Age-related thermal response: the cellular resilience of juveniles

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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 hours 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.

Introduction

In a changing world, our abilities 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, not only in terms 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 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 long-term, but loses essential biological functions, such as the ability to bury in sediment, much earlier. 50% of animals fail to rebury within 24 hours at 2.5°C, which is only 1-2°C over 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 hour) 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 10-18m in January 2010 at Hangar Cove, Rothera Point, Adelaide Island, Antarctic Peninsula (67°34'07°S, 68°07'30°W) (water temperature: 0.5°C ± 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 (with lengths ranging around 60mm and mature gonads) and small animals (lengths ranging around 30mm, with no gonads present), the sizes of which equated to average ages of 16 and 7 years respectively (Watson, unpublished) (Table 1). This species begins to produce gonads at 35-40mm in length (MS Clark, pers. obs.) 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, 10 old and 10 young animals were transferred to a 60 litre 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 hour period. This temperature was then maintained for a further 12 hours, 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 (Figure 1) and immediately snap frozen in liquid nitrogen and stored at -80°C. The siphon is a joint fused inhalant/exhalent 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 (Figure 1) and portions of the gills were randomly taken from both sides. The sampling regime was repeated on 10 old and 10

young animals that had been maintained in the flow-through aquarium for the same time period at 0°C (control animals).

Array hybridization

RNA was extracted from all samples using TriSure (Bioline, UK), according to 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 5 animals for each group (old treated, young treated, old control and young control) were used in the array hybridization experiments. The construction of the 8,448 clone *L. elliptica* array has been previously described in Truebano et al. (2010). PCR amplified labelled cDNA targets were prepared from 1µg total RNA using the protocol described in Petalidis et al. (2003) and hybridizations performed as described in Purać et al. (2008) with modifications according to Truebano et al. (2010). 5 biological replicates were used for each experiment with 3 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 < 3x 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 2 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 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 (Pertea et al. 2003) was used to assemble sequences. These sequences were then Blastx sequence similarity searched against NCBI non-redundant database with a cut-off 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 cut-off 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,

159 particularly in the young animals. The number of clones up-regulated in young animals varied from 0
160 in mantle to a maximum of 14 in siphon (out of 8,928 clones on the array). The older animals
161 showed a wider ranging effect, with higher numbers of significantly up-regulated clones in each
162 tissue; from 6 in gill to 71 in Siphon (Figure 2). When sequenced, these 71 clones mapped to 35
163 contigs and ESTs, with putative annotation for 13 (Table 2). The annotation indicated that the
164 majority (70%) of these transcripts were associated with either enhanced mitochondrial respiration
165 or protein production. This was probably due to the thermal dependency of biochemical reactions,
166 with the higher temperature increasing the general metabolic rate of the animals (Schmidt-Nielsen,
167 1991; Dahlhoff and Somero, 1993). In both age cohorts, the siphon showed the greatest effect in
168 terms of the numbers of clones showing up-regulation, with relatively little effect in gill and foot, as
169 evidenced by the low numbers of up-regulated microarray clones for both tissues and by the tight
170 clustering of gill and foot profiles in the tissue-specific PCA analysis (Supplementary Figure S1).
171
172 In the analysis described above, the effect of thermal stress was identified by comparing treated
173 animals at 3°C with the same age animals at 0°C as controls. In a second analysis, the expression
174 profiles of the treated young and treated old animals were directly compared to identify those
175 transcripts associated with age (Figure 3). Again relative expression levels were similar and low in
176 the foot and especially the gill, with most differences seen in the mantle and the siphon. These two
177 tissues showed conflicting patterns, with more clones relatively up-regulated in the siphon tissue in
178 the younger animals (382 clones, which mapped to 62 contigs, with 26 putative annotations) (Table
179 3) compared with only 136 clones up-regulated in older animals (mapping to 63 contigs, with
180 putative annotations for 16) (Table 4). The expression profiles of the younger animals indicated an
181 active cellular “stress” response with the up-regulation of transcripts putatively involved in protein
182 folding (peptidyl-prolyl cis-trans isomerase and a member of the 70kDa heat shock protein family
183 (the 78 kDa glucose-regulated protein: GRP78) and combating reactive oxygen species (ROS)
184 (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 protein kinase and apolipophorin-like transcripts) (Table 5). In the mantle tissue of the younger animals 47 clones were up-regulated which mapped to 31 contigs, of which 8 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. Again a tissue-specific pattern was identified (Table 6) and as per the previous results, 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 2, 3 and 4). In each tissue, the putative MAP kinase-interacting

211 serine/threonine protein kinase transcript, and in two out of these three tissues, the putative
212 apolipophorins-like transcript were up-regulated, both of which were found in the expression
213 profiles of old treated tissues. It was interesting to note that putative trancripts involved in protein
214 folding (GRP78 and peptidyl prolyl cis-trans isomerase were up-regulated in at least one of each of
215 three older tissues analysed in detail (Supplementary Tables 2, 3 and 4), but this was not a universal
216 response across the tissues.
217
218 **Discussion**
219 The aim of this study was to investigate the tissue and age-specific response to an acute thermal
220 stress in the Antarctic clam, *Laternula elliptica* using a custom-made microarray. Different tissues in
221 the same animal showed very little similarity in the complements of transcripts which were up-
222 regulated in each tissue in response to the increased water temperature. Hence, there was not a
223 single transcript which could be ascribed as a universal gene biomarker of heat stress. However,
224 analysis of the expression profiles using age as the variable factor did show significant differences
225 between young and old animals. These data emphasised the importance of age underlying
226 environmental stress responses, as identified in previous experiments involving the environmental
227 challenges of sediment deposition, iceberg scour, physical injury, microbial infection, hypoxia and
228 heat (Philipp et al. 2011; Husmann et al. 2011, 2014; Clark et al. 2013; Peck et al. 2013).
229
230 The temperature used for this thermal stress, although acute was not lethal in the medium term and
231 this was reflected in the gene expression profiles. 3°C was chosen because 50% of animals
232 (particularly the large ones) fail to re-bury within 24 hours at this temperature, thus indicating at
233 least the onset of a physiological stress in some of the animals (Peck et al. 2007). Experiments which
234 kept these animals at 3°C for 5 days did not have any mortalities, but did show a permanent increase
235 in metabolic rate, as measured by oxygen consumption. In the same experiment heartbeat rate
236 returned to normal within 12-24 hours and there was some tissue-specific accumulation of

anaerobic end products (succinate) in siphon tissue after the five 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 months at this temperature (SA Morley, pers. obs). 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, pers obs), it was expected that all tissues of the animals would be equilibrated to 3°c within 10-15 minutes and 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), which 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 2 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, 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

263 the experiment described here was performed in January in the Antarctic with animals used almost
264 directly from the field, whilst those of Truebano et al. (2010) were returned to Cambridge in a
265 recirculating transport aquarium and acclimated in tanks for several months before the experiments
266 were performed. It is also possible that the time of day when the animals were sampled was
267 different in each experiment and circadian effects could have influenced gene expression, but this is
268 unlikely to play a major role, especially when compared with potential seasonal effects. Finally, we
269 changed the age cohort of the animals used in this experiment. We specifically targeted young pre-
270 reproductive animals (30-33 mm in size) and older reproductively mature animals (62-69 mm) (Table
271 1) to examine the effect of age on the response, whilst the previous cohort of animals (Truebano et
272 al. 2010) was intermediate between these two with an average size of 51mm and around 12 years of
273 age. We had previously demonstrated that age affected the expression profiles in this species in
274 response to hypoxia (Clark et al. 2013) and these data, using a different environmental challenge,
275 supported this finding. However, again, as with the previous thermal challenge experiment, there is
276 very little overlap in the transcripts described here when compared with oxygen deprivation, with
277 only a single gene (calponin) shared between all three experiments.
278
279 It was notable that none of the “classical” stress response genes, such as heat shock proteins or
280 antioxidants such as the glutathione-S-transferases, were identified as up-regulated in the treated
281 animals of either age. However, to a certain extent, the response is constrained by the clones on the
282 microarray. A mix of tissues (gill, mantle and siphon) from 12 year old animals kept at both 0°C and
283 3°C was used to make the array. Therefore the most relevant transcripts should be present on the
284 array, given we exposed the animals in this experiment to 3°C. Sequencing was only carried out on
285 those clones demonstrating differential expression. In total 1570 transcripts were sequenced over
286 three experiments (this one, Truebano et al. 2010 and Clark et al. 2013), which comprised
287 approximately 19% of the clones on the microarray. Of the clones sequenced two showed Blast
288 sequence similarity matches below e 10^{-10} to GRP78 and HSP70, whilst a further two matched a

289 peroxidase-like gene and microsomal glutathione-S-transferase. GRP78 showed up-regulation under
290 certain conditions in this experiment, but the other three showed no significant change in expression
291 levels. Thus it is not possible to define whether, under these conditions, *L. elliptica* lacks the classical
292 heat shock response *per se*, or whether the result is constrained by clone coverage. It may require an
293 exposure of longer than 12 hours at 3°C for the cells to demonstrate a response to oxidative stress,
294 provoking the up-regulation of these gene families. In fact previous attempts to demonstrate a
295 laboratory-induced heat shock response in this species required the far more acute challenge of
296 exposure to 10-15°C (Clark et al. 2008). An additional factor influencing the response may be the
297 ability of *L. elliptica* to modify its' metabolism. It has previously been demonstrated that *L. elliptica*
298 can enter a hypometabolic state, closing the siphon for periods of several hours during winter and
299 reducing metabolic rate (Morley et al. 2007). This was suggested as a measure of conserving energy
300 during the winter when their algal food supply is scarce (Morley et al. 2007). It is entirely feasible
301 that such an approach can also be adopted, at least in the short term, during periods of stressful
302 environmental conditions. Indeed it has been shown that metabolism decreases in older *L. elliptica*
303 in response to sedimentation events (Philipp et al. 2011). Such behaviour would impact gene
304 expression profiles, with the expectation of a reduction in sensitivity to the external conditions. In a
305 similar vein, in all expression profile comparisons of gill and foot tissue, there was a relatively weak
306 signal of response to the thermal challenge, even in the older animals. This may have been because
307 the thermal challenge was relatively short at 12 hours and these internal organs were more
308 protected from the immediate effects, possibly due to closure of the siphon and a reduction in
309 metabolism, at least in the short term.
310
311 Although none of the animals treated at 3°C showed a significant cellular stress response, when
312 compared to 0°C controls, the expression patterns changed markedly when treated animals of
313 different ages were directly compared i.e. old animals at 3°C compared with young animals at 3°C.
314 The expression profile in the mantle tissue of the younger animals was particularly distinctive, with

315 the up-regulation of several transcripts with cytoprotective roles (Table 3). These included
316 transcripts with high sequence similarity to a reactive oxygen species modulator 1, which is involved
317 in redox homeostasis; manganese superoxide dismutase, an antioxidant and the two chaperone
318 proteins, peptidyl-prolyl cis-trans isomerise and the heat shock protein GRP78. The latter is
319 particularly interesting as previous thermal tolerance experiments have only seen induction of this
320 gene in response to chronic thermal stress and temperature change in the field (Clark and Peck,
321 2009). This implies that there are generally higher constitutive levels of this transcript in younger
322 animals. These data fit with those of previous investigations into the immune response of young and
323 old *L. elliptica*, with young animals shown to have a higher basal level of ROS generation per cell and
324 more rapid stimulation in challenge experiments (Husmann et al. 2011). Whilst GRP78 is the major
325 chaperone in the endoplasmic reticulum, it has also been shown to have an anti-apoptotic role and
326 is a key regulator of ER stress transducers (Yu et al. 1999; Bertolotti et al. 2000). It is one of the few
327 transcripts which was also up-regulated in siphon tissue. Thus enhanced activity of this transcript in
328 the external facing tissues of young clams may help explain their more robust defence to thermal
329 challenge.
330
331 There was also up-regulation of transcripts with high sequence similarity to C1q, which has been
332 associated with an immune response in *Mytilus* (Gestal et al. 2010) and the actin cytoskeleton (actin
333 and calponin) (Table 3). The latter system is being increasingly associated with an important role in
334 stress response signalling and an indicator of general cell health (Leadsham and Gourlay, 2008;
335 Tomanek, 2011). It was interesting to note that transcripts putatively involved in shell and
336 extracellular matrix production (nacrein-like 1, carbonic anhydrase, perlucin and an endochitinase)
337 were also upregulated in young animals (with both nacrein-like 1 and carbonic anhydrase also
338 upregulated in siphon tissue). Chitinase genes have been shown to be upregulated in response to
339 injury, with roles in immunity, apoptosis and tissue remodelling (Lee et al. 2011). Nacrein (and by
340 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, but 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 apolipophorin. 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. 1998). 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 50.1-83.5mm (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 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

367 metabolism in response to a challenge, rather than producing an active cellular response. In other
368 experiments, younger animals also have a better respiratory response to sedimentation, they rebury
369 faster and survive better after injury, with an enhanced immune response and survive to higher
370 upper lethal temperatures (Philipp et al. 2011; Husmann et al. 2011; Peck et al. 2013). Hence the
371 expression data described here not only support the physiological results, but also those of previous
372 expression studies, in which the younger animals were shown to display a more rapid and active
373 cellular response to stress (Husmann et al. 2014; Clark et al. 2013). With any defence response,
374 there is a cost to the cell, additional to that of homeostasis. Previous biochemical analyses showed
375 that younger animals had a higher cellular energy charge than older animals (Clark et al. 2013) and
376 thus have a greater capacity to respond, at least in the short term, to changed environmental
377 conditions. Enhanced cellular energy levels would provide them with the extra capacity to produce
378 energetically costly proteins such as heat shock proteins (Sørensen and Loeschcke, 2007) as part of
379 their defence.
380
381 Comparison of our data with results in other species is complex. Experimental conditions vary
382 widely, such that there is rarely overlap in gene complements between the expression profiles of
383 different species even when subjected to similar stresses. Often certain categories of genes can be
384 similar, such as transcription, translation and protein turnover which are important for generalised
385 cellular functioning, whilst others such as antioxidants and chaperones are indicative of a “stress”
386 response (Kültz, 2005; Tomanek, 2011). However, data are increasingly showing that significant
387 differences exist in the stress responses of different tissue within the same species (Buckley et al.
388 2006; Pantzartzi et al. 2010) and also between species (Walker et al. 2000; Buckley et al. 2006;
389 Lockwood et al. 2010), which may drive the invasiveness of some mollusc species (Fields et al. 2012).
390
391 In this example of the Antarctic clam there are clear tissue-specific differences in the response to a
392 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 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.

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Figure Legends

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

Figure 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.

546 **Figure 3:** Graph showing the number of age-related clones relatively up-regulated in response to
547 temperature. The expression profiles of old animals at 3°C and young animals at 3°C are compared
548 with each other according to tissue type. Red = old animals and blue = young animals.
549

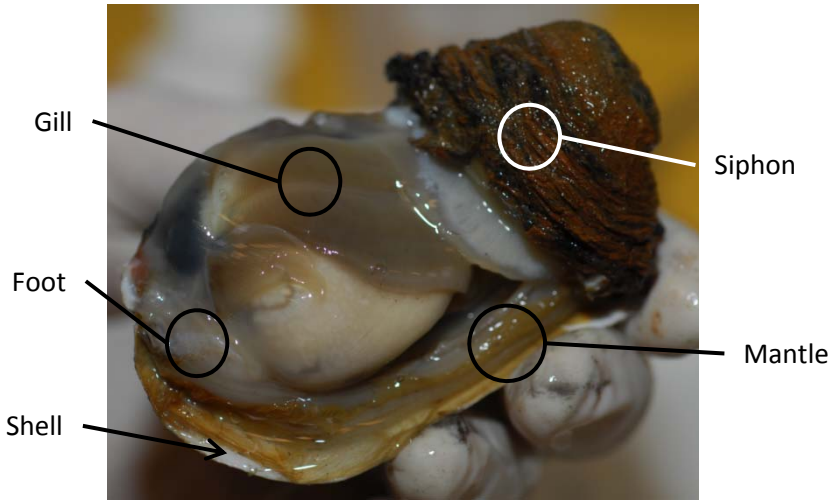


Figure 1

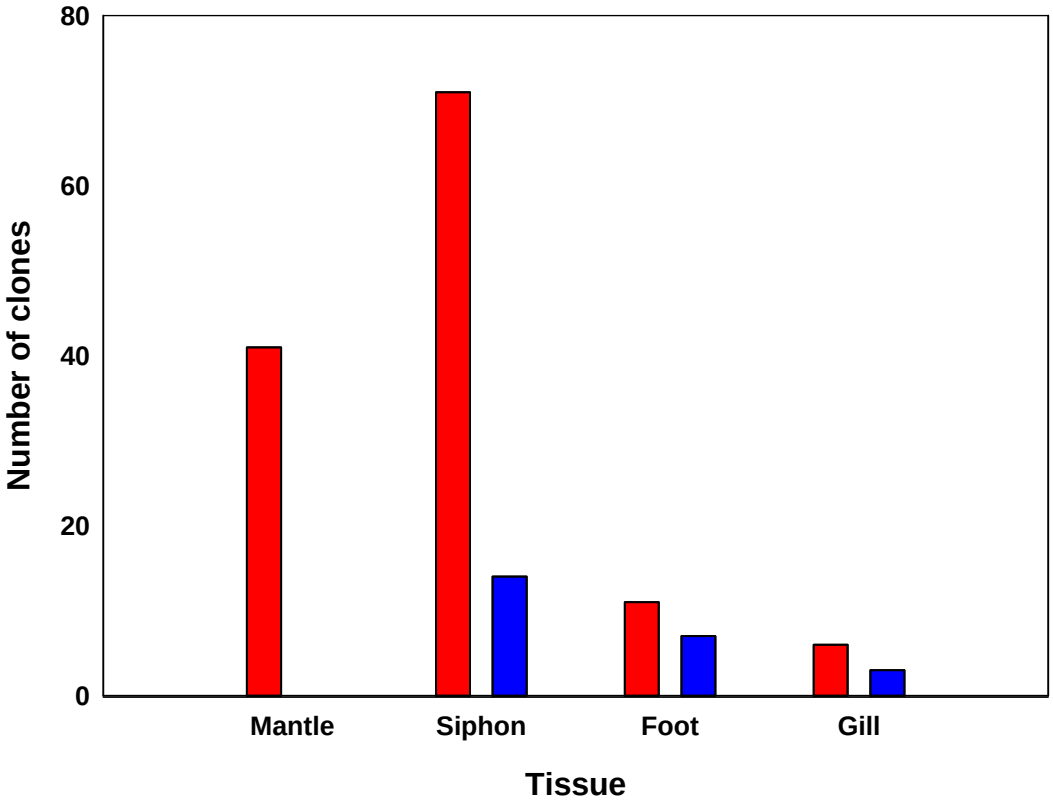


Figure 2

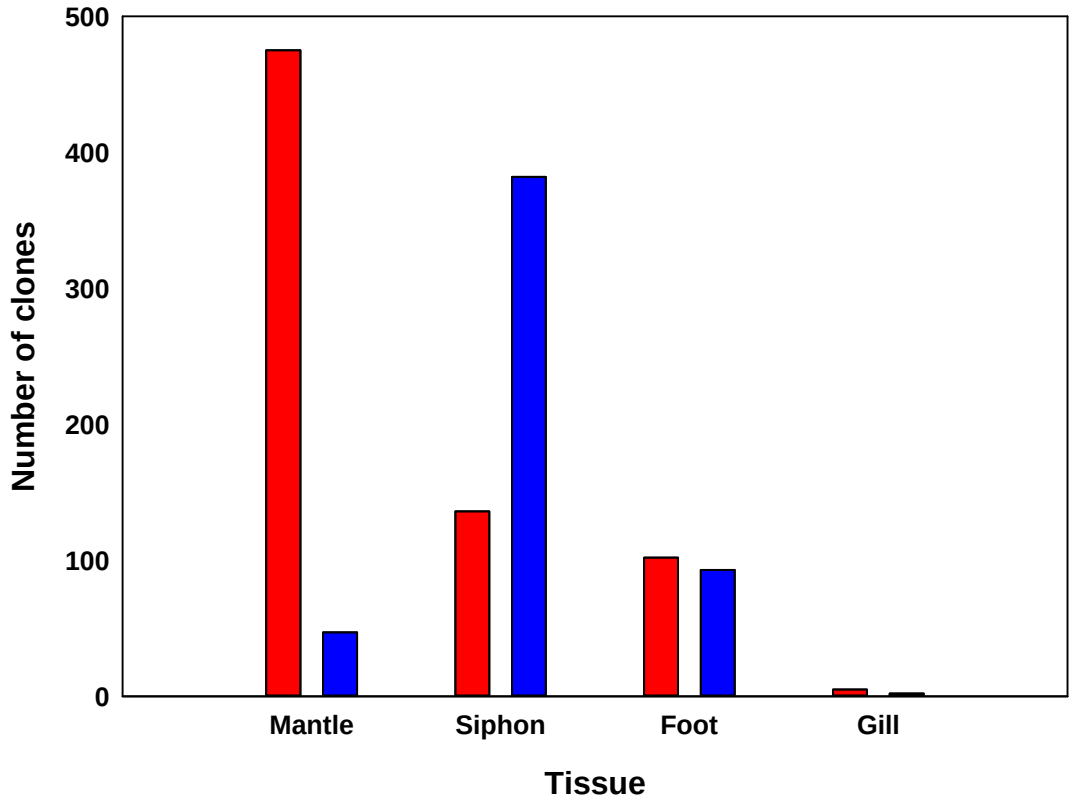

500
400
300
200
100
0
Number of clones
Mantle
Siphon
Foot
Gill
Tissue

Figure 3

Table 1: Size and average age data for the *L. elliptica* used in the microarray hybridizations. 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

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	E 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 (ubiqinone) 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

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	E 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 cis-trans 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 cis-trans isomerase B	$1.19e^{-61}$	Protein folding
Contig04203	40s ribosomal protein	$2.31e^{-27}$	Translation
Contig04781	Glucose regulated protein 78kDa	$1.23e^{-83}$	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.

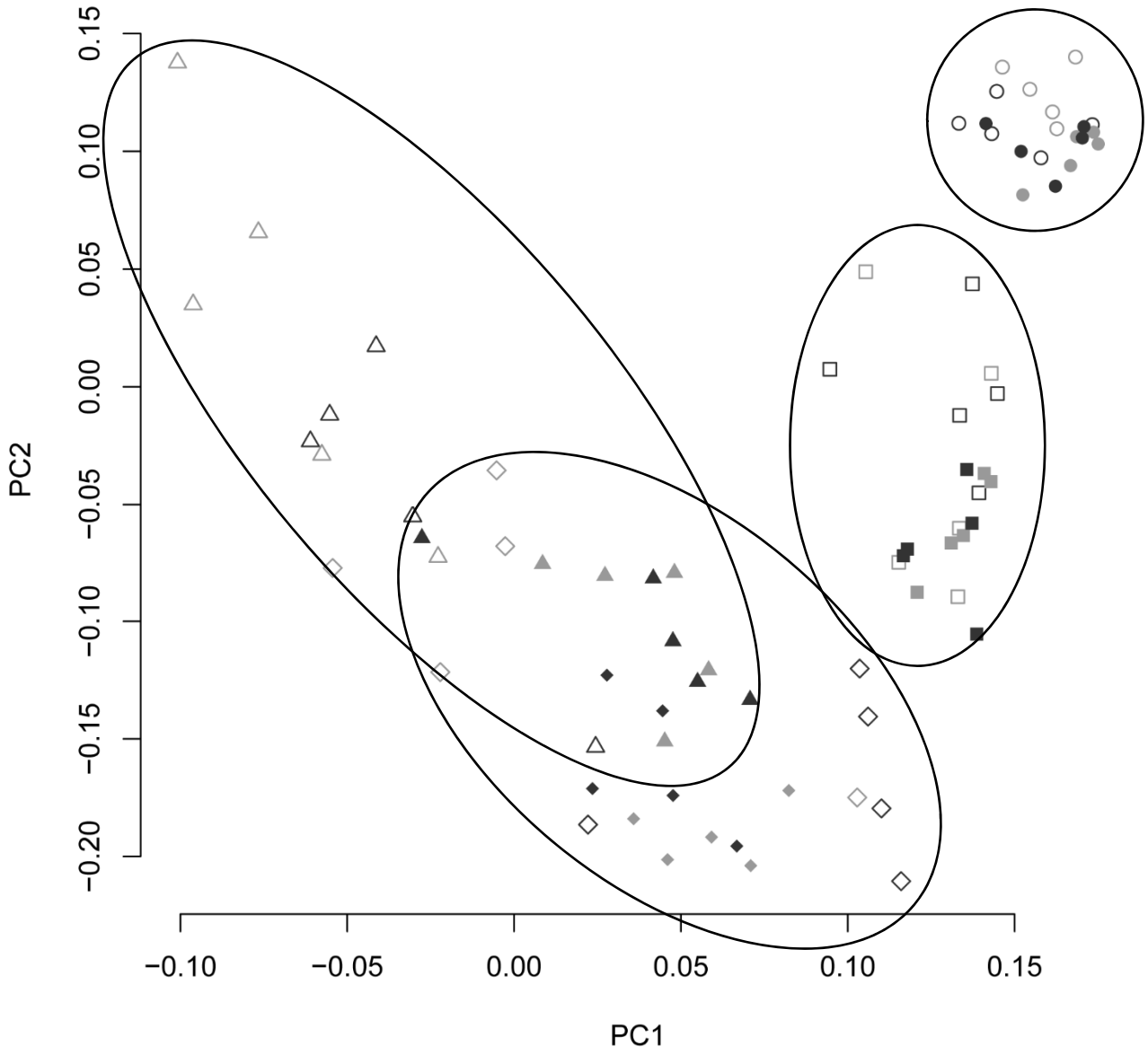
Contig/EST ID	Putative annotation	E value	Function
Contig00111	Phosphoenolpyruvate carboxykinase	0.0	Gluconeogenesis
Contig00447	MAP kinase-interacting serine/threonine protein kinase 1	$3.36e^{-174}$	May have a role in response to environmental stress
Contig00484	Apolipophorins-like	$1.39e^{-17}$	Immune/lipid 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

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	E 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	Apolipophorins-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 ribsosomal 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	No of clones up-regulated	No of mappings to contigs	No 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



Supplementary Figure S1:: PCA analysis for the different tissues. Key to symbols:
Foot: squares; Mantle: triangles; Siphon: diamonds; Gill: circles.
Open symbols: old tissues; Filled symbols: young tissues
Grey: control tissues; Black: treated tissues.

Supplementary Table S1: Age effect: Putative annotation of transcripts up-regulated in mantle tissue in controls: young animals at 0°C compared with older animals at 0°C.

Contig/EST ID	Putative annotation	E value	Function
Contig00464	Ribosomal protein L22	4.15 e^{-40}	Translation
Contig00634	Tyramine beta hydroxylase	7.29 e^{-127}	Neurotransmitter
Contig00731	Collagen α-2 (I) chain	2.66 e^{-24}	Cytoskeletal
Contig00796	60s ribosomal protein L37a	4.30 e^{-23}	Translation
Contig00823	Muscle LIM protein M1p84B	1.09 e^{-43}	Multifunctional
Contig00873	Histone H3	2.06 e^{-68}	Transcription
Contig01056	Nacrein-like protein 1	1.51e^{-30}	Shell production
Contig01069	Actin	1.15e^{-80}	Cytoskeleton
Contig01365	Predicted serine/threonine protein phosphatase 2A	2.07 e^{-61}	Multifunctional
Contig01549	CGI_10002181	4.02 e^{-165}	Immune
Contig02011	Cytochome C1	9.09 e^{-96}	Mitochondrial respiratory chain
Contig02214	Putative nuclear hormone receptor HR3	2.36 e^{-141}	Transcription
Contig02265	Carbonic anhydrase	1.11e^{-32}	Shell production
Contig02381	C1q	2.36e^{-23}	Immune
Contig02569	NADH dehydrogenase subunit 6	6.46 e^{-48}	Mitochondrial respiratory chain
Contig03185	Janus kinase and microtubule interacting protein	2.02 e^{-33}	Signalling
Contig04781	Glucose regulated protein 78kDa	1.23e^{-83}	Protein folding
Contig06350	RNA binding protein NOB1	1.02 e^{-60}	RNA processing
Contig07293	UspA domain containing protein	3.17 e^{-30}	Response to stress
Contig16108	Tropomyosin	9.91 e^{-28}	Cytoskeletal
Contig17207	LOTGIDRAFT_171372	4.98 e^{-21}	Transcription
Contig18173	α tubulin	7.16 e^{-39}	Cytoskeletal

Supplementary Table S2: Age effect: Putative annotation of transcripts up-regulated in mantle tissue in controls: older animals at 0°C compared with younger animals at 0°C.

Contig/EST ID	Putative annotation	E value	Function
Contig00103	NADH dehydrogenase subunit 1	$1.34\ e^{-129}$	Mitochondrial respiratory chain
Contig00191	α tubulin	0.0	Cytoskeletal
Contig00447	MAP kinase-interacting serine/threonine protein kinase 1	$3.36e^{-174}$	May have a role in response to environmental stress
Contig00484	Apolipophorins-like	$1.39e^{-17}$	Immune/lipid metabolism
Contig00619	Sushi	$3.57\ e^{-48}$	Immune
Contig00635	Von Willebrand factor D and EGF domain-containing protein	$1.12e^{-15}$	Multifunctional
Contig01309	Actin, cytoplasmic	$1.33\ e^{-104}$	Cytoskeletal
Contig01361	CGI_10023765	$2.95\ e^{-27}$	Protease inhibitor
Contig01573	Calreticulin	$1.70\ e^{-159}$	Protein folding
Contig01591	Peptidyl prolyl cis-trans isomerase	$3.46\ e^{-42}$	Protein folding
Contig01734	Lipoma HMGIC fusion partner-like 3	$9.68\ e^{-41}$	Uncharacterised
Contig02135	Perlucin	$4.66e^{-16}$	Shell production
Contig02449	LOTGIDRAFT_223715	$1.14\ e^{-71}$	Transporter
Contig03463	ATP-dependent RNA helicase DDX5	$1.32e^{-169}$	Transcriptional regulation
Contig03532	Thioester-containing protein B	$6.27\ e^{-113}$	Protease inhibitor
Contig04062	Actin	$6.45e^{-113}$	Cytoskeleton
Contig05358	Quinone oxidoreductase	$5.35\ e^{-73}$	oxidoreductase
Contig06040	EF-hand domain containing protein	$6.15\ e^{-65}$	Calcium binding/signalling
Contig08959	CGI_10028476	$2.81e^{-34}$	Unknown
Contig10367	Coiled-coil domain containing protein 47-like	$6.00\ e^{-66}$	Unknown
Contig16118	Bcl-2 like protein	$4.95\ e^{-11}$	Anti-apoptotic

Supplementary Table S3: Age effect: Putative annotation of transcripts up-regulated in controls: foot tissue in older animals at 0°C compared with younger animals at 0°C.

Contig/EST ID	Putative annotation	E value	Function
Contig00447	MAP kinase-interacting serine/threonine protein kinase 1	$3.36e^{-174}$	May have a role in response to environmental stress
Contig00449	Uncharacterised *Aplysia californica*	$1.65\ e^{-138}$	Unknown
Contig01055	Predicted coagulation factor XII-like	5.96 e-54	Immune
Contig01786	Peptidyl-prolyl cis-trans isomerase-like protein		Protein folding
Contig04780	Glucose regulated protein 78kDa	$1.23e^{-83}$	Protein folding
Contig05978	Predicted HSP75	$3.94\ e^{-130}$	Stress response
Contig12424	Serine threonine protein kinase PAK1	$7.99\ e^{-25}$	Multifunctional
Contig13689	Predicted SURF-1 like protein	$6.22\ e^{-14}$	Mitochondrial respiratory chain
Contig16715	BTG-1	$5.70\ e^{-43}$	Anti-proliferation protein
Contig16896	Hypothetical *Lottia gigantea*	$1.87\ e^{-63}$	Cell proliferation/cytoskeleton
Contig17546	Ubiquitin-domain containing protein	$8.57\ e^{-16}$	Protein degradation

Supplementary Table S4: Age effect: Putative annotation of transcripts up-regulated in controls: siphon tissue in older animals at 0°C compared with younger animals at 0°C.

Contig/EST ID	Putative annotation	E 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	Apolipophorins-like	$1.39e^{-17}$	Immune/lipid metabolism
Contig00635	Von Willebrand factor D and EGF domain-containing protein	$1.12e^{-15}$	Multifunctional
Contig02265	Carbonic anhydrase	$1.11e^{-32}$	Shell production
Contig02856	Deleted in malignant brain tumours 1	$3.58\ e^{-17}$	Immune
Contig04062	Actin, cytoplasmic 2 isoform XI	$6.45\ e^{-113}$	Cytoskeleton
Contig04781	Glucose regulated protein 78kDa	$1.23e^{-83}$	Protein folding
Contig05366	Eukaryotic peptide chain release factor	$3.28\ e^{-27}$	Translation
Contig08959	CGI_10028476	$2.81e^{-34}$	Unknown
Contig11055	Zinc metalloproteinase nas 1- like	$3.11\ e^{-14}$	Peptidase
Contig12424	Serine threonine protein kinase PAK1	$7.99\ e^{-25}$	Multifunctional