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The Greenland shark: A new challenge for the oxidative stress theory of ageing?

David Costantini [a,b,*], Shona Smith [b], Shaun S. Killen [b], Julius Nielsen [c], John F. Steffensen [c]

[a] Department of Biology, University of Antwerp, Universiteitsplein 1, 2610 Wilrijk, Belgium
[b] Institute for Biodiversity, Animal Health and Comparative Medicine, University of Glasgow, Glasgow, G12 8QQ, UK
[c] Marine Biological Section, Department of Biology, University of Copenhagen, Strandpromenaden 5, DK-3000 Helsingør, Denmark

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ABSTRACT

The free radical theory of ageing predicts that long-lived species should be more resistant to oxidative damage than short-lived species. Although many studies support this theory, recent studies found notable exceptions that challenge the generality of this theory. In this study, we have analysed the oxidative status of the Greenland shark (*Somniosus microcephalus*), which has recently been found as the longest living vertebrate animal known to science with a lifespan of at least 272 years. As compared to other species, the Greenland shark had body mass-corrected values of muscle glutathione peroxidase and red blood cells protein carbonyls (metric of protein oxidative damage) above 75 percentile and below 25 percentile, respectively. None of the biochemical metrics of oxidative status measured in either skeletal muscle or red blood cells were correlated with maximum lifespan of species. We propose that the values of metrics of oxidative status we measured might be linked to ecological features (e.g., adaptation to cold waters and deep dives) of this shark species rather to its lifespan.

1. Introduction

Some species live for hundreds of years; the lifetime of some other species is limited to only a few hours. This surprising variation in longevity has intrigued biologists for decades. A key issue that has generated an overwhelming body of research is the underlying cause of senescence and of this dramatic variation in maximum lifespan. In 1956, Denham Harman suggested that the production of free radicals might be responsible for cell senescence because free radicals can damage vital biomolecules like proteins, lipids and nucleic acids, causing cell senescence. The free radical theory of ageing has since been refined and a number of iterations have been presented, including the mitochondrial theory of ageing (Harman, 1956) and the oxidative stress hypothesis of ageing (Yu and Yang, 1996).

One commonly used method of unraveling the factors linked to species longevity is to compare a given biochemical metric of oxidative status across species exhibiting variation in maximum lifespan. In this context, many studies concluded that long-lived species are more resistant to oxidative damage (e.g., cell composition with biomolecules less sensitive to oxidation) than short-lived species and that long-lived species have lower antioxidant levels than short-lived species because they produce less free radicals (Hulbert et al., 2007; Pamplona and Costantini, 2011). As with all apparently general patterns in biology, there are important exceptions that challenge the generality of the theory. Contrary to the predictions of the free radical theory of ageing, naked mole rats (*Heterocephalus glaber*), a long-living rodent, do not have exceptionally high levels of antioxidant protection (Andziak et al., 2005) and have elevated damage to DNA, lipids and proteins (Andziak et al., 2006) as compared to similar-sized mice. Another example is the olm (*Proteus anguinus*), a small cave lifespan of over 100 years. Surprisingly, neither its basal metabolism nor oxidative damage and activities of antioxidant enzymes explain why this species sits as an outlier in the relationship between amphibian size and longevity (Issartel et al., 2009; Voituron et al., 2011). These and other experimental findings led some authors to challenge the oxidative stress theory of ageing (Speakman and Selman, 2011).

The Greenland shark (*Somniosus microcephalus*) is one of the world's largest predatory sharks (maximum body length > 5 m), mainly distributed in Arctic and subarctic regions of the North Atlantic (Bigelow and Schroeder, 1948; Nielsen et al., 2014). Surprisingly little is known about the general biology of Greenland shark (Hansen, 1952, 1963), but a recent study based on radiocarbon dating of the sharks eye lens found a lifespan of at least 272 years and that it has a very long phase before sexual maturity (around 130 years until maturity for females, Nielsen et al., 2016), which makes the Greenland shark the longest living vertebrate animal known to science. The Greenland shark is clearly suitable for evaluating whether its oxidative status explains its lifespan

* Corresponding author at: Department of Evolutionary Ecology, Leibniz Institute for Zoo and Wildlife Research, Alfred-Kowalke-Str. 17, 10315 Berlin, Germany.
E-mail address: davidcostantini@libero.it (D. Costantini).

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or is rather a consequence of its ecology (e.g., exposure to repeated episodes of hypoxia associated with deep water dives). We have therefore measured two metrics of oxidative status in both blood and muscle of Greenland sharks and compared their levels to those of species with different maximum lifespans.

2. Material and methods

2.1. Tissue collection from sharks

Samples from non-sexually mature Greenland sharks (n = 11 individuals, 2 males and 9 females; Table 1) were collected in 2012 in Ammassalik Fjord, south-eastern Greenland from the Danish research vessel Dana. Sharks were caught on bottom long lines at depths between 300 and 500 m. Immediately after capture, a sample of blood (ca. 100 ml) was collected, centrifuged for 3 min at 10,000 rpm and stored at −18 °C while on the field. Samples of skeletal muscle (white muscle) were collected from the dorsal side above the gills and stored at −18 °C while on the field. All samples were then stored at −80 °C. Body mass of each individual shark was measured on a digital scale except for four sharks. For these sharks body mass was calculated according to Nielsen et al. (2014). Animals were either released with tags or euthanized for research purposes. Sampling of sharks was carried out in accordance with laws and regulations and with authorization from the Government of Greenland (Ministry of Fisheries, Hunting & Agriculture, document number 935119).

2.2. Laboratory analyses

Samples of skeletal muscle were homogenised in ice cold PBS (supplemented with 20% (v/v) glycerol and 0.2 mM phenylmethylsulfonyl fluoride as an inhibitor of proteases) using a pestle. Samples were then sonicated for 10 min and then centrifuged for 10 min at 10,000 rpm. The supernatant was split into different aliquots, which were stored at −80 °C for later analyses of protein carbonyls (metric of protein oxidative damage) and activity of glutathione peroxidase, two metrics that were found to be linked to species lifespan (Pamplona and Costantini, 2011; Halliwell and Gutteridge, 2015). Protein carbonyls (PCs) were measured according to Levine et al. (1990). Carbonyls (C═O) are introduced into proteins from free radicals or via reactions with lipid peroxidation products (malondialdehyde and hydroxynonenal) or carbohydrates; protein carbonylation is mostly irreversible (Halliwell and Gutteridge, 2015). Nucleic acids were removed by adding 1 volume of a 10% solution of streptomycin sulfonate to 9 volumes of sample. PCs were then derivatised to 2,4-dinitrophenylhydrazone by reaction with 2,4-dinitro-phenylhydrazine (DNPH). The Ransel assay (RANDOX Laboratories, Crumlin, UK) was used to quantify the activity of glutathione peroxidase (GPX). Glutathione peroxidase uses the reduced form of glutathione to reduce peroxides and hydroperoxides to water and alcohols, respectively (Halliwell and Gutteridge, 2015). The Ransel assay is based on the method of Paglia and Valentine (1967) using cumene hydroperoxide as a substrate. Measures of protein carbonyls and GPX were standardised by expressing the concentrations per mg of proteins as measured by the Bradford protein assay (Bio-Rad Laboratories, Hercules, USA) using a standard curve of bovine serum albumin. Hence, protein carbonyls were expressed as nmol/mg proteins, while GPX was expressed as Units/mg proteins.

Table 1
Descriptive statistics of Greenland sharks included in our study. X = the biological matrix collected from a single specimen. RBCs = red blood cells; PCs = protein carbonyls (expressed as nmol/mg proteins); GPX = glutathione peroxidase (expressed as Units/mg proteins). * For these sharks body mass was calculated according to Nielsen et al. (2014). Mean total length (TL) was 327 cm (SD = 38; n = 11) and mean body mass (BM) was 343 kg (SD = 123; n = 11).

Sex	TL (cm)	BM (kg)	RBC PCs	Muscle PCs	RBC GPX	Muscle GPX	RBCs	Muscle
F	346	416	7.0		0.008		×	
F	306	246	3.1	10.5	0.007	0.091	×	×
F	264	168	1.9	4.5	0.006	0.099	×	×
F	386	560	7.0	3.1	0.004	0.118	×	×
F	365	430		1.8		0.059		×
F	336	338	3.3	3.4	0.007	0.070	×	×
F	355	435*	6.9		0.013		×	
F	351	452	2.8	6.3	0.011	0.075	×	×
F	302	262*	7.8		0.007		×	
M	290	231*	3.3	9.2	0.006	0.142	×	×
M	291	234*	3.6		0.006		×	

2.3. Data collection from literature

A literature search for studies in which protein carbonyls and glutathione peroxidase (GPX) were analysed using our same protocols, respectively, was conducted on both Web of Science and Scopus using a combination of keywords ("protein carbonyls", "GPX", "muscle", "red blood cells"). Data are reported in Table 2.

Data on body mass and maximum lifespan were collected from genomics.senescence.info/species/ or fishbase.org/ when not reported in the articles selected for this study. Longevity of *Hypophtalmichthys molitrix* and of *Hoplias malabaricus* was obtained, respectively, from animaldiversity.ummz.umich.edu/accounts/Hypophthalmichthys_molitrix/ and Novaes and Carvalho (2011).

2.4. Statistical analyses

All statistical analyses were performed using SPSS version 22 (Chicago, USA). Values of biochemical metrics, body mass and maximum lifespan (Table 2) were transformed as log (x) or log (x + 1) before analyses in order to improve normality of distribution. Similar results were obtained including untransformed variables in all models (data not shown). To describe the distribution of PCs and GPX across species while controlling for multiple sources of variation, we extracted residuals of each biochemical measure (included in the models as dependent variables) from Linear Mixed Models (LMMs) including 'study' as a random factor (because some articles analysed multiple species for muscle PCs and GPX and for red blood cell GPX) and two independent fixed factors: 1) origin of the animals (i.e. wild or cultured; humans were included in the group of artificially selected strains); and 2) taxonomic class (fish, amphibians, reptiles, birds or mammals). Mean adult body mass of each species was included as a covariate to control for any allometric effects. Given that body mass data were obtained from different sources, we opted to run additional LMMs where body mass was not included in the models in order to control for any bias. In order to assess whether species that live longer have lower production of PCs and lower levels of GPX in both skeletal muscle and red blood cells, we calculated correlations between residuals of biochemical metrics and residuals of maximum lifespan obtained from a linear regression of log-transformed maximum lifespan onto log-transformed body mass. Given the moderate sample size, we present results from Spearman test. Note that results were similar where Pearson test was used. In preliminary analyses, we also used LMMs to test the covariation between a given biochemical metric and maximum lifespan while controlling for potential sources correlations for ease of presentation.

3. Results

Compared to other species included in this analysis, the Greenland shark has particularly high values of PCs and GPX in muscle (both above 75 percentile; Table 3). When among species differences in body mass are corrected for, compared to other species, the Greenland shark has high muscle GPX (above 75 percentile) and low values of red blood cell PCs (below 25 percentile; Table 3). In contrast, values of

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