Age-related thermal response: the cellular resilience of juveniles

M. S. Clark¹ · M. A. S. Thorne¹ · G. Burns¹ · L. S. Peck¹

Received: 2 June 2015 / Revised: 27 August 2015 / Accepted: 2 September 2015 / Published online: 12 September 2015
© Cell Stress Society International 2015

Abstract Understanding species' responses to environmental challenges is key to predicting future biodiversity. However, there is currently little data on how developmental stages affect responses and also whether universal gene biomarkers to environmental stress can be identified both within and between species. Using the Antarctic clam, *Laternula elliptica*, as a model species, we examined both the tissue-specific and age-related (juvenile versus mature adult) gene expression response to acute non-lethal warming (12 h at 3 °C). In general, there was a relatively muted response to this sub-lethal thermal challenge when the expression profiles of treated animals, of either age, were compared with those of 0 °C controls, with none of the “classical” stress response genes up-regulated. The expression profiles were very variable between the tissues of all animals, irrespective of age with no single transcript emerging as a universal biomarker of thermal stress. However, when the expression profiles of treated animals of the different age groups were directly compared, a very different pattern emerged. The profiles of the younger animals showed significant up-regulation of chaperone and antioxidant transcripts when compared with those of the older animals. Thus, the younger animals showed evidence of a more robust cellular response to warming. These data substantiate previous physiological analyses showing a more resilient juvenile population.

Keywords Heat shock protein · GRP78 · Superoxide dismutase · Immune · MAP kinase · Tissue-specific

Introduction

In a changing world, our ability to accurately predict the effect of environmental perturbation on ecosystems is limited. Ecological observations can record shifts in species ranges and regime changes associated with climate change events (Drinkwater 2009); however, these are a posteriori observations. To provide a priori predictions, we need to understand species' responses to change, in terms not only of their abilities to adapt and potentially survive but also the developmental, physiological and biochemical trade-offs that may occur as a result of the animals coping with change (Gunter and Degan 2008; Somero 2010). It is with the latter that molecular biology can impact most significantly on the very distantly related field of ecosystem monitoring and future predictions. Transcriptome analyses allow us to describe a complex suite of responses at the cellular level far more accurately than whole animal physiological observations, leading to the identification of putative gene biomarkers (Truebano et al. 2010). Such analyses are increasingly common, but in a complex organism, there is always the question of which tissue(s) to sample and also which developmental stage or the age of the adults (particularly pertinent in long-lived species). This is often the result of either investigator choice or merely what animals are available and how much of each tissue can be reproducibly sampled in sufficient quantities. This then leads to the question of whether any putative biomarkers are universally expressed across all tissues and ages or whether future samplings need to be similarly targeted.

We previously characterised the short-term response to thermal stress and hypoxia in the Antarctic clam, *Laternula*

Electronic supplementary material The online version of this article (doi:10.1007/s12192-015-0640-x) contains supplementary material, which is available to authorized users.

✉ M. S. Clark
mscl@bas.ac.uk

¹ British Antarctic Survey, Natural Environment Research Council, High Cross, Madingley Road, Cambridge CB3 0ET, UK

elliptica, using a custom-built microarray (Truebano et al. 2010; Clark et al. 2013). The first study indicated that genes involved in antioxidant production and calcium signalling represented potential biomarkers of the physiological state of this organism under thermal stress. However, this study only used mantle tissue which is the shell secreting organ of the animal, and hence the heat-induced calcium signalling may have been a direct reflection of this tissue's functional response (Truebano et al. 2010). The latter study examined both gill and siphon tissue but identified age as an over-riding factor, with a differing tissue-specific response (Clark et al. 2013). These two studies both used different tissues, and therefore it was not possible to directly compare the results with regard to the identification of universal putative biomarkers to differing stresses or to define a constrained tissue-specific response.

In terms of ecology, *L. elliptica* is highly abundant with a circumpolar distribution (Dell 1972), and as an infaunal filter-feeder, it plays a significant role in benthopelagic coupling (Arntz et al. 1994; Ahn 1994). It is one of the best characterised Antarctic marine invertebrates and the largest individual mollusc in the Southern Ocean with regard to live weight (Ralph and Maxwell 1977) with several tissues that are easy to dissect for tissue-specific gene expression analyses. *L. elliptica* is also one of the more sensitive Antarctic marine species. It suffers significant mortalities at 4–5 °C at long term but loses essential biological functions, such as the ability to bury in sediment, much earlier. Some 50 % of animals fail to rebury within 24 h at 2.5 °C, which is only 1–2 °C over the current summer maximum sea water temperatures (Peck et al. 2004; 2009). Hence, it represents an ideal candidate for examining the tissue-specific effects of thermal challenge and whether universal biomarkers to environmental challenge can be identified in any one species.

In this study, we subjected *L. elliptica* to an acute (12 h) 3 °C heat shock. A microarray was used to characterise the effects of this on the expression profiles of four different tissues (mantle, siphon, gill and foot) in both young (juveniles) and older reproductively mature animals. The aim was to identify the effects of thermal stress on the different tissues, how this was affected by age and whether any gene(s) was/were universally expressed in response to environmental challenge.

Materials and methods

Animal collection and sampling

Specimens of *L. elliptica* were collected by scuba divers at a depth of 10–18 m in January 2010 at Hangar Cove, Rothera Point, Adelaide Island, Antarctic Peninsula (67°34'07" S, 68°07'30" W) (water temperature 0.5±0.09 °C SE). The Antarctic is not privately owned, and collections were not

made from any of the protected sites within Antarctica. The field studies did not involve endangered or protected species. Collections were made within Antarctic Act Permits numbers S7-06/2011 and S7-02/2010 as granted under sections 12 and 13 of the Antarctic Act 1994. Specimens were collected in two size classes: large animals (lengths ranging around 60 mm and with mature gonads) and small animals (lengths ranging around 30 mm, with no gonads present), the sizes of which equated to average ages of 16 and 7 years, respectively (Watson, unpublished manuscript) (Table 1). This species begins to produce gonads at 35–40 mm in length (MS Clark, personal observation), and these sizes correspond to mature adults and large juveniles, but lack of maturity in the juveniles was confirmed on dissection by lack of gonads. These two groups were termed “old” and “young”, respectively. The clams were maintained in a flow-through aquarium and allowed to acclimate to laboratory conditions for 2 weeks before experimentation. At the end of the acclimation period, ten old and ten young animals were transferred to a 60-l jacketed tank with aerated sea water, connected to a thermocirculator (Grant LTD 20g, Grant Instruments Ltd, Cambridge, UK). The sea water temperature was gradually raised from 0 °C to +3 °C over a 12-h period. This temperature was then maintained for a further 12 h before sampling the animals. The animals were not fed during this time and were not kept on sediment and so could not rebury. Tissue samples were dissected from siphon, mantle, foot and gill (Fig. 1) and immediately snap-frozen in liquid nitrogen and stored at –80 °C. The siphon is a joint fused inhalant/exhalant siphon, and this was sampled towards the posterior end, away from the siphon holes. The mantle tissue was sampled across all folds of the mantle (Fig. 1), and portions of the gills were randomly taken from both sides. The sampling regime was repeated on ten old and ten young animals that had been maintained in the flow-through aquarium for the same time period at 0 °C (control animals).

Array hybridisation

RNA was extracted from all samples using TriSure (Bioline, UK), according to the manufacturer's instructions, with subsequent RNA purification using Qiagen RNeasy minikit spin columns, which included an on-column DNase treatment. The quantity of RNA was measured by spectrophotometry using a NanoDrop (ND1000) and quality-checked on an agarose gel. RNAs from mantle, siphon, foot and gill from each of five animals for each group (old treated, young treated, old control and young control) were used in the array hybridisation experiments. The construction of the 8448 clone *L. elliptica* array has been previously described in Truebano et al. (2010). PCR-amplified labelled cDNA targets were prepared from 1 µg total RNA using the protocol described in Petalidis et al. (2003), and hybridisations were performed as described

Table 1 Size and average age data for the *L. elliptica* used in the microarray hybridisations ($N=5$ for each category)

	Mean shell length (mm)	Range of shell lengths (mm)	SE	Approximate age (years)
Young controls	33.5	31.3–35.0	0.68	7
Old controls	62.1	51.9–76.4	5.09	16–17
Young treated	30.3	24.8–34.0	1.69	7–8
Old treated	69.5	58.8–86.3	5.87	17–18

in Purac et al. (2008) with modifications according to Truebano et al. (2010). Five biological replicates were used for each experiment with three dye swaps (young control foot, old control mantle and old treated foot) included for quality assurance purposes.

Data acquisition, normalisation and analysis

Data were extracted using the Genepix Pro software v 6.0.1 (MDS Analytical Technologies, Berkshire, UK). Anomalous features were excluded following visual inspection. Low-intensity features (median foreground intensity $< 3 \times$ median background intensity) were also excluded. The R (R Development Core Team 2005) limma microarray package (Smyth and Speed 2003; Smyth 2004; 2005; Smyth et al. 2005; Richie et al. 2007) was used for data analysis. Background subtraction (half) and within (print-tip loess) and between (R quantile) normalisations were conducted across the arrays. Treatments were compared using a reference design-based linear model (Smyth 2004). Differentially expressed clones were selected at an adjusted p -value of < 0.01 (Benjamini and Hochberg 1995) and a minimum two-fold change, as used previously in Clark et al. (2013), which ensures that the most highly expressed transcripts are highlighted. PCA analysis (Mardia et al. 1979; Venables and Ripley 2002) was also performed. The array had been validated on two previous occasions using Q-PCR of 11 clones with a Pearson correlation of 0.69, $p=0.005$, as described in Truebano et al. (2010) and Clark et al. (2013). The array

design and experiment are in Array Express: Experiment name: A-MEXP-1676; ArrayExpress accession numbers: Gill: E-MTAB-3280; Foot: E-MTAB-3282; Mantle: E-MTAB-3283; Siphon: E-MTAB-3284.

Sequencing of differentially expressed clones and data analysis

The inserts from all cDNAs of interest were PCR-amplified and sequenced following Truebano et al. (2010), and sequence runs were performed by Source Bioscience (Nottingham, UK). Trace2dbest (Parkinson et al. 2004) was used to remove and trim poor quality and vector sequence. The TGI clustering tool (Perteau et al. 2003) was used to assemble sequences. These sequences were then searched for Blastx sequence similarity against NCBI non-redundant database with a cutoff level for annotation of less than 10^{-10} . But they were also Blast-searched against the *Laternula* contigs generated from Clark et al. (2010) (SRA accession number: 011054) to identify longer reads, where possible, for more accurate annotation. These contigs were then annotated using Blastx (Altschul et al. 1997) against the non-redundant GenBank database (Bairoch et al. 2007) with a cutoff level for annotation of less than 10^{-10} . All sequences are available from GenBank (accession numbers JK991088–JK993117).

Results

There were no mortalities or abnormal behavioural responses recorded during this experiment. When the data were analysed using separate pairwise comparisons of each tissue and developmental stage for the effect of thermal stress, relatively little effect was identified, particularly in the young animals. The number of clones up-regulated in young animals varied from 0 in mantle to a maximum of 14 in siphon (out of 8928 clones on the array). The older animals showed a wider ranging effect, with higher numbers of significantly up-regulated clones in each tissue; from six in gill to 71 in siphon (Fig. 2). When sequenced, these 71 clones mapped to 35 contigs and ESTs, with putative annotation for 13 (Table 2). The annotation indicated that the majority (70 %) of these transcripts were associated with either enhanced mitochondrial respiration or protein production. This was probably due to the thermal

Fig. 1 Dissected *L. elliptica* showing the internal organs and sampling points. Photograph copyright permission obtained from Erwan Amice

Fig. 2 Graph showing the number of clones up-regulated in response to temperature, with the expression profiles of animals at 3 °C compared with control animals at 0 °C. Results defined in terms of tissue and age where *red*=old animals and *blue*=young animals

dependency of biochemical reactions, with the higher temperature increasing the general metabolic rate of the animals (Schmidt-Nielsen 1991; Dahlhoff and Somero 1993). In both age cohorts, the siphon showed the greatest effect in terms of the numbers of clones showing up-regulation, with relatively little effect in gill and foot, as evidenced by the low numbers of up-regulated microarray clones for both tissues and by the tight clustering of gill and foot profiles in the tissue-specific PCA analysis (Supplementary Fig. S1).

In the analysis described above, the effect of thermal stress was identified by comparing treated animals at 3 °C with same-age animals at 0 °C as controls. In a second analysis, the expression profiles of the treated young and treated old

animals were directly compared to identify those transcripts associated with age (Fig. 3). Relative expression levels were again similar and low in the foot and especially the gill, with most differences seen in the mantle and the siphon. These two tissues showed conflicting patterns, with more clones relatively up-regulated in the siphon tissue in the younger animals (382 clones, which mapped to 62 contigs, with 26 putative annotations) (Table 3) compared with only 136 clones up-regulated in older animals (mapping to 63 contigs, with putative annotations for 16) (Table 4). The expression profiles of the younger animals indicated an active cellular “stress” response with the up-regulation of transcripts putatively involved in protein folding (peptidyl-prolyl cis-trans isomerase and a member of the 70-kDa heat shock protein family (the 78-kDa glucose-regulated protein: GRP78)) and combating reactive oxygen species (ROS) (manganese superoxide dismutase) (Table 3). Interestingly, several transcripts putatively involved in shell production were also identified (namely, perlucin, nacrein-like 1 and carbonic anhydrase). In contrast, the transcripts up-regulated in the older animals compared with younger ones had very similar functions to those when the analysis was carried out using old animals at 0 °C (Tables 2 and 4), but with the addition of some putative immune genes and a putative MAP-kinase interacting serine/threonine protein kinase transcript. The latter has been shown in other species to be involved in response to environmental stress. Conversely, in the mantle, there were more clones up-regulated in older animals (364 clones, mapped to 68 contigs with putative annotations for 11) compared with younger animals (Table 5). The annotations of the genes up-regulated in older animals were not very informative as three of them were identical to those identified in siphon (phosphoenolpyruvate carboxykinase, MAP-kinase interacting serine/threonine

Table 2 Putative annotation of transcripts up-regulated in response to temperature in older animals (3 °C animals compared with 0 °C controls of the same age)

Contig/EST ID	Putative annotation	<i>E</i> value	Function
Contig01571	60s ribosomal protein L13	2.04e ⁻⁷³	Translation
Contig02011	Cytochrome C1	9.09e ⁻⁹⁶	Mitochondrial respiratory chain
Contig02083	Calpain-A	1.42e ⁻⁵²	Protease: multifunctional
Contig02569	NADH dehydrogenase subunit 6 mitochondrial	6.46e ⁻⁴⁸	Mitochondrial respiratory chain
Contig03760	NADH dehydrogenase (ubiquinone) iron sulfur protein 3	7.56e ⁻⁹³	Mitochondrial respiratory chain
Contig04203	40s ribosomal protein S3	2.31e ⁻²⁷	Translation
Contig04568	U6 sn RNA associated Sm-like protein	2.45e ⁻²¹	RNA processing
Contig13913	Ubiquitin-like protein FUB1 isoform X3	4.85e ⁻¹⁴	Protein degradation
Contig15516	Metalloendopeptidase	1.04e ⁻¹⁶	Peptide hydrolysis
A02_06B01	LOAG_17945	1.04e ⁻¹⁶	Translation
A02_24E01	40s ribosomal protein S21-like isoform X2	1.34e ⁻³²	Translation
Contig17114	Adenosylhomocysteinase A	0.0	Metabolism
Contig17205	Cytochrome b-c1 complex subunit	1.01e ⁻²⁷	Mitochondrial respiratory chain

Fig. 3 Graph showing the number of age-related clones relatively up-regulated in response to temperature. The expression profiles of old animals at 3 °C and young animals at 3 °C are compared with each other according to tissue type. *Red* old animals; *blue* young animals

protein kinase and apolipoprotein-like transcripts) (Table 5). In the mantle tissue of the younger animals, 47 clones were up-regulated which mapped to 31 contigs, of which eight were putatively annotated (data not shown). Of these, three

annotations were present in both mantle and siphon tissue (nacrein-like 1; carbonic anhydrase and GRP78), with GRP78, a key chaperone protein (Table 3).

As a comparison, analyses were also carried out comparing the significantly up-regulated transcripts in the tissues of control animals held at 0 °C between young and old animals. A tissue-specific pattern was again identified (Table 6), and as per the previous results, the mantle showed significant differential expression in both age cohorts. Interestingly, there was relatively little up-regulation in the other tissues of the young animals, with a higher level of differential expression in older animals also identified in siphon and foot. In general, there were relatively few transcripts up-regulated in gill tissue for either age cohort. In the young mantle tissue, 22 annotated transcripts were up-regulated compared with older tissues and showed putative functions associated with normal growth and metabolism (Supplementary Table S1). There were variable levels of annotation for the number of transcripts up-regulated in the mantle, siphon and foot older tissues (21, 12 and 11, respectively) (Supplementary Tables S2, S3, and S4). In each tissue, the putative MAP kinase-interacting serine/threonine protein kinase transcript, and in two out of these three tissues, the putative apolipoprotein-like transcript was

Table 3 Age effect: putative annotation of transcripts up-regulated in the siphon tissue of younger animals at 3 °C compared with older animals at 3 °C

Contig/EST ID	Putative annotation	<i>E</i> value	Function
Contig00005	Tyramine beta hydroxylase	2.58e ⁻²⁰	Neurotransmitter
Contig00041	Endochitinase	1.39e ⁻⁶⁵	Shell production
Contig00045	Endochitinase	1.95e ⁻⁴⁸	Shell production
Contig00905	Reactive oxygen species modulator 1	1.41e ⁻¹⁸	Redox homeostasis
Contig01056	Nacrein-like protein 1	1.51e ⁻³⁰	Shell production
Contig01069	Actin	1.15e ⁻⁸⁰	Cytoskeleton
Contig01340	Protocadherin—Fat 4	1.20e ⁻⁴¹	Cell adhesion
Contig01359	Tyrosine kinase-like	2.17e ⁻¹¹⁰	Signalling
Contig01361	Thioester containing protein B	2.95e ⁻²⁷	Protease inhibitor
Contig01552	Manganese superoxide dismutase	7.07e ⁻¹²¹	Antioxidant
Contig01591	Peptidyl-prolyl <i>cis-trans</i> isomerise	3.46e ⁻⁴²	Protein folding
Contig01923	Ras-related C3 botulinum toxin substate	2.03e ⁻⁴¹	Signal transduction
Contig02135	Perlucin	4.66e ⁻¹⁶	Shell production
Contig02265	Carbonic anhydrase	1.11e ⁻³²	Shell production
Contig02381	C1q	2.36e ⁻²³	Immune
Contig03241	Calponin 2	3.14e ⁻¹⁸	Cytoskeleton
Contig03532	Thioester-containing protein B	6.27e ⁻¹¹³	Protease inhibitor
Contig03833	Organic cation transporter protein	1.74e ⁻⁴⁶	Membrane transport
Contig04163	Peptidyl prolyl <i>cis-trans</i> isomerase B	1.19e ⁻⁶¹	Protein folding
Contig04203	40s ribosomal protein	2.31e ⁻²⁷	Translation
Contig04781	Glucose regulated protein 78 kDa	1.23e ⁻⁸³	Protein folding
Contig06531	Cytochrome C oxidase subunit 5B	1.46e ⁻²²	Mitochondrial respiratory chain
Contig07127	40s ribosomal protein S14	2.51e ⁻³⁶	Translation
Contig09062	CGI_10016952	5.4e ⁻¹³	Involved in cell matrix
Contig12332	Odr-4-like protein	1.48e ⁻²¹	Accessory protein

Table 4 Age effect: putative annotation of transcripts up-regulated in siphon tissue in older animals at 3 °C compared with younger animals at 3 °C

Contig/EST ID	Putative annotation	<i>E</i> value	Function
Contig00111	Phosphoenolpyruvate carboxykinase	0.0	Gluconeogenesis
Contig00447	MAP kinase-interacting serine/threonine protein kinase 1	3.36e ⁻¹⁷⁴	May have a role in response to environmental stress
Contig00484	Apolipoproteins-like	1.39e ⁻¹⁷	Immune/lipid metabolism
Contig00490	Complement component C3	8.38e ⁻¹⁹	Immune
Contig00492	Complement component C3	3.71e ⁻⁷⁴	Immune
Contig00552	Cytochrome C oxidase subunit IV, mitochondrial	8.50e ⁻³²	Mitochondrial respiratory chain
Contig01055	Coagulation-like factor	5.96e ⁻⁵⁴	Immune
Contig02083	Calpain-A	1.42e ⁻⁵²	Protease: multifunctional
Contig02569	NADH dehydrogenase subunit 6	6.46e ⁻⁴⁸	Mitochondrial respiratory chain
Contig03760	NADH dehydrogenase ubiquinone iron-sulfur protein	7.56e ⁻⁹³	Mitochondrial respiratory chain
Contig04203	40s ribosomal protein	2.31e ⁻²⁷	Translation
Contig04568	U6 snRNA associated Sm-like protein	2.46e ⁻²¹	RNA processing
Contig13913	Ubiquitin-like protein FUB1 isoform X3	4.85e ⁻¹⁴	Protein degradation
Contig15516	Metalloendopeptidase	5.00e ⁻¹⁸	Peptide hydrolysis
Contig17114	Adenosylhomocysteinase A	0.0	Metabolism
Contig17205	Cytochrome b-c1 complex	1.02e ⁻²⁷	Mitochondrial respiratory chain

up-regulated, both of which were found in the expression profiles of old treated tissues. It was interesting to note that putative transcripts involved in protein folding (GRP78 and peptidyl prolyl *cis-trans* isomerase) were up-regulated in at least one of each of the three older tissues analysed in detail (Supplementary Tables S2, S3, and S4), but this was not a universal response across the tissues.

Discussion

The aim of this study was to investigate the tissue- and age-specific response to an acute thermal stress in the Antarctic clam,

L. elliptica, using a custom-made microarray. Different tissues in the same animal showed very little similarity in the complements of transcripts which were up-regulated in each tissue in response to the increased water temperature. Hence, there was not a single transcript which could be ascribed as a universal gene biomarker of heat stress. However, analysis of the expression profiles using age as the variable factor did show significant differences between young and old animals. These data emphasised the importance of age underlying environmental stress responses, as identified in previous experiments involving the environmental challenges of sediment deposition, iceberg scour, physical injury, microbial infection, hypoxia and heat (Philipp et al. 2011; Husmann et al. 2011, 2014; Clark et al. 2013; Peck et al. 2013).

Table 5 Age effect: putative annotation of transcripts up-regulated in mantle tissue in older animals at 3 °C compared with younger animals at 3 °C

Contig/EST ID	Putative annotation	<i>E</i> value	Function	Up-regulated in siphon
Contig00111	Phosphoenolpyruvate carboxykinase	0.0	Gluconeogenesis	X
Contig00447	MAP kinase-interacting serine/threonine protein kinase 1	3.36e ⁻¹⁷⁴	May have a role in response to environmental stress	X
Contig00484	Apolipoproteins-like	1.39e ⁻¹⁷	Immune/lipid metabolism	X
Contig00635	Von Willebrand factor D and EGF domain-containing protein	1.12e ⁻¹⁵	Multifunctional	
Contig01361	Thioester-containing protein B	2.95e ⁻²⁷	Protease inhibitor	
Contig03463	ATP-dependent RNA helicase DDX5	1.32e ⁻¹⁶⁹	Transcriptional regulation	
Contig04062	Actin	6.45e ⁻¹¹³	Cytoskeleton	
Contig04597	Proteasomal ubiquitin receptor ADRM1	1.33e ⁻⁶⁴	Protein degradation	
Contig07305	60s ribosomal RPL31	3.86e ⁻¹¹	Translation	
Contig08959	CGI_10028476	2.81e ⁻³⁴	Unknown	
Contig17802	40s ribosomal S12	8.37e ⁻³⁰	Translation	

Table 6 Comparison of the number of clones and annotation levels of young animals with old animals under control conditions at 0 °C

Tissue	Age	Number of clones up-regulated	Number of mappings to contigs	Number of annotations
Mantle	Young	132	66	23
	Old	791	114	23
Siphon	Young	46	21	5
	Old	371	78	14
Foot	Young	32	21	6
	Old	101	35	12
Gill	Young	31	22	6
	Old	37	23	6

The temperature used for this thermal stress, although acute, was not lethal in the medium term, and this was reflected in the gene expression profiles. Then, 3 °C was chosen because 50 % of animals (particularly the large ones) fail to re-bury within 24 h at this temperature, thus indicating at least the onset of a physiological stress in some of the animals (Peck et al. 2007). Experiments which kept these animals at 3 °C for 5 days did not have any mortalities but did show a permanent increase in metabolic rate, as measured by oxygen consumption. In the same experiment, heartbeat rate returned to normal within 12–24 h, and there was some tissue-specific accumulation of anaerobic end-products (succinate) in siphon tissue after 5 days (Peck et al. 2002). So, in the short term, these animals can cope with being at 3 °C, but it is chronically lethal, and they do not survive for months at this temperature (SA Morley, personal observation). Although there was a big size difference between the young and old animals in this experiment, with the older ones approximately twice the size of the young ones (Table 1), this was not expected to be a contributing factor to the age-specific gene expression patterns. Water is a very good conductor of heat, and the clams regularly pump water through the mantle cavity. They have a circulating haemolymph which is in intimate contact with large areas of soft tissue within this cavity, and based on previous data examining temperature equilibration in limpets (LS Peck, personal observation), it was expected that all tissues of the animals would be equilibrated to 3 °C within 10–15 min; therefore, the thermal stress on both young and old animals would be of the same magnitude.

This experiment used the same methodology and source population as Truebano et al. (2010), who also examined the response to thermal challenge in *L. elliptica*. In those previous results, 294 clones were up-regulated in mantle tissue which mapped to 160 transcripts with annotation for 33. Whilst a comparison of the annotations in both pieces of work showed shared functions such as protein synthesis and cytoskeletal elements, only two transcripts were shared (actin and

calponin, an actin-binding protein). This lack of concordance may not be entirely surprising as, in retrospect, there were a number of biological factors which almost certainly influenced the animals' responses, all of which were difficult, if not impossible, to constrain within a repeated experimental design. Interannual and seasonal variability could have influenced the expression profiles. These experiments were conducted 2 years after those of Truebano et al. (2010), and the animals will have been subjected to slightly different environmental conditions in the field, such as ice cover in winter, summer temperatures and food availability, which affected their condition. Very strong inter-annual variation in biological characters, such as reproductive investment, has been demonstrated in several Antarctic marine invertebrates, e.g. the brittle star *Ophionotus victoriae* (Grange et al. 2004). Also, the experiment described here was performed in January in the Antarctic with the animals used almost directly from the field, whilst those of Truebano et al. (2010) were returned to Cambridge in a recirculating transport aquarium and acclimated in tanks for several months before the experiments were performed. It is also possible that the time of day when the animals were sampled was different in each experiment, and circadian effects could have influenced gene expression, but this is unlikely to play a major role, especially when compared with potential seasonal effects. Finally, we changed the age cohort of the animals used in this experiment. We specifically targeted young pre-reproductive animals (30–33 mm in size) and older reproductively mature animals (62–69 mm) (Table 1) to examine the effect of age on the response, whilst the previous cohort of animals (Truebano et al. 2010) was intermediate between these two with an average size of 51 mm and around 12 years of age. We had previously demonstrated that age affected the expression profiles in this species in response to hypoxia (Clark et al. 2013), and these data, using a different environmental challenge, supported this finding. However, again, as with the previous thermal challenge experiment, there is very little overlap in the transcripts described here when compared with oxygen deprivation, with only a single gene (calponin) shared among all three experiments.

It was notable that none of the “classical” stress response genes, such as heat shock proteins or antioxidants such as glutathione-*S*-transferase, were identified as up-regulated in the treated animals of either age. However, to a certain extent, the response is constrained by the clones on the microarray. A mix of tissues (gill, mantle and siphon) from 12-year-old animals kept at both 0 and 3 °C was used to make the array. Therefore, the most relevant transcripts should be present on the array, given that we exposed the animals in this experiment to 3 °C. Sequencing was only carried out on those clones demonstrating differential expression. In total, 1570 transcripts were sequenced over three experiments (this one, Truebano et al. 2010 and Clark et al. 2013), which comprised

approximately 19 % of the clones on the microarray. Of the clones sequenced, two showed Blast sequence similarity matches below $e 10^{-10}$ to GRP78 and HSP70, whilst a further two matched a peroxidase-like gene and microsomal glutathione-*S*-transferase. GRP78 showed up-regulation under certain conditions in this experiment, but the other three showed no significant change in expression levels. Thus, it is not possible to define whether, under these conditions, *L. elliptica* lacks the classical heat shock response per se or whether the result is constrained by clone coverage. It may require an exposure of longer than 12 h at 3 °C for the cells to demonstrate a response to oxidative stress, provoking the up-regulation of these gene families. In fact, previous attempts to demonstrate a laboratory-induced heat shock response in this species required the far more acute challenge of exposure to 10–15 °C (Clark et al. 2008). An additional factor influencing the response may be the ability of *L. elliptica* to modify its metabolism. It has previously been demonstrated that *L. elliptica* can enter a hypometabolic state, closing the siphon for periods of several hours during winter and reducing metabolic rate (Morley et al. 2007). This was suggested as a measure of conserving energy during the winter when their algal food supply is scarce (Morley et al. 2007). It is entirely feasible that such an approach can also be adopted, at least in the short term, during periods of stressful environmental conditions. Indeed it has been shown that metabolism decreases in older *L. elliptica* in response to sedimentation events (Philipp et al. 2011). Such behaviour would impact gene expression profiles, with the expectation of a reduction in sensitivity to the external conditions. In a similar vein, in all expression profile comparisons of gill and foot tissue, there was a relatively weak signal of response to the thermal challenge, even in the older animals. This may have been because the thermal challenge was relatively short at 12 h, and these internal organs were more protected from the immediate effects possibly due to closure of the siphon and a reduction in metabolism, at least in the short term.

Although none of the animals treated at 3 °C showed a significant cellular stress response, when compared to 0 °C controls, the expression patterns changed markedly when treated animals of different ages were directly compared, i.e. old animals at 3 °C compared with young animals at 3 °C. The expression profile in the mantle tissue of the younger animals was particularly distinctive, with the up-regulation of several transcripts with cytoprotective roles (Table 3). These included transcripts with high sequence similarity to a reactive oxygen species modulator 1, which is involved in redox homeostasis; manganese superoxide dismutase, an antioxidant and the two chaperone proteins, peptidyl-prolyl *cis-trans* isomerase and the heat shock protein GRP78. The latter is particularly interesting as previous thermal tolerance experiments have only seen the induction of this gene in response to chronic thermal stress and temperature change in the field (Clark and Peck

2009). This implies that there are generally higher constitutive levels of this transcript in younger animals. These data fit with those of previous investigations into the immune response of young and old *L. elliptica*, with young animals shown to have a higher basal level of ROS generation per cell and more rapid stimulation in challenge experiments (Husmann et al. 2011). Whilst GRP78 is the major chaperone in the endoplasmic reticulum, it has also been shown to have an anti-apoptotic role and is a key regulator of ER stress transducers (Yu et al. 1999; Bertolotti et al. 2000). It is one of the few transcripts which was also up-regulated in siphon tissue. Thus, enhanced activity of this transcript in the external facing tissues of young clams may help explain their more robust defence to the thermal challenge.

There was also up-regulation of transcripts with high sequence similarity to C1q, which has been associated with an immune response in *Mytilus* (Gestal et al. 2010) and the actin cytoskeleton (actin and calponin) (Table 3). The latter system is being increasingly associated with an important role in stress response signalling and an indicator of general cell health (Leadsham and Gourlay 2008; Tomanek 2011). It was interesting to note that transcripts putatively involved in shell and extracellular matrix production (nacrein-like 1, carbonic anhydrase, perlucin and an endochitinase) were also up-regulated in young animals (with both nacrein-like 1 and carbonic anhydrase also up-regulated in siphon tissue). Chitinase genes have been shown to be up-regulated in response to injury, with roles in immunity, apoptosis and tissue remodelling (Lee et al. 2011). Nacrein (and by similarity, nacrein-like 1 transcript) and carbonic anhydrase are classic markers of shell production. Although they have clearly identified structural roles, like the chitinases, they may also have other functions involved in tissue damage repair. Alternatively, increased shell production can be a thermal defence mechanism as thicker shells impede heat transfer to the inner organs. This association of thicker shells with warmer thermal regimes has previously been characterised in the sea snails *Littoraria pallescens* and *Littorina striata* (Cook and Freeman 1986; de Wolf et al. 1998), but these are both inter-tidal species and therefore regularly exposed to air. It is more likely in our infaunal marine species that the expression of these transcripts was indicative of perturbation of intracellular calcium due to thermal stress (Drummond et al. 1986).

The older animals showed a more passive cellular response when warmed, both when compared with control animals of the same age and also young treated animals (Tables 2, 4, and 5). There was a potential immune response with the up-regulation of putative transcripts encoding complement component C3, a coagulation-like factor and an apolipoprotein. This immune response was also identified in older animals in the hypoxia experiment (Clark et al.

2013). Interestingly, there was also up-regulation of a MAP kinase interacting serine/threonine protein (Tables 4 and 5). This transcript has a long-recognised role in response to environmental stress in some species (Waskiewicz et al. 1997). In *L. elliptica*, it may also play a more general role as it was the most highly expressed sequence in the 454 transcriptome, which was constructed from mature animals ranging in size from 50.1 to 83.5 mm (6–20 years old) (Clark et al. 2010). However, the general lack of a strong response in terms of expression profile is similar to previous experiments examining response to siphon injury. Older animals produced very few gene expression changes, even though they were clearly physiologically affected. They are generally sluggish, less mobile and less active in filtration when injured (Husmann et al. 2014). The analysis of the up-regulation transcripts in control animals did show some expression of protein folding genes (Supplementary Tables S2, S3 and S4), but these were not expressed when the animals were subjected to the thermal challenge. Given the expression profiles for the older animals at 3 °C, it is almost as if the older animals are shutting down their metabolism in response to a challenge rather than producing an active cellular response. In other experiments, younger animals also have a better respiratory response to sedimentation, they rebury faster and survive better after an injury, with an enhanced immune response and survive to higher upper lethal temperatures (Philipp et al. 2011; Husmann et al. 2011; Peck et al. 2013). Hence, the expression data described here support not only the physiological results but also those of previous expression studies in which the younger animals were shown to display a more rapid and active cellular response to stress (Husmann et al. 2014; Clark et al. 2013). With any defence response, there is a cost to the cell, additional to that of homeostasis. Previous biochemical analyses showed that younger animals had a higher cellular energy charge than older animals (Clark et al. 2013) and thus have a greater capacity to respond, at least in the short term, to changing environmental conditions. Enhanced cellular energy levels would provide them with the extra capacity to produce energetically costly proteins such as heat shock proteins (Sørensen and Loeschcke 2007) as part of their defence.

Comparison of our data with results in other species is complex. Experimental conditions vary widely, such that there is rarely overlap in gene complements between the expression profiles of different species even when subjected to similar stresses. Often, certain categories of genes can be similar, such as transcription, translation and protein turnover, which are important for generalised cellular functioning, whilst others such as antioxidants and chaperones are indicative of a “stress” response (Kultz 2005; Tomanek 2011). However, data are increasingly showing that significant differences exist in the stress responses of different

tissues within the same species (Buckley et al. 2006; Pantartzzi et al. 2010) and also between species (Walker et al. 2000; Buckley et al. 2006; Lockwood et al. 2010), which may drive the invasiveness of some mollusc species (Fields et al. 2012).

In this example of the Antarctic clam, there are clear tissue-specific differences in the response to a thermal challenge which clearly need to be taken into account for transcriptomic analyses when monitoring responses to change. The over-riding effect, however, was that of age. The younger animals mount a more robust physiological defence in response to the environmental challenge (Peck et al. 2004, 2013; Philipp et al. 2011; Husmann et al. 2011; 2014), which can be seen at the cellular level. The younger animals transcribe higher levels of some of the “classical” stress response genes, namely, chaperones and antioxidants, when challenged, which enables them to actively manage cellular homeostasis, at least in the short term. This difference in the environmental resilience at different life history stages clearly needs to be addressed in any future biodiversity models, particularly those of long-lived species in the polar regions, areas of the planet which are subject to rapid rates of regional warming under climate change.

Acknowledgments This paper was funded by NERC core funding to BAS within the Polar Sciences for Planet Earth Programme. We would like to thank the Rothera Dive Team for help in collecting animals. The NERC National Facility for Scientific Diving (Oban) provided overall diving support. We would also like to thank three anonymous reviewers for their very constructive comments and additional references, which have greatly improved the manuscript.

References

- Ahn I-Y (1994) Ecology of the Antarctic bivalve *Laternula elliptica* (King and Broderip) in Collins Harbour, King George Island: benthic environment and adaptive strategy. Mem Natl Inst Polar Res Spec Issue 50:1–10
- Altschul SF, Madden TL, Schaffer AA, Zhang JH, Zhang Z et al (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucl Acids Res 25:3389–3402
- Amtz WE, Brey T, Gallardo VA (1994) Antarctic zoobenthos. In: Ansell AD, Gibson RN, Barnes M, editors. Oceanography and Marine Biology, An Annual Review. 32: 241–304
- Bairoch A, Bougueleret L, Altairac S, Amendolia V, Auchincloss A et al (2007) The universal protein resource (UniProt). Nucl Acids Res 35: D193–D197
- Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate—a practical and powerful approach to multiple testing. J Roy Stat Soc B 57:289–300
- Bertolotti A, Zhang YH, Hendershot LM, Harding HP, Ron D (2000) Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. Nat Cell Biol 2:326–332
- Buckley BA, Gracey AY, Somero GN (2006) The cellular response to heat stress in the goby *Gillichthys mirabilis*: a cDNA microarray and protein-level analysis. J Exp Biol 209:2660–2677

- Clark MS, Peck LS (2009) Triggers of the HSP70 stress response: environmental responses and laboratory manipulation in an Antarctic marine invertebrate (*Nacella concinna*). *Cell Stress Chaperones* 14:649–660
- Clark MS, Fraser KPP, Peck LS (2008) Antarctic marine molluscs do have an HSP70 heat shock response. *Cell Stress Chaperones* 13: 39–49
- Clark MS, Thorne MAS, Vieira FA, Cardoso JCR, Power DM et al (2010) Insights into shell deposition in the Antarctic bivalve *Laternula elliptica*: gene discovery in the mantle transcriptome using 454 pyrosequencing. *BMC Genomics* 11:362
- Clark MS, Husmann G, Thorne MAS, Burns G, Truebano M et al (2013) Hypoxia impacts large adults first: consequences in a warming world. *Glob Chang Biol* 19:2251–2263
- Cook LM, Freeman PM (1986) Heating properties of morphs of the mangrove snail *Littoraria pallescens*. *Biol J Linn Soc* 29:295–300
- Dahlhoff E, Somero GN (1993) Effects of temperature on mitochondria from abalone (genus *Haliotis*): adaptive plasticity and its limits. *J Exp Biol* 185:151–168
- De Wolf H, Backeljau T, Verhagen R (1998) Spatio-temporal genetic structure and gene flow between two distinct shell morphs of the planktonic developing periwinkle *Littorina striata* (Mollusca: Prosobranchia). *Mar Ecol Prog Ser* 163:155–163
- Dell RK (1972) Antarctic benthos. *Adv Mar Biol* 10:1–216
- R Development Core Team (2005) R: a language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria. <http://www.R-project.org>
- Drinkwater K (2009) Comparison of the response of Atlantic cod (*Gadus morhua*) in the high-latitude regions of the North Atlantic during the warm periods of the 1920s–1960s and the 1990s–2000s. *Deep-Sea Res II* 56:2087–2096
- Drummond IAS, McClure SA, Poenie M, Tsein RY, Steinhart RA (1986) Large changes in intracellular pH and calcium observed during heat shock are not responsible for the induction of heat shock proteins in *Drosophila melanogaster*. *Mol Cell Biol* 6:1767–1775
- Fields PA, Zuzow MJ, Tomanek L (2012) Proteomic responses of blue mussel (*Mytilus*) congeners to temperature acclimation. *J Exp Biol* 215:1106–1116
- Gestal C, Pallavicini A, Venier P, Novoa B, Figueras A (2010) MgC1q, a novel C1q-domain-containing protein involved in the immune response of *Mytilus galloprovincialis*. *Dev Comp Immunol* 34:926–934
- Grange LJ, Tyler PA, Peck LS, Cornelius N (2004) Long-term interannual cycles of the gametogenic ecology of the Antarctic brittle star *Ophionotus victoriae*. *Mar Ecol Prog Ser* 278:141–155
- Gunter HM, Degan BM (2008) Impact of ecologically relevant heat shocks in Hsp developmental function in the vetigastropod *Haliotis asinina*. *J Exp Zool* 310B:450–464
- Husmann G, Philipp EER, Rosenstiel P, Vazquez S, Abele D (2011) Immune response of the Antarctic bivalve *Laternula elliptica* to physical stress and microbial exposure. *J Exp Mar Biol Ecol* 398: 83–90
- Husmann G, Abele D, Rosenstiel P, Clark MS, Kraemer L et al (2014) Age-dependent expression of stress and antimicrobial genes in the hemocytes and siphon tissue of the Antarctic bivalve, *Laternula elliptica*, exposed to injury and starvation. *Cell Stress Chaperones* 19:15–32
- Kultz D (2005) Molecular and evolutionary basis of the cellular stress response. *Ann Rev Physiol* 67:225–257
- Leadsham JE, Gourlay CW (2008) Cytoskeletal induced apoptosis in yeast. *Biochim Biophys Acta, Mol Cell Res* 1783:1406–1412
- Lee CG, Da Silva CA, Dela Cruz CS, Ahangari F, Ma B et al (2011) Role of chitin and chitinase/chitinase-like proteins in inflammation, tissue remodeling, and injury. *Ann Rev Physiol* 73:479–501
- Lockwood BL, Sanders JG, Somero GN (2010) Transcriptomic responses to heat stress in invasive and native blue mussels (genus *Mytilus*): molecular correlates of invasive success. *J Exp Biol* 213: 3548–3558
- Mardia KV, Kent JT, Bibby JM (1979) Multivariate analysis. Academic, London
- Morley SA, Peck LS, Miller AJ, Portner HO (2007) Hypoxia tolerance associated with activity reduction is a key adaptation for *Laternula elliptica* seasonal energetics. *Oecologia* 153:29–36
- Pantartz C, Drosopoulou E, Yiangou M, Drozdov I, Tsoka S et al (2010) Promoter complexity and tissue-specific expression of stress response components in *Mytilus galloprovincialis*, a sessile marine invertebrate species. *PLoS Comp Biol* 6, e1000847
- Parkinson J, Anthony A, Wasmuth J, Schmid R, Hedley A et al (2004) PartiGene—constructing partial genomes. *Bioinformatics* 20:1398–1404
- Peck LS, Portner HO, Hardewig I (2002) Metabolic demand, oxygen supply, and critical temperatures in the Antarctic bivalve *Laternula elliptica*. *Physiol Biochem Zool* 75:123–133
- Peck LS, Webb KE, Bailey DM (2004) Extreme sensitivity of biological function to temperature in Antarctic marine species. *Func Ecol* 18: 625–630
- Peck LS, Morley SA, Portner HO, Clark MS (2007) Thermal limits of burrowing capacity are linked to oxygen availability and size in the Antarctic clam *Laternula elliptica*. *Oecologia* 154:479–484
- Peck LS, Clark MS, Morley SA, Massey A, Rossetti H (2009) Animal temperature limits and ecological relevance: effects of size, activity and rates of change. *Func Ecol* 23:248–256
- Peck LS, Souster T, Clark MS (2013) Juveniles are more resistant to warming than adults in 4 species of Antarctic marine invertebrates. *PLoS One* 8, e66033
- Perteau G, Huang XQ, Liang F, Antonescu V, Sultana R et al (2003) TIGR Gene Indices clustering tools (TGICL): a software system for fast clustering of large EST datasets. *Bioinformatics* 19:651–652
- Petalidis L, Bhattacharyya S, Morris GA, Collins VP, Freeman TC et al (2003) Global amplification of mRNA by template-switching PCR: linearity and application to microarray analysis. *Nucl Acids Res* 31, e142
- Philipp EER, Husmann G, Abele D (2011) The impact of sediment deposition and iceberg scour on the Antarctic soft shell clam *Laternula elliptica* at King George Island. *Antarct Antarct Sci* 23:127–138
- Purac J, Burns G, Thorne MAS, Grubor-Lajsic G, Worland MR et al (2008) Cold hardening processes in the Antarctic springtail, *Cryptopygus antarcticus*: clues from a microarray. *J Insect Physiol* 54:1356–1362
- Ralph R, Maxwell JGH (1977) Growth of 2 Antarctic Lamellibranchs—*Adamussium colbecki* and *Laternula elliptica*. *Mar Biol* 42:171–175
- Richie ME, Silver J, Oshlack A, Holmes M, Diyagama D, Holloway A, Smyth GK (2007) A comparison of background correction methods for two colour microarrays. *Bioinformatics* 23:2700–2707
- Schmidt-Nielsen K (1991) Animal physiology: adaptation and environment. Cambridge University Press, Cambridge
- Smyth GK (2004) Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* 3:3
- Smyth GK (2005) Limma: linear models for microarray data. Gentleman R, Carey VJ, Huber W, Irizarry RA, Dudoit S, editors. 397–420 p
- Smyth GK, Speed T (2003) Normalization of cDNA microarray data. *Methods* 31:265–273
- Smyth GK, Michaud J, Scott HS (2005) Use of within-array replicate spots for assessing differential expression in microarray experiments. *Bioinformatics* 21:2067–2075
- Somero GN (2010) The physiology of climate change: how potentials for acclimatization and genetic adaptation will determine ‘winners’ and ‘losers’. *J Exp Biol* 213:912–920
- Sørensen JG, Loeschcke V (2007) Studying stress responses in the post-genomic era: its ecological and evolutionary role. *J Biosci* 32:447–456

- Tomanek L (2011) Environmental proteomics: changes in the proteome of marine organisms in response to environmental stress, pollutants, infection, symbiosis, and development. *Ann Rev Mar Sci* 3:373–399
- Truebano M, Burns G, Thorne MAS, Hillyard G, Peck LS et al (2010) Transcriptional response to heat stress in the Antarctic bivalve *Latemula elliptica*. *J Exp Mar Biol Ecol* 391:65–72
- Venables WN, Ripley BD (2002) *Modern applied statistics*. Springer, Berlin
- Walker ST, Mantle D, Bythell JC, Thomason JC (2000) Oxidative-stress: comparison of species specific and tissue specific effects in the marine bivalves *Mytilus edulis* (L.) and *Dosinia lupinus* (L.). *Comp Biochem Physiol B* 127:347–355
- Waskiewicz AJ, Flynn A, Proud CG, Cooper JA (1997) Mitogen activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2. *EMBO J* 16:1909–1920
- Yu ZF, Luo H, Fu WM, Mattson MP (1999) The endoplasmic reticulum stress-responsive protein GRP78 protects neurons against excitotoxicity and apoptosis: suppression of oxidative stress and stabilization of calcium homeostasis. *Exp Neurol* 155: 302–314