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Resistance to oxidative stress is not associated with the exceptional longevity of the freshwater pearl mussel, Margaritifera margaritifera nor three unionid species

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RESEARCH ARTICLE

Resistance to oxidative stress is not associated with the exceptional longevity of the freshwater pearl mussel, *Margaritifera margaritifera* nor three unionid species

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Abstract Bivalve molluscs are newly discovered models of successful ageing. Here, we test the hypothesis that extreme longevity of freshwater mussels is associated with an enhanced resistance to oxidative stress. We assess whether resistance to oxidative stress might be causally involved in the exceptional longevity exhibited by the freshwater pearl mussel *Margaritifera margaritifera*. We compared resistance to oxidative stress and total haemocyte counts, a health status biomarker in *M. margaritifera* (maximum lifespan potential 190 years) with three other freshwater bivalve species spanning a range of longevities. Previous studies of the comparative stress resistance and longevity of marine bivalves provide evidence for the hypothesis that an association exists between longevity and not only an enhanced resistance to oxidative stress but also a general resistance to multiplex stressors. We compared baseline total haemocyte counts, age-related changes, and responses to exposure to the oxidative stressor *tert*-butyl hydroperoxide (TBHP). Surprisingly our data does not support the premise that extreme longevity in *M. margaritifera* is associated with enhanced resistance to oxidative stress. In comparison with its shorter-lived counter parts *M. margaritifera* was the least resistance to oxidative stress. Following TBHP exposure, no association between longevity and resistance to oxidative stress-induced mortality nor a marked resistance to oxidative stress-induced declines in total haemocyte counts were observed. The results suggest longevity evolved separately in freshwater mussels and this group warrants further attention from biogerontologists because such study may provide novel insights not detected through the study of the marine members of the class, where most attention is currently focused.

Keywords *Margaritifera margaritifera* · Ageing · Oxidation · Longevity · Stress resistance

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Introduction

Bivalves have been subject to increasing attention from gerontologists over the past 5 years and this interest has been heightened since *Arctica islandica* was confirmed as the world's longest-lived non colonial animal known to science, with a lifespan of over 500 years (Butler et al. 2013). However, despite the recent attention, there has been scant attention focused on the ageing biology of the freshwater mussels, especially *Margaritifera margaritifera*, another of the world's longest-lived non-colonial animals (Bauer 1992). Depending on latitude and environmental conditions, *M. margaritifera* has a life span of between 100–200 years. Exhibiting a strong latitudinal trend in maximum lifespan the difference in maximum age varies 3–7 fold between Southern populations, with a maximum (A_{max}) of 28–40 years, and northern Arctic populations, with A_{max} of 114–190 years (Ziuganov 2004; Fernandez et al. 2009). Three major traits make bivalves ideal models for ageing research. Firstly, the class Bivalvia

contains more species with lifespans in excess of 100 years than any other animal group. Secondly, bivalves can be accurately aged through their annual shell growth rings (Neves and Moyer 1988)—enabling the relationship between physiological state and chronological age in wild populations to be established. Thirdly, bivalves are considered to be genetically intermediate between the classical invertebrate models of ageing (e.g. worms and flies) and mammals (Buttemer et al. 2010), providing an opportunity to understand the evolution of stress response pathways and ageing (Austad 2009; Philipp and Abele 2010).

The oxidative stress theory of ageing predicts long-lived animals will have less cumulative damage resulting from mismatches in the production of reactive oxidative species (ROS) and antioxidants and may also have structural characteristics that make them more resistant to oxidative damage (Harman 1956; Buttemer et al. 2010). Here we examine the hypothesis that exceptional longevity is associated with an enhanced resistance to oxidative stress as documented in marine bivalves (Ungvari et al. 2011a). Although the theory is much maligned, to the point that some authors now debate its demise (Pérez et al. 2009), it has a dominant status as a plausible mechanistic theory to explain variations in rates of ageing in different organisms. The strongest support for the oxidative stress theory of ageing is from comparisons of longevity and resistance to oxidative stress in phylogenetically diverse, shorter, and longer-living animals, including mammals (Kapahi et al. 1999), birds (Ogburn et al. 2001) and bivalves (Ungvari et al. 2011a).

A central tenet of the theory is that long-lived species should be more resistant to oxidative stress than their shorter-lived counterparts (Buttemer et al. 2010). In a seminal paper Ungvari et al. (2011a) documented that the exceptional longevity of *Arctica islandica* was associated with an enhanced resistance to stress, and further documented the extreme longevity of *A. islandica* is associated with a multi-stress resistance phenotype, not just oxidative stress (Ungvari et al. 2013).

In this paper we investigate the hitherto rarely studied subject of ageing in freshwater mussels, despite their well-documented longevity (Ziuganov 2004). We compare the resistance to oxidative stress of four species of freshwater mussels spanning a range of longevities, including the exceptionally long-lived *M. margaritifera.* We document the survival of the species following exposure to a powerful oxidative stressor and also changes in their total haemocyte count, a biomarker of health status (Galloway and Depledge 2001). We also document the basic demographics of each population investigated through basic catch curve analysis to provide crude estimations of maximum size, growth rate, longevity, and mortality.

Methods

Animal collection

Four field sites for sampling each of the species were chosen with the assistance of the Maine Department of Inland Fisheries and Wildlife, USA. All sites were non-polluted, clean flowing rivers in Maine, USA, which were deemed by the Department of Inland Fisheries and Wildlife to enable sustainable collections without harming the species' presence at that location. The four sites were the Sebasticook river at Waterville for *Lampsilis radiata* (44.57°, 69.56° W) (0.5–1 m depth), the Penobscot river in Orono for *Elliptio complanata* (44.93°, 68.64° W) (0.5–1 m depth), the Narraguagus River at Beddington for *Anodonta implicata* (44.81°, 68.03° W) (0.5–1 m depth) and the Sunkhaze stream for *Margaritifera margaritifera* [(44.99°, 68.49° W) (0.3–0.6 m) depth].

At each location an initial survey was undertaken to establish suitable sampling locations. To establish the population demographics at each location 100 individuals, covering the size range available, were collected, their shell length recorded and returned to the environment. A further 50 animals were removed and taken back to the aquaria at the University of Maine, Orono, for further studies. The shells from 30 of these animals were then sent back to the UK and aged using appropriate sclerochronological techniques (See Ridgway et al. 2012).

Measurements of the 100 live collected individuals of each species were undertaken to determine the population size frequency distribution. Shell height (maximum distance from umbo to the ventral margin) was measured to the nearest 0.01 mm with a digital vernier caliper and a size frequency distribution constructed. A selection of 20–30 animals of each species spanning a range of sizes from the collection were analysed to produce growth curves and von Bertalanffy parameters for the populations under investigation.

Age determination

The age and growth rate of the species were determined from acetate peels of the sectioned shells (see Richardson 2001). Clean, dry shells were embedded in resin and sectioned along the major growth axis using a rotating diamond saw. The cut surface was ground on increasingly finer grades of wet and dry paper, polished and then etched for 5 min in 0.1 M Hydrochloric acid. Acetate peel replicas were prepared using the methodology described by Richardson (1989) and Wanamaker et al. (2008) and viewed in a transmitted light microscope. The ages were estimated by counting the number of clear annual growth lines present in the hinge region of acetate peels of the shell sections and

Table 1 Estimations of parameters for each of the four species

	Youngest (years)	Oldest (years)	Longevity (years)	Mortality (Z)	L_∞ (mm)	K	OGP	Phi Prime
Margaritifera margaritifera	9	40	62.80	0.086	146.8	0.037	5.068	2.902
Elliptio complanata.	8	23	28.67	0.198	103.7	0.1053	5.070	3.054
Lampsilis radiata	8	26	34.25	0.1782	84.8	0.164	5.000	3.072
Anodonta implicata	12	16	17.6	0.260	99.4	0.34	5.524	3.526

The Von Bertalanffy growth function (VBGF) was fitted to the shell length-at-age data, and the growth constant (K) and asymptotic maximum shell length (L_∞) determined using the fisheries programme Fishparm. OGP and Phi prime were estimated from these. Longevity and Mortality were estimated via catch curve analysis

the population age structure constructed. For the purpose of this study, what is classified as old and young for each species is documented in Table 1.

Analysis of population demographics

The Von Bertalanffy growth function (VBGF) ($L_t = L_\infty (1^{-e-k}(t - t^0))$) was fitted to the shell length-at-age data, and the growth constant (K) and asymptotic maximum shell length (L_∞) determined using the fisheries program Fishparm (Prager et al. 1989). These parameters were then used to estimate the age of the 100 animals measured at the field locations. Due to the asymptotic growth nature displayed in bivalves, this method provides only a crude estimation of age. However, using small sub-samples of shells from each location allowed the estimation of the demographics of the populations without large-scale removal and death of many individuals that are required for accurate sclerochronological analysis of the shell. Large-scale collection of these freshwater mussels was not possible due to their conservation status. However, such methods are typically used by those studying the demographics of freshwater mussels (Hastie 2006).

Growth data estimated in this study were compared with each other and with data available from the literature. Instantaneous natural mortality rate (M) was estimated on the basis of age-frequency distributions (Ricker 1975). As each field site that was believed to have no history of commercial harvesting it was assumed that total mortality (Z) was equivalent to natural mortality. Z was estimated by calculating the slope of the regression between the natural log of the frequency at age data. The analysis was restricted on the descending right limb of the age-frequency curve. This is likely an artifact of size selectivity of the sampling and documented absence of young mussels in the environment. Longevity was also estimated through catch curve analysis, with the intercept on the X-axis providing a population level estimate of longevity. Longevity and mortality estimates were obtained from analysis of age frequencies based on ages estimated from length at age curves for each species, using the Von Bertalanffy Growth Function (VBGF).

Due to the non-linearity of the growth process, the comparison of growth amongst different organisms is often complex (Moura et al. 2009). To overcome this problem, several growth performance indices have been used, namely, the overall growth performance (P) and the growth performance index phi prime (φ') (Pauly 1979; Munro and Pauly 1983). The overall growth performance P ($P = \log(K \times L_\infty^3)$ and the phi prime index (φ) were used as a measure of growth performance:$-(\varphi' = \log K + 2 \times \log L_\infty)$, and used to compare growth data obtained during the present study with that obtained from the literature.

Resistance to oxidative stress

To assess resistance to oxidative stress the mussels were exposed to various concentrations of *Tert*-Butyl Hydroperoxide (TBHP) in freshwater (tap water) at ambient temperature (15 °C). An organic peroxide, TBHP is highly stable in aqueous solutions and documented to induce apoptosis in a wide variety of eukaryotic cells by damaging DNA, lipids, and proteins and is a useful tool to assess cellular oxidative stress resistance (Ungvari et al. 2011a). To study organismal resistance to oxidative stress, the survival of the four species of mussel exposed to 6×10^{-3} mol/L TBHP was recorded for 7 days. Death was recorded at the point the animals gaped, an indicator used in previous studies (Ungvari et al. 2011a, b, 2013). All comparisons of stress resistance were undertaken on animals estimated to be of the sexually mature young adult life history stage, around 25 % of their maximum lifespan potential (MLSP), which could be estimated through growth curve analysis. Death was recorded at the time the shell valves of the animal 'gaped'. Periodically, aquaria were checked to observe siphons, which indicated that the stressors were inhaled. Additionally to ensure infiltration of the stressor (TBHP) a section of the ventral margin of the shell of each species was carefully removed, minimizing damage to tissues, using a handheld rotary saw. This prevented the animals sealing themselves off from the external environment, which they can do for prolonged periods of time (Ungvari et al. 2013).

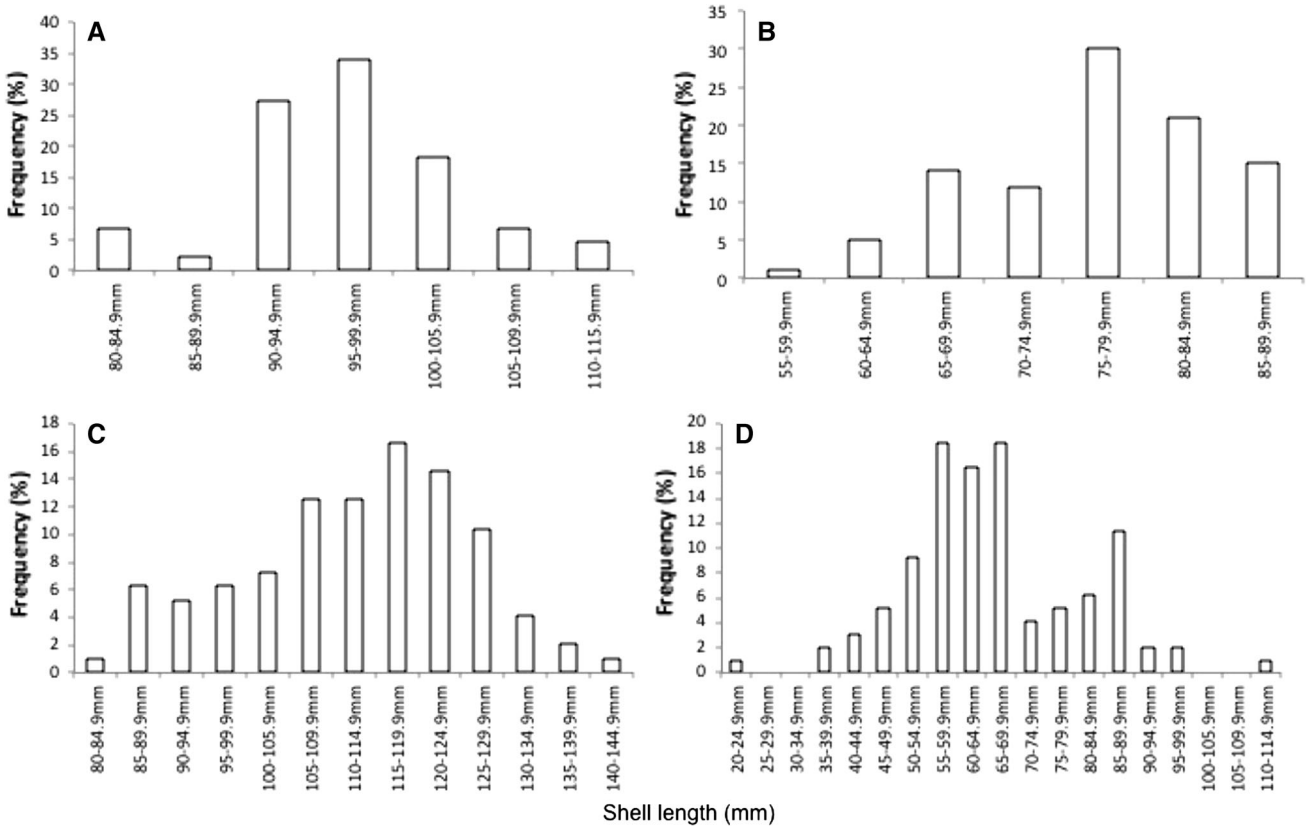

Fig. 1 Size distribution of the four species at each of the sampling locations: **a** *Anodonta implicata*, **b** *Lampsilis radiata*, **c** *Margaritifera margaritifera* and **d** *Elliptio complanata*

Analysis of immuno-competence

To assess the health status, total haemocyte count was analysed at baseline, following exposure to TBHP, and with age-related changes. Briefly, haemolymph was withdrawn from the adductor muscle haemolymph sinus (with a 25-gauge needle attached to a 1 ml syringe) and mixed with a formalin solution (1:3 10 % freshwater formalin [100 ml formalin (40 % aqueous solution of formaldehyde), 900 ml distilled water)]. Counting of haemocytes was performed using a haemocytometer (improved Neubauer counting chamber) and standard procedures (Baker et al. 1966). Prior to counting, the solution was briefly vortex-mixed to suspend the cells.

Data analysis

Statistical analyses of data were performed by Student's *t* test or by analysis of variance followed by the Tukey post hoc test, as appropriate. Survival curves were compared using the log-rank test, using GraphPad Prism 4.0 software. $p < 0.05$ was considered statistically significant. Data are expressed as mean ± SEM, unless otherwise indicated.

Results

Population demographics

Basic population demographics of each of the four species investigated are shown in Table 1. The size–frequency histograms for three of the species (Fig. 1) show a unimodal peak in shell height, and subsequent steep decline in size-class abundance. *Ellipito complanata* however demonstrates a bimodal peak in size class abundance. There was an absence of small individuals in all four species investigated.

The age of a sub-sample of 20–30 shells was determined and used to create a population von Bertalanffy (VBGF) growth curve that could then be used to estimate the age of a mussel solely from the size data of measured individuals. The growth curves and age at size data are illustrated in Fig. 2. Counting the number of internal growth increments provided age estimates ranging between 9 and 40 years old for *M. margaritifera*, between 12 and 16 years old for *Anodonta implicata*, between 8 and 26 years old for *Lycoris radiata* and between 8 and 23 years old for *E. complanata*. The population VBGF growth equation fitted using data from the annual internal growth increments provided

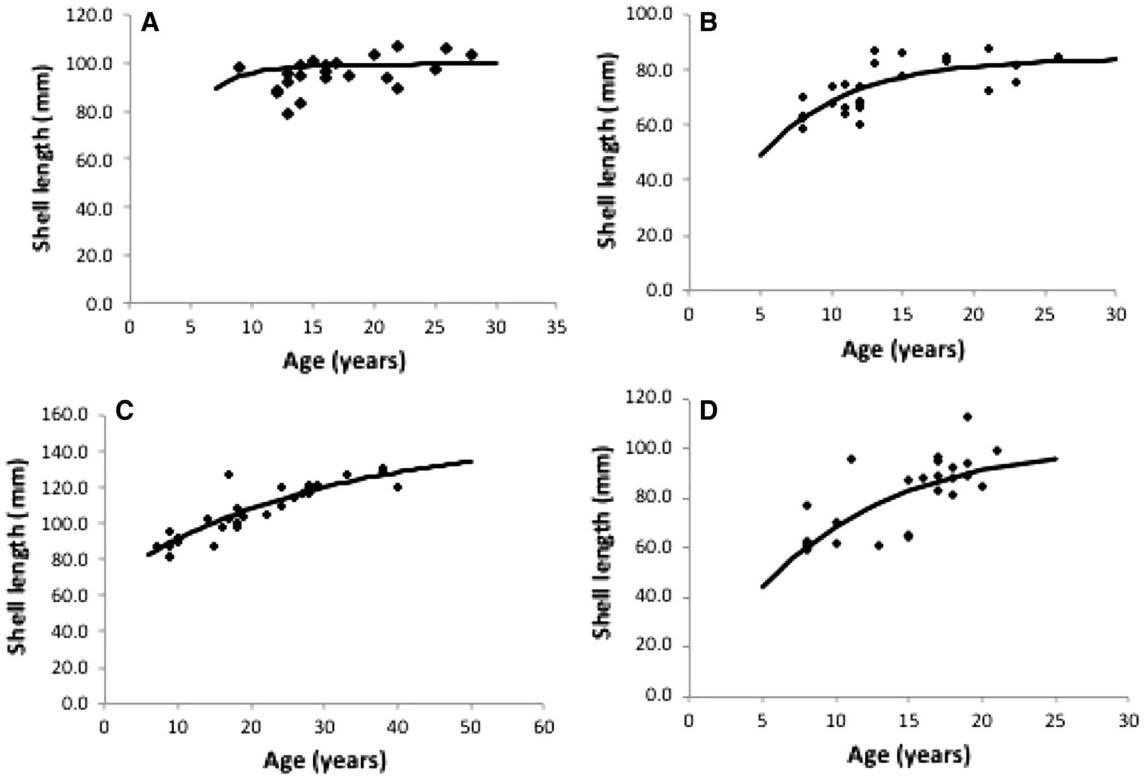

Fig. 2 Variation in maximum shell height with age for each species: **a** *Anodonta implicata*, **b** *Lampsilis radiata,* **c** *Margaritifera margaritifera* and **d** *Elliptio complanata.* The growth curve fitted using the Von Bertalanffy Growth Function

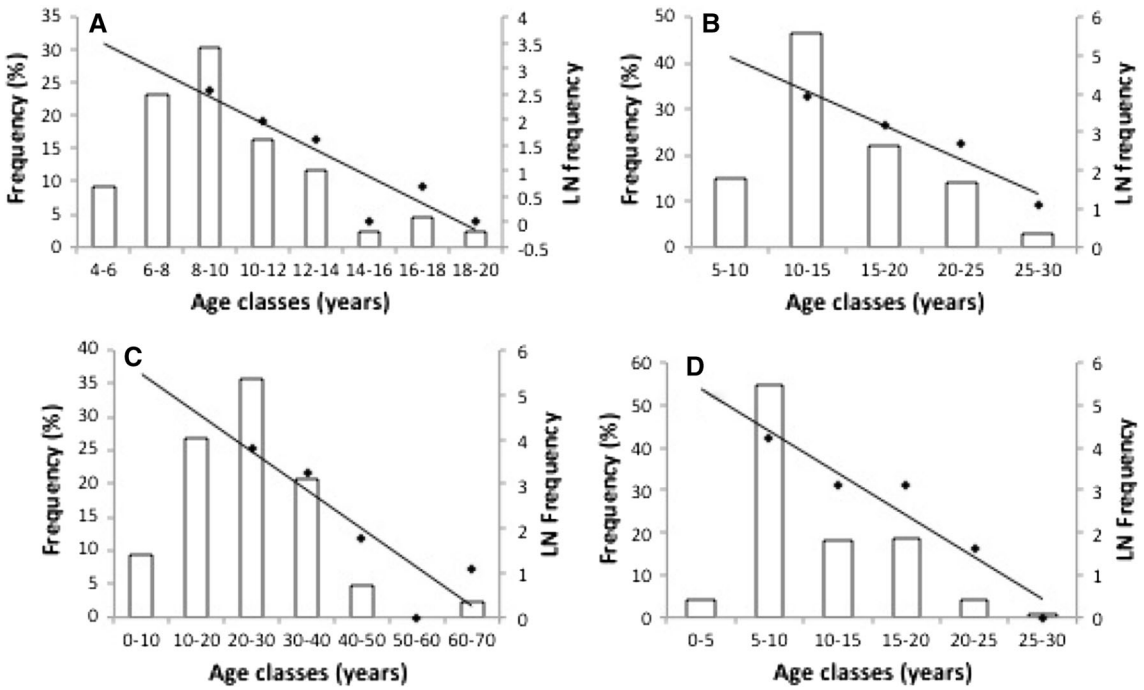

Fig. 3 Age frequency distribution for each species: **a** *Anodonta implicata* (n = 100), **b** *Lampsilis radiata* (n = 100), **c** *Margaritifera margaritifera* (n = 100) and **d** *Elliptio complanata* (n = 100). These graphs are based on the ages estimated from size, using the VBGF. Regression analysis of the LN frequency data provides estimations of Longevity (the X axis intercept) and mortality (the slope)

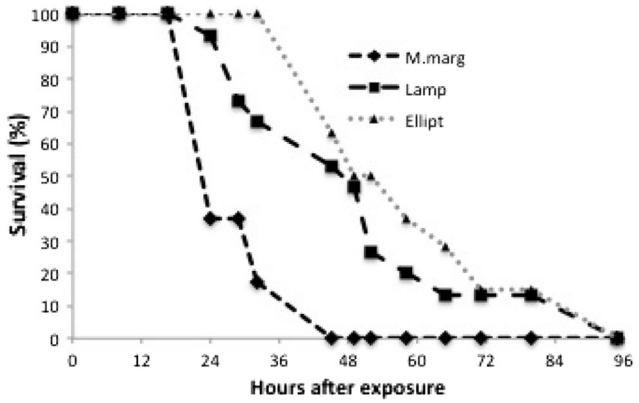

Fig. 4 Survival analysis of the three species *Lampsilis radiata, Margaritifera margaritifera and Elliptio complanata* exposed to 10^{-3} mol/L *tert*-butyl hydroperoxide (TBHP). Data pertaining to *Anodonta implicata* was eliminated for reasons explained in the text

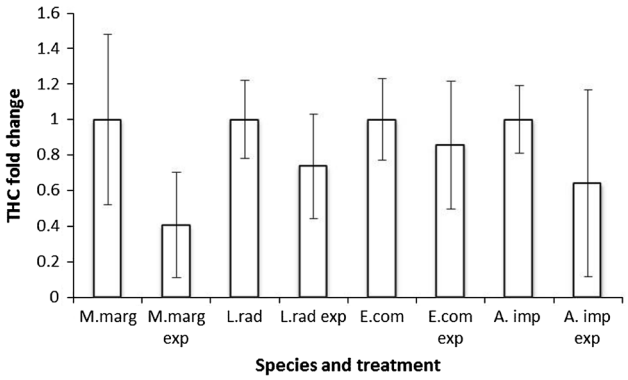

Fig. 5 Fold changes in total haemocyte counts between control unexposed animals and animals exposed overnight to 10^{-3} mol/L *tert*-butyl hydroperoxide (TBHP) for each of the four species

estimates of L_∞ and growth rate (K), which are listed for each species in Table 1. All four species had similar growth curves (Fig. 2) depicting a period of rapid growth after which the growth rate declined. *Margaritifera margaritifera* has the lowest growth rate (K, Table 1) and *A. implicata* the fastest. The parameters of the VBGF were then used to estimate the age of the 100 randomly sampled individuals from each population. Catch curve analysis (Fig. 3) from these individuals with their age-determined-from size, provided population level estimates of mortality and longevity for each species that are documented in Table 1.

Resistance to oxidative stress

To assess resistance to oxidative stress, we obtained survival curves of the clams in the presence of TBHP. Analysis of the survival curves revealed both *L. radiata* and *E. complanata* both survived significantly longer than *M. margaritifera* (Fig. 4). Unfortunately, the valves of *A. implicata* often did not 'gape' upon death. This was only realized part way through the investigation, so survival may have been over estimated as some individuals may have been counted as alive when in fact they were dead. For this reason data pertaining to *A. implicata* were not used in the comparison.

Analysis of immuno-competence

To assess the health status of the freshwater mussels following a short duration of a non-fatal exposure to TBHP we assessed total haemocyte count (THC) at baseline and following exposure. Results are presented in Fig. 5; Due to interspecific variability in THC we report findings as fold change (the ratio of the final value from the initial value) from baseline figures for each respective species. Following exposure we only observed significant declines in THC in *M. margaritifera* (F-test; $F_{1,10} = 7{,}855$, $p = 0.021$). No other significant differences were observed, however in all four species we did see a consistent trend in declining THC with oxidative stress. In all four species we also compared age related changes, comparing young and old of each species at baseline (data not presented) and no significant differences between age groups were observed.

Discussion

Contrary to our predictions based on the oxidative stress hypothesis of ageing the present study provides evidence suggesting resistance to oxidative stress and exceptional longevity are not associated in freshwater mussels. An observation in stark contrast to previous work on marine bivalves by Ungvari et al. (2011a). The current investigation presents preliminary data so further work is required to investigate whether the longer-lived *M. margaritifera* is more resistance to other forms of cellular stress, cytotoxic and genotoxic stressors, as has been documented in other animal groups (Harper et al. 2007; Salmon et al. 2009; Ungvari et al. 2011a). The absence of stable primary cell cultures for bivalves is also a stumbling block for further ageing research on this class of animals and future research efforts should concentrate on this problem. The use of THC as an in expensive non-fatal biomarker of oxidative stress was effective, however the results were obscured by large intra-species variability in THC; a typical problem with the measure (Ridgway et al. 2006). However, they accurately demonstrated, at chronic stress level, TBHP was still causing significant stress to *M. margaritifera.*

Ecologically these results may also explain the absence of the freshwater pearl mussel in polluted waters, particularly where the pollutants may be oxidative stressors such as heavy metals and pesticides. Young (1991) reviewed the status of the freshwater pearl mussel (*M.*

margaritifera) in the British Isles and Continental Europe. Despite its almost complete protection in most European countries this mussel continues to decline in numbers throughout its European range, apparently caused mainly by pollution (especially by organic enrichment). Although overfishing is also a concern, the increased organic load, which can occur in most rivers, contains many ultrafine particles (UFP) known to induce oxidative stress (Li et al. 2003). These UFP, heavy metals (Pytharopouloua et al. 2011) and pesticides (El-Shenawy et al. 2009) are all known to induce oxidative stress in animal tissues and will have resulted in significant levels of oxidative stress in the rivers of Western Europe and North America over the past 50 years and exasperate the decline of the freshwater pearl mussel throughout its range. Suspension-feeding sessile invertebrates, such as freshwater mussels, provide vital ecosystem functions, firstly in clearing the water of particulates and secondly providing further substrata as settlement surfaces and ecological niches to further increase biodiversity (Vaughn and Hakenkamp 2001). Therefore, if suitable water quality can be provided the species will further assist in the restoration of the impoverished biodiversity of the aquatic ecosystems of Western Europe and North America.

Estimates of L_{∞}, K, longevity and mortality for the four populations under investigation are also provided. Although comparisons between different species in differing environments are troublesome, documenting such life history traits for all populations studied, especially for a knowledge-sparse domain like freshwater mussels, enables meta-analysis of such traits to be undertaken in the future. In the present study we estimated the longevity of the *M. margaritifera* population to be around 60 years, this is less than the reported A_{max} for the species in the literature; however the population under investigation was sampled from a narrow, shallow stream in a temperate environment and the species is documented to exhibit strong latitudinal trends in lifespan (Bauer 1992). At the latitude of the river sampled in the present study (44.57°N) the reported species A_{max} is around 40–60 years (Bauer 1992), it is not until the higher latitudes of 60°N (Bauer 1992) or even the Arctic (66°N) (Ziuganov 2004) are the individuals older than 150 years found. Hastie (2006) reported typical modal age groups of 20-30 years for a number of rivers in Scotland, much wider than the river sampled in the present study. In freshwater populations a general pre-dominance of middle-aged individuals is generally apparent (Hastie 2006; Negus 1966).

Through internal shell analysis we provided estimates of longevity for *L. radiata* and *E. complanata*. Estimates of their longevity vary greatly. Previous studies using mark and recapture (Anthony et al. 2001) have indicated that *E. complanata* and species of the *Lampsilis* genera may live in excess of one century but Haag (2009) believes these to be overestimations of age. Our current work here, determining age through sclerochronological analysis, indicates both populations of each species had estimated longevities of around 25–30 years. Anthony et al. (2001) using mark-recapture growth data to estimate mussel age and estimated mean maximum ages of 149 years for *E. complanata* and 167 years for *Lampsilis siliquoidea*. These estimates are an order of magnitude higher than estimates of longevity for these species based on shell rings (Ghent et al. 1978; Grier 1922). Haag (2009) believes these estimations of extreme longevity are subject to multiple, additive sources of bias and therefore cannot be considered accurate representations of life span and furthermore cannot be used to conclude that traditional methods of bivalve ageing by interpretation of shell rings are flawed.

We also document an absence of small/young animals in all populations under investigation. Irregular recruitment into freshwater populations is well documented (Negus 1966), however there is also a gap in the literature with regard to the juvenile life stages of freshwater species between the glochidial larval stage and recruitment of the young adults into the population (Bauer 1987).

The oxidative stress theory of ageing predicts successfully ageing species have increased tolerance to oxidative stress-induced injury through superior cellular antioxidant defense mechanisms or increased elimination/repair of damaged macromolecules (Buttemer et al. 2010; Ungvari et al. 2011a). Although the theory is currently under significant scrutiny with some significant debate and the theory may, in fact, be defunct (see Pérez et al. 2009), it continues to be among the most commonly used mechanistic theories to explain variations in the rate of ageing. The strongest support for the oxidative stress theory of ageing is obtained from comparisons of longevity and resistance to oxidative stress in phylogenetically diverse, shorter, and longer-living mammals (Kapahi et al. 1999), birds (Ogburn et al. 2001) and bivalves (Ungvari et al. 2011a).

Whilst early genetic manipulations up-regulating antioxidants extended lifespan provided strong support for this theory (Sohal and Weindruch 1996), however, retrospective analyses suggest suboptimal conditions and/or the use of short-lived, unhealthy stocks may have been a contributing factor (Sohal et al. 2002). In fact, increasing the expression of genes for cellular antioxidants and antioxidant enzymes in a range of organisms has consistently failed to increase lifespan (Mockett et al. 2003; Huang et al. 2000; Doonan et al. 2008). Additionally, 'knocking out' the gene for cellular antioxidants and antioxidant enzymes did not result in reduced lifespan in other species (Doonan et al. 2008; Salmon et al. 2009).

In 2009, Gems and Doonan stated it is time to start thinking about ageing in new ways and a new theory of

ageing is rapidly gaining a great deal of support (Gems and Doonan 2009). Maintenance of protein homeostasis and protein stability is now hypothesised to be a critical determinant of life span (Pérez et al. 2009; Salmon et al. 2009), thus further studies are warranted to determine whether into the exceptional longevity of bivalves is due to protein homeostasis, for example greater stability of the proteins or such factors as enhanced protein recycling activities (Ungvari et al. 2011b).

In summary, two important points are raised. Firstly, there appears to be no association between the exceptional longevity and resistance to oxidative stress in the four species of freshwater mussels investigated, including one of the oldest non-colonial animals known to science, the freshwater pearl mussel *M. margaritifera.* Secondly, *M. margaritifera* is a species under special conservation across much of its range and this study for the first time may explain its sensitivity to polluted waters compared to its more tolerant relatives. Care should therefore focus on limiting the release of oxidative stressors into the aquatic system if the species is to return to its past distribution and abundance.

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