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Age-related cellular changes in the long-lived bivalve *A. islandica*

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Abstract One of the biggest challenges to studying causes and effects of aging is identifying changes in cells that are related to senescence instead of simply the passing of chronological time. We investigated two populations of the longest living non-colonial metazoan, *Arctica islandica*, with lifespans that differed sixfolds. Of four investigated parameters (nucleic acid oxidation, protein oxidation, lipid oxidation, and protein instability), only nucleic acid oxidation increased with age and correlated with relative lifespan. Nucleic acid oxidation levels increased significantly faster and were significantly higher in the shorter-lived than the longer-lived population. In contrast, neither protein oxidation, lipid oxidation, nor protein stability changed over time. Protein resistance to unfolding stress when treated with urea was significantly lower overall in the shorter-lived population, and lipid peroxidation levels were higher in the longer-lived population. With the exception of nucleic acid oxidation, damage levels of *A. islandica* do not change with age, indicating excellent cellular maintenance in both populations. Since correlations between nucleic acid oxidation and age have also been shown previously in other organisms, and nucleic acid oxidation accumulation rate correlates with relative age in both investigated populations, nucleic acid oxidation may reflect intrinsic aging mechanisms.

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Introduction

While the primary causes of aging are not yet fully understood, several hypotheses propose mechanisms that may cause aging. The free radical theory of aging (FRTA) suggests that destructive reactive oxygen species (ROS) cause cellular and organismal decline (Harman 1956). Many studies have shown correlations between oxidative damage and aging in support of the FRTA (Philipp et al. 2005a, b; Vermeulen et al. 2005; Csiszar et al. 2007; Ungvari et al. 2008, 2013). Due to contradictory experimental results with antioxidants and ROS production in animal studies, the FRTA was refined to the mitochondrial free radical theory of aging (MFRTA) (Harman 1972). The MFRTA suggests that increased ROS production by mitochondria with age primarily attack mitochondria themselves. This hypothesis explains reduced numbers of mitochondria in cells and increased mitochondrial fragility with increasing age. In contrast, the target of rapamycin (TOR)-centered model of aging suggests that continuing developmental programs cause cell hyperfunctioning and that cell aging is quasi-programmed development (Blagosklonny 2006). During quasi-programmed development, TOR upregulates various cellular pathways (e.g., cell growth, autophagy, protein synthesis), eventually leading to organismal aging. The TOR-centered model of aging predicts that ROS act as secondary messengers in regulatory pathways, and ROS concentrations can therefore incidentally increase in cells with age (Blagosklonny 2008). Additional hypotheses propose decreasing proteome functionality or the reduction of telomeres with age as mechanistic causes of aging (Walton 1982; Pérez et al. 2009). Because each hypothesis predicts different mechanisms of aging and different types and amounts of damage accumulation with age, observations of damage can lend support to or refute aging hypotheses.

Damage markers are cellular parameters that change according to the rate of aging. These markers can predict an organism's biological age and may also allow for comparison of biological age between organisms. Biological age is the loss of functional capacity of a cell or organism over time; an organism's biological age is not always equal to its chronological age. Damage markers, including oxidation of proteins, lipids and nucleic acids, protein stability, telomere length, and telomerase activity, correlate with chronological age in many species (Campisi 1996; Turturro et al. 1999; Proctor and Kirkwood 2002; Butler et al. 2004; Philipp et al. 2005a, b, 2006; Buffenstein 2008; Pérez et al. 2009; Austad 2010). Species with long lifespans also exhibit lower levels of cellular damage than species with short lifespans (Philipp et al. 2005a, b, 2006; Ungvari et al. 2008; Pérez et al. 2009; Austad 2010). One notable exception to these observations is a higher level of lipid and protein oxidation in the naked mole rat, a rodent, which has a long lifespan and exhibits high levels of cellular damage (Andziak et al. 2006). Observed correlations between damage markers and chronological age may be unrelated to biological age or the aging process and instead simply accumulate with time. To identify true markers of aging-related damage, individuals from the same species with different aging rates must be compared.

We measured putative damage markers in two *Arctica islandica* populations with lifespans that differ sixfolds: Icelandic *A. islandica* and Baltic Sea *A. islandica* (maximum lifespan (MLSP) of 226 and 36 years, respectively). The bivalve *A. islandica* (the ocean quahog) is the longest-living non-colonial animal currently known to science and can experience a fourfold longer lifespan than humans (MLSP over 500 years compared to human MLSP of 122 years) (Gampe et al. 2010; Butler et al. 2013). In addition, genetic analyses classify both *A. islandica* populations into the same species regardless of MLSP (Dahlgren et al. 2000; Glöckner et al. 2013). Long-lived ocean quahogs show little decline in telomere maintenance, protein carbonylation, and antioxidant capacities with age; they also have low susceptibility to genotoxic stresses, including topoisomerase inhibitors (Strahl et al. 2007; Abele et al. 2008; Ungvari et al. 2011, 2013; Gruber et al. 2014). The short-lived Baltic Sea population experiences high environmental stress throughout the year (e.g., fluctuations in temperature, oxygen availability, salinity), while the long-lived Icelandic population lives in a relatively constant environment (Basova et al. 2012). Stressful conditions may harm the shorter-lived population and lead to higher mortality rates (Begum et al. 2010). We compared nucleic acid oxidation, lipid oxidation, protein oxidation, and protein stability between the long-lived and the short-lived *A. islandica* populations. We use maximum lifespan as a proxy for aging rate because there is no evidence for extrinsic mortality

in *A. islandica* adults, and we assume a steady rate of aging, due either to internal processes or accumulating environmental stress (Philipp and Abele 2009).

We measured *A. islandica* damage markers in a cross-sectional analysis of wild-caught ocean quahogs from both populations. Individual clams were caught and measured during a single winter. While a single sampling limits observations of seasonal variability, valid aging-markers should not be influenced by seasonal fluctuations and instead should accumulate over age in any tissue of the organism. In addition, we sampled individuals when their environments were the most stable: bivalves experience low external stresses (e.g., temperature and salinity fluctuations, fresh water inflow from melting ice, plankton blooms) and internal stresses (e.g., lower metabolism and gonadal production) in the winter months (Mann 1982; Swaileh 1996; Baumard et al. 1999) (www.ices.dk). We measured highly proliferating gill tissue (Strahl and Abele 2010) because we expected it to be one of the first tissues showing severe, permanent, and nonseasonal changes in a true aging marker.

We collected *A. islandica* individuals covering a large part of the total age range of each population, measured individual ages by counting shell growth rings, and measured each potential marker of aging. We then tested for correlations between age and damage levels and for differences in damage levels between the two populations. We expected true aging markers to accumulate relative to a population's lifespan, irrespective of an individual's chronological age.

Material and methods

Animal sampling and age determination

We sampled 160 Baltic Sea *A. islandica* from the station "Süderfahrt" in February 2010 and 179 Icelandic *A. islandica* from northeast Iceland in March 2010 (54° 32.6 N, 10° 42.1 E and 66° 01.5 N, 14° 50.9 W, respectively). Both populations were sampled at a depth of 20 m with a hydraulic dredge and subsequently kept under stable laboratory conditions with fresh natural habitat water flowthrough. Flowthrough water was approximately 5 °C for both populations, 20–25 practical salinity units (PSU) for Baltic Sea clams and 35 PSU for Icelandic clams. Baltic Sea animals were kept at the GEOMAR Helmholtz Centre for Ocean Research (Kiel, Germany) and Icelandic animals, at the Suðurnes University Research Centre (Sandgerði, Iceland). After 5 days of recovery from sampling stress, animals were dissected and gill tissues were sampled, shock-frozen in liquid nitrogen, and stored at −80 °C. Ages were determined as in Gruber et al. (2014) by counting each shell's annual growth rings (Witbaard et al. 1994; Schöne et al. 2005).

Nucleic acid oxidation

We measured levels of RNA and DNA oxidation in the gill tissue of 21 Baltic Sea and 20 Icelandic *A. islandica* (Table 1). Ratios of 8-oxo-7,8-dihydroguanosine to guanosine and 8-oxo-7,8-2′-deoxyguanosine to deoxyguanosine were measured using high-performance liquid chromatography coupled to electrochemical detection (HPLC-ECD) and used as proxies for total RNA and DNA oxidation, respectively (Hofer et al. 2006). Gill tissue was ground in liquid nitrogen and homogenized on slush ice using a glass-glass Duall homogenizer in 0.6 ml (1:10 *w*:*v*) buffer (3 M guanidine thiocyanate, 0.2 %*w*/*v*N-lauroylsarcosinate, 20 mM Tris, pH 7.5, 10 mM deferoxamine mesylate (DFOM)). After transferring the solution into phase-lock gel (PLG) tubes, an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, pH 6.7) was added and the samples were vortexed for 10 s at 0 °C to completely release nucleic acids. After centrifugation, the aqueous phase was transferred into a new PLG tube and mixed with an equal volume of chloroform/isoamyl alcohol (24:1). Samples were hand-shaken and centrifuged, and the aqueous phase was collected. Nucleic acids were precipitated from the aqueous phase by addition of an equal volume of isopropanol and stored in a −80 °C freezer overnight. After centrifugation, the pellet containing nucleic acids was washed with 70 %*v*/*v* ethanol, centrifuged, dried, dissolved in 150 μl of water containing 30 μM DFOM, and hydrolyzed using 9 U nuclease P_1 and 4 U alkaline phosphatase in buffer (30 mM sodium acetate, 20 μM $ZnCl_2$, pH 5.3) at 50 °C for 60 min. All centrifugations were for 10 min and at 10,000×*g* at 0 °C.

After filtration, samples were analyzed using high-performance liquid chromatography coupled to electrochemical and UV detection (HPLC-ECD/UV). The HPLC system consisted of an EAS Model 582 pump (ESA Inc., Chelmsford, MA, USA), a chilled (4 °C) model 542 autosampler (ESA), a temperature-controlled (35 °C) column oven holding two Delta-Pak (150×3.9 mm inner

Table 1 Sample attributes and age ranges and numbers of individuals (*N*) investigated for each parameter

Population	N_{total}	Age range [years]	Size range [mm]	DNA/RNA oxidation: age range; *N*	Lipid peroxidation: age range; *N*	Protein carbonyls: age range; *N*	Protein stability: age range; *N*
Baltic Sea	148	10–36	33.16–60.6	10–36; 21	11–31; 15	11–35; 15	11–35; 15
Iceland	141	6–226	22.11–105.02	11–226; 20	11–226; 25	11–226; 25	11–226; 25

diameter, 5 μm) C-18 reversed-phase columns (Waters, Milford, MA, USA), a SpectraSYSTEM UV1000 detector (Thermo Electron Corp., San Jose, CA, USA) set at 290 nm and an electrochemical detector (Coulochem III, ESA) with a 5011A Analytical Cell (2 nA; screen electrode, +200 mV; analytical electrode, +300 mV). Chromatograms were recorded using EZChrom Elite (Scientific Software Inc., Pleasanton, CA, USA).

Lipid peroxidation

F_2-isoprostanes (F_2-IsoPs) were measured in the gill tissue of 15 Baltic Sea and 25 Icelandic animals as markers of lipid peroxidation (Table 1). F_2-IsoPs were quantified using stable isotope dilution followed by gas chromatography/negative-ion chemical ionization/mass spectrometry detection as previously described (Morrow and Roberts 1999). Briefly, 100–200 mg tissue was homogenized in ice-cold Folch solution (chloroform/methanol 2:1) containing 5 mg/100 ml butylated hydroxytoluene and lipids were extracted. Extracted lipids were then chemically hydrolysed with 15 % KOH. After acidification with 1 M HCL to pH 3, isotope-labeled 8-*iso*-prostaglandin $F_{2\alpha}$-d_4 was added as an internal standard. Following cleaning using C-18 and silica Sep-Pak cartridges, the eluted compounds were dried under N_2, converted to pentafluorobenzyl esters, and purified using thin-layer chromatography. Purified F_2-IsoPs were converted to trimethylsilyl ether derivatives and dissolved in undecane for quantification by GC/MS. We performed negative ion chemical ionization MS using Agilent 6890 GC and Model 5975 MSD instruments with selected ions monitored for the 8-*iso*-prostaglandin $F_{2\alpha}$-d_4 internal standard (*m*/*z* 573) and F_2-IsoPs (*m*/*z* 569).

Protein oxidation

We measured the ratio of protein carbonyls to total protein content as a proxy for protein oxidation in 15 Baltic Sea and 25 Icelandic animals (Table 1). Soluble and insoluble protein carbonyls were quantified by staining gill tissue with fluorescein-5-thiosemicarbazide (FTC), and FTC-stained protein carbonyls were compared against total cellular proteins stained with Coomassie Brilliant Blue (Chaudhuri et al. 2006). To extract proteins, 150–200 mg gill tissue was homogenized with 350 μl potassium phosphate buffer (20 mM KPO_4, 0.5 mM $MgCl_2$, 1 mM EDTA, pH 6, 1 tablet/10 ml cOmplete Mini Protease Inhibitor (Roche, Mannheim, Germany)) using zirconium silicate beads in a Retsch MM 301 homogenizer (2 min with 30/s). The lysate was centrifuged in a Beckmann TL-100 ultracentrifuge (1 h, 100,000×*g* at 4 °C), and the supernatant was transferred to a small reaction tube and kept on ice. Pellets were resuspended in 200 μl potassium phosphate buffer (20 mM KPO_4, 0.5 mM $MgCl_2$, 1 mM EDTA, 2 % SDS, 0.5 % IGEPAL, 0.5 % sodium deoxycholate, 1 tablet/10 ml cOmplete Mini Protease Inhibitor, 1 mM dithiothreitol, pH 6). Resuspended pellets were sonicated (Bandelin: Sonopuls HD200, 10 s on ice) and ultracentrifuged (10 min, 100,000×*g* at room temperature (RT)). Protein concentrations were measured using Pierce BCA assays (Thermo Scientific, Rockford, IL, USA).

We then measured protein carbonyl concentrations and total protein content using FTC and Coomassie brilliant blue staining. First, 500 μg protein was added to 500 μl KPO_4 buffer containing 0.3 M guanidine and 1 mM FTC label and incubated at 37 °C for 2 h. After incubation, we precipitated proteins by adding 500 μl 20 % chilled trichloroacetic acid in KPO_4 buffer and incubating mixtures on ice for 15 min. After centrifugation (15 min, 16,000×*g* at 4 °C), supernatants were removed and pellets were repeatedly washed in 1:1 ethanol/ethyl acetate. Tubes were kept on ice at all times to prevent protein dissolving. After the last wash, excess ethanol/ethyl acetate was removed and protein pellets were dissolved in 50 μl of 8 M Urea in KPO_4 buffer and incubated at 37 °C for 30 min. Dissolved pellets were repeatedly sonicated and centrifuged (2 min, 16,000×*g* at room temperature). Protein content was again

measured using the BCA assay. An equal amount of protein (6.66 μg) from each sample was separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Fluorescent protein images were captured with a Typhoon 9400 (GE Healthcare, Munich, Germany) using an excitation wavelength of 488 nm and an emission filter at 520 nm with a PMT value of 460. Gels were then washed with water and stained with Bio-Safe Coomassie (BioRad, Munich, Germany) to visualize total protein concentration. Before capturing the Coomassie image with a visible spectrum camera (Molecular Imager ChemiDoc XRS, Bio-Rad, Munich, Germany), the gel was washed with water until all residual stain had disappeared. Fluorescence and Coomassie images were analyzed using ImageJ 1.45S, and data were expressed in ratios of florescent to Coomassie-stained protein (F/C).

Protein stability

We measured protein resistance to unfolding with and without urea stress in 15 Baltic Sea and 25 Icelandic animals (Table 1). Denatured proteins were stained with 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid dipotassium salt (BisANS) and compared with total protein content as described by Pierce et al. (2006). To extract cytosolic proteins, 150–200 mg gill tissue was homogenized in 350 μl labeling buffer (50 mM Tris-HCl, 10 mM $MgSO_4$, pH 7.4, 1 tablet/10 ml cOmplete Mini Protease Inhibitor) using zirconium silicate beads in a Retsch MM 301 homogenizer (2 min with 30/s). Lysates were then ultracentrifuged in a Beckmann TL-100 ultracentrifuge (1 h, 100,000×*g* at 4 °C). Protein concentrations of supernatants were determined using Pierce BCA assays, and extracts were diluted to 1 mg/ml. Control extracts were stained immediately, while stress-treated extracts were incubated on ice for 1 h with 1 M urea. To stain extracts, 100 μM BisANS was added and samples were incubated for 1 h on ice under direct exposure to a 115-V 0.16-A handheld longwave UV lamp (365 nm, UVL-56, UVP, Cambridge, UK). Following photoincorporation, samples were subjected to SDS-PAGE. SDS-PAGE gels were then illuminated with 365 nm UV light and BisANS fluorescence was measured using the Molecular Imager ChemiDoc XRS Imaging System with Quantity One 4.6.8 software (BioRad, Munich, Germany). We measured total protein content using Coomasie brilliant blue as described above.

Statistical analyses

We used linear regressions to investigate the relationships between clam age and each damage parameter (nucleic acid oxidation, F_2-IsoPs, protein carbonyls, and protein stability) within each population. When a damage parameter significantly changed with clam age, we followed up with analyses looking for differences between the two populations. We first tested for equivalent slopes between populations using two-slope *t* tests. When slopes were not significantly different, we tested for equivalent intercepts using ANCOVAs. If a linear regression showed no change in a parameter with clam age, we tested for different damage levels between populations using a *t* test. Finally, we tested for different protein stability levels between populations before and after urea treatment using a two-way ANOVA followed by Tukey's post hoc tests for multiple comparisons. F_2-isoprostane levels were $\log_{10}$ transformed to normalize values. All analyses were performed using GraphPad Prism 6 Version 6.04. Outliers were identified using Grubb's test with an alpha=0.05 and discarded.

Results

We sampled individuals covering 90 % of the maximum reported age range of Baltic Sea *A. islandica* (MLSP 40 years) (Begum et al. 2010) and 45 % of the maximum reported age range of Icelandic *A. islandica* (MLSP 507 years) (Butler et al. 2013) (Table 1). Overall, Baltic Sea individuals ranged from 10 to 36 years old, while Icelandic individuals ranged from 6 to 226 years. Approximately 70 % of the 148 Icelandic individuals exceeded the maximum observed lifespan of 36 years in 141 sampled Baltic Sea individuals.

RNA and DNA oxidation

DNA and RNA oxidation increased significantly with age in both populations (Fig. 1a, b; Table 1), and oxidation accumulated significantly faster in the Baltic Sea than in the Icelandic population ($p_{DNA,\ slopes}<0.0005$, $t=3.6$, $d.f.=37$; $p_{RNA,\ slopes}<0.0001$, $t=5.37$, $d.f.=37$; Fig. 1a, b). When scaled to relative observed lifespan (BS: 36 years=100 %, IC: 226 years=100 %), damage accumulated at the same pace in both populations (Fig. 1c, d, $p_{relDNA,\ slopes}=0.4$, $t=0.26$, $d.f.=37$; $p_{relRNA,\ slopes}=0.41$, $t=0.22$, $d.f.=37$). Scaled RNA oxidation was generally

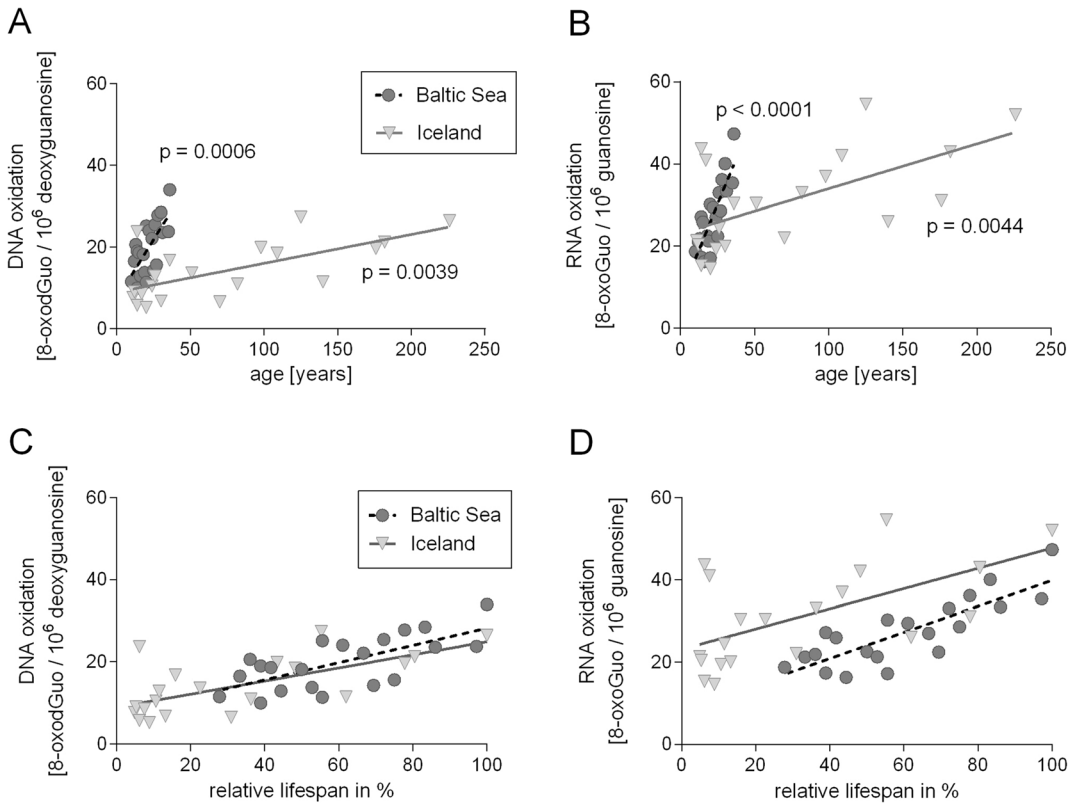

Fig. 1 Nucleic acid damage in gill tissue of Baltic Sea (*circles*) and Icelandic (*triangles*) animals. **a** DNA and **b** RNA oxidation in both populations over age. Significance of the slopes, i.e., increase of oxidation with increasing age is displayed within the graphs. **c** DNA and **d** RNA oxidation calculated over the relative lifespan of both populations

higher in the Icelandic population than in the Baltic Sea population, as indicated by a significantly higher intercept ($p<0.0001$, $F(1,38)=22.76$). Linear regression equations are listed in supplementary Table S1.

Lipid peroxidation and protein oxidation

Both F_2IsoPs and protein carbonyl content remained stable over all investigated ages (Figs. 2 and 3a, b; supplementary Table S1). Mean levels of F_2IsoPs across ages were higher in Icelandic individuals than Baltic Sea individuals ($p<0.0001$, $t=4.55$, $d.f.=37$). Oxidation levels of soluble and insoluble proteins did not differ between populations (soluble proteins: $p=0.19$, $t=1.35$, $d.f.=38$; insoluble proteins: $p=0.99$, $t=0.02$, $d.f.=17$).

Protein stability

To measure protein stress resistance, proteins extracted from gill tissues were stressed with urea and assayed with a probe specific for partially folded protein intermediates. No age-related changes in protein sensitivity were observed for either population (Fig. 3c, d; supplementary Table S1). A two-way ANOVA revealed significant differences between populations ($p<0.0075$, $F(1,74)=7.6$) and stress treatments ($p<0.0001$, $F(1,74)=20.03$; supplementary Table S2). Proteins treated with urea were significantly more denatured than non-stressed controls, indicating that the stress treatment was effective. Proteins of Baltic Sea clams displayed significantly greater unfolding in response to stress than proteins of Icelandic clams, and folding levels of unstressed Baltic Sea proteins were not significantly different from those of stressed Icelandic proteins (Fig. 3e; Tukey's test for multiple comparisons, $p<0.05$, $q=1.68$, $d.f.=74$; supplementary Table S3).

Discussion

We compared physiological damage markers (protein oxidation, lipid oxidation, nucleic acid oxidation, and

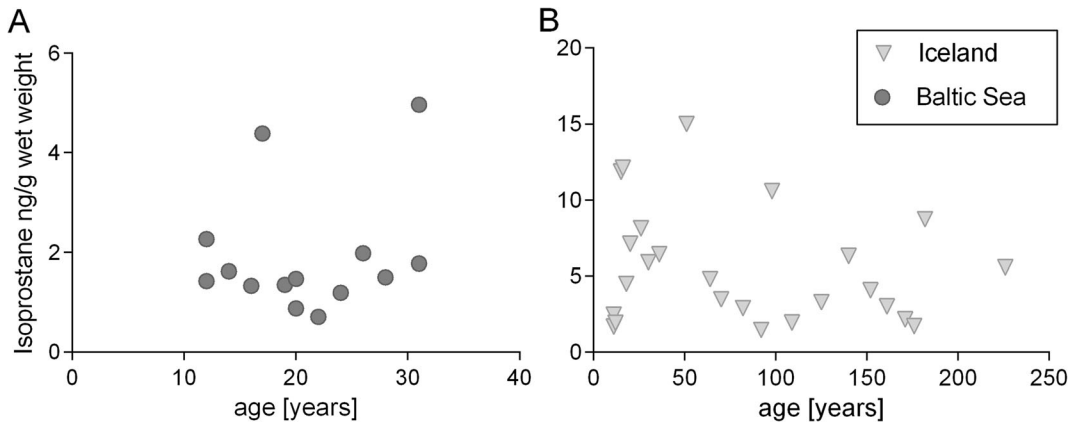

Fig. 2 Lipid peroxidation in gill tissue of Baltic Sea and Icelandic *A. islandica*. Lipid peroxidation over age quantified as F_2-isoprostanes relative to total wet weight in **a** the Baltic Sea (*circles*) and **b** the Icelandic (*triangles*) population (scales are different for each population)

protein instability) among *A. islandica* individuals to investigate intrinsic causes of aging. Damage was compared in a cross-sectional snapshot covering the entire age ranges of two populations that differed dramatically in MLSP (226 years northeast of Iceland and 36 years in the Baltic Sea). Nucleic acids contrast with proteins and lipids because their levels of oxidation increased significantly with age and correlated with relative age within each population (i.e., nucleic acid oxidation increased faster in the shorter-lived Baltic Sea ocean quahogs). In contrast, we detected no increase in lipid or protein oxidation with age in either population, despite previous observations of protein carbonyl accumulation in other organisms, including humans and clams (Sohal 2002; Mutlu-Türkoğlu et al. 2003; Philipp et al. 2005a, b).

In some cases, overall damage differed between the two *A. islandica* populations. While the long-lived Icelandic population showed more lipid and RNA damage than the Baltic Sea population, it also showed less protein instability when challenged with urea stress. Relatively higher lipid and RNA oxidation levels in the long-lived population suggest that oxidation does not impact the ocean quahog's extraordinarily long lifespan. Instead, accumulating RNA oxidation may be an effect, but not a cause, of the aging process. Differences in *A. islandica* protein stability may be due to posttranslational modifications, chaperone compositions, and chaperone activities (Pride et al. 2015). Relatively high protein stability in the gills of long-lived *A. islandica* is consistent with previous observations of high protein stability in *A. islandica* foot and adductor muscle compared to other clams, and higher protein stability in long-lived than short-lived vertebrates (Pérez et al. 2009; Salmon et al. 2009; Treaster et al. 2014). However, protein stability does not change over the lifetime of either *A. islandica* population.

Observed differences in damage parameters between populations may result from different extrinsic stresses in the Icelandic and Baltic Sea environments. The Baltic Sea environment is characterized by large environmental fluctuations, while the Icelandic environment is relatively stable (Basova et al. 2012). For example, Baltic Sea animals must adapt their metabolic behavior to recurring hypoxic and anoxic events, which can induce changes in gene expression levels that potentially lower protein stability (Conley et al. 2007, 2009; Philipp et al. 2012). Relatively stable proteins in Icelandic *A. islandica* support previous findings showing high stress resistance in *A. islandica* (Ungvari et al. 2011, 2013). Membrane peroxidation in clams can also be influenced by diet and seasonal temperature (Munro and Blier 2014). We speculate that higher observed F_2IsoPs in Icelandic *A. islandica* may be the result of higher total lipid uptake in the Icelandic environment. Many clams get most of their polyunsaturated fatty acids from their diets (Langdon and Waldock 1981; Parent et al. 2008), and Icelandic *A. islandica* may have a richer available diet than Baltic Sea *A. islandica* (Ridgway and Richardson 2010). Analyses and comparisons of lipid and proteome compositions of the two investigated *A. islandica* populations could give more insights into underlying mechanisms of lipid peroxidation and protein stability.

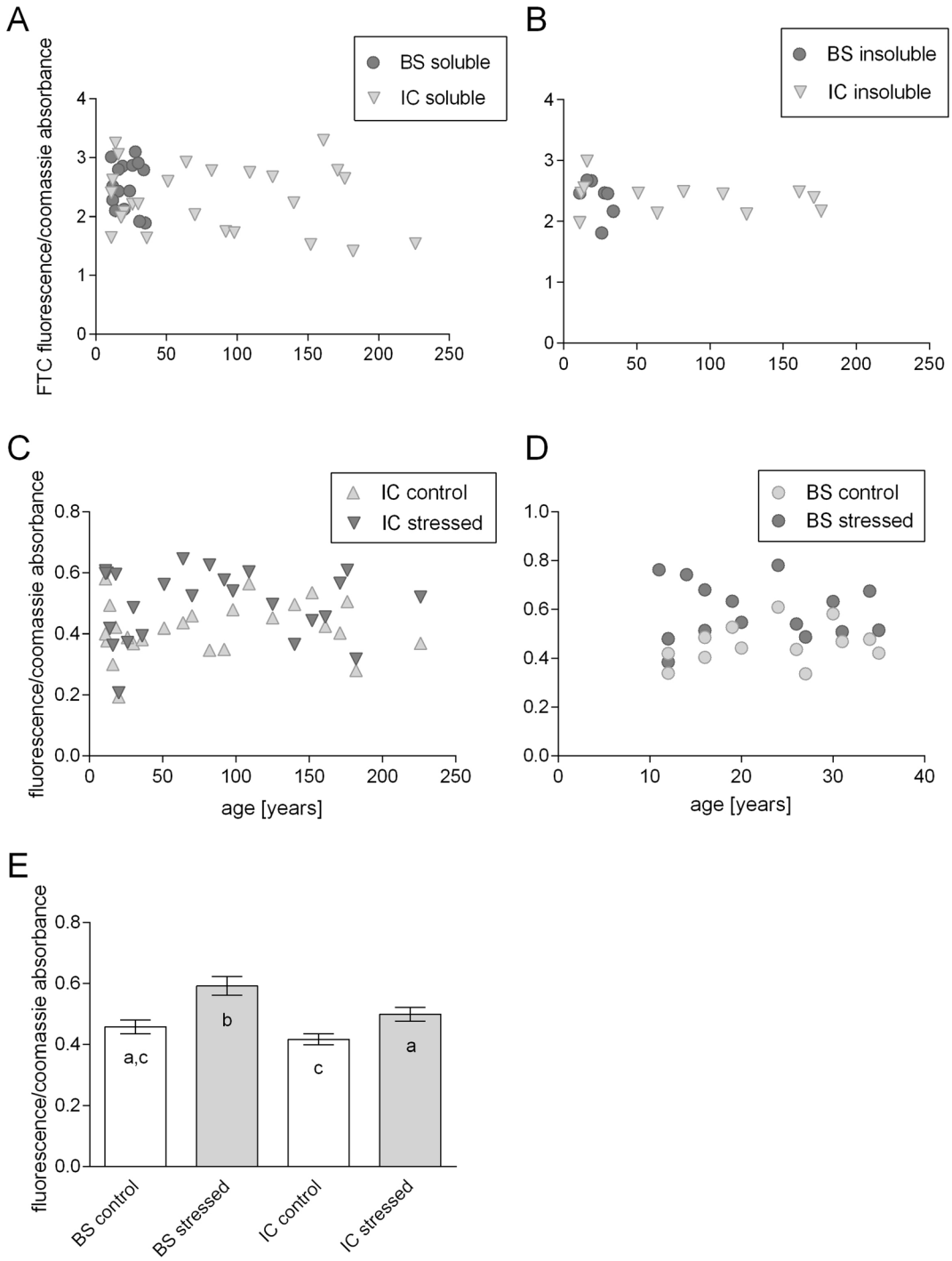

Fig. 3 Protein damage in gill tissue of Baltic Sea (*circles*) and Icelandic (*triangles*) *A. islandica*. Relative quantification of protein carbonyls (in FTC fluorescence) to total protein content (Coomassie absorbance) in **a** soluble and **b** insoluble proteins. **c** Protein resistance with age to 1 M urea unfolding stress measured as BisANS-coupled fluorescent units relative to total protein content in Icelandic samples. **d** Protein resistance to 1 M urea stress of Baltic Sea animals. **e** Mean levels of protein resistance to urea stress of all measured BS and IC individuals±SEM. *Equal letters* indicate nonsignificant differences among populations and treatments, whereas *different letters* indicate significances at the $p \leq 0.05$ level

Nucleic acid oxidation—a true aging-marker?

Nucleic acid oxidation in the clams increases according to relative age within each population instead of merely accumulating over chronological time. Oxidized nucleic acids accumulated significantly faster over time in the shorter-lived Baltic Sea than in the Icelandic population (Fig. 1). But when standardized to population lifespan, oxidation of each nucleic acid accumulated at the same rate in both populations. Nucleic acid oxidation can be compared between populations because both populations are members of the same species, and the only difference between populations is lifespan. Here, the use of 226 years as maximal lifespan for Icelandic *A. islandica* may be more realistic than the previously reported 507 years because individuals with ages around 200 years were found in previous studies, but only one individual with an age >375 years has been found so far (Schöne et al. 2005; Butler et al. 2013). One caveat to our nucleic acid observations is that it is possible that only individuals with high oxidative damage reach old age. A potential mechanism for oxidative damage promoting survival is that damage-inducing ROS can serve as a beneficial antimicrobial defense mechanism. We consider this unlikely because bivalves are expected to experience low extrinsic mortality once they reach adulthood (Philipp and Abele 2009). Instead, we consider nucleic acid oxidation to be a true marker of biological age because it accumulates in both long-lived and short-lived *A. islandica* at the same rate over biological age.

Further evidence for a correlation between nucleic acid oxidation and biological age comes from comparisons across species. DNA oxidation levels in *A. islandica* increase at rates of 6.73 and 9.25 % per year in gill tissue of Icelandic and Baltic Sea individuals, respectively. Nucleic acid oxidation rate in adult human muscle tissue is 3.34 % per year (Mecocci et al. 1999) which is of the same order of magnitude as in *A. islandica*. Likewise, human lifespan is of the same order of magnitude as observed *A. islandica* lifespan (122 and 36–226 years, respectively). In contrast, DNA oxidation increases much faster in short-lived rodents: 21–370 % over a rodent's 18-month lifespan in several tissues (Hamilton et al. 2001). Because the biologies of humans, rodents, and clams differ, our results stress the advantage of intraspecific damage-marker comparison between populations with different maximum lifespans. While nucleic acid oxidation is a genuine marker for primary aging, its causes are not yet completely understood.

Aging hypotheses

While correlations between cellular changes and aging have been observed frequently, researchers have not yet been able to determine which, if any, are intrinsic causes or effects of the primary aging process (Turturro et al. 1999; Butler et al. 2004; Philipp et al. 2005a, b, 2006; Buffenstein 2008; Austad 2010). The question remains as to whether there are universal mechanisms for primary aging in all animals, but several mechanisms of cellular aging have been proposed. Increases in nucleic acid oxidation with biological age in *A. islandica* can support or contradict several aging hypotheses.

Both the FRTA and the MFRTA propose increased oxidation of cellular macromolecules with organism age (Harman 1956, 1972). These theories of aging suggest that an imbalance between intrinsic ROS production and elimination by antioxidants or other ROS scavenging mechanisms results in oxidative damage of nucleic acids, proteins, and lipids and that the resulting oxidated molecules are mechanistic causes of aging (Barja 2013). While the FRTA does not specify the source of oxidative damage, the MFRTA specifically indicates that mitochondrial production of destructive ROS increases with age and promotes oxidative damage in nearby long-lived macromolecules (e.g., collagen, elastin, DNA) (Harman 1972). Both hypotheses predict increases of oxidated proteins, lipids, and nucleic acids with time and age within an organism. Observed increases in *A. islandica* 8-oxoGuo levels with biological age may result from decreasing base excision repair rates, decreasing antioxidants, or increasing ROS production rate by mitochondria with age and support both free-radical hypotheses. While the FRTA predicts no mechanism for ROS production and does not specify molecular targets of oxidation, the MFRTA predicts that oxidation should occur in long-lived macromolecules, including proteins and lipids, in close proximity to mitochondrial ROS production. We did not observe increased protein or lipid oxidation with biological age and consider our observations of increasing nucleic acid oxidation with biological age to give more support to the FRTA than the MFRTA. However, further analyses of mitochondrial components are needed to give support to the MFRTA in *A. islandica*.

The TOR-centered model of aging predicts that developmental programming continuation can, but does not always, incidentally increase ROS when ROS act as secondary messengers in regulatory pathways

(Blagosklonny 2008). In the TOR-centered model, ROS do not cause aging or senescence; instead, quasi-development, mediated by TOR, causes aging-related diseases before ROS reaches lethal levels. Observed correlations between nucleic acid oxidative damage and biological age in *A. islandica* can therefore neither support nor refute the TOR-centered model. Longitudinal studies are extremely difficult to do in long-lived organisms such as *A. islandica*. However, if further experimental data, such as observations of extended *A. islandica* longevity after rapamycin treatment, support the TOR-centered model of aging, our observations of increasing nucleic acid oxidation with age could provide supplemental evidence that the TOR pathway incidentally increases nucleic acid oxidative damage.

Neither decreased telomere maintenance with time nor decreased protein stability over time are likely explanations for *A. islandica* aging. While our observations of higher deoxyguanosine oxidation over time would otherwise suggest shortening of G-rich telomeric repeat sequences with number of cell divisions, telomeric maintenance capacity is stable with age in both *A. islandica* populations (Gruber et al. 2014). In addition, our data do not support the hypothesis that protein maintenance is impaired by chronic oxidative stress leading to protein misfolding, protein oxidation, and decreasing protein stability with age (Pérez et al. 2009). We observed no increases in protein oxidation or instability with time in either *A. islandica* population. However, the proteome may generally be more stable in longer-lived than shorter-lived organisms, as suggested by higher protein stability in Icelandic than in Baltic Sea *A. islandica*.

Conclusion

Of the four measured damage markers (protein oxidation, lipid oxidation, nucleic acid oxidation, and protein stability), only nucleic acid oxidation accumulates with time in *A. islandica*. In addition, nucleic acid oxidation accumulates at a faster rate in the longer-lived bivalve population, correlating with biological instead of chronological age. Nucleic acid oxidation is frequently caused by oxidative damage from ROS, and high ROS production may be a cause or effect of aging. While nucleic acid oxidation is mechanistically linked with primary aging, its accumulation is consistent with several hypotheses promoting aging mechanisms. In *A. islandica*, nucleic acid oxidation is a true marker of biological aging. Further investigations of cellular aging processes will be necessary to understand the underlying causes for and consequences of the faster nucleic acid oxidation in the shorter-lived *A. islandica* population.

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Conflict of interest The authors declare that they have no competing interests.

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