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Orginal Contribution

Oxidative damage and cellular defense mechanisms in sea urchin models of aging

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ABSTRACT

The free radical, or oxidative stress, theory of aging proposes that the accumulation of oxidative cellular damage is a major contributor to the aging process and a key determinant of species longevity. This study investigates the oxidative stress theory in a novel model for aging research, the sea urchin. Sea urchins present a unique model for the study of aging because of the existence of species with tremendously different natural life spans, including some species with extraordinary longevity and negligible senescence. Cellular oxidative damage, antioxidant capacity, and proteasome enzyme activities were measured in the tissues of three sea urchin species: short-lived *Lytechinus variegatus,* long-lived *Strongylocentrotus franciscanus,* and *Strongylocentrotus purpuratus,* which has an intermediate life span. Levels of protein carbonyls and 4-hydroxynonenal measured in tissues (muscle, nerve, esophagus, gonad, coelomocytes, ampullae) and 8-hydroxy-2′-deoxyguanosine measured in cell-free coelomic fluid showed no general increase with age. The fluorescent age pigment lipofuscin, measured in muscle, nerve, and esophagus, increased with age; however, it appeared to be predominantly extracellular. Antioxidant mechanisms (total antioxidant capacity, superoxide dismutase) and proteasome enzyme activities were maintained with age. In some instances, levels of oxidative damage were lower and antioxidant activity higher in cells or tissues of the long-lived species compared to the short-lived species; however, further studies are required to determine the relationship between oxidative damage and longevity in these animals. Consistent with the predictions of the oxidative stress theory of aging, the results suggest that negligible senescence is accompanied by a lack of accumulation of cellular oxidative damage with age, and maintenance of antioxidant capacity and proteasome enzyme activities may be important mechanisms to mitigate damage.

Aging is a progressive decline in biological function accompanied by an increased risk of degenerative disease and death over time. It is a complex and multifactorial process and there have been many theories proposed to explain this phenomenon at the molecular, cellular, systemic, and evolutionary levels [1]. The oxidative stress, or free radical, theory of aging is one of the most studied hypotheses for the molecular basis of aging. This theory proposes that the accumulation of cellular damage caused by reactive oxygen species (ROS) plays a key role in the aging process, as well as in determining organismal longevity [2—4]. ROS are partially reduced derivatives of oxygen that are highly reactive with major components of the cell such as proteins, lipids, and DNA. They are continually generated as by-products of a number of cellular processes and are also produced when the cells are exposed to various external stimuli such as ultraviolet light, ionizing radiation, and environmental toxins [3]. ROS are required for proper cell function because of their role in cell signaling and the immune response; however, when ROS exceed a level of homeostasis, oxidative stress ensues. Oxidative stress results from an imbalance between the production of ROS and the cell's ability to mitigate damage through antioxidant pathways or mechanisms that repair or eliminate damaged molecules.

Over the years, there has been much evidence in support of the oxidative stress theory of aging and numerous studies have shown that oxidative damage increases with age in the cells and tissues of many organisms [5]. In addition, several studies comparing animals with different life spans suggest that long-lived species tend to show reduced oxidative damage, reduced mitochondrial ROS production, increased antioxidant defenses, and/or increased resistance to oxidative stress [6]. However, the status of this theory is far from certain as some studies have found a lack of correlation between oxidative damage and life span, and genetic manipulation of antioxidant pathways in invertebrate models (e.g., the nematode worm *Caenorhabditis elegans* and the fruit fly

Abbreviations: 8-OHdG, 8-hydroxy-2′-deoxyguanosine; HNE, 4-hydroxynonenal; ROS, reactive oxygen species; SOD, superoxide dismutase; TAC, total antioxidant capacity.

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Drosophila melanogaster) have yielded variable results on life span, whereas studies in higher animals (e.g., the mouse, *Mus musculus*) have generally not supported a role for oxidative stress in modulating longevity [6,7]. Nevertheless there is convincing evidence that a reduction in oxidative stress positively influences biomarkers of health, physiological function, and disease resistance, and therefore it remains an important aspect of the aging process [6].

Many studies of the oxidative stress theory have focused on humans and short-lived model organisms such as *C. elegans, D. melanogaster,* and *M. musculus,* all animals that exhibit well-characterized organismal senescence; however, less is known about the role of oxidative stress in animals that exhibit negligible senescence. Negligible senescence is a term used to describe animals that do not show an increase in mortality rate or a decrease in fertility, physiological function, or disease resistance with age [8,9]. In this respect, sea urchins offer an interesting model to explore because of the existence of species with very different natural life spans, including some that display extreme longevity and negligible senescence. The red sea urchin (*Strongylocentrotus franciscanus*) is one of the earth's longest living animals, living in excess of 100 years with no age-related increase in mortality rate or decrease in reproductive capacity [10,11]. In contrast, *Lytechinus variegatus* has an estimated maximum life expectancy of approximately 4 years [12,13], and the maximum life expectancy of *Strongylocentrotus purpuratus* is estimated to be approximately 50 years [14,15]. Thus sea urchins provide a unique model to investigate cellular mechanisms underlying life-span determination and negligible senescence. From a phylogenetic viewpoint, sea urchins are also interesting as they are more closely related to humans than other invertebrate models used for aging research (e.g., *C. elegans* and *D. melanogaster*). Sequencing of the genome of *S. purpuratus* confirmed the close genetic relationship between sea urchins and humans, revealing that sea urchins have an estimated 23,300 genes, including representatives of nearly all vertebrate gene families and orthologs of many human disease-associated genes [16]. Age-related gene expression profiles in the tissues of *S. purpuratus* have revealed some interesting distinctions from aging animals. There was no age-related decline in expression of genes involved in energy production and, in some tissues, an increase in expression of genes involved in protein homeostasis, apoptosis, and signaling pathways that play a role in tissue regeneration [17]. No previous studies have investigated oxidative cellular damage or defense mechanisms with respect to age in sea urchins.

When proteins are oxidized by ROS, some amino acid residues are modified, which can lead to conformation changes and functional loss. One of the most commonly studied oxidative products is the formation of carbonyl derivatives of amino acids such as lysine, arginine, proline, and threonine [18]. When cellular membranes are exposed to ROS, the lipids containing polyunsaturated fatty acids undergo a series of chain reactions to yield end products such as malondialdehyde and 4-hydroxynonenal (HNE) [19]. Levels of oxidative DNA damage can be assessed by measuring products of modified nucleosides such as 8-hydroxy-2′-deoxyguanosine (8-OHdG) in tissues or bodily fluids [20—22].

To prevent oxidative damage to proteins, lipids, and DNA, the generation of ROS is counterbalanced by various antioxidant systems. These include enzymes such as superoxide dismutase (SOD) and catalase and nonenzymatic antioxidants such as glutathione, tocopherols, and thioredoxin [19,23]. In addition to antioxidant mechanisms, the degradation of oxidized proteins by the proteasome and autophagy—lysosomal pathways also constitutes an important part of the cell's defense against oxidative stress [24,25]. In particular, the autophagic pathways have been shown to remove damaged mitochondria and oxidatively damaged macromolecules from the cytosol, resulting in the accumulation of aggregates of the highly autofluorescent pigment lipofuscin in the lysosomal lumen [26,27]. Lipofuscin accumulation is one of the most recognized hallmarks of aging in a wide range of vertebrate and invertebrate animals.

To understand the role of the oxidative stress theory of aging in animals with a life history different from that of humans and short-lived invertebrate models, we characterized age-related oxidative cellular damage, antioxidant capacity, and proteasome enzyme activities in tissues of three sea urchin species with different life spans (*L. variegatus, S. purpuratus,* and *S. franciscanus*).

Materials and methods

Collection and age determination of sea urchin species

L. variegatus were collected from Flatt's Inlet, Bermuda (32° 10.375'N and 64° 44.216'W) in January 2012. *S. purpuratus* were collected in Mission Bay, San Diego, California, USA (32° 46.833'N and 117° 14.557'W) in April 2012. *S. franciscanus* were collected near Kendrick Island in Gabriola Pass, British Columbia, Canada (49° 07.554'N and 123° 41.461'W) in July 2011 and July 2012. After collection, the sea urchins were kept in flow-through aquaria to ensure all urchins were exposed to a uniform environment before tissue dissections.

L. variegatus ages were estimated using test (shell) diameter and comparisons with previously established growth curves generated from size frequency and growth-band counting data collected in Florida and Bermuda [12,13]. *S. purpuratus* ages were estimated from test diameter using growth curves previously generated from the Tanaka parameters from tetracycline-tagging experiments conducted at Mission Bay, San Diego ($f = 1.30757$, $d = 2.80184$, $a = 0.16314$) [28]. *S. franciscanus* age estimates were based on growth curves generated from tetracycline-tagged sea urchins in Oregon and Washington State using the Tanaka function ($f = 0.22929$, $d = 6.07531$, $a = 0.19906$) [29,30].

Preparation of tissue and cell extracts

The tissues examined in this study include muscle taken from the jaw structure, which is referred to as Aristotle's lantern muscle; the radial nerve; the esophagus; the gonad; and the ampullae, which is part of the sea urchin's water vascular system. These tissues were dissected from *L. variegatus, S. purpuratus,* and *S. franciscanus* and stored at −70 °C until analysis. Ampullae were not harvested from *S. franciscanus* collected during July 2011. Coelomocytes were harvested from 1.5—3 ml of coelomic fluid by centrifuging at 6000 *g* for 5 min, and both the cell pellets and cell-free coelomic fluid were frozen until further analysis. Tissue homogenates for the oxidative damage and antioxidant assays were prepared from 30—50 mg of tissue or coelomocyte cell pellets by homogenization in 100—200 μl of protein homogenization buffer (50 mM HEPES, 125 mM KCl, 1.25 mM EDTA, 0.6 mM $MgSO_4$) [31] supplemented with protease inhibitors (40 μg/ml pepstatin A, 5 μg/ml antipain, and 100 μg/ml phenylmethylsulfonyl fluoride) using pestles designed for 1.5-ml microcentrifuge tubes. The tissue homogenates were left on ice for 10 min and then were centrifuged at 9300 *g,* 4 °C for 5 min. The supernatant was harvested and protein concentrations were determined by the Bradford assay as previously described [32]. The homogenates were stored in aliquots at −70 °C before subsequent analyses.

Measurement of 8-OHdG in cell-free coelomic fluid

As in indicator of oxidative DNA damage, 8-OHdG was measured in 50 μl cell-free coelomic fluid, prepared as described above, from sea urchins of all three species using the OxiSelect

oxidative DNA damage ELISA kit (8-OHdG quantitation) assay (Cell Biolabs, San Diego, CA, USA). The signals generated by the colorimetric assay were detected at 450 nm on a Versa Max microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Measurement of oxidative damage to proteins and lipids

Protein oxidation in the sea urchin cell and tissue extracts was analyzed by measuring protein carbonyls using the OxiSelect protein carbonyl ELISA kit (Cell Biolabs) with 1 μg input protein for each cell or tissue homogenate. The lipid peroxidation by-product, 4-hydroxynonenal histidine protein adduct, was measured using the OxiSelect HNE—His adduct ELISA kit (Cell Biolabs) using 1 μg input protein. The signals generated by the colorimetric assays were detected at 450 nm on a Versa Max microplate reader (Molecular Devices).

Measurement of total antioxidant capacity and antioxidant enzyme activities

Total antioxidant capacity in the tissue and cell extracts was assayed using the OxiSelect total antioxidant capacity assay kit (Cell Biolabs) using 10 μg input protein for each sample. Activities of three antioxidant enzymes in the extracts were measured using the superoxide dismutase activity assay kit, the catalase activity assay kit, and the glutathione reductase activity assay kit (BioVision, Milpitas, CA, USA), according to the manufacturer's protocols, using 5, 20, and 20 μg input protein, respectively. The assay reactions were conducted in 96-well plates and the colorimetric signals were detected using a Versa Max microplate reader (Molecular Devices).

Measurement of proteasome activity

Proteasome activity was evaluated using the Proteasome-Glo trypsin-like, chymotrypsin-like, and caspase-like assays (Promega, Madison, WI, USA), following the manufacturer's guidelines and using 5 μg input protein for each sample. For these assays, the tissue or cell homogenates were prepared as described above except that the protease inhibitors were omitted from the homogenization buffer. The assay reactions were placed into a 96-well optical bottom plate (Thermo Fisher Scientific, Waltham, MA, USA) and the luminescence produced was detected and recorded as relative light units using a SpectraMax M2 microplate reader (Molecular Devices).

Evaluation of lipofuscin by autofluorescence

Tissues (Aristotle's lantern muscle, radial nerve, and esophagus) were dissected and placed into 4% paraformaldehyde in phosphate-buffered saline (PBS) and sent to Histopathology Services at The Jackson Laboratory (Bar Harbor, ME, USA), where they were embedded in paraffin and sectioned (5 μm). Paraffin was removed from the slides by immersion in toluene for 5 min followed by successive treatments with 100, 95, and 70% ethanol for 5 min each. Slides were then rinsed in 1 × PBS and left to dry in a 37 °C oven for 20 min. Autofluorescence was assessed using an Olympus Provis AX70 Electrofluorescence microscope (Olympus, Tokyo, Japan) with a 40 × objective by excitation with narrow green light (530—545 nm) and emission at 610—675 nm. Images were captured with a Toshiba IK-TU40A CCD camera (Irvine, CA, USA) or a Retiga EXi digital camera (Qimaging, Surrey, BC, Canada).

For each tissue section, 12 locations were randomly selected and images captured under bright-field and fluorescent illumination. Autofluorescence levels were quantified using Image Pro Plus, version 4.0 (Media Cybernetics, Bethesda, MD, USA) using the integrated optical density (IOD) measurement tool. In this analysis, lipofuscin granules were discriminated from the surrounding tissues in the digital images by light intensity range selection after background subtraction based on a constant background level set for each tissue. Image Pro's measurement tool was then used to determine the total tissue area in each image using the bright-field image. The average IOD per tissue area for each tissue was calculated after removing the highest and lowest values and averaging the remaining 10 images.

Statistical analysis

Outliers in the data were detected by the Grubbs test, using QuickCalcs (GraphPad Software, La Jolla, CA, USA). Species- and age-specific differences were analyzed using the general linear model for comparison within each tissue type (Statgraphics Centurion; Statpoint Technologies, Warrenton, VA, USA). Comparisons between the young and the old groups within a tissue for each sea urchin species were conducted using Statgraphics Centurion and Excel using *t* tests for equal or unequal variances as appropriate after confirming that the data were normally distributed or using the Mann—Whitney test if the data were not normally distributed. Species-specific differences were evaluated using one-way ANOVA for normally distributed data and the Kruskal—Wallis test if the data were not normally distributed, followed by multiple range post hoc tests (Statgraphics Centurion). Differences of $p < 0.05$ were considered statistically significant. Data are expressed as the mean ± standard error of the mean.

Results

Age estimates for sea urchins

Ages of individual sea urchins were estimated from test (shell) diameter using previously established growth curves generated for sea urchins that were analyzed at or near our collection sites. The sea urchins were divided into two age groups (young and old) and their average test diameter, the number of animals in each group, and estimated ages are shown in Table 1. As growth rate can be affected by environmental factors (e.g., food availability) our age estimates serve only as a guideline and not absolute values. However, studying animals within a defined study site should give a reasonable indication of relative age within the population. For our study we selected animals at the upper and lower size ranges for the populations under study.

8-OHdG levels in cell-free coelomic fluid of L. variegatus, S. purpuratus, and S. franciscanus

Cell-free coelomic fluid from *L. variegatus, S. purpuratus,* and *S. franciscanus* was used to assess the level of 8-OHdG as a marker of oxidative DNA damage. Levels of 8-OHdG in cell-free coelomic fluid showed no significant difference in levels between age groups or between species (age, $F_{1,31} = 0.01$, $p = 0.93$; species, $F_{2,31} = 0.68$, $p = 0.51$) (Fig. 1).

Protein oxidation and lipid peroxidation

The protein carbonyl content in cell and tissue extracts from the three sea urchin species is shown in Fig. 2. Ampullae were not harvested from *S. franciscanus* collected during July 2011 and so indicators of oxidative damage were not assayed in this tissue. The general linear model showed significant species-specific differences in protein carbonyl levels in all tissues and age-specific differences in esophagus and gonads. Post hoc tests comparing

Table 1
Estimated ages of young and old *L. variegatus, S. purpuratus,* and *S. franciscanus* used for oxidative damage, antioxidant, proteasome, and lipofuscin analyses.

Sample	Test diameter (mm)	Est. age (years)	Ref.
Oxidative damage, antioxidant, proteasome assays			
Lv, young	40.2 ± 0.3 (6)	1.3 ± 0.1 (6)	[12]
Lv, old	73.2 ± 0.4 (6)	4.0 ± 0.1 (6)	
Sp, young	32.2 ± 0.8 (6)	1.9 ± 0.1 (6)	[28]
Sp, old	73.7 ± 1.7 (6)	41.0 ± 9.1 (6)	
Sf[a], young	48.2 ± 4.1 (6)	3.7 ± 0.2 (6)	[29,30]
Sf[a], old	159.5 ± 1.3 (6)	128.1 ± 7.4 (6)	
Sf[b], young	34.5 ± 0.1 (7)	3.1 ± 0.1 (7)	[29,30]
Sf[b], old	159.0 ± 0.1 (5)	124.7 ± 6.6 (5)	
Lipofuscin assay			
Lv, young	40.8 ± 0.4 (6)	1.4 ± 0.1 (6)	[12]
Lv, old	73.2 ± 0.4 (6)	4.0 ± 0.1 (6)	
Sp, young	28.7 ± 1.1 (6)	1.7 ± 0.1 (6)	[28]
Sp, old	73.7 ± 1.7 (6)	41.0 ± 9.1 (6)	
Sf[b], young	39.4 ± 0.2 (7)	3.3 ± 0.1 (7)	[29,30]
Sf[b], old	159.0 ± 0.1 (5)	124.7 ± 6.6 (5)	

The estimated ages are expressed as the mean ± standard error and the numbers in parentheses represent the number of samples (n) in each group.

[a] *S. franciscanus* collected in July 2011, used for oxidative damage and antioxidant analyses.

[b] *S. franciscanus* collected in July 2012, used for proteasome and lipofuscin analyses.

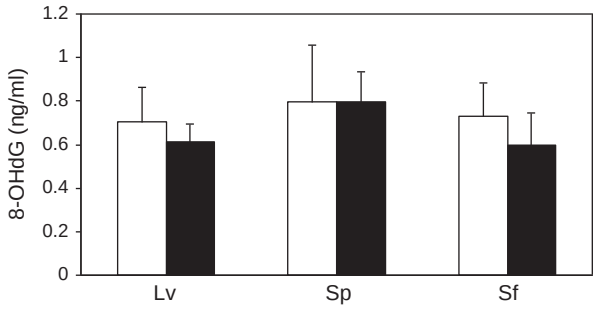

Fig. 1. 8-Hydroxy-2′-deoxyguanosine (8-OHdG) levels in cell-free coelomic fluid of *L. variegatus* (Lv), *S. purpuratus* (Sp), and *S. franciscanus* (Sf). The white bars represent the average 8-OHdG values for the young sea urchins and the black bars represent levels for the old sea urchins. All values are presented as the mean ± standard error of the mean.

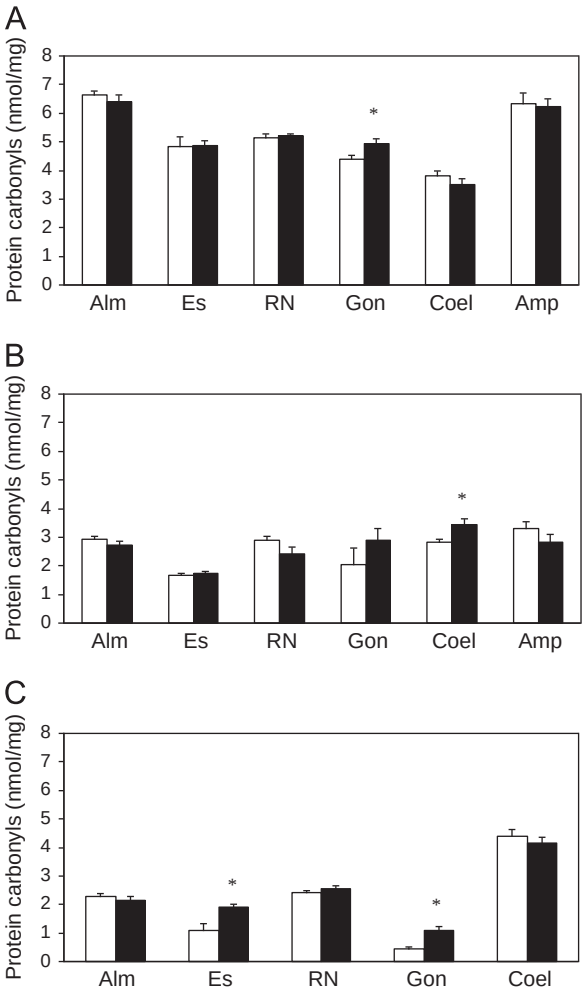

Fig. 2. Protein carbonyl content in tissue extracts from the three sea urchin species: (A) *L. variegatus,* (B) *S. purpuratus,* and (C) *S. franciscanus.* The tissues assayed were muscle (Alm), esophagus (Es), radial nerve (RN), gonad (Gon), coelomocytes (Coel), and ampullae (Amp). The white bars represent the average protein carbonyl values for the young sea urchins and the black bars represent levels for the old sea urchins (expressed as nmol/mg protein). All values are the mean ± standard error of the mean. *$p < 0.05$, significantly different between the young and the old sea urchins.

young and old within each species confirmed that most tissues did not show increased protein carbonyls with age. There was no significant age-related change in protein carbonyl content in muscle, esophagus, nerve, ampullae, or coelomocytes from *L. variegatus;* however, there was a small but statistically significant increase with age in gonad tissue (1.12-fold, $p = 0.03$) (Fig. 2A). For *S. purpuratus,* there was no significant age-related change in protein carbonyl content in muscle, esophagus, nerve, gonad, or ampullae tissues, but a statistically significant increase with age in protein carbonyl content was found in coelomocytes (1.22-fold, $p = 0.02$) (Fig. 2B). There was no significant age-related change in protein carbonyl content in muscle, nerve, or coelomocytes from *S. franciscanus* (Fig. 2C). A statistically significant age-related increase in protein carbonyl content was observed in esophagus (1.76-fold, $p < 0.01$) and gonad (2.43-fold, $p < 0.01$) of *S. franciscanus;* however, overall levels in these tissues remained very low. One-way ANOVA or the Kruskal—Wallis test and post hoc analyses confirmed that protein carbonyls were significantly higher in all *L. variegatus* tissues (except coelomocytes) compared to the corresponding tissues from the other two long-lived species (> 1.7-fold, $p < 0.05$ in each case). Comparisons between the two longer lived species showed significantly lower levels of protein carbonyls in both young and old muscle tissue (1.3-fold, $p < 0.05$) and gonad (> 2.5-fold, $p < 0.05$) of *S. franciscanus* compared to *S. purpuratus,* whereas protein carbonyls in coelomocytes of *S. franciscanus* were significantly higher than in both *L. variegatus* and *S. purpuratus* (> 1.2-fold, $p < 0.05$, in each case).

The same extracts used for protein carbonyl analysis were used to measure levels of HNE—His as an indicator of lipid peroxidation (Fig. 3). The general linear model showed significant species-specific differences in HNE—His levels in all tissues except gonads and age-specific differences in muscle and radial nerve. Post hoc tests comparing young and old within each species confirmed that most tissues did not show increased HNE with age. There was no significant age-related change in HNE—His adduct level in all the tissues from *L. variegatus* except the radial nerve, which showed a statistically significant increase of 1.44-fold ($p < 0.01$) with age (Fig. 3A). Similarly, there was no significant age-related change in HNE—His adduct level in all the tissues examined from *S. purpuratus* except radial nerve, which showed a 1.54-fold increase ($p = 0.03$; Fig. 3B). In *S. franciscanus,* a statistically significant age-related increase in HNE—His adduct level in muscle (1.23-fold, $p < 0.01$) and nerve (1.50-fold, $p < 0.01$) was observed (Fig. 3C). However, a statistically significant age-related decrease (1.25-fold, $p = 0.03$) in HNE—His adduct level in coelomocytes was observed. One-way

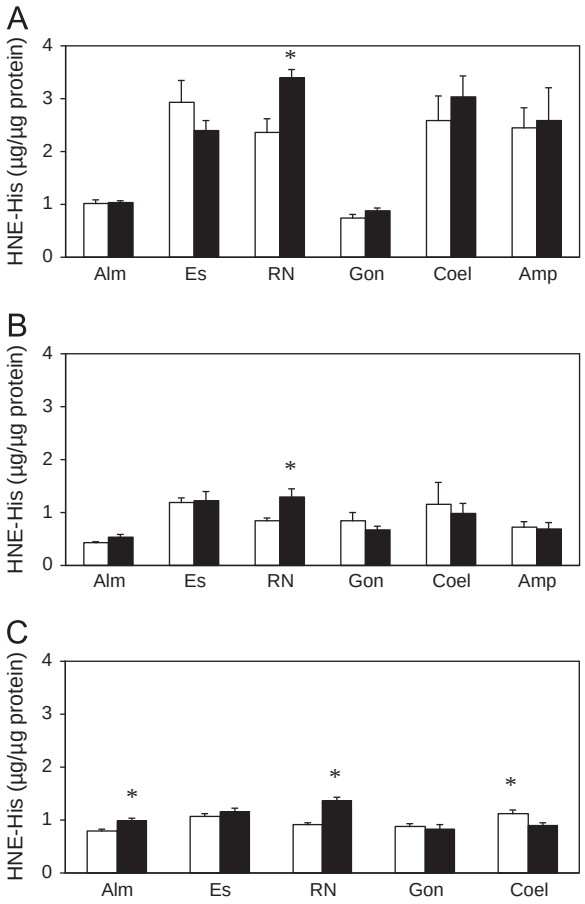

Fig. 3. HNE—histidine adduct levels in tissue extracts from the three sea urchin species: (A) *L. variegatus,* (B) *S. purpuratus,* and (C) *S. franciscanus.* The tissues assayed were muscle (Alm), esophagus (Es), radial nerve (RN), gonad (Gon), coelomocytes (Coel), and ampullae (Amp). The white bars represent the average HNE—histidine values for the young sea urchins and the black bars represent levels for the old sea urchins (expressed as μg/μg protein). All values are the mean ± standard error of the mean. *$p < 0.05$, significantly different between the young and the old sea urchins.

ANOVA or Kruskal—Wallis test and post hoc analyses were used for interspecies comparisons, which revealed significantly higher levels of HNE—His adducts in esophagus, radial nerve, coelomocytes, and ampullae for *L. variegatus* compared to the corresponding tissues from the long-lived species (> 1.9-fold, $p < 0.05$ in all cases). Levels of HNE—His were significantly lower in young and old muscle tissue of *S. purpuratus* compared to *S. franciscanus* (> 1.8-fold and $p < 0.05$) and also *L. variegatus* (> 1.9-fold and $p < 0.05$).

Total antioxidant capacity

Total antioxidant capacity (TAC) was evaluated in tissue extracts from the three sea urchin species by measuring the reduction of Cu^{2+} to Cu^{+}. Ampullae were not harvested from *S. franciscanus* collected during July 2011 and therefore antioxidant capacity was not assayed in this tissue. The general linear model showed significant species-specific differences in TAC levels in all tissues except esophagus and gonads and age-specific differences in esophagus, radial nerve, and coelomocytes. Post hoc tests revealed no significant age-related change in TAC in all the tissues examined from *L. variegatus* and *S. franciscanus* (Fig. 4A, C, and D). In *S. purpuratus,* there was a statistically significant age-related increase in TAC in muscle (1.19-fold, $p = 0.05$), esophagus (2.29-fold, $p < 0.01$), and radial nerve (1.77-fold, $p < 0.01$; Fig. 4B). However, there was a significant age-related decrease in coelomocytes (1.77-fold, $p < 0.01$; Fig. 4D). It was observed that the TAC of coelomocytes was much higher than that of the other tissues for all three of the sea urchin species examined and thus it was plotted using a different scale (Fig. 4D). One-way ANOVA or Kruskal—Wallis test and post hoc analyses revealed that the coelomocytes from *S. purpuratus* and *S. franciscanus* exhibited statistically higher TAC (> 5-fold, $p < 0.05$) than the coelomocytes from *L. variegatus*. Although there was no significant difference between TAC in coelomocytes of young *S. franciscanus* and *S. purpuratus,* the coelomocytes from old *S. franciscanus* had higher TAC than those from old *S. purpuratus* (1.5-fold, $p < 0.05$). Interspecies comparisons also revealed a significantly lower level of TAC in young and old muscle of *S. franciscanus* compared to *L. variegatus* and *S. purpuratus* (> 1.5-fold, $p < 0.05$) and a higher level of TAC in young and old ampullae of *L. variegatus* compared to *S. purpuratus* (> 1.6-fold, $p < 0.05$).

Antioxidant enzyme activities

SOD activity of various tissues from the three sea urchin species is shown in Fig. 5. Ampullae were not harvested from *S. franciscanus* collected during July 2011 and therefore antioxidant enzyme activity was not assayed in this tissue. The general linear model showed significant species-specific differences in SOD levels in all tissues except radial nerve and age-specific differences in muscle and radial nerve. Post hoc tests revealed no significant age-related change in SOD activity in all the tissues examined from *L. variegatus* (Fig. 5A). In *S. purpuratus,* there was no significant age-related change in SOD activity in all the tissues examined except radial nerve and muscle tissue, which each showed a significant age-related increase of 1.19-fold ($p < 0.01$ for nerve and $p = 0.03$ for muscle; Fig. 5B). A significant age-related increase (1.26-fold, $p < 0.01$) in SOD activity was observed in the radial nerve from *S. franciscanus,* but no other age-related changes were observed in this species (Fig. 5C). One-way ANOVA or Kruskal—Wallis test and post hoc analyses revealed some species-specific differences in all tissues with the most notable being gonad and coelomocyte. SOD activity was significantly higher (> 2-fold, $p < 0.05$) in young and old gonad of *S. purpuratus* and old gonad of *S. franciscanus* compared to *L. variegatus*. Coelomocytes from both young and old *S. purpuratus* and *S. franciscanus* exhibited statistically higher SOD activity (> 2-fold, $p < 0.05$) than the coelomocytes from *L. variegatus.*

Catalase and glutathione reductase assays were also conducted with all tissue extracts except coelomocytes. Catalase activity was undetectable in most of the tissues examined in the three sea urchin species when 20 μg of total protein was used in the assay. Catalase activity was detected in the gonad of the three sea urchin species but no significant age-related change was observed (data not shown). Glutathione reductase activity was detectable in all the tissues examined in *L. variegatus* with no age-related difference (data not shown). In the long-lived species, glutathione reductase activity was undetectable in several tissues when 20 μg of total protein was used in the assay. Glutathione reductase activity was detected in the esophagus and gonad of *S. purpuratus* and the esophagus, gonad, and nerve of *S. franciscanus* but no significant age-related change was observed (data not shown).

Proteasome enzyme activity in sea urchin tissues

Proteins that are damaged by ROS are recognized and degraded by the proteasome complex, which exhibits trypsin-like, chymotrypsin-like, and caspase-like activities. These activities were measured in tissue extracts for the three sea urchin species. Coelomocytes were not assayed for proteasome activity owing to a limited amount of material for these samples. Although proteasome activity has been reported to

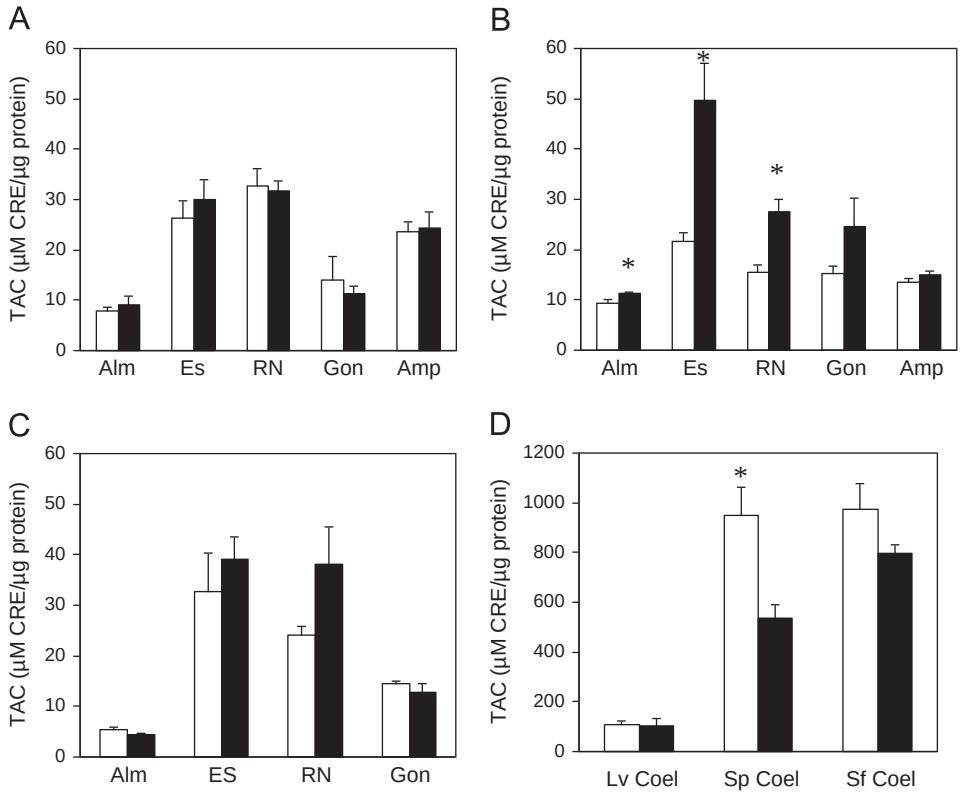

Fig. 4. Total antioxidant capacity (TAC) in tissue extracts from the three sea urchin species: (A) *L. variegatus,* (B) *S. purpuratus,* and (C) *S. franciscanus.* (A), (B), and (C) show the results for muscle (Alm), esophagus (Es), radial nerve (RN), gonad (Gon), and ampullae (Amp). (D) The results for coelomocytes from the three species. The white bars represent the average TAC values for the young sea urchins and the black bars represent levels for the old sea urchins. TAC is expressed as μM copper-reducing equivalents (CRE)/μg input protein for each tissue extract. All values are the mean ± standard error of the mean. *$p < 0.05$, significantly different between the young and the old sea urchins.

decline with age in other organisms [25] there was no consistent decrease across sea urchin tissues with age (Fig. 6). Trypsin-like activity did significantly decrease in radial nerve of *S. franciscanus* (2.55-fold, $p = 0.01$) and the caspase-like activity in esophagus of *S. purpuratus* decreased 1.33-fold ($p = 0.02$) with age. Otherwise, there was a significant increase with age for trypsin-like activity in *L. variegatus* muscle tissue (1.22-fold, $p < 0.01$). Chymotrypsin-like activity was increased with age in *S. purpuratus* ampullae (2.75-fold, $p = 0.02$) and nerve (1.81-fold, $p < 0.01$) as well as *S. franciscanus* ampullae (1.61-fold, $p < 0.01$) and nerve (2.38-fold, $p = 0.01$). Caspase-like activity increased with age in muscle tissue of all three species: *L. variegatus* (1.25-fold, $p = 0.04$), *S. purpuratus* (1.44-fold, $p < 0.01$), and *S. franciscanus* (1.37-fold, $p < 0.01$). Caspase-like activity also increased slightly with age in *L. variegatus* nerve tissue (1.16-fold, $p = 0.04$). The general linear model showed significant species-specific differences in all tissues except trypsin-like activity of esophagus. One-way ANOVA or Kruskal—Wallis test and post hoc analyses revealed the most notable difference in radial nerve and ampullae tissue, with the short-lived *L. variegatus* having higher levels of trypsin-like and chymotrypsin-like activities than the longer lived species (Fig. 6A—C). For ampullae, trypsin-like activity was significantly higher in young and old *L. variegatus* compared to young and old *S. purpuratus* and *S. franciscanus* (> 2.5-fold, $p < 0.05$ in all cases) and chymotrypsin-like activity was significantly higher in young *L. variegatus* compared to young tissue from *S. purpuratus* and *S. franciscanus* (> 5.7-fold, $p < 0.05$, in all cases). For radial nerve, trypsin-like activity was significantly higher in young and old *L. variegatus* compared to young and old *S. purpuratus* and old *S. franciscanus* (> 1.77-fold, $p < 0.05$, in all cases). Also for radial nerve, chymotrypsin-like activity of *L. variegatus* was significantly higher than in young and old *S. franciscanus* and young *S. purpuratus* (> 1.8-fold, $p < 0.05$, in each case) and chymotrypsin-like activity of young and old *S. purpuratus* was significantly higher than that of young and old *S. franciscanus* (> 3.82-fold, $p < 0.05$, in each case).

Lipofuscin accumulation in sea urchin tissues

Lipofuscin is an autofluorescent pigment that accumulates in cells and tissues of both vertebrate and invertebrate animals with age. Autofluorescence was evaluated in muscle, esophagus, and nerve tissue of young and old sea urchins as an indicator of lipofuscin levels (Fig. 7). Analysis of the data using the general linear model revealed both age and species differences for muscle and nerve and species-specific differences in esophagus. Although all tissues showed an increase in autofluorescence with age, post hoc tests revealed this reached significance in muscle tissue of *L. variegatus* ($p = 0.02$) and *S. purpuratus* ($p = 0.02$), esophagus tissue of *S. purpuratus* ($p = 0.02$), and nerve tissue of *L. variegatus* ($p = 0.03$), *S. purpuratus* ($p = 0.04$), and *S. franciscanus* ($p = 0.05$). For young muscle tissue, autofluorescence was significantly higher in *L. variegatus* than in both *S. purpuratus* and *S. franciscanus* (Kruskal—Wallis, $H = 8.02$, $df = 2$, $n = 19$, $p = 0.02$); however, for old muscle, autofluorescence was higher in *S. franciscanus* than in *L. variegatus* and *S. purpuratus* (Kruskal—Wallis, $H = 11.88$, $df = 2$, $n = 17$, $p < 0.01$). For nerve, there was no species-specific difference between the young tissues ($H = 3.07$, $df = 2$, $n = 19$, $p = 0.22$) but autofluorescence was significantly higher in old nerve tissue of *S. franciscanus* compared to that of *L. variegatus* and *S. purpuratus* ($H = 11.7$, $df = 2$, $n = 17$, $p < 0.01$). For esophagus, autofluorescence was significantly higher in both young and old sea urchins of *L. variegatus* compared to *S. purpuratus* and *S. franciscanus* (Kruskal—Wallis, young, $H = 15.61$, $df = 2$, $n = 19$,

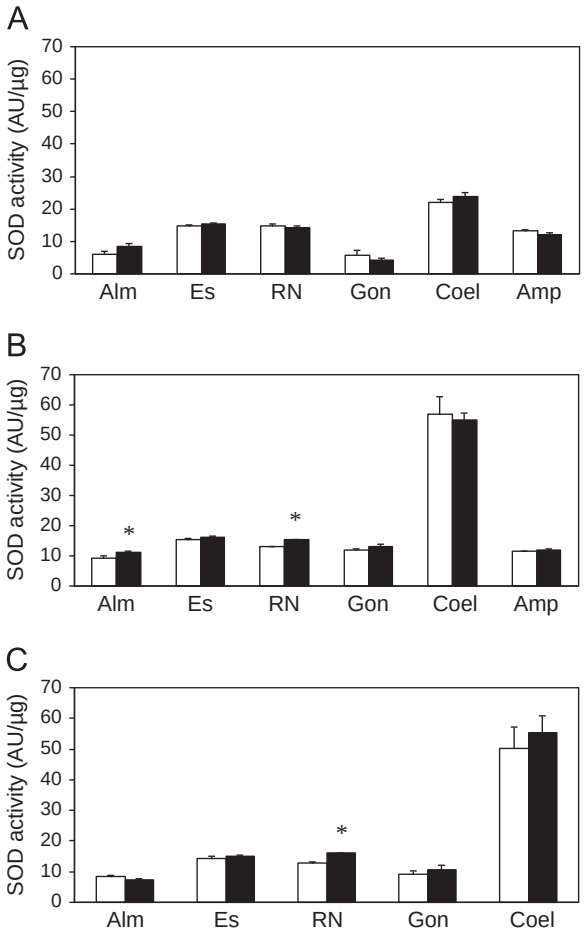

Fig. 5. Superoxide dismutase (SOD) activity in tissue extracts from the three sea urchin species: (A) *L. variegatus,* (B) *S. purpuratus,* and (C) *S. franciscanus.* The tissues assayed were muscle (Alm), esophagus (Es), radial nerve (RN), gonad (Gon), coelomocytes (Coel), and ampullae (Amp). The white bars represent the average SOD values for the young sea urchins and the black bars represent levels for the old sea urchins. SOD activity is expressed as activity units (AU)/μg protein in each tissue extract. All values are the mean ± standard error of the mean. *$p < 0.05$, significantly different between the young and the old sea urchins.

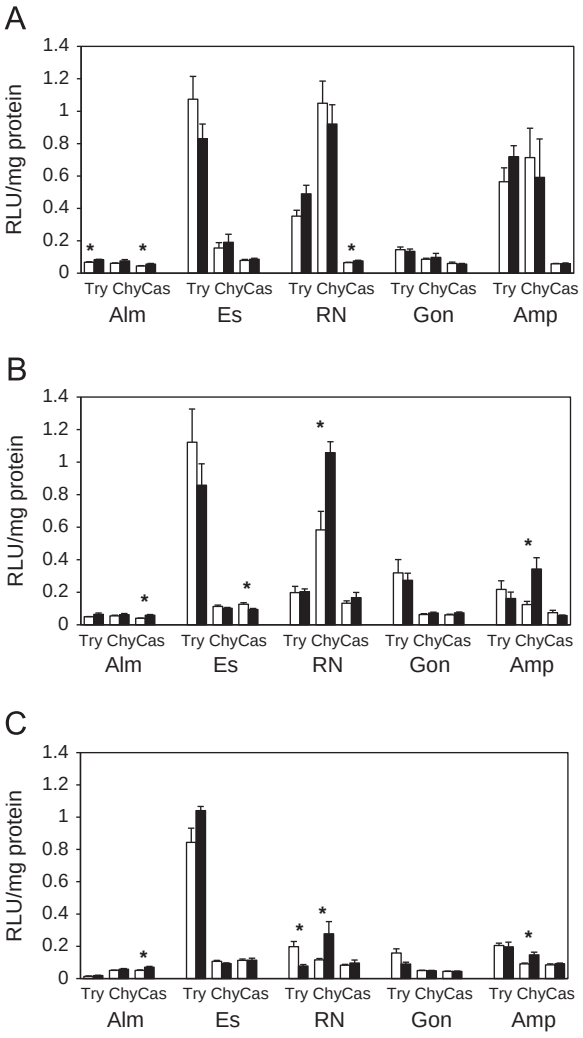

Fig. 6. Proteasome enzyme activities in tissue extracts from the three sea urchin species: (A) *L. variegatus,* (B) *S. purpuratus,* and (C) *S. franciscanus*. The proteasome enzyme activities (trypsin-like (Try), chymotrypsin-like (Chy), and caspase-like (Cas)) are shown for muscle (Alm), esophagus (Es), radial nerve (RN), gonad (Gon), and ampullae (Amp). The white bars represent the average enzyme activity for the young sea urchins and the black bars represent levels for the old sea urchins. Enzyme activity is expressed as relative light units (RLU)/mg protein in each tissue extract. All values are the mean ± standard error of the mean. *$p < 0.05$, significantly different between the young and the old sea urchins.

$p < 0.01$, and old, $H = 7.76$, $df = 2$, $n = 17$, $p = 0.02$). It was evident from these analyses that autofluorescence was not evenly distributed throughout the tissues but appeared in scattered patches. When tissues were treated with the DNA dye 4′,6-diamidino-2-phenylindole, it revealed that areas of autofluorescence did not colocalize with nuclear staining but were found in areas devoid of cellularity. It was also noted that there was a high degree of variability in autofluorescence between individuals as reflected in the large error bars shown in Fig. 7.

Discussion

Although many studies have shown that oxidative damage increases with age in the cells and tissues of a variety of organisms [5], a general age-related increase in some classic markers of oxidative damage was not observed in sea urchin tissues. In humans, levels of oxidative DNA damage have been assessed by measuring products of modified nucleosides such as 8-OHdG in tissues and bodily fluids, and increased levels of 8-OHdG have been reported with respect to age, smoking, inflammatory diseases, and cancer [20—22]. Measuring 8-OHdG in the bodily fluids (e.g., serum or urine) gives an indication of the overall level of damage in the whole body and has the advantage that it avoids artifactual oxidation of DNA that can be introduced during sample preparation [22]. Similar to the blood of higher animals, coelomic fluid of sea urchins is in direct contact with internal cells and tissues and can provide an overall profile of the biological status of the organism. Contrary to what is seen in aging animals, we did not detect an increase in 8-OHdG in cell-free coelomic fluid with age in sea urchins.

Markers of protein oxidation (carbonyls) and lipid peroxidation (HNE) did not show an age-related increase in most cells and tissues of sea urchins. Consistent with these observations, a study on aging in the long-lived ocean quahog clam *Arctica islandica* showed no significant difference in the level of proteins carbonyls with age in gill and mantle tissues [31]. A statistically significant increase in levels of HNE was observed in radial nerve tissues of all three sea urchin species examined, indicating that certain tissues are susceptible to some forms of oxidative damage. It is interesting that the magnitude of the increase is similar in all three species (approximately 1.5-fold), even though *S. purpuratus* and *S. franciscanus* are estimated to be much longer lived, indicating a lower

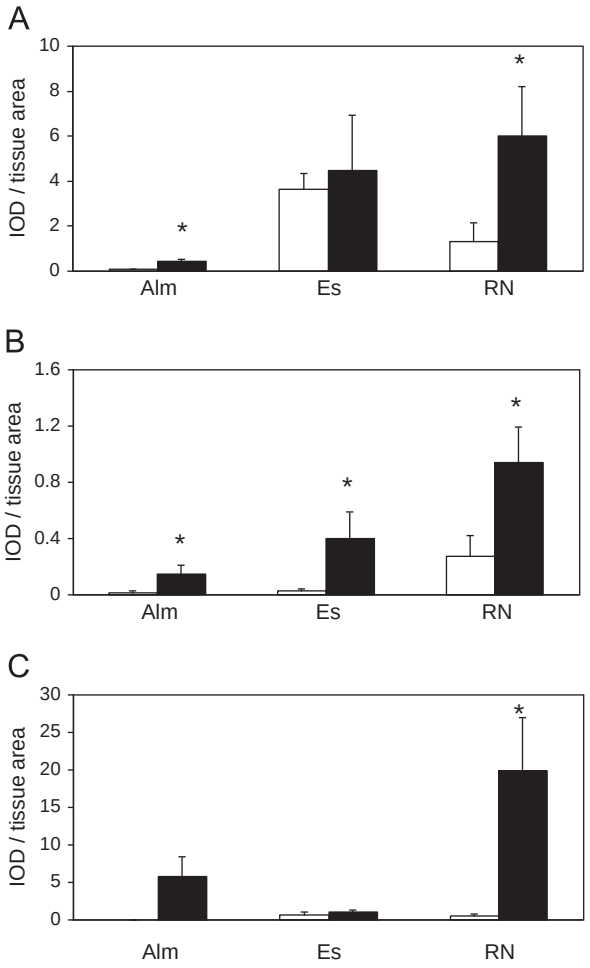

Fig. 7. Autofluorescence levels in muscle (Alm), esophagus (Es), and radial nerve (RN) tissue of (A) *L. variegatus,* (B) *S. purpuratus,* and (C) *S. franciscanus*. Autofluorescence is expressed as integrated optical density (IOD) per tissue area. The white bars represent the average autofluorescence for the young sea urchins and the black bars represent levels for the old sea urchins. All values are the mean ± standard error of the mean. $^{*}p < 0.05$, significantly different between the young and the old sea urchins.

rate of accumulation of damage in these species. It has been reported that protein carbonyl levels are lower in the tissues of long-lived *A. islandica* compared to tissues of short-lived clams (*Mya arenaria* and *Laternula elliptica*) or scallops (*Aequitpecten opercularis* and *Adamassium colbecki*) [31]. Similarly, our results indicated that there was generally less damage in tissues of long-lived species than in the short-lived species of sea urchin. Although interesting, these data should be viewed with caution as the sea urchins used in this study were collected from different geographical locations and it has been shown that variable environmental factors (e.g., temperature, light, pollution) can stochastically modify the production of ROS and the accumulation of damage in animals from different locations [33,34]. Therefore, it is difficult to conclude that differences across species are solely related to life span. Further studies could address this point by examining sea urchin species with different life spans collected from the same geographic location.

To explore potential mechanisms for the lack of accumulation of oxidative damage with age, we investigated the activity of a variety of cellular antioxidant systems: direct ROS-scavenging enzymes SOD and catalase, as well as glutathione reductase, a rate-limiting enzyme in glutathione recycling. In addition, TAC was measured by the ability of the cell and tissue extracts to reduce Cu^{2+} to Cu^{+}. In sea urchin tissues, there was generally no age-related change in TAC or antioxidant enzyme activity and little difference between the species with different life spans. We did observe a high level of antioxidant activity in coelomocytes, which may be important to combat high levels of ROS produced during the immune response of the phagocytotic cells present in this cell mixture. It is also interesting that there were much higher levels of TAC and SOD in coelomocytes of the long-lived species compared to *L. variegatus* but whether this reflects a more robust immune response and higher ROS levels in long-lived species remains to be determined. Other studies of age-dependent changes in antioxidant activities have not shown a consistent trend as the activities of some antioxidants decline, whereas others remain unchanged or even increase with age depending on enzyme, tissue, or species [5]. In addition, long-lived animals often have lower levels of tissue antioxidants than short-lived animals and experimentally altering antioxidants through dietary supplementation or transgenic techniques does not, in most cases, affect maximum life span [23]. It is now thought that the overall level of production of ROS is more important than the modulation of ROS by antioxidant activities in the context of the oxidative stress theory of aging [23] and it will be important to measure ROS production in sea urchin tissues in future studies.

The degradation of oxidized proteins by the proteasome pathway constitutes another important part of the cell's defense against oxidative stress by preventing the accumulation of damaged proteins [24,25]. There is evidence that proteasome activity decreases with age in human tissues and model systems, which may be causally related to aging and several age-related diseases [24,25]. In contrast, we found no age-related decrease in the activity of proteasome enzymes except for trypsin-like activity in *S. franciscanus* radial nerve tissue and caspase-like activity in *S. purpuratus* esophagus tissue. In all other tissues trypsin-, chymotrypsin-, and caspase-like activities were unchanged or, in some cases, showed a significant increase with age. This suggests a general maintenance in proteasome function with age, which may contribute to the lack of increase in protein carbonyls observed in this study. It was noted that some tissues of the short-lived *L. variegatus* had higher proteasomal activities than the longer lived species and *S. franciscanus* generally had the lowest levels of activity. When proteasome enzyme activities were measured in tissues of clams with different life spans, there were no interspecies differences between trypsin-like and chymotrypsin-like activity in gills but caspase was significantly lower in gills of the long-lived *A. islandica* compared to short-lived *Mercenaria mercenaria* [35]. However, a recent study has demonstrated that nonproteasomal proteases in crude cell or tissue extracts can contribute to the activity measured in the proteasomal assay and that this contribution varies between species and tissue types [36]. Therefore it is essential to distinguish between proteasomal and nonproteasomal activities using proteasome-specific inhibitors before one can make generalizations about interspecies differences.

Another major pathway of catabolism in eukaryotic cells is the autophagy—lysosomal system, and it is thought that this pathway is responsible for the accumulation of lipofuscin, one of the most recognized hallmarks of aging in a wide range of vertebrate and invertebrate animals [26,27]. Many studies have reported the negative effects of accumulating lipofuscin on lysosomal activity and cellular function, but more recent studies have suggested that lysosomes can play a protective role in the oxidative stress response and suggest that lipofuscin accumulation is indicative of cellular maintenance through autophagic activity [26,27,37]. In sea urchins, an age-related accumulation of lipofuscin was seen in all tissues examined (muscle, nerve, and esophagus), which reaches significance in muscle tissue of *L. variegatus* and *S. purpuratus,* esophagus tissues of *S. purpuratus,* and nerve tissue of all three species. Lipofuscin staining was not evenly distributed and not punctate as one would expect if it was confined to

lysosomes, but rather appeared as patches of autofluorescence scattered throughout the tissues in areas devoid of nuclear staining. There was a high level of interindividual variation in the levels of lipofuscin, consistent with previous studies in which lipofuscin, measured in the gonads of the sea urchin *Strongylocentrotus intermedius,* was also found to be highly variable between individuals such that no strong correlation was observed between lipofuscin levels and exposure to pollution (DDT, oil hydrocarbons, and heavy metals) [38].

Lipofuscin has been shown to accumulate with age in the tissues of other marine invertebrates such as bivalves and crustaceans [31,39—42]. In *A. islandica,* lipofuscin levels were significantly different in three tissues examined (gill > mantle > muscle) and increased significantly with age in all three tissues [31]. The authors report that lipofuscin granules were located primarily in connective tissues and interstitial spaces, presumably where they are less likely to impair metabolic processes of the tissues. Lipofuscin accumulation in the neural tissues of lobsters (*Homarus gammarus* (European lobsters) and *Panulirus argus* (Caribbean spiny lobsters)) has been used to determine age and is reported to provide a more accurate estimate of age than carapace length [40—42]. However, levels of lipofuscin have been shown to be affected by environmental factors such as seasonal temperature oscillations and metal exposure [34,39,40], and age-accumulated lipofuscin can be reversed presumably through exocytosis [43]. Similar to sea urchins, lipofuscin granules were shown to be mainly extracellular in the eye stalk of lobster neural tissue [40]; however, it remains to be determined if the accumulation of lipofuscin affects tissue function in these animals.

The overall findings of this study show that markers of cellular oxidative damage did not generally increase with age in the tissues of sea urchin species with different life spans. Antioxidant capacity and proteasomal enzyme activities were maintained with age, which may contribute to the lack of accumulation of oxidative damage; however, future studies should examine if the production of ROS differs between tissues and between species. This study is the first to look at age-related oxidative damage in sea urchins with different life spans collected from their natural environment. It should be noted that we have investigated only two time points during the life span of these animals and therefore may be missing important trends that occur over the lives of these animals. In addition, although the sea urchins investigated represent the oldest and youngest in the populations under study, we cannot definitely say that the oldest animals are approaching their maximum limit. Further studies are required to examine sea urchins with different life spans from the same environment to see if the negative correlation between damage and longevity observed in this study is confirmed. The absence of an age-related increase in oxidative damage in animals that lack senescence and low levels of damage in long-lived species are both consistent with the oxidative stress theory of aging. The data from this study support the idea that negligible senescence is accompanied by maintenance of antioxidant and proteasome activities and mitigation of some forms of oxidative damage and provides a foundation to further explore the connections between oxidative damage, negligible senescence, and longevity.

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