



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



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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.

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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 on several fundamental aspects of Greenland shark biology (MacNeil et al., 2012). 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 (at least 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

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explains its lifespan 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 $-18\text{ }^{\circ}\text{C}$ while on the field. Samples of skeletal muscle (white muscle) were collected from the dorsal side above the gills and stored at $-18\text{ }^{\circ}\text{C}$ while on the field. All samples were then stored at $-80\text{ }^{\circ}\text{C}$. 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 $-80\text{ }^{\circ}\text{C}$ 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

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		×	

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.

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 *Hypophthalmichthys 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

Table 2

Summary information of species and articles included in the comparative work. MLS = maximum lifespan; rbcs = red blood cells; PCs = protein carbonyls (expressed as nmol/mg proteins); GPX = glutathione peroxidase (expressed as Units/mg proteins).

Class	Scientific name	Origin	Body mass (g)	MLS (years)	PCs muscle	PCs rbcs	GPX muscle	GPX rbcs	Article
Amphibians	<i>Pelophylax ridibundus</i>	Wild	39.5	11			0.0186		Feidantsis et al., 2013
Amphibians	<i>Proteus anguinus</i>	Captivity	10.6	102			0.0041		Issartel et al., 2009
Amphibians	<i>Calotriton asper</i>	Captivity	4.3	26			0.0041		Issartel et al., 2009
Birds	<i>Taeniopygia guttata</i>	Captivity	15.1	12		7.594			Costantini, unpublished data
Birds	<i>Bubo bubo</i>	Wild	2686	68				0.6110	Espín Luján, 2013
Birds	<i>Gyps fulvus</i>	Wild	7436	41.4				0.4490	Espín Luján, 2013
Birds	<i>Coturnix japonica</i>	Captivity	235.4	6		9.608		0.0072	Marasco et al., 2013
Birds	<i>Fulica atra</i>	Wild	734	20.6				0.7240	Martinez- Haro et al., 2011
Birds	<i>Aythya ferina</i>	Wild	823	23.2				0.2640	Martinez- Haro et al., 2011
Birds	<i>Anas platyrhynchos</i>	Wild	1048	29.1				0.3080	Martinez-Haro et al., 2011
Birds	<i>Gallinula chloropus</i>	Wild	392	18.6				0.5800	Martinez-Haro et al., 2011
Birds	<i>Columba livia</i>	Captivity	417	35	0.380				Montgomery et al., 2011
Birds	<i>Melopsittacus undulatus</i>	Captivity	26	21	0.295				Montgomery et al., 2012
Birds	<i>Nymphicus hollandicus</i>	Captivity	83.2	35	0.823				Montgomery et al., 2012
Birds	<i>Coturnix japonica</i>	Captivity	235.4	6	0.884				Montgomery et al., 2012
Birds	<i>Coturnix chinensis</i>	Captivity	45.4	5	0.819				Montgomery et al., 2012
Birds	<i>Agapornis sp.</i>	Captivity	48.9	16.1	1.528				Montgomery et al., 2012
Birds	<i>Aptenodytes patagonicus</i>	Wild	14,000	26			0.0081		Rey et al., 2008
Birds	<i>Cyanistes caeruleus</i>	Wild	11.08	14.6		3.824		0.1257	Smith, 2016
Fish	<i>Scophthalmus maximus</i>	Captivity	113.3	26	6.288				Abele et al., 2007
Fish	<i>Cyprinus carpio</i>	Captivity	10,600	47		11.030			García- Medina et al., 2013
Fish	<i>Morone saxatilis</i>	Captivity	31,350	30			0.0105		Grim et al., 2013
Fish	<i>Carassius auratus</i>	Captivity	52.5	41	3.370				Kubrak et al., 2012
Fish	<i>Hoplias malabaricus</i>	Wild	246.32	9.3	3.000				Monteiro et al., 2013
Fish	<i>Oncorhynchus mykiss</i>	Captivity	663	11	0.260				Passi et al., 2004
Fish	<i>Dicentrarchus labrax</i>	Wild	1217	15	0.240				Passi et al., 2004
Fish	<i>Somniosus microcephalus</i>	Wild	343,000	272	5.540	4.675	0.0936	0.0074	Present study
Fish	<i>Barbus barbus</i>	Captivity	12,000	15			0.0190		Radi et al., 1985
Fish	<i>Cyprinus carpio</i>	Captivity	10,600	47			0.0050		Radi et al., 1985
Fish	<i>Carassius carassius</i>	Captivity	3000	10			0.0700		Radi et al., 1985
Fish	<i>Ctenopharyngodon idella</i>	Captivity	1450	21			0.0019		Radi et al., 1985
Fish	<i>Hypophthalmichthys molitrix</i>	Captivity	450	20			0.0250		Radi et al., 1985
Fish	<i>Sparus aurata</i>	Captivity	9460	11			0.0023		Sanz et al., 2012
Mammals	<i>Mus musculus</i>	Captivity	20.5	4	0.995				Kaczor et al., 2007
Mammals	<i>Lepus europaeus</i>	Wild	4175	10.7			0.2100		Linsak et al., 2013
Mammals	<i>Homo sapiens</i>	Captivity	62,000	122.5		4.670			Luqman and Rizvi, 2006
Mammals	<i>Mus musculus</i>	Captivity	17.6	4		3.150			Maity et al., 2013
Mammals	<i>Rattus norvegicus</i>	Captivity	343	3.8	0.696				Montgomery et al., 2011
Mammals	<i>Homo sapiens</i>	Captivity	62,000	122.5	3.444				Pansarasa et al., 1999
Mammals	<i>Rattus norvegicus</i>	Captivity	240	3.8		1.100			Qujeq et al., 2005
Mammals	<i>Equus caballus</i>	Captivity	421,000	57			0.0469		Williams et al., 2004
Mammals	<i>Spermophilus tridecemlineatus</i>	Captivity	230	7.9	0.567				Woods and Storey, 2005
Reptiles	<i>Lacerta vivipara</i>	Wild	2.94	11			0.0006		Voituron et al., 2006
Reptiles	<i>Trachemys scripta</i>	Captivity	240	41.3			0.0130		Willmore and Storey, 1997

muscle PCs now sit in the middle of the whole variation of our sample. Activity of GPX in red blood cells sits in the middle of the whole variation of our sample irrespective of whether body mass is controlled for or is not.

None of the biochemical metrics of oxidative status were related to species maximum lifespan (Spearman correlation coefficients: range -0.18 to 0.18 , $p \geq 0.30$), regardless of whether body mass was or was not included in the models (Fig. 1).

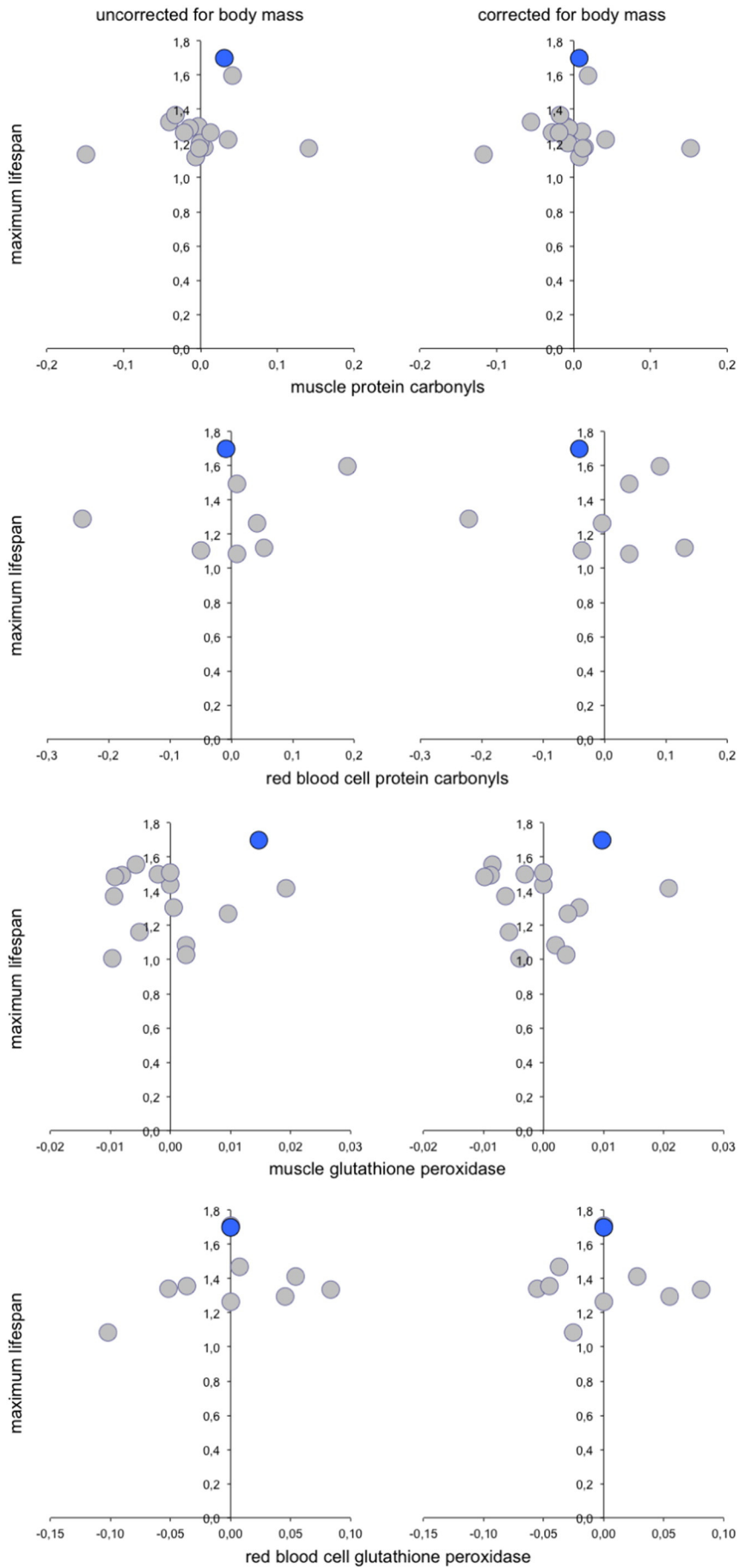
4. Discussion

Results of our comparative study indicated that the oxidative status of Greenland shark appears to differ from that of other species. This is particularly evident from body mass-corrected values of muscle GPX and red blood cells PCs, which were above 75 percentile and below 25 percentile, respectively. These characteristics of the Greenland shark do not seem, however, to be the reason for its long lifespan as compared

Table 3

Descriptive statistics of biochemical metrics of oxidative status included in this study. Values of Greenland shark (GS) are also reported separately for comparison with the whole variation of our sample. N = number of vertebrate species data were collected from; SD = standard deviation; rbcs = red blood cells; PCs = protein carbonyls (expressed as nmol/mg proteins); GPX = glutathione peroxidase (expressed as Units/mg proteins); res = residuals; NoBM = no correction for body mass; BMcorr = correction for body mass.

	PCs muscle	PCs rbcs	GPX muscle	GPX rbcs	resPC muscle NoBM	resPC muscle BMcorr	resPCr bcs NoBM	resPCr bcs BMcorr	resGP Xmuscle NoBM	resGP Xmuscle BMcorr	resGP Xrbcs NoBM	resGP Xrbcs BMcorr
N	16	8	15	10	16	16	8	8	15	15	10	10
Mean	1.8	5.7	0.032	0.312	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
SD	1.9	3.4	0.056	0.267	0.058	0.054	0.121	0.107	0.009	0.008	0.054	0.044
Min	0.2	1.1	0.001	0.007	-0.149	-0.118	-0.242	-0.221	-0.010	-0.010	-0.102	-0.056
Max	6.3	11.0	0.210	0.724	0.141	0.152	0.189	0.131	0.019	0.021	0.083	0.081
Perc. 25	0.4	3.3	0.004	0.037	-0.019	-0.018	-0.040	-0.039	-0.008	-0.006	-0.040	-0.039
Perc. 50	0.9	4.7	0.011	0.286	-0.001	0.000	0.009	0.018	0.000	0.000	0.000	0.000
Perc. 75	3.3	9.1	0.025	0.588	0.027	0.014	0.050	0.078	0.003	0.004	0.047	0.035
GS	5.5	4.7	0.094	0.007	0.031	0.007	-0.009	-0.040	0.015	0.010	0.000	0.000



to other species. In fact, none of the metrics of oxidative status in either skeletal muscle or red Greenland sharks have high muscle GPX and low red blood cell PCs. The Greenland shark inhabits cold waters, mostly in the range -1.8 to 10 °C (Stokesbury et al., 2005).

Moreover, data from 25 years of surveys show that Greenland sharks are usually caught at 400–700 m, but were found at all depths between 100 and 1200 m (Nielsen et al., 2014). Hence, the oxidative status of the Greenland shark might have been shaped to some degree by adaptation to a life in the cold Arctic waters and to deep dives. For example, many species that are faced with repeated episodes of hypoxia/reoxygenation (as typical of diving animals) tend to have high basal levels of antioxidant defences in order to protect them against high free radical production that occurs during reoxygenation (Costantini, 2014). This might be one reason that explains why Greenland sharks have high GPX (an antioxidant enzyme) as compared to the other species included in this study.

Estimates of maximum lifespan include both the pre- and post-reproductive phases. Hence, maximum lifespan of long-lived species may be inflated with the addition of a period of life over which individuals are under a waning force of selection (Reznick et al., 2006). Conversely, evolution would have shaped antioxidant mechanisms to impact on the organism only in the period of life where selection is stronger. This is particularly relevant for the Greenland shark. It has in fact a very long phase before sexual maturity (Nielsen et al., 2016) during which selection would be expected to be particularly long and sustained. Hence, individuals poorly protected against oxidative stress might be rapidly eliminated by natural selection. All Greenland shark individuals included in this study were not sexually mature. However, growth does not appear to be a reason for the high GPX because sharks have an indeterminate growth, i.e., they keep growing all their life long. Also many studies found juveniles to have lower GPX than adults (Costantini, 2014). Further studies will clearly be needed to unravel the reasons for the high activity of GPX in skeletal muscle and the low level of protein damage in red blood cells. Although both protein carbonyls and GPX were previously found to be linked to species lifespan (Pamplona and Costantini, 2011; Halliwell and Gutteridge, 2015), it will be important to collect data on other metrics of oxidative status (e.g., DNA damage and repair) that might reveal routes through which Greenland sharks pave the way to their exceptional longevity. Species-based approaches may, however, hide within-species variation in the association between oxidative status and longevity that has been found in several vertebrate species (Costantini, 2014). Thus, a proper understanding of the oxidative stress theory of ageing will require much further study, in particular a combination of comparative species- and individual-based approaches tailored to elucidate the association between oxidative status and longevity, and whether the nature of the association is species-specific rather than general.

In conclusion, our study showed that levels of GPX and oxidative damage to proteins (protein carbonyls) in muscle and red blood cells were not associated with species lifespan, suggesting that these two metrics of oxidative status do not explain the exceptional longevity of the Greenland shark. The high and low values of muscle GPX and red blood cells PCs, respectively, we found in the Greenland shark as compared to other vertebrate species might be explained by other ecological features of this shark species, such as the adaptation to cold waters and deep dives.

Competing interests

The authors declare no competing financial interests.

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Fig. 1. Scatterplots of the residuals of maximum lifespan (corrected for body mass in all panels) against those of metrics of oxidative status (see text). None of the biochemical metrics of oxidative status were significantly correlated to maximum lifespan, regardless of whether body mass was or was not included in the models. The Greenland shark is shown as a blue dot in each graph. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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