Hypoxia impacts large adults first: consequences in a warming world.

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Abstract

Future oceans are predicted to contain less oxygen than at present. This is because oxygen is less soluble in warmer water and predicted stratification will reduce mixing. Hypoxia in marine environments is thus likely to become more widespread in marine environments and understanding species-responses is important to predicting future impacts on biodiversity. This study used a tractable model, the Antarctic clam, *Laternula elliptica*, which can live for 36 years, and has a well characterised ecology and physiology to understand responses to hypoxia and how the effect varied with age. Younger animals had a higher condition index, higher adenylate energy charge and transcriptional profiling indicated that they were physically active in their response to hypoxia, whilst older animals were more sedentary, with higher levels of oxidative damage and apoptosis in the gills. These effects could be attributed, in part, to age-related tissue scaling; older animals had proportionally less contractile muscle mass and smaller gills and foot compared with younger animals, with consequential effects on the whole-animal physiological response. The data here emphasize the importance of including age effects, as large mature individuals appear less able to resist hypoxic conditions and this is the size range that is the major contributor to future generations. Thus the increased prevalence of hypoxia in future oceans may have marked effects on benthic organisms abilities to persist and this is especially so for long-lived species when predicting responses to environmental perturbation.

Key words: mollusc, sarcopenia, antioxidant, protein oxidation, tissue scaling

Introduction

The effects of climate change on oceans have many consequences for marine organisms. The main factors usually highlighted are higher temperatures which are predicted to affect both distributions (e.g. Russell *et al*. 2012) and survival of populations or species (e.g. Walther *et al*. 2001; Thomas *et al.* 2004). The main driver of climate warming is elevated CO_2 in the atmosphere derived from anthropogenic sources. The oceans are absorbing, and have absorbed roughly one third of the extra CO_2 from these sources in recent decades (Takahashi *et al.* 2002). This has produced significant concern over the acidification of the oceans for the survival of marine invertebrates (e.g. Byrne 2011), and this is especially so for early reproductive stages (e.g. Dupont et al. 2009; Watson *et al.* 2009).

Ocean warming has both direct and indirect effects on organisms. The direct effects via increases in metabolic rates and in relation to thermal limits have been relatively well studied (e.g. Peck *et al.* 2009; Somero, 2010). However, the indirect effects are less well understood. One of these that is becoming of increasing concern is hypoxia (e.g. Grantham *et al.* 2004). The solubility of oxygen in seawater varies inversely with temperature and a 2°C rise in temperature reduces the oxygen content at saturation by around 5% (Benson & Krause 1984, Peck & Uglow 1990). In excess of this stratification of the oceans is expected to become markedly increased (Capotondi *et al.* 2012). Both of these factors reduce oxygen availability for marine species, while higher temperatures increase the demand for ectotherms through elevated metabolic rates. Chronic hypoxia, or hypoxic events will thus be increasingly likely in marine environment as a consequence of climate change, yet the effects of this on animal populations and life history characteristics such as age and maturity are poorly understood.

Resilience (or sensitivity) to environmental change may vary over the life history of an animal (Peck, 2011; Philipp & Abele, 2010) and it is particularly important to understand this for long-lived species, where deferred maturity results in reduced generational turnover, and therefore phenotypic plasticity will be more important in terms of adjusting to environmental change, rather than genetic adaptation. Organisms particularly affected include those inhabiting high latitudes, regions which are currently experiencing rapid change, specifically the Arctic and the Antarctic Peninsula (IPCC, 2007), as many polar species have long life spans and have evolved under stable temperature regimes for millenia.

To date, studies examining the responses of marine species to environmental perturbation have concentrated on adults (cf. Peck *et al.*, 2009). However, to gain a holistic picture of climate change effects on species, studies on different life history stages and across a spectrum of adult ages are needed (Abele, 2012). The paradigm is that early life history stages, particularly larvae, are the most vulnerable to environmental perturbation (Pechenik, 1999) and many studies in the Ocean Acidification field have concentrated on this area (cf. Kurihara, 2008). The impact of age and reproductive maturity on physiological resilience is rarely examined despite physiological capacities often decreasing with age (cf. Kirkwood & Austad, 2000). Many Antarctic ectotherms show delayed maturity and tend to have longer lifespans and grow to larger adult sizes than related temperate species. As fecundity in ectotherms increases with body size (Angilletta et al. 2004), older animals provide the reproductive stock to ensure population continuity. It is therefore essential to understand the effects of environmental perturbation on adults of different ages.

In this respect, the Antarctic clam, *Laternula elliptica*, presents as an ideal candidate. It is highly abundant with a circumpolar distribution and as an infaunal filter-feeder it plays a

significant role in benthopelagic coupling (Arntz *et al.*, 1994; Momo *et al.*, 2002). This species can live for 36 years (Philipp *et al.*, 2005a) with deferred reproduction until the second quartile of its lifespan and continuous gonad production until death (Urban & Mercuri, 1998; Clark and Peck, unpub). It possesses distinctive annual growth bands in the shell and whereby individuals can be aged relatively easily (Philipp *et al.*, 2008). Indeed, *L. elliptica* has been proposed as a model species for understanding cellular events associated with ageing (Abele et al., 2009). It has also been shown that older clams fail first in short term acute stress tolerance experiments (Peck *et al.*, 2002; 2007; Philipp *et al.*, 2011). Hence the older, sexually mature animals, which produce the next generation are less resistant to environmental change compared with younger immature animals, certainly in the context of increasing water temperatures. Because of the previous studies showing large individuals to fail in warming experiments before smaller specimens the aim here was to test the hypothesis that larger mature animals would be less resistant to hypoxia than juveniles, and to put this into context of consequences for population persistence.

Biochemical assays were conducted, evaluating condition index, tissue energy status, accumulation of oxidised proteins and apoptotic activity on treated animals of different ages. These represent *a priori* assumptions of biochemical pathways known to be affected under environmental stress in different species. However to uncover novel pathways and expand our knowledge of the biochemical and physiological effects of severe hypoxia in low temperature adapted animals, molecular analyses using a custom-made microarray were also used. Such an approach has previously provided a finer scale detail on molecular responses to environmental challenge in this species (Truebano *et al.*, 2010). Finally tissue scaling was measured to evaluate whether muscle wasting with age occurs in *L. elliptica* and contributes to the effects seen on organism resilience.

Materials and Methods

Animal collection and sampling

L. elliptica were sampled by divers in January 2006 – February 2007 in Potter Cove, King George Island, Antarctica (62°13.511`S, 058°39.575`W). After sampling animals were kept at constant temperature (1°C) with running ambient seawater for one week before experiments were started. Two non-overlapping size classes were investigated: small sexually immature animals (33-50mm) with a mean age of 3 years and large sexually mature animals with a mean age of 19 years (73-92mm). Ages of the animals used were calculated from shell length using a Von Bertalanffy growth model (VBGM) based on length-at-age data for the Potter Cove population taken from Philipp *et al.* (2008).

Tolerance to the absence or very low concentrations of oxygen (LT_{50})

To provide the background data for the main experiment, the tolerance of *L. elliptica* oxygen deprivation was determined as the time of 50 % survival (LT_{50}) hypoxia (PO_2 level of 2kPa: 2 % O_2 in nitrogen, equivalent to severe hypoxia) and also anoxia. For the anoxia experiment, animals (shell length: 74.9mm mean ± 1.5mm (SEM)) were kept in individual sealed glass jars which were flushed with N_2 (AirLiquide, Germany) for 1h prior to inserting the animal. This system had been previously tested to ensure that all oxygen was depleted. After inserting the animals the jars were flushed with N_2 for 45min daily to ensure constant anoxic conditions. For the severe hypoxia LT_{50} experiment a similar system was used (mean shell length: 75.2mm ± 1.7mm (SEM)), but the seawater was constantly bubbled with oxygen at 2kPa PO_2 (AirLiquide, Germany) or air for controls (mean shell length: 74.6mm ± 2.9mm). All experiments were run in water baths maintained at 1°C using heater/cooler units (Julabo, Germany). Every morning animal survival was assessed by touch-responsiveness of the

siphon and mantle. Unresponsive individuals were classified as dead. Ammonia (Tetra*test* NH_3/NH_4^+, Tetra, Germany) and nitrite (Tetra*test* No_2^-, Tetra Germany) levels were monitored, and water was changed with pre-gassed water for the respective treatment when values were >0.26mg/l for NO_2^-. Values for NH_3/NH_4^+ were always ≤0.3mg/l. Animal size was determined after death or termination of the experiment (shell length: range: 67.1mm-84.8mm; mean 76.mm ± 0.8mm SEM; mean age 15 years) and did not differ between the different treatments.

Severe hypoxia experiment on different sized individuals

Based on the LT_{50} result of 17 days for severe hypoxia (oxygen level of 2kPa), a more extensive hypoxia experiment was designed to last 16 days. Younger/small (36.9mm mean shell length ± 0.5 SEM; mean age 3 years) and older/large (80.2mm mean shell length ± 1.0 SEM, mean age 19 years) individuals were used. Animals were kept in 2 aquaria per treatment bubbled with nitrogen at 2kPa or normal air and large and small animals were equally mixed. NH_3/NH_4^+ and NO_2^- were monitored regularly. Small and large individuals were sampled at the start of the experiment and after 16 days of severe hypoxia and normoxia (controls). Animals were dissected into different tissues (gill, siphon and mantle). Each tissue was weighed and snap frozen in liquid nitrogen and stored at -80°C until required. The target tissues (which were not necessarily used in all experiments) were chosen with gills as the most hypoxia relevant target and siphon and mantle as large organs in contact with the external environment to demonstrate tissue specificity of effect and the latter potentially acting to buffer internal acidification via shell carbonate mobilisation. Shells were dried for at least 24h and then weighed and length measured.

Morphometric parameters

Condition indices: CI = (soft tissue weight (g) / shell weight (g)) * 100 (Davenport & Chen, 1987). For the severe hypoxia experiment, shell weight to shell length ratios: SWSr = shell weight (g) / shell length (mm) were also calculated to assess whether shell carbonate was used to buffer internal acidification due to anaerobic metabolite accumulation under hypoxic conditions as found in *Mytilus edulis* by Michaelidis *et al.* (2005):

Biochemical analyses

Tissue energy charge: This involved the measuring of adenylates (AMP, ADP, ATP) and nicotinamide adenines (NAD, NADH, NADP, NADPH) by HPLC using the method after Lazzarino *et al.* (2003) described in detail in Philipp *et al.* (2005b). The tissue energy charge (EC) of the adenylates and the adenylate pool were calculated after Ataullakhanov & Vitvitsky (2002).

Protein oxidation: Measures of oxidative damage using protein carbonyls and lipid peroxidation were employed. The detection of protein carbonyl groups as a measure of protein oxidative modifications was carried out after Levine *et al.* (1990) and as described in detail in Philipp *et al.* (2005a). Sample protein contents were determined by the Bradford method using bovine serum albumin as a standard. The marker for lipid peroxidation malondialdehyde (MDA) were measured by HPLC after Lazzarino *et al.* (2003) and described in detail in Philipp *et al.* (2005b).

Apoptotic activity: Activities of key members of the apoptotic pathway (caspases 3 and 7) were determined in gill and siphon tissue. Frozen tissues were ground in liquid nitrogen and processed according to a modified protocol of Liu *et al.* (2004) using the Caspase-Glo 3/7 Assay (Promega, Madison, USA). Tissue homogenates (1:100 w/v) were prepared in

198 extraction buffer (25mM HEPES, pH 7.5, 5mM $MgCl_2$, 1mM EGTA and 1µg*ml^{-1} of each of
199 pepstatin, leupeptin and aprotinin). Following centrifugation (15 min, 13000 rpm at 4°C),
200 equal volumes of supernatant and freshly prepared assay reagent were gently mixed in white
201 96-well plates. After incubation at 25°C for 60 min, luminescence was measured using a
202 Microplate Reader (TriStar Multimode Reader LB 941, Berthold technologies GmbH & Co
203 KG, Germany). Results were measured as protein concentrations in the supernatants
204 following (Bradford, 1976). Activities of caspases 3/7 were expressed as relative
205 luminescence units (RLU) * mg^{-1} protein.
206
207 *Biochemical statistical analysis*
208 Survival curves were only produced from older animals with a mean age of 15 years.
209 Statistical analysis was carried out using GraphPad Prism software (version 5.01). Survival
210 curves were compared using log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests.
211 Animals taken out of the experiment during the LT_{50} experiment were included in the
212 calculation and graph generation. Non-parametric Kruskal-Wallis with Dunns PostHoc tests
213 were used to identify significant differences between three or more groups. Differences
214 between two groups were detected with Mann-Whitney t-tests.
215
216 *Gene expression analyses*
217 *Pilot molecular analyses:* Expression levels of the inducible heat shock protein genes
218 (HSP70A and HSP70B) were evaluated in 16 day normoxia younger (shell length 37.8mm ±
219 1.3 SEM) and older (shell length 80.1mm ± 1.4 SEM) controls against 16 day younger (shell
220 length 36.8mm ±1.0 SEM) and older (79.6mm ± 2.8 SEM) hypoxia samples (n=6). Q-PCR
221 using HSP70A and HSP70B primers sets with β actin as a control sequence were used and
222 analysed following Clark *et al.* (2008). These data were used as a preliminary proof of

concept, prior to the microarray experiment, that significantly different gene expression profiles would be generated under the more extensive molecular analyses.

Microarray hybridization

Gene expression was analysed in siphon and gill tissue of a sub-set of the animals described above (n = 6 for each treatment) with ages for normoxic animals: younger (shell length 36.1mm ± 0.92 SEM, mean age 3 years) and older (shell length 80.3mm ±1.58 SEM, mean age 19 years) and hypoxic animals: younger (shell length 37.6mm ± 1.14 SEM, mean age 3 years) and older (shell length 80.0mm ± 3.34 SEM, mean age 18 years). RNA was extracted from all individuals using TriSure (Bioline, UK), following manufacturer's instructions, with subsequent RNA purification using Qiagen Rneasy minikit spin columns. PCR amplified labelled cDNA targets were prepared from 1μg total RNA using protocols in Petalidis *et al.* (2003) and hybridizations to an 8448 clone cDNA array performed following Purac *et al.* (2008) with modifications according to Truebano *et al.* (2010).

Microarray 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 & Speed, 2003; Smyth, 2004; 2005; Smyth *et al.*, 2005; Richie *et al.*, 2007) was used for data analysis. Background subtraction (half), and within (printtiploess) and between (Rquantile) 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 & Hochberg, 1995) and a minimum two

248 fold change. The array design and experiment have been submitted to Array Express:
249 Experiment name: Laternula elliptica siphon hypoxia treatment ArrayExpress accession: E-
250 MEXP-3613; Experiment name: Laternula elliptica gill hypoxia treatment ArrayExpress
251 accession: E-MEXP-3611.
252
253 *Sequencing of differentially expressed clones and data analysis*
254 The inserts from all cDNAs of interest were PCR amplified and sequenced following
255 Truebano *et al.* (2010) and sequence runs performed by Source Bioscience Lifescience
256 (Nottingham, UK). Trace2dbest (Parkinson *et al.*, 2004) was used to remove and trim poor
257 quality and vector sequence. The TGI clustering tool (Pertea *et al.*, 2003) was used to
258 assemble sequences, and Blastx (Altschul *et al.*, 1997) was used to annotate against the non-
259 redundant GenBank database and Swissprot (Bairoch *et al.*, 2007). All sequences have been
260 submitted to GenBank (Accession numbers JK991088-JK993117).
261
262 *Validation of differentially expressed genes by quantitative PCR (Q-PCR)*
263 The microarray was validated previously in Truebano *et al.* (2010). The current array
264 experiments were further validated using 6 primer pairs (Supplemental Table 1) tested against
265 either older versus younger hypoxic animals or older normoxic versus older hypoxic animals,
266 as appropriate (n=5) using Q-PCR methodology as detailed in Clark *et al.* (2008).
267
268 *Tissue scaling related to age*
269 52 animals ranging from 8mm (<1 year old), through to 101.7 mm length (≥18 years old)
270 were collected by scuba divers at depths of 10-18m in 2011 at Hangar Cove, Rothera Point,
271 Adelaide Island, Antarctic Peninsula (67°34’07°S, 68°07’30°W). Despite the geographical
272 distance to King George Island, where the hypoxia experiment was performed, AFLP

analyses show both cohorts are genetically undifferentiated (Harper *et al.*, 2012). Animals ≥ 30mm length were dissected into six separate tissues: siphon, mantle, adductor muscle, gill, foot and then remaining tissue (largely composed of digestive gland and gonad which could not be separated) was treated as a single sample. Animals ≤ 30mm were dissected into five separate tissues: siphon, mantle, adductor muscle, foot, and remaining tissue (gill could not be separated from digestive gland and gonads were not present). Tissue dry and ash-free dry masses were evaluated following (Peck, 1993). Shell lengths were measured using vernier callipers. Contractile tissue was defined as siphon, mantle, adductor muscle and foot. All statistics and regression analyses were calculated using Minitab v15.0.

Results and Discussion

Tolerance to very low concentrations or absence of oxygen

L. elliptica showed a considerable capacity to survive reduced oxygen conditions (Supplemental Figure S1). The LT_{50} for anoxia was 10 days, whilst this was extended to 17 days under severe hypoxia at 2kPa oxygen. Only one animal (out of 12) died during 17 days in the normoxic control treatment, indicating aquarium conditions were suitable for long-term culture. As a result of these data, an experimental duration of 16 days was chosen for the main hypoxia experiment. Compared to other bivalves, the *L. elliptica* LT_{50} of 10 days in anoxia was not unusual; *Mya arenaria*, a temperate clam has an LT_{50} of 16 days, whilst *Mytilus* can survive 15-30 days of anoxia, and these are not the most hypoxia/anoxia tolerant bivalves on record (Theede *et al.,* 1969). Lower temperatures, especially below 10ºC can prolong hypoxic survival in temperate bivalves (Theede *et al.,* 1969). Hence *L. elliptica* is principally hypoxia tolerant at low temperatures, but certainly more sensitive than many temperate and even sub-Antarctic species. This may be because *L. elliptica* regularly adopts hypometabolic strategies

298 to reduce energy costs, for example, in winter when food resources are low (Morley *et al.*,
299 2007), and also spontaneously reduces oxygen uptake (only large old specimens) in response
300 to environmental challenge e.g. high sediment loads from glacial melt waters (Philipp *et al*.,
301 2011). These abilities of bivalve molluscs to tolerate significant levels of hypoxia are
302 adaptations conferring resistance to reduced oxygen and make them ideal for studying
303 predicted increases in hypoxia, as they represent a robust group and hence effects and
304 conclusions drawn here should be conservative .
305
306 *Morphometric parameters*
307 Animal condition indices (CI) were used as a metric of animal health. These did not vary in
308 any of the treatments during the experiment, thus data sets of all animals within the different
309 age groups were taken together and age-specific differences analysed. Older individuals had
310 CI values of 1.99 ± 0.05 SEM (n=37), whilst the CI of younger individuals was over 10%
311 higher and this difference was significant(2.21 ± 0.05 SEM; n=45) (t-test, $p<0.05$). CI varies
312 with stored food reserves and usually follows a seasonal pattern of increase in spring/summer
313 and decrease in winter. In filter feeders it often correlates with the phytoplankton abundance
314 (Bayne *et al.,* 1976; Norkko & Thrush, 2006). Bivalves in particular, use energy reserves over
315 winter in an effort to maintain size, and temporary reductions of bulk are often replaced by
316 water (Bayne *et al.,* 1976). Smaller animals are most efficient at converting food to body mass
317 at low levels of food availability. As this study was carried in early to mid-summer it is
318 highly probable that the older animals in this study were still replacing food reserves depleted
319 over the previous winter. The higher CI in smaller animals therefore is likely to indicate a
320 healthier state which may contribute to their higher stress tolerance (Bayne *et al*., 1976).
321 However, such factors will be highly relevant in future periods of oxygen stress, as in many
322 environments these will vary seasonally and capacities to replenish reserves after winter will

be critical in marginal habitats. The better performance of smaller individuals in spring would also make them more resilient in a warming world as this is the time when temperatures are increasing and hypoxic events more likely. Shell weight-length ratios did not change over the 16 days of hypoxic exposure (data not shown), indicating shell bicarbonate ions were not mobilized to regulate internal acid-base balance under hypoxic exposure. Additional biochemical analyses were employed to understand the details underlying the hypoxia response at the tissue level.

Tissue energy status

Adenylate energy charge (AEC) of tissues/organs has been proposed as a direct measure of organism energetic state (Atkinson, 1977). It usually ranges from 1 in the fully charged, healthy state to 0.3-0.4, the critical values for survival. Adenylate concentrations in this study were both tissue and age-specific. Younger *L. elliptica* had higher AEC and ATP values and a higher adenylate pool in the investigated tissues (Figure 1 and Supplemental Tables 2 and 3), indicating more cellular energy was available per unit tissue, which correlates with findings of decreased mitochondrial respiratory capacity with age (Philipp *et al.*, 2005b). With regard to tissue-specific differences, in all investigated animals AMP concentration was highest and ATP and energy charge was lowest in gills compared with mantle and siphon tissue, as evidenced by large differences in ATP:AMP ratios (Supplemental Table 2). Moreover the adenylate pool was lowest in gill tissue. Whilst tissue-specific differences have been found in other species (Giesy, 1988), the results for gill are intriguing. Lower ATP and higher AMP indicate high energy turnover, probably for ciliary activity. Thus gills might have a higher requirement for cycling ATP and ADP. Cycling of ADP in gill cells is presumably through adenylate kinase (AK) activity. It is typically found in ciliated epithelia where a special AK isoform catalyses ADP transphosphorylation: 2ADP to ATP + AMP + Pi. AK is not inhibited

348 by high AMP levels and covers the intimate energy demand of synchronised ciliary
349 movements (Dzeja &Terzic, 2009). Indeed, the gill is a large surface area where both ciliary
350 water pumping for ventilation and feeding and active ion pumping for osmotic homeostasis
351 occur. A higher energy demand in a tissue is likely to translate into greater oxygen
352 requirement, suggesting that the gills in species like *L. elliptica* may be expected to be a
353 critical tissue in a warmer more hypoxic ocean. However, unlike ATP:AMP ratios under the
354 hypoxia treatment, there were only significant changes in adenylate concentrations in the
355 mantle tissue of older individuals (Figure 1, Supplemental Table 3). A similar trend was
356 observed in siphon tissue, but the changes were not significant, whereas in gills no effect of
357 the 16 days hypoxia treatment was visible (Figure 1 and Supplemental Table 3). Thus in gill
358 tissue higher cycling of adenylates may prevent reduced AEC under hypoxic conditions. This
359 might not only occur during environmental low oxygen conditions but also under functional
360 hypoxia during increased ciliary activity. The lower adenylate pool might therefore be
361 sufficient for physiological functioning in some tissues, whereas in mantle and siphon tissues
362 higher levels are needed to buffer sudden hypoxic events.
363
364 ATP formation is tightly coupled to the oxidation and reduction of NADH/NAD+, with a shift
365 to the reduced state (more NADH) expected under environmental hypoxia (Shofer &
366 Tjeerdema, 1998). Overall NAD and NADH tissue concentrations declined in the same order
367 (siphon>mantle>gills) as the ATP and overall adenylate levels, and also showed the same
368 pattern in the age groups (younger>older) of untreated individuals (Table 1). Conversely
369 NADP concentrations in all age groups were highest in gill tissue followed by mantle and
370 siphon tissue, whereas the NADPH concentrations had no tissue specific pattern. The overall
371 NADP/NADPH and NAD/NADH ratio was again highest in gill tissue. Nicotinamide
372 nucleotide concentrations did not change in either age group incubated under severe hypoxia

(Supplemental Table S4) suggesting that anaerobic pathways were not used to generate new energy (Shofer & Tjeerdema, 1998). This suggests that metabolic suppression rather than anaerobic energy production is the adaptation to reduced oxygen in groups like *L. elliptica*. In a generally lower oxygen world this would lead to a lowered overall level of performance across the year, the consequences of which would depend on the time of year and duration of any event.

Oxidative damage and apoptosis

As the tissues become energetically compromised (e.g. during prolonged hypoxic exposure), protein turnover decreases as an energy saving strategy related to the overall depression of metabolic rate (Hochachka *et al.,* 1996). Under these conditions, cells may fail to efficiently remove cellular oxidative damage products, such as protein carbonyls (oxidised proteins) and MDA (malonedialdehyde: an initial product of lipid peroxidation) and accumulation of these products is bound to occur as metabolic rates decline and autophagic and proliferative activities become reduced (summarized by Philipp and Abele, 2010). MDA concentrations were similar in younger and older *L. elliptica* and this did not change under severe hypoxia (data not shown). Conversely, protein carbonyls significantly increased in gill tissue of older individuals under severe hypoxia compared to normoxic controls (Figure 2). Hence, there is an age-dependent effect of hypoxia on protein carbonyl turnover which is slowed in old *L. elliptica*. The age effect on autophagic cell clearance has already been observed in other bivalves even under unstressed conditions, for example as seen in in older cohorts of the scallop *Argopecten ventricosus* which presents as failure to remove protein carbonyls from gill tissue (Guerra *et al.,* 2012). Accumulation of oxidised proteins and the formation of fluorescent age pigment (lipofuscin) aggregates in cells have been indicated to enhance cellular senescence through the inhibition of 20S proteasome in a cycle of progressive protein

damage accumulation (Sitte *et al.,* 2000). This in turn relates to the induction of apoptotic cell death by dysregulation of pro-apoptotic proteins (Powell *et al.,* 2005). If this fails, cell death results (Zhang *et al.,* 2008). We therefore measured severe hypoxia effects on the intensity of apoptosis in gills and siphon in both age groups, and there was a significant induction of apoptotic cell death in gills of hypoxia treated older animals, which corresponds with the increased tissue carbonyl levels (Supplemental Figure S2). Thus older individuals, and especially their respiratory tissues, seem more susceptible to hypoxic exposure and less capable of controlling damage accumulation resulting in enhanced necessity for apoptotic removal of terminally damaged cells. These data showing large individuals enter apoptotic states earlier and have poorer abilities to remove cellular oxidative damage products suggests that such materials would likely accumulate chronically in adults under increasing hypoxia or more frequent hypoxic events. This would mean large adult performance will probably decline well before small individuals in future change scenarios, with consequences for amounts of energy available for other physiological processes, especially growth and reproduction.

Heat shock protein (HSP70) expression

An initial molecular study investigated expression of HSP70 genes as another indicator of cellular stress. These analyses were restricted to gill tissue and the two duplicate forms of the HSP70 genes, based on a previous, more extensive, survey of tissues and HSP70 gene family members (data not shown). Hypoxia-induced HSP70A expression was marginally significant in older animals ($p=0.058$) and HSP70B expression was significant in younger animals ($p=0.04$) (Supplemental Table 5). Interestingly HSP70 has an anti-apoptotic function and is up-regulated under stress to reduce apoptotic cell death. A 2-way ANOVA of age versus gene showed no significant effect of age on gene expression ($F_{1,1}=12.26$, $p=0.177$), although from

this limited sampling the younger animals showed only 30% to 50% of the expression levels of the samples from older animals.

Microarray results

Two tissues were screened on the array: gill, as a hypoxia relevant target, compared with siphon, to examine any tissue-specific effects. Expression profiles of transcripts were partitioned into the effects of treatment (hypoxia versus normoxia) and age (younger versus older animals) as the major variables. In surveying overall numbers of clones that were significantly up-regulated, the initial results were surprising because animal age had a far greater effect than hypoxia (616 compared with 335) (Table 2).

The effect of environmental treatment

A custom-made microarray was employed to identify gene pathways involved in the hypoxia response in a discovery lead approach, in addition to the targeted biochemical analyses. Taking older animal gill tissue as the reference point for the description of the severe hypoxic response, 75 clones were up-regulated in hypoxia when compared with older animals under normoxia (Supplemental Table 6). 25 clones were annotated using sequence similarity searching, with descriptions assigned to 17 putatively different functions (Supplemental Table 6). These annotations indicated that the animals mount a complex defence response to reduced oxygen conditions. Up-regulation of transcripts with putative functions was associated with combatting reactive oxygen species, the unfolded protein response and activation of the immune system. In terms of oxidoreductases, the main active transcript was represented by thioredoxin peroxidase (= peroxiredoxin), and quinone reductase. The identification of a small heat shock protein (with potential anti-apoptotic activity) and peptidyl-prolyl cis-trans isomerase (PID) indicated an enhanced requirement for protein

448 folding, with potential mobilisation or redistribution of energy reserves shown by the up-
449 regulation of PCK2, involved in glucose homeostasis and the regulator of lipid storage gene.
450 The immune response comprised the activation of the innate immune system via F-type
451 lectins (fucolectin) (Kawabata & Iwanaga, 1999) and the complement system (adioponectin).
452 The latter protein has several domains and the *L. ellipitca* clone aligned with the C1q domain,
453 a sub-unit of the C1 enzyme complex that activates the serum complement system and is
454 involved in immune functioning of *Mytilus galloprovincialis* (Gerdol *et al.,* 2011; Philipp *et*
455 *al.,* 2012). Immune response changes with age have previously been demonstrated in *L.*
456 *elliptica* in both the presence and absense of environmental stress. Older animals have more
457 hemocytes but produce a lower oxidative burst response (normalized to cell numbers)
458 compared with small individuals (Husmann *et al.,* 2011). Consequently, older animals
459 exhibited higher mortality rates after injury compared to younger specimens (Philipp *et al.,*
460 2011). Additionally two transcription factors were identified; an NF-kappa-B inhibitor and
461 AP-1 protein. The latter is strongly up-regulated in hypoxia responses in some mammals
462 (Papandreou *et al.,* 2005). NF-kappa-B inhibitor is highly conserved, ubiquitously expressed,
463 and is normally bound to NF-kappa-B, maintaining this potent signaling molecule in an inert
464 form (Montagnani *et al.,* 2008). NF-kappa-B has an immune function, but is also involved in
465 cell atrophy (Salminen *et al.,* 2008). Up-regulation of the inhibitor may represent an attempt
466 to slow down hypoxia-induced apoptosis, which occurred in the gills of the older animals
467 from the apoptosis analyses. Attempts to combat apoptosis were supported by the up-
468 regulation of transcripts with sequence similarity to tenascin, cadherin, B cell translocation
469 gene and a tissue-type plasminogen gene, all of them involved in cell adhesion interactions
470 and cellular differentiation events. Increased expression of NF-kappa-B inhibitor and the
471 antioxidant, quinone reductase, in hypoxic animals were both confirmed by Q-PCR
472 (Supplemental Figure S3). Younger animals appeared to respond more effectively to hypoxia

(when transcripts from hypoxia-treated younger animals were compared with animals of a similar age under normoxia), with additional transcripts putatively involved in immune responses, antioxidant activities (glutathione-s-transferase and tyrosinase) and the unfolded protein response accompanied by degradation of damaged proteins via ubiquitin and skeletrophin, which has an E3 ubiqutin-protein ligase activity (Supplemental Table 7). This supports previous data indicating decreased protein turnover with age in *L. elliptica* (Philipp *et al.,* 2005a) and also our study which showed younger animals to accumulate less protein carbonyls. Similar patterns of up-regulated gene expression were identified in siphon tissue (data not shown).

Age-related response

In these analyses, older animals under severe hypoxia were directly compared with younger animals under severe hypoxia to examine the effect of age. In the siphon experiments 75 clones were up-regulated under hypoxia in older animals compared with younger animals under hypoxia, but sequence similarity searches primarily revealed matches to proteins with low complexity repeats. The clones with putative annotation could all be ascribed to elevated immune system functioning (data not shown). The gene expression pattern in the siphon of younger animals was completely different to those in older animals under severe hypoxia. These analyses identified 165 up-regulated transcripts compared with older hypoxic animals, of which 34 had putative functionality ascribed via sequence similarity searching (Supplemental Table 8). The vast majority of these (dynein, myosin, tropomyosin, actin, LIM domain protein and calponin) are involved in cytoskeletal structuring, muscle structure and function. These were accompanied by transcripts for isocitrate dehydrogenase, ATP synthase and arginine kinase, which indicated enhanced energy production (validated by Q-PCR (Supplemental Figure S3). These findings were further supported by the adenylate data

presented above showing that ATP (and whole adenylate content) decreases in older animals under hypoxic exposure. Few age effects were evident in gill tissue with only 25 clones up-regulated under hypoxia (data not shown). When the transcription profiles of older animals under normoxia were compared with younger animals under normoxia, the older animals showed weak signals of up-regulation of immune genes and the younger animals, more muscle genes (data not shown), but not to the extent seen under the severe hypoxia treatment. Hence, the severe hypoxia transcription profiles demonstrate and magnify the enhanced susceptibility of older animals and the very different response of the younger animals.

Overall these molecular data highlight very different age-specific hypoxic responses in different tissues, with siphon more affected than gill (Figure 3). This contrasts with the biochemical results for hypoxia, where gill was the most sensitive tissue in some tests (oxidised proteins and apoptosis). The adenylate data, however, showed more hypoxia sensitivity in siphon and mantle than gill tissue. It may be that gill cells progress more rapidly to self-destruction under stress, possibly due to the relatively high energy turnover and strategy of mitochondrial autophagy to reduce ROS (Zhang *et al.*, 2008), whereas cells in other tissues are more programmed to resist? Adenylate biochemistry data showed a higher energy charge in all tissues of younger animals indicating a better phosphorylation capacity and better conservation of energy reserves under stressful conditions. This more efficient, robust cellular physiology is corroborated in the microarray data, where the hypoxic response of younger animals included up-regulation of energy provision and muscle genes, whereas older animals rather displayed enhanced immune defenses.

Increased expression of muscle genes in younger animals (and therefore, by implication, more muscle activity) under severe hypoxia is intriguing and links directly to published

523 experiments. It had previously been noted that older *L. elliptica* lose critical biological
524 functions (the ability to bury in sediment) when warmed, (Peck *et al.*, 2002; 2007). In a
525 warming, more hypoxic world, therefore, larger adults in species like *L. elliptica* are likely to
526 suffer a double problem of poorer capacities to perform activity at elevated temperature and
527 reduced tissue energy availability due to hypoxia.
528
529 Smaller animals show much faster re-burrowing ability compared to larger individuals, which
530 may relate to body size, but also to decreased muscle activity with increasing age (Philipp *et*
531 *al.*, 2011). In our severe hypoxia experiment, sediment was not provided and burying was not
532 possible, therefore increased muscle activity could be a result of either increased water
533 pumping to enhance oxygen delivery, or attempted movement away from the stress. The fact
534 is, however, that older animals express fewer muscles genes and are physically less active
535 than young specimens as a consequence of ageing. It has been well documented from
536 nematodes to humans that older individuals are less active than younger specimens and that
537 this is accompanied by sarcopenia, the progressive loss of skeletal muscle mass and strength
538 with age (Nair, 2005; Grotewiel *et al.*, 2005; Wolkow, 2006).
539
540 *Tissue mass and ageing*
541 AFDM was derived for 5-6 tissues from each of 52 individuals. Whole animal AFDM
542 increased with size with a regression scaling coefficient of 3.68 (Supplemental Table 9). This
543 was not consistent with isometric scaling and implied shape changes with size. Indeed shell
544 dimensions also deviated from isometric scaling where shell height increases more than
545 length with age ($p = 0.035$) (SupplementalTable 9). This species thus becomes rounder and
546 wider with age, increasing more in volume than would occur with isometric scaling. It is
547 unknown why the change in shell shape occurs, but we hypothesize that a larger volume may

548 be needed for reproductive tissues, or to minimise shell production costs at lower calcium
549 carbonate saturation states (Watson et al. 2012)..
550
551 Beneath this overall relationship there were differences in scaling between tissues. Combined
552 contractile tissue (siphon, mantle, adductor muscle and foot) AFDM scaled against length,
553 had a slope of 3.55 (Supplemental Table 9). Consequently the percentage of contractile tissue
554 in the animal decreased with length (age), with a negative regression slope of -0.221
555 (Figure 4, Supplemental Table 9). Thus the smallest individuals were composed of around
556 75% contractile tissue, but this decreased by >2% for every 10mm increase in length. The
557 major reason for the decline in contractile tissue was a reduction in the relative size of the
558 foot. GLM analyses using tissue as a covariate showed that regressions with size for foot and
559 gill were significantly different to those of other tissues ($P < 0.0001$ (data not shown)).
560 Whereas the tissue scaling relationships with age for the main tissues were between 3.3 and
561 3.6, that of the foot was only 2.9 (Supplemental Table 9). Similar scaling patterns have been
562 demonstrated in another soft shell clam, *Mya arenaria* (Checa & Cadee, 1997). This result
563 enhances the observations of Peck *et al.* (2002; 2007) and; Philipp *et al.* (2011) where older
564 animals more often fail to re-bury compared with younger individuals. A proportionally
565 smaller foot in older animals makes re-burying into the sediment more difficult and more
566 energetically costly per unit foot tissue, especially as they have to re-bury deeper than smaller
567 animals. The frequency with which an animal has to re-bury also affects their capacity for
568 reburial. In a comparison of burying behaviour of *L. elliptica* from sites with different
569 incidences of ice-berg disturbance, animals from sites where disturbance was common
570 reburied faster than those from relatively undisturbed sites, indicating an additional
571 behavioural or training effect (Philipp *et al.*, 2011; Harper *et al.*, 2012). Younger animals are
572 also more likely to re-bury frequently as they live much closer to the sediment surface and are

573 less anchored than larger animals, which can bury to depths exceeding 50cm (Ralph &
574 Maxwell, 1977). Thus clams, like many other animals, have reduced muscle mass and a more
575 sedentary life style as they age. The biochemical data can be further elucidated by comparison
576 with the tissue scaling data. The gills scale with a slope of only 2.51 (Supplemental Table 9),
577 so older animals have a proportionally smaller gill surface for oxygen extraction. Older
578 animals have a lower metabolic rate, but tissue scaling probably contributes towards the age-
579 related stress effects seen in older animals.
580
581 Our data indicate that for a wide range of metrics, including tissue energy status, cellular
582 senescence and apoptosis, immune function and cellular stress, older animals will be
583 compromised in future more hypoxic marine environments. This problem is further
584 exacerbated by the poorer performance of older individuals in warmer conditions (e.g. Peck *et*
585 *al.* 2004). Hence, the older animals comply with the disposable-soma-theory theory of ageing
586 that predicts that in species reproducing all their lives, aged specimens divert energy from
587 tissue maintenance to reproduction (Abele *et al*., 2009). Hence age must become an
588 important factor in predictions of population level responses to environmental perturbation.
589 This likely applies not only to *L. elliptica,* but also other very long-lived polar marine species,
590 such as the brachiopod *Liothyrella uva* (>50 years (Peck & Brey, 1996), and the bivalves
591 *Yoldia eightsii* (80-100 years (Scourse, pers. comm)) and *Adamussium colbecki* (>100 years
592 (Berkman *et al.*, 2004)), where climate change is impacting most rapidly (IPCC, 2007). In
593 long-lived marine species older individuals often contribute progressively more to population
594 reproductive effort (Grahame 1973; Peck *et al.* 1987; Chockley & Mary 2003; Birkeland &
595 Dayton 2005). Size also often provides a refuge from predation (e.g. Harper et al. 2009),
596 producing left skewed size distributions and populations dominated by mature animals. The
597 loss of the oldest half of the mature individuals in a population would cause a much larger

impact on numbers of embryos produced and hence recruitment. This would be one of the likely outcomes in a warming more hypoxic ocean, especially for long lived slow growing species. Whether adaptations producing younger reproductively capable individuals can be entrained fast enough, or sufficient early maturing individuals survive will depend on the rate of change and intensity of the combined warming and hypoxic conditions.

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837

838 **Figure Legends**

839

840 **Figure 1:** Adenylate energy charge (EC) in mantle, siphon and gill tissue of younger and
841 older *L. elliptica* individuals sampled at the beginning of the experiment (controls) and
842 incubated for 16 days under normoxic (16 days N) or hypoxic (16 days H; 2% O_2). Different
843 letters between treatments with one age group indicate significant differences (non-parametric
844 one-way ANOVA; $p<0.05$). * indicate differences between younger and older control
845 individuals (Mann-Whitney U test). N=4-6 per group.

846

847 **Figure 2:** Concentration of protein carbonyls in gill tissue of older *L. elliptica* individuals
848 incubated for 16 days under normoxic (16 days N) or hypoxic (16 days H; 2% O_2) conditions.
849 * indicate significant differences ($p<0.05$, Mann-Witney t-test). N=8-12 per group.

850

851 **Figure 3:** Schematic diagram summarising hypoxia effects on a general population of *L.*
852 *elliptica* and the specific responses of younger and older animals.

853

854 **Figure 4:** Graph showing percentage of contractile tissue in individual animals (as derived
855 from the AFDM of siphon, mantle, foot and adductor muscle tissue) plotted against the length
856 of shell. Shell length is a proxy of age, with the smallest animals at 8mm being less than a
857 year old and the largest animals at around 100mm being 18 years or older.

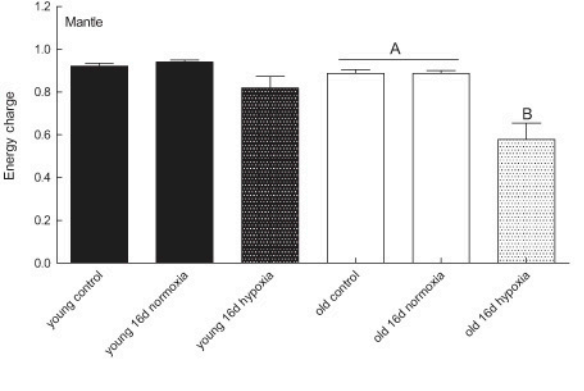
858

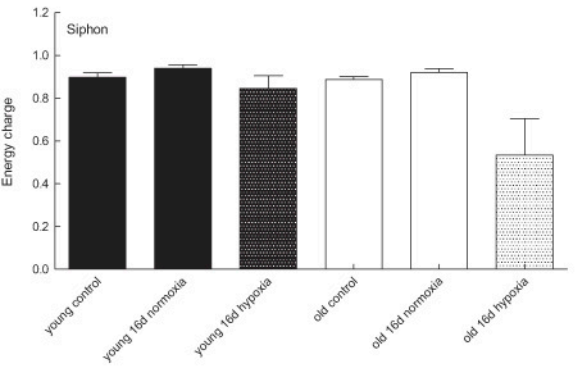
	Tissue	Young		Older	
		Mean	**SEM**	**Mean**	**SEM**
NAD	Mantle	**189.10^{A}***	3.49	**150.30^{A}***	6.50
nmol*gwwt	Siphon	**214.30^{B}***	10.85	**169.60^{B}***	7.89
	Gills	158.40	36.99	142.50	8.51
NADH	Mantle	11.70	1.55	9.81	0.71
nmol*gwwt	Siphon	19.72	3.85	11.24	3.98
	Gills	8.94	0.89	8.41	1.80
	Mantle	22.80	7.40	15.46	0.74
NAD/NADH	Siphon	13.24	2.534	20.73	5.628
	Gills	20.29	5.54	21.17	6.79
NADP	Mantle	**32.48^{A}***	2.01	**24.62^{A}***	1.78
nmol*gwwt	Siphon	26.09	2.81	27.26	3.89
	Gills	**94.96^{B}**	5.01	**81.82^{B}**	6.09
NADPH	Mantle	10.84	0.83	6.27	1.76
nmol*gwwt	Siphon	8.50	2.68	11.46	3.11
	Gills	6.61	1.15	18.58	6.25
	Mantle	**3.09^{A}**	0.31	4.88	1.23
NADP/NADPH	Siphon	8.718	5.088	3.545	1.569
	Gills	**16.05^{B}**	2.02	7.22	3.35

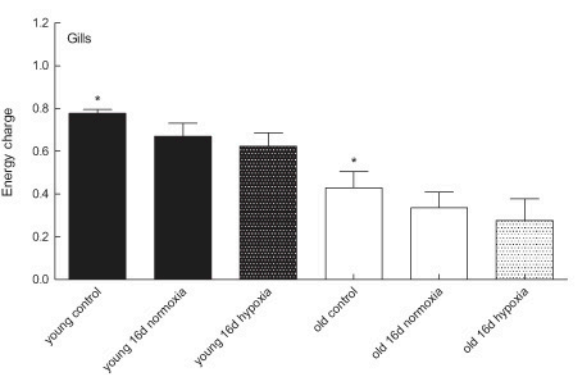
Table 1: Nicotinamide nucleotide concentration (nmol*gram wet weight) and ratios (NAD/NADH; NADP/NADPH) in the mantle, siphon and gill tissue of the control individuals of the younger and older animals. Different letters between tissues within one age group and parameters indicate significant differences (non-parametric one-way ANOVA, $p<0.05$). * indicate differences between younger and older individuals within one parameter (Mann-Whitney U test). N = 5-6 (younger individuals) or 4 (older individuals).

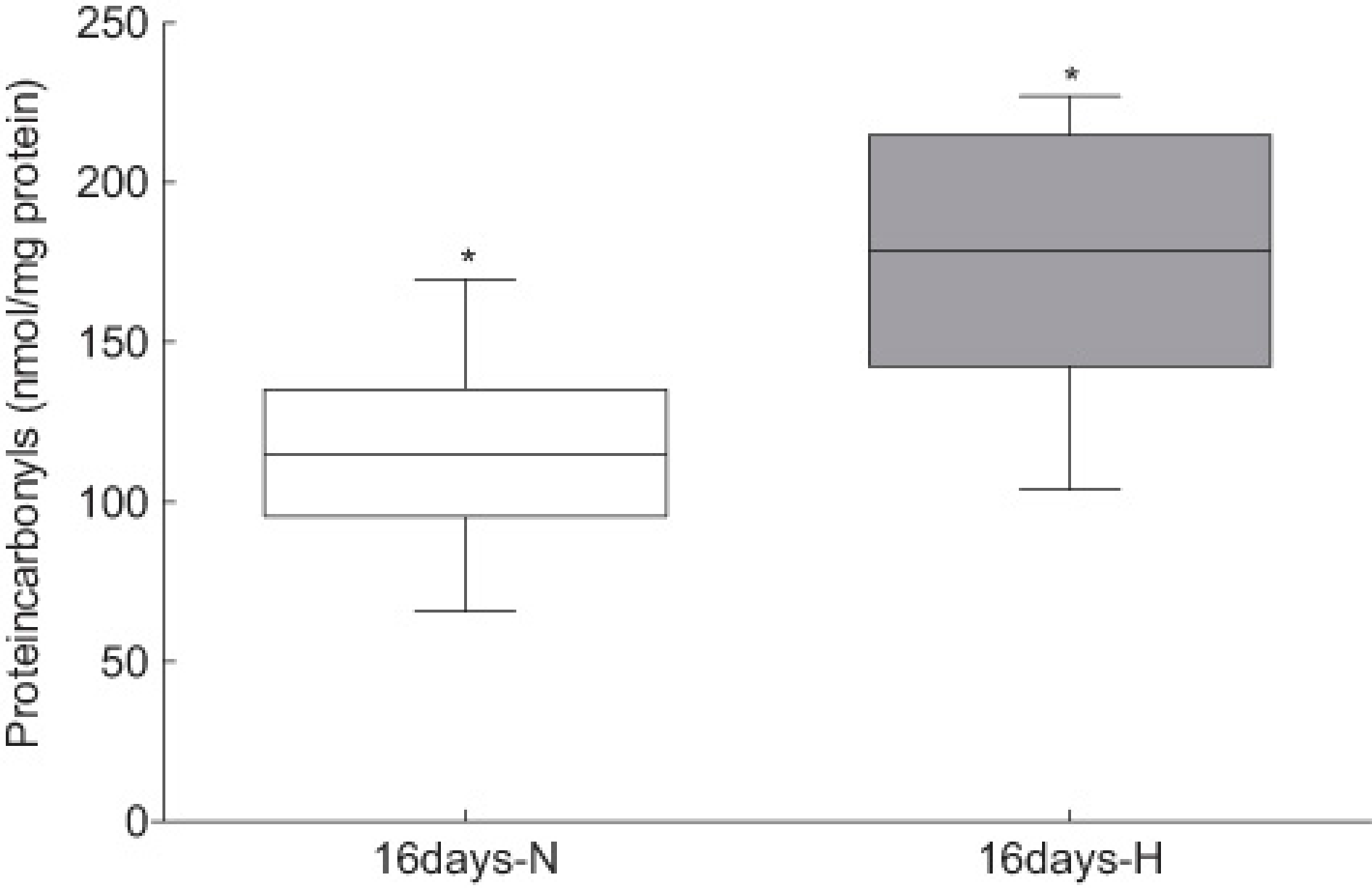
Effect of environmental condition					
	Siphon		**Gill**		
	Older	**Younger**	**Older**	**Younger**	
Upregulated in normoxia	36 (14)	8 (1)	43 (6)	9 (7)	
No change	5057	5091	5098	5117	
Upregulated in hypoxia	40 (11)	34 (15)	75 (25)	90 (31)	
Total differentially expressed	**76 (25)**	**42 (16)**	**118 (36)**	**99 (38)**	**335 (115)**
Effect of age					
	Siphon		**Gill**		
	Hypoxia	**Normoxia**	**Hypoxia**	**Normoxia**	
Upregulated in younger	165 (34)	145 (15)	12 (3)	8 (6)	
No change	4893	4835	5191	5163	
Upregulated in older	75 (8)	153 (9)	13 (6)	45 (1)	
Total differentially expressed	**240 (34)**	**298 (49)**	**25 (9)**	**53 (7)**	**616 (104)**

Table 2: Summary of transcripts differentially expressed in the microarray experiments. Results are partitioned into the effect of environmental condition and age. Numbers in brackets indicate the number of transcripts that showed a significant match on Blast sequence similarity searching to genes in other species with ascribed functions.


Mantle
Energy charge
1.2
1.0
0.8
0.6
0.4
0.2
0.0
A
B
young control
young 16d normoxia
young 16d hypoxia
old control
old 16d normoxia
old 16d hypoxia


Siphon
Energy charge
1.2
1.0
0.8
0.6
0.4
0.2
0.0
young control
young 16d normoxia
young 16d hypoxia
old control
old 16d normoxia
old 16d hypoxia


Gills
Energy charge
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
young control
young 16d normoxia
young 16d hypoxia
old control
old 16d normoxia
old 16d hypoxia


250
200
150
100
50
0
Proteincarbonyls (nmol/mg protein)
*
*
16days-N
16days-H

HYPOXIA

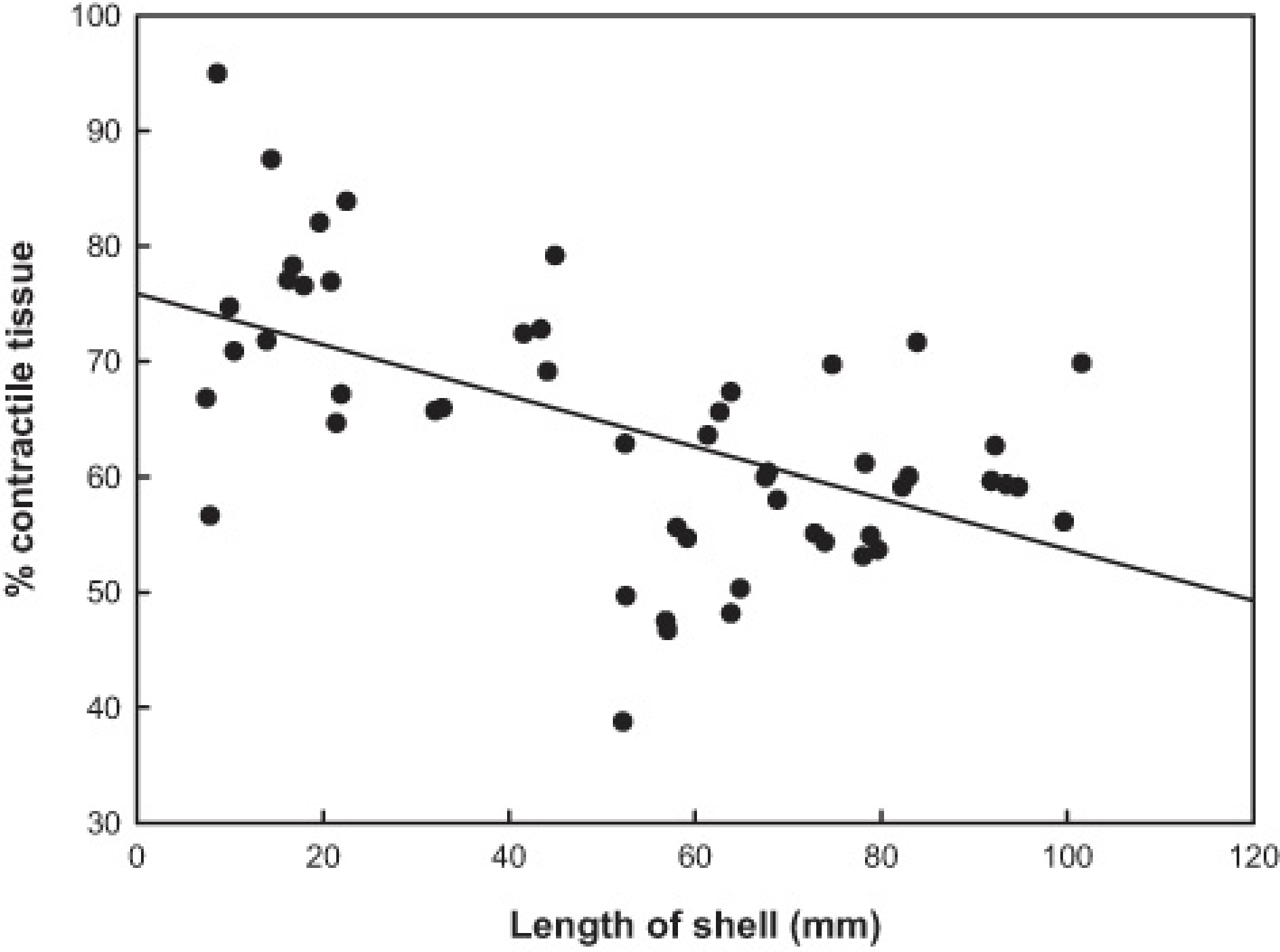
ANTIOXIDANTS
IMMUNE SYSTEM
UNFOLDED PROTEIN RESPONSE

YOUNG

CYTOSKELETON & MUSCLE
FUNCTION
ENERGY PRODUCTION

OLD

IMMUNE SYSTEM


100
90
80
70
60
50
40
30
% contractile tissue
0
20
40
60
80
100
120
Length of shell (mm)

Gene ID	Clone ID	Primer sequence	RSq	Efficiency
Arginine kinase	Le_A01_07C03 - F	GACGCCGTCACGGAGATGATGAAC	0.997	108.3%
	Le_A01_07C03 - R	AAAGGCTGCCTCCTCTAAACCCGT		
Quinone reductase	Le_A01_08G05 - F	TCCCCTCCCTGACAGTCTGACCTT	0.997	104.0%
	Le_A01_08G05 - R	TGACGCTCCGAGGGAGGTTACAAG		
NF-kappa-B	Le_A01_11H12 - F	ATTGAACCGGACGAAGACGGGGAT	0.993	106.8%
	Le_A01_11H12 - R	TAAGCTGGCCCCTGCACAGATCAA		
Isocitrate dehydrogenase	Le_A03_01A11 - F	CATCAAGTGTGCCACCATCACCCC	0.979	96.1%
	Le_A03_01A11 - R	CCGAAAGCGTGACGACCAATGACA		
ATP synthase	Le_A03_03B11 - F	CACTGAGGGAGGAGTACCTCGTGA	0.999	118.6%
	Le_A03_03B11 - R	TGTTCCCACTGCAAGGTGCTTCAA		
Tropomyosin	Le_A03_28G03 - F	AAACATTCGCTGAACTGGCTGGCT	0.997	118.0%
	Le_A03_28G03 - R	ATGTCGACAGCAAGAAAAGGGCGG		
Control	Le_A02_24A10 - F	GCCCGAGGTCAGAAAAGCTCAACG	0.997	107.3%
	Le_A02_24A10 - R	TTTATCGTTTGCCACCGACACGGG		

Supplemental Table S1: Primer sequences used in Q-PCR validation.

	Tissue	Young		Old	
		Mean	**SEM**	**Mean**	**SEM**
ATP	Mantle	2.24	0.08	1.76	0.20
µmol*gwwt	Siphon	**3.41^{A}**	0.30	**2.67^{A}**	0.16
	Gills	**1.19^{B}**	0.04	**0.40^{B}**	0.12
ADP	Mantle	**0.28^{A}**	0.02	0.34	0.04
µmol*gwwt	Siphon	**0.56^{B}**	0.08	0.57	0.08
	Gills	0.36	0.04	0.34	0.03
AMP	Mantle	**0.06^{A}**	0.02	**0.07^{A}**	0.01
µmol*gwwt	Siphon	0.13	0.04	0.10	0.02
	Gills	**0.22^{B}***	0.02	**0.59^{B}***	0.13
Adenylate pool	Mantle	2.57	0.07	2.17	0.19
µmol*gwwt	Siphon	**4.09^{A}**	0.28	**3.33^{A}**	0.19
	Gills	**1.77^{B}***	0.05	**1.32^{B}***	0.11
AEC	Mantle	**0.92^{A}**	0.01	**0.89^{A}**	0.02
	Siphon	0.90	0.02	0.89	0.01
	Gills	**0.78^{B}***	0.02	**0.43^{B}***	0.08
ATP:AMP ratio					
	Mantle	37.0		25.0	
	Siphon	26.0		26.7	
	Gills	5.4		0.7	

Supplemental Table S2: Adenylate concentrations (µmol*gram wet weight) and energy charge (EC) in mantle, siphon and gill tissue of young and old *L. elliptica* control individuals. Different letters between tissues within one age group and parameter mark significant differences (non-parametric one-way ANOVA, $p<0.05$). N = 6 (young individuals) or 4 (old individuals). * marks differences between young and old individuals of the hypoxia experiment within one parameter (Mann-Whitney U test). The ATP:AMP ratios are also given, these are calculated from the mean values.

	Treatment	Young			Old		
Mantle		**N**	**Mean**	**SEM**	**N**	**Mean**	**SEM**
ATP	Normoxia	5	2.18	0.11	5	**1.81^{A}**	0.16
µmol*gwwt	Hypoxia	5	1.71	0.29	5	**0.60^{B}**	0.16
ADP	Normoxia	5	0.25	0.03	5	**0.32^{A}**	0.03
µmol*gwwt	Hypoxia	5	0.48	0.11	5	**0.54^{B}**	0.04
AMP	Normoxia	5	**0.03^{A}**	0.00	5	**0.08^{A}**	0.01
µmol*gwwt	Hypoxia	5	**0.16^{B}**	0.07	5	**0.32^{B}**	0.07
Adenylate pool	Normoxia	5	2.46	0.08	5	**2.22^{A}**	0.13
µmol*gwwt	Hypoxia	5	2.36	0.16	5	**1.45^{B}**	0.11
Siphon		**N**	**Mean**	**SEM**	**N**	**Mean**	**SEM**
ATP	Normoxia	4	3.45	0.47	5	2.53	0.32
µmol*gwwt	Hypoxia	5	3.21	0.51	4	1.17	0.66
ADP	Normoxia	4	0.29	0.03	5	0.35	0.05
µmol*gwwt	Hypoxia	5	0.90	0.32	4	0.72	0.22
AMP	Normoxia	4	0.07	0.04	5	0.05	0.02
µmol*gwwt	Hypoxia	5	0.19	0.14	4	0.87	0.42
Adenylate pool	Normoxia	4	3.82	0.47	5	2.92	0.29
µmol*gwwt	Hypoxia	5	4.30	0.20	4	2.76	0.31
Gill		**N**	**Mean**	**SEM**	**N**	**Mean**	**SEM**
ATP	Normoxia	5	0.80	0.09	5	0.27	0.07
µmol*gwwt	Hypoxia	5	0.72	0.19	4	0.13	0.08
ADP	Normoxia	5	0.34	0.04	5	0.29	0.04
µmol*gwwt	Hypoxia	5	0.39	0.05	4	0.28	0.07
AMP	Normoxia	5	0.33	0.09	5	0.69	0.12
µmol*gwwt	Hypoxia	5	0.32	0.07	4	0.52	0.13
Adenylate pool	Normoxia	5	1.47	0.11	5	1.24	0.05
µmol*gwwt	Hypoxia	5	1.43	0.19	4	0.92	0.15

Supplemental Table S3: Adenylate concentrations (µmol*gram wet weight) in mantle, siphon and gill tissue of young and old *L. elliptica* individuals incubated for 16 days under normoxic (16 days-N) or hypoxic (16 days_H; 2% O_2) conditions. Different letters between treatments within one age group mark significant differences (non-parametric one-way ANOVA; $p<0.05$).

	Treatment	Young			Old		
		N	Mean	SEM	N	Mean	SEM
Mantle							
	controls	5	189.10	3.49	4	150.30	6.50
NAD	16 days-N	5	186.80	5.40	5	160.90	4.07
nmol*gwwt	16 days-H	5	200.80	12.39	6	131.00	14.65
	controls	5	11.70	1.55	4	9.81	0.71
NADH	16 days-N	5	11.12	1.48	5	10.44	1.77
nmol*gwwt	16 days-H	4	13.39	0.71	6	8.56	1.31
	controls	5	22.80	7.40	4	15.46	0.74
NAD/NADH	16 days-N	5	17.99	2.37	5	16.95	2.48
	16 days-H	4	15.87	0.34	6	17.58	3.90
	controls	6	32.48	2.01	4	24.62	1.78
NADP	16 days-N	5	27.48	2.17	5	22.47	2.15
nmol*gwwt	16 days-H	5	26.29	1.59	6	19.92	2.60
	controls	6	10.84	0.83	4	6.27	1.76
NADPH	16 days-N	5	15.37	1.99	5	9.64	2.13
nmol*gwwt	16 days-H	5	9.13	1.72	6	8.60	2.77
	controls	6	3.09	0.31	4	4.88	1.23
NADP/NADPH	16 days-N	5	1.97	0.42	5	2.79	0.56
	16 days-H	5	4.02	1.61	6	7.46	3.66
Siphon							
	controls	6	214.30	10.85	4	169.60	7.89
NAD	16 days-N	5	199.40	47.07	5	148.00	15.88
nmol*gwwt	16 days-H	5	251.20	20.88	5	173.00	17.85
	controls	6	19.72	3.85	4	11.24	3.98
NADH	16 days-N	5	14.56	1.02	5	16.52	3.01
nmol*gwwt	16 days-H	5	16.57	3.62	5	14.05	1.98
	controls	6	13.24	2.534	4	20.73	5.628
NAD/NADH	16 days-N	5	14.75	4.28	5	9.842	1.593
	16 days-H	5	25.21	12.79	5	14.12	3.627
	controls	6	26.09	2.81	4	27.26	3.89
NADP	16 days-N	5	24.15	3.44	5	20.33	1.88
nmol*gwwt	16 days-H	5	25.19	3.70	5	21.06	1.07
	controls	6	8.50	2.68	4	11.46	3.11
NADPH	16 days-N	5	9.18	4.98	5	17.54	2.02
nmol*gwwt	16 days-H	5	11.01	3.57	5	8.22	2.62
	controls	6	8.718	5.088	4	3.545	1.569
NADP/NADPH	16 days-N	4	6.985	4.2	5	1.202	0.1491
	16 days-H	5	5.33	3.174	5	11.77	9.066
Gill							
	controls	6	158.40	36.99	4	142.50	8.51
NAD	16 days-N	5	131.70	13.44	5	102.90	10.59
nmol*gwwt	16 days-H	5	91.03	20.34	6	91.26	19.02

	controls	6	8.94	0.89	4	8.41	1.80
NADH	16 days-N	5	5.78	1.36	5	5.69	0.84
nmol*gwwt	16 days-H	5	9.27	1.28	6	5.45	0.92
	controls	6	20.29	5.54	4	21.17	6.79
NAD/NADH	16 days-N	4	20.18	3.81	5	20.35	4.19
	16 days-H	5	12.50	5.24	6	21.33	6.42
	controls	6	94.96	5.01	4	81.82	6.09
NADP	16 days-N	5	95.67	17.22	5	58.67	4.32
nmol*gwwt	16 days-H	5	74.74	15.25	6	39.35	5.35
	controls	6	6.61	1.15	4	18.58	6.25
NADPH	16 days-N	5	16.57	5.22	5	20.34	4.59
nmol*gwwt	16 days-H	5	12.64	2.64	6	14.71	4.43
	controls	6	16.05	2.02	4	7.22	3.35
NADP/NADPH	16 days-N	5	7.47	2.00	5	3.35	0.56
	16 days-H	5	10.50	5.94	6	4.28	1.44

Supplemental Table S4 : Nicotinamide nucleotide concentration (nmol*gram wet weight) and ratios (NAD/NADH; NADP/NADPH) in the mantle, siphon and gill tissue of young and old *L. elliptica* individuals incubated under normoxic, hypoxic (2% O_2) and anoxic conditions.

Gene	Age	p-value	Relative fold increase in gene expression	Range
HSP70A	Old	0.058	+3.962	0.877-17.893
HSP70B		0.174	+10.769	1.008-115.034
HSP70A	Young	0.761	+1.194	0.495-2.881
HSP70B		**0.004**	+5.530	1.966-15.554

Supplemental Table S5: Changes in HSP expression in gill tissue of younger and older individuals incubated for 16 days under hypoxic or normoxic condition. N = 6 per group.

Signature clone	Other clones	Putative ID	Accession Number	Expect value
Le_A01_04B04		Tenascin	Q0O546	$2.0e^{-49}$
Le_A01_08A11		Thioredoxin peroxidase (peroxiredoxin)	P0CB50	$4.0e^{-25}$
Le_A01_08B07		AP-1protein	P54864	$1.0e^{-12}$
Le_A01_08G05		Quinone reductase	O97764	$2.0e^{-65}$
Le_A01_10D07	Le_A01_13A03	Probable chaperone (HSP31)	Q04432	$1.0e^{-27}$
Le_A01_11H12		NF-kappa-B inhibitor	Q91974	$1.0e^{-25}$
Le_A01_18H12		Translation elongation factor 2	Q96X45	$3.0e^{-16}$
Le_A01_19H12		Myosin	P05945	$3.0e^{-50}$
Le_A02_04E04	Le_A02_04F02	Peptidyl-prolyl cis-trans isomerase	Q7Q1V1	$4.0\ e^{-47}$
Le_A02_05B08		Similar to tissue-type plasminogen	Q28198	$5.0e^{-14}$
Le_A02_10A11		B cell translocation gene	Q63073	$1.0e^{-30}$
Le_A02_21A07	Le_A02_24A09; Le_A02_27C05; Le_A02_30B05; Le_A03_09G10 Le_A03_31E10	Fucolectin	Q91927	$2.0e^{-04}$
Le_A02_21A01		Cadherin	A9U1A7	$1.0e^{-05}$
Le_A02_28A08		Adioponectin	F0V477	$3.\ 0e^{-10}$
Le_A02_35F01		PCK2	F6SMX0	0.0
Le_A03_16A06		Regulator of lipid storage	A9YVJ0	$1.0e^{-49}$
Le_A03_24H10		G-protein coupled receptor family 1	Q0MUS4	$7.0e^{-09}$
Matches to uncharacterised proteins		Le_A01_01A05; Le_A01_05D06; Le_A02_11D05;Le_ A03_21H02		

Supplemental Table S6 Clones with putatively ascribed functions identified in gill tissue from older animals under hypoxic conditions.

Signature clone	Other clones	Putative ID	Accession Number	Expect value
Le_A01_03G06	Le_A01_12A05	Ubiquitin	P0CG71	$1.0e^{-105}$
Le_A01_06H06		Thioredoxin peroxidase	P0CB50	$4.0e^{-25}$
Le_A01_06H11	Le_A01_18F06	Mnk	Q27SZ8	$1.0e^{-164}$
Le_A01_08G05		Quinone reductase	O97764	$2.0e^{-65}$
Le_A01_10D07	Le_A01_13A03	Probable chaperone (HSP31)	Q04432	$1.0e^{-27}$
Le_A01_11H12		NF-kappa-B inhibitor	Q91974	$1.0e^{-25}$
Le_A01_13A05	Le_A02_31E06; Le_A03_18F10	Glutathione-s -transferase	Q9CPU4	$2.0e^{-29}$
Le_A02_05B08		Similar to tissue-type plasminogen	Q28198	$5.0e^{-14}$
Le_A02_10A11		B cell translocation gene	Q63073	$1.0e^{-30}$
Le_A02_18C12	Le_A02_24A09; Le_A02_27C05; Le_A02_30B05; Le_A03_09G10	Fucolectin	Q91927	$2.0e^{-04}$
Le_A02_30A12	Le_A02_30C01	Tyrosinase	Q19673	$3.0e^{-20}$
Le_A02_35F01		PCK2	F6SMX0	0.0
Le_A03_13H07		Skeletrophin	B7P3H6	$5.0\ e^{-15}$
Le_A03_16A06		Adipocyte differentiation-related protein	A9YVJ0	$1.0\ e^{-49}$
Le_A03_22H09	Le_A03_27A05	Thioester-containing protein	D5FT49	$3.0e^{-41}$
Le_A03_24H10		G-protein coupled receptor family 1	Q0MUS4	$7.0e^{-09}$

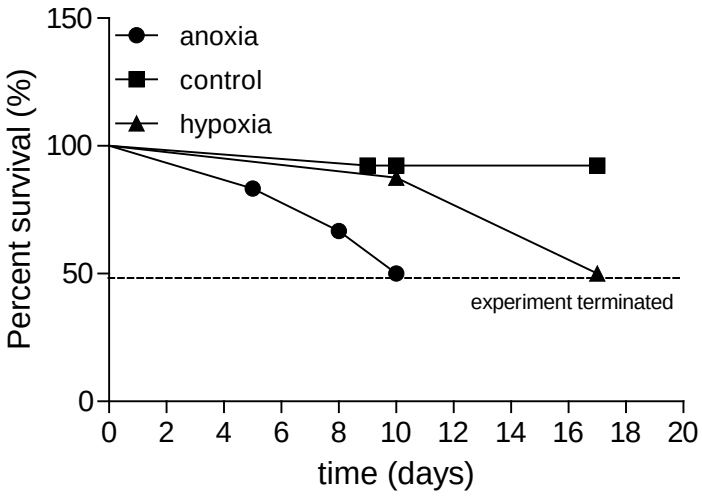
Supplemental Table S7: Clones with putatively ascribed functions identified in gill tissue from young animals under hypoxic conditions.

Signature clone	Other clones	Putative ID	Accession Number	Expect value
Le_A01_06B09		Dynein light chain	Q78P75	$8.0e^{-45}$
Le_A01_21F01		Autophagy-related protein	A5A6N3	$1.0e^{-19}$
Le_A02_04A03	Le_A02_05F11; Le_A02_34G09	Calponin	Q966V3	$5.0e^{-22}$
Le_A02_12G02	Le_A02_14A08; Le_A0319D10	PIF (aragonite binding protein)	C7G0B5	$2.0e^{-36}$
Le_A02_17E09	Le_A02_29A05; Le_A02_32H04; Le_A03_10D04; Le_A03_17F08; Le_A03_23A03; Le_A03_24A08; Le_A03_26H06; Le_A03_33H02	Myosin	P05945	$3.0e^{-50}$
Le_A02_20D04	Le_A02_21H08; Le_A03_03H04; Le_A03_14H03; Le_A03_14G05; Le_A03_28G03; Le_A03_28G08	Tropomysin	Q9GZ71	$3.0e^{-40}$
Le_A03_13A01	A03_27F07	Actin	Q7ZZZ0	$3.0e^{-10}$
Le_A03_33C04		LIM protein	Q2XT33	$4.0e^{-68}$
Le_A03_01A11	A03_17F04	Isocitrate dehydrogenase	Q5QGY7	$2.0e^{-84}$
Le_A01_07C03	A03_30D08	Arginine kinase	Q8N0P4	$5.0e^{-07}$
Le_A03_03B11	A03_17F04	ATP synthase	P19483	$3.0e^{-16}$
Matches to uncharacterised proteins		Le_A03_06F07		

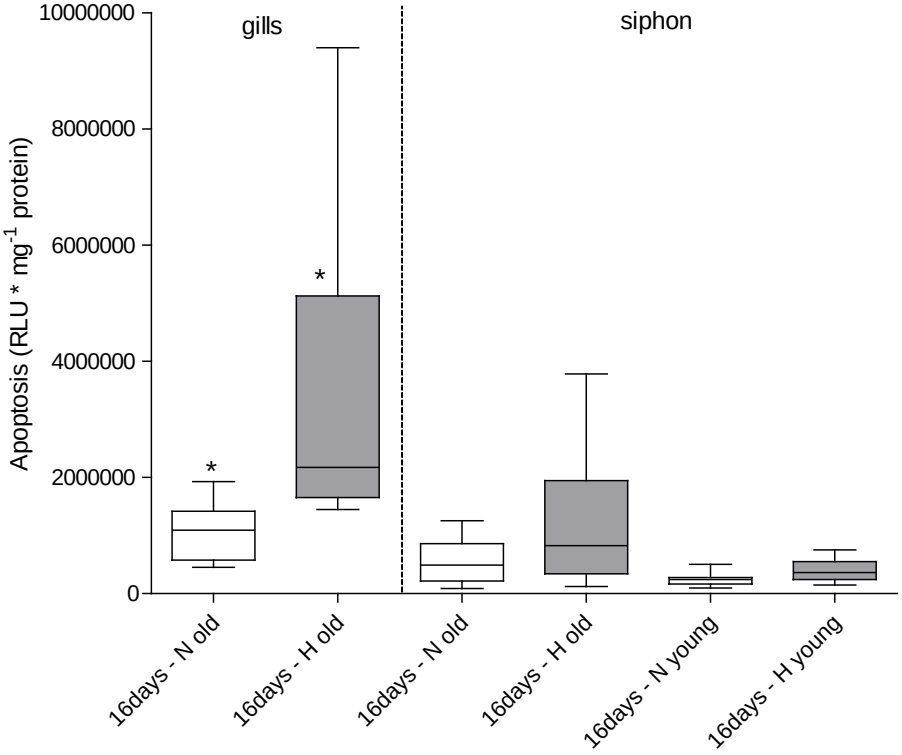
Supplemental Table S8: Clones with putatively ascribed functions identified in siphon tissue from young animals under hypoxic conditions.

	Intercept	(±SE)	Slope	(±SE)	R2	F	DF	P
Whole animal	-13.7	(0.37)	+3.68	(0.10)	0.97	1422	51	<0.0001
Contractile tissue	-13.7	(0.38)	+3.55	(0.10)	0.96	1306	51	<0.0001
Shells								
height	-1.76	(0.11)	+1.06	(0.03)	0.97	1375	44	<0.0001
width	-0.53	(0.07)	+1.03	(0.02)	0.99	3282	44	<0.0001
Tissue								
Mantle	-15.1	(0.34)	+3.41	(0.09)	0.97	1497	51	<0.0001
Siphon	-14.3	(0.32)	+3.62	(0.08)	0.97	1871	51	<0.0001
Adductor	-15.3	(0.39)	+3.35	(0.10)	0.96	1089	51	<0.0001
Gill	-12.0	(1.34)	+2.51	(0.32)	0.63	62	36	<0.0001
Foot	-14.6	(0.34)	+2.90	(0.09)	0.96	1110	51	<0.0001
The rest	-15.8	(0.37)	+3.91	(0.10)	0.97	1595	51	<0.0001

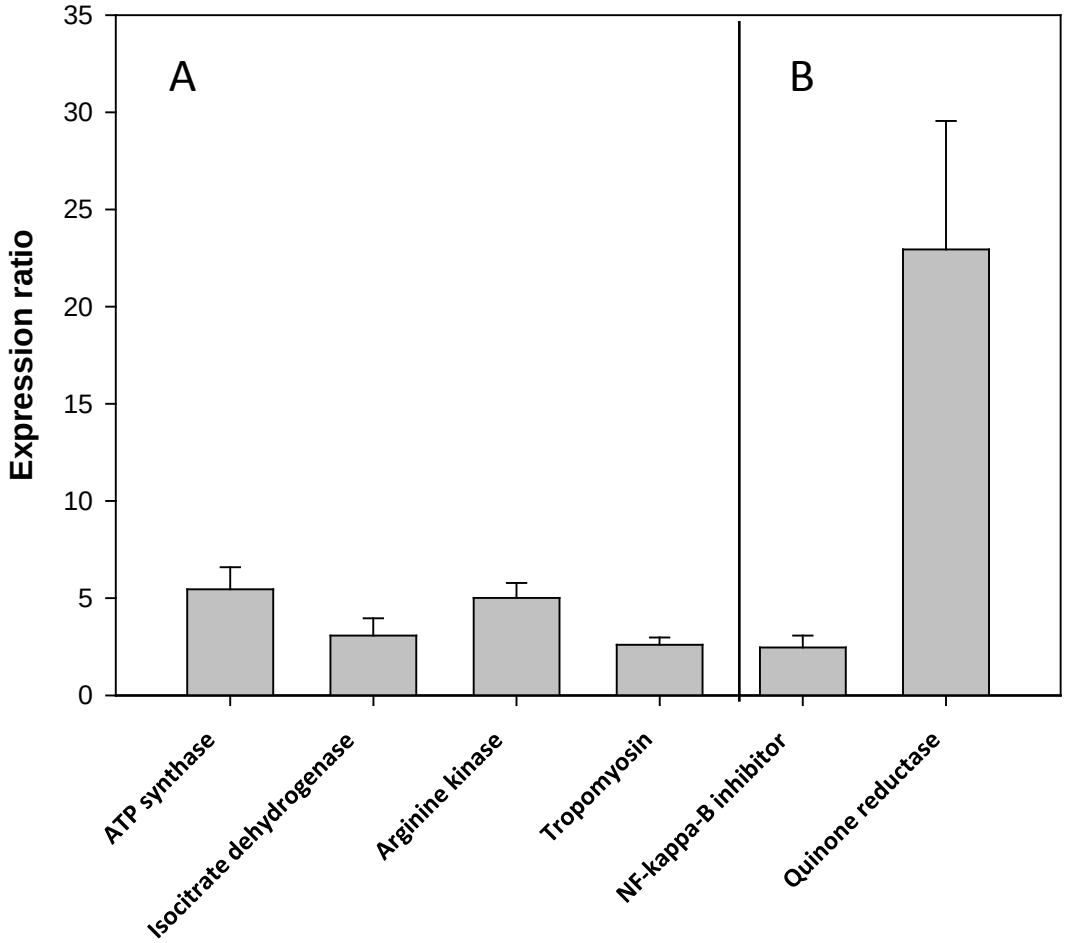
Supplemental Table S9: Regression data for the shell and tissue scaling in *L. elliptica.* All measurements were converted to natural logs and compared with Ln_length.



Supplemental Figure S1: Survival curves of *L. elliptica* incubated under normoxic, hypoxic (2% O_2) and anoxic (0% O_2) conditions. LT_{50}: 10days for anoxia and 17days for hypoxia. N=6 for anoxia, 8 animals for hypoxia and 12 for normoxia.



Supplemental Figure S2: Apoptotic activity in gill tissue of older *L. elliptica* individuals incubated for 16 days under normoxic (16 days N) or hypoxic (16 days H; 2% O_2) conditions and siphon tissue of older and younger individuals under the same treatment. * indicate significant differences ($p<0.05$, Mann-Witney t-test). N=8-12 per group.



Supplemental Figure S3: Q-PCR results showing relative gene expression in siphon tissue
A: younger versus older hypoxic animals, with up-regulation in young animals of all genes tested
B: Older hypoxic versus older normoxic animals, with the genes being up-regulated in hypoxic animals.