SeaActinF	ACCGACTACYTSAKKAAGATCCT	N/A	N/A
		SeaActinRev	GAVGCVAGGATGGAGCCRCC		
Odontaster validus	GRP78	Ova2F2	ATCGGACGCACATGGGATGA	0.998	93.9%
		Ova2Rev2	GCACTGATTTCCTCAGCAGCA		
	HSP70	Ova9F	AATTTGTCGATAGTGCGGTGC	0.977	104.9%
		Ova9Rev	AGCTGATCTCCTCAGCGAAG		
	Actin	OvaActinF	GAGCGTGGCTACTCTTTCACC	0.994	65.9%
		OvaActinRev	TCGTAGCTCTTCTCCAGGGAG		
Paraceradocus gibber	HSP70	Pgi1F	CTAAAGCCCAGGCATCTTGGA	0.996	171.0%
		Pgi1Rev	AATTTCAGGATCATCATACTTGC		
	HSP70	Pgi2F	AAAAGCTCAAGGAGCCGTGAAT	0.998	133.7%
		Pgi2Rev2	CACAGTGGGGTCGTCATACC		
	HSP70	Pgi9F	TAAAGCTCAGGCGTCTTGGAA	0.994	89.1%
		Pgi9Rev	AGCATCAGGATCATCATACTTGC		
	GRP78	Pgi11F	CCAAGAACCAGCTTACGACAA	0.999	94.1
		Pgi11Rev	AACGGACTTCTCGTTCCAGTC		
	Actin	PgiActinF	GAACTTCCCGACGGTCAGGT	1.000	88.9
		PgiActinRev	GCGAAGAGATCCTTACGGATAT		

Table 2

Organism	Gene	Temp	p-value	Relative Gene Expression	Range	Gene Regulation
Paraceradocus gibber	HSP70A	10	0.878	1.20	0.03-47.05	up
	HSP70B	10	0.910	1.22	0.22-6.59	up
	HSP70C	10	0.558	0.46	0.03-7.05	-2.14
	GRP78	10	0.651	0.54	0.08-3.32	-1.82
	HSP70A	15	0.356	3.72	0.05-273.48	up
	HSP70B	15	0.423	1.89	0.07-49.21	up
	HSP70C	15	0.301	1.96	0.16-23.41	up
	GRP78	15	0.587	1.19	0.32-4.37	up
Odontaster validus	HSP70	10	0.465	0.52	0.06-4.35	-1.92
	GRP78	10	0.553	3.46	0.03-7.96	up
	HSP70	15	0.611	1.81	0.18-18.15	up
	GRP78	15	0.685	1.98	0.07-55.84	up

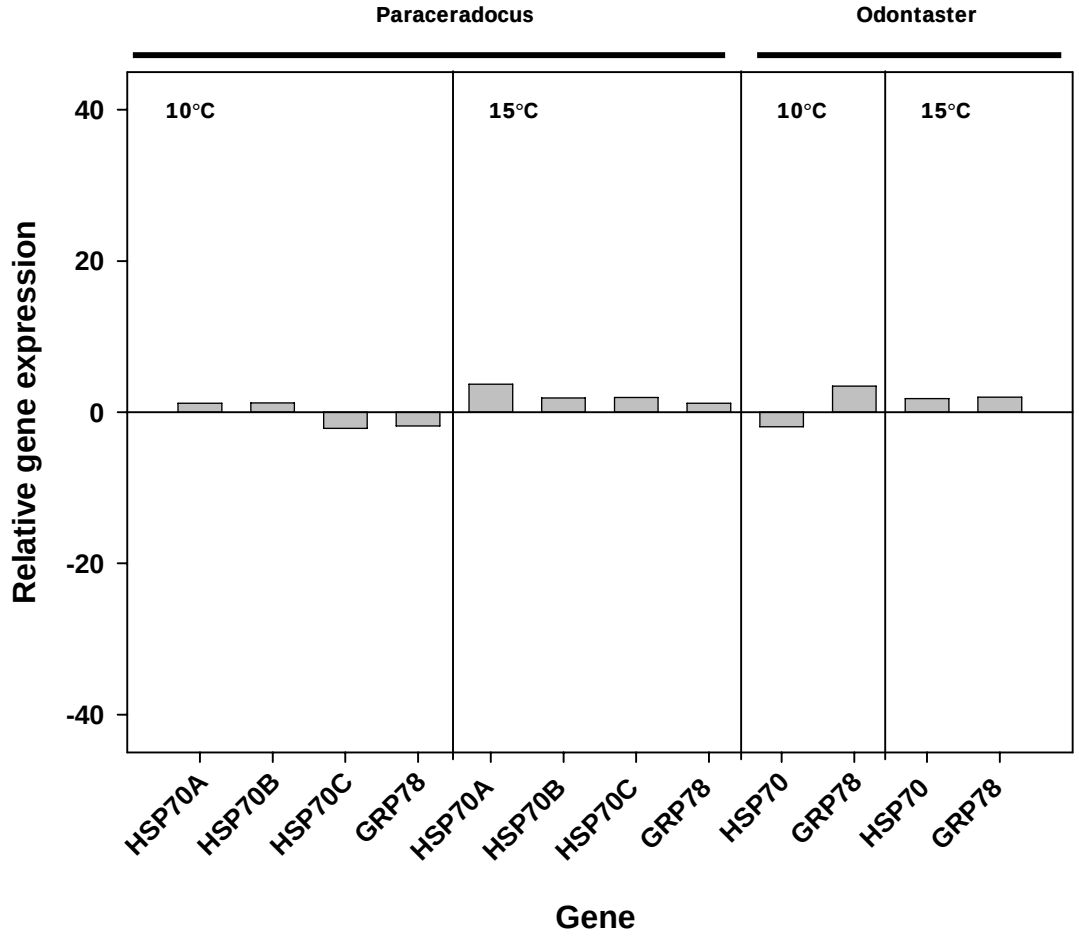

Figure 1

Gene	Temp	Time (hours)	p-value	Relative Gene Expression	Range	Gene Regulation
GRP78	2	2	0.114	0.02	0.002-0.23	-40.4
	2	6	0.165	0.10	0.01-0.60	-9.42
	2	12	0.943	1.14	0.17-7.59	up
	2	24	0.110	0.17	0.01-1.88	-5.61
	2	48	0.441	0.40	0.04-3.31	-2.45
	2	168	0.930	1.57	0.16-14.71	up
HSP70	2	2	0.181	0.14	0.01-1.06	-6.90
	2	6	0.253	0.21	0.03-1.24	-4.66
	2	12	0.899	0.55	0.12-2.58	-1.78
	2	24	0.426	0.50	0.07-3.59	-1.96
	2	48	0.515	0.43	0.06-3.06	-2.27
	2	168	0.986	0.68	0.17-2.71	-1.46
GRP78	6	2	0.947	1.49	0.15-14.15	up
	6	6	0.820	2.64	0.44-15.83	up
	6	12	0.625	2.17	0.16-28.42	up
	6	24	0.712	3.75	0.34-40.30	up
	6	48	0.546	5.68	0.86-37.26	up
	6	168	0.383	3.81	0.97-14.89	up
HSP70	6	2	0.981	1.11	0.28-4.38	up
	6	6	0.751	3.55	0.59-21.05	up
	6	12	0.878	0.88	0.12-6.34	-1.13
	6	24	0.850	2.18	0.29-16.34	up
	6	48	0.818	2.10	0.96-4.56	up
	6	168	0.542	2.13	0.19-23.78	Up

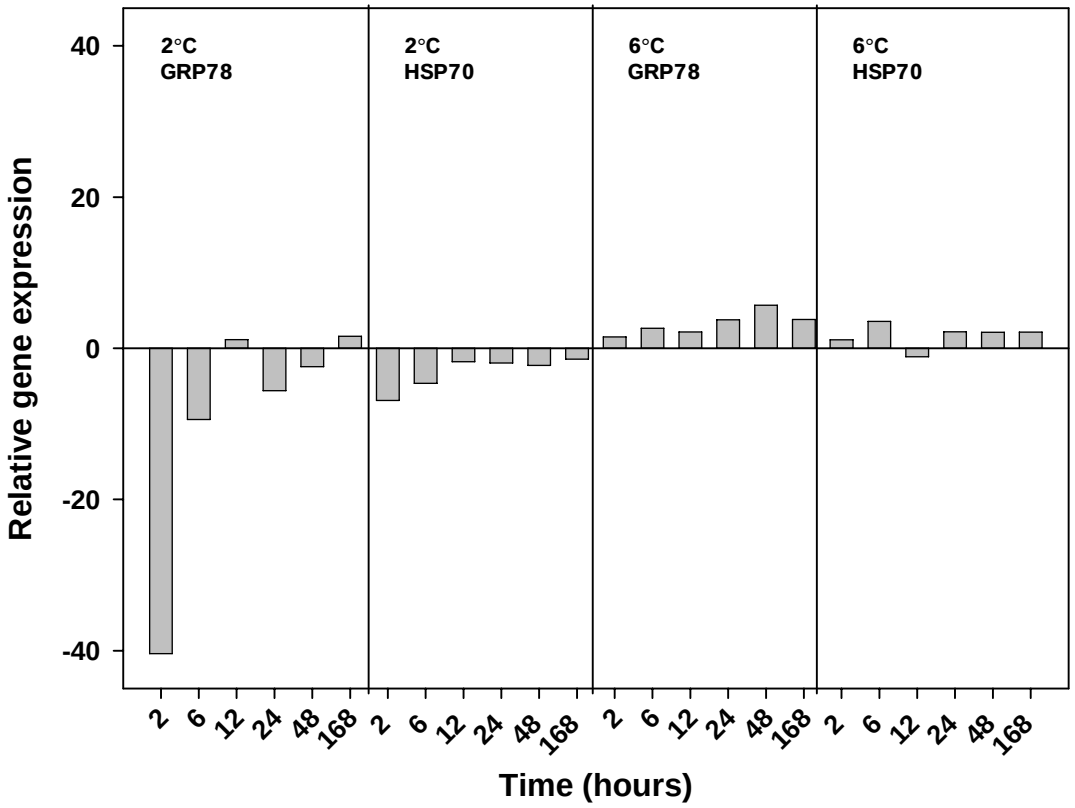

Figure 2