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Title

The longest-lived metazoan, *Arctica islandica*, exhibits high mitochondrial H_2O_2 removal capacities.

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Abstract

A greater capacity of endogenous matrix antioxidants has recently been hypothesized to characterize mitochondria of long-lived species, curbing bursts of reactive oxygen species (ROS) generated in this organelle. Evidence for this has been obtained from studies comparing the long-lived naked mole rat to laboratory mice. We tested this hypothesis by comparing the longest-lived metazoan, the marine bivalve *Arctica islandica* (MLSP=507 y), with shorter-lived and evolutionarily related species. We used a recently developed fluorescent technique to assess mantle and gill tissue mitochondria's capacity to consume hydrogen peroxide (H_2O_2) in multiple physiological states *ex vivo*. Depending on the type of respiratory substrate provided, mitochondria of *Arctica islandica* could consume between 3-14 times more H_2O_2 than shorter-lived species. These findings support the contention that a greater capacity for the elimination of ROS characterizes long-lived species, a novel property of mitochondria thus far demonstrated in two key biogerontological models from distant evolutionary lineages.

Keywords: mitochondria, reactive oxygen species (ROS), longevity, hydrogen peroxide, *Arctica islandica*, bivalves

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A greater capacity of endogenous matrix antioxidants has recently been hypothesized to characterize mitochondria of long-lived species, curbing bursts of reactive oxygen species (ROS) generated in this organelle. Evidence for this has been obtained from studies comparing the long-lived naked mole rat to laboratory mice. We tested this hypothesis by comparing the longest-lived metazoan, the marine bivalve *Arctica islandica* (MLSP=507 y), with shorter-lived and evolutionarily related species. We used a recently developed fluorescent technique to assess mantle and gill tissue mitochondria's capacity to consume hydrogen peroxide (H_2O_2) in multiple physiological states *ex vivo*. Depending on the type of respiratory substrate provided, mitochondria of *Arctica islandica* could consume between 3-14 times more H_2O_2 than shorter-lived species. These findings support the contention that a greater capacity for the elimination of ROS characterizes long-lived species, a novel property of mitochondria thus far demonstrated in two key biogerontological models from distant evolutionary lineages.

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1. Introduction

The idea that the endogenous production of reactive oxygen species (ROS) in animal cells drives cellular senescence, and therefore organismal decay, dates back to the middle of 20th century [1]. A recent refinement of this hypothesis, the mitochondrial oxidative stress theory of aging (MOSTA, [2, 3]) focuses on these organelles and suggests that they are both the source (FeS centres of respiratory complexes) and target (mitochondrial membranes lipids, proteins and DNA) of ROS of major importance, driving the aging process. Despite this refinement helping in framing research around mitochondria, the MOSTA continues to receive both support [2, 4] and rebuttal [5, 6]. These contrary results are likely due to the complex nature of ROS management and its impact on cellular stress and on the regulation of signalling pathways. For example, SOD and catalase enzymes operate at different range of ROS concentrations corresponding to signalling or stress, which can explain why suppression of SOD in *C. elegans* and overexpression of mitochondrial catalase in mouse both led to significant extensions of lifespan [7, 8]. This makes it necessary to better tease apart the role of mitochondrial ROS management in aging.

Since the late 1980s and until recently, a classical series of comparative studies provided support for the MOSTA, as they suggested mitochondria of long-lived species had lower ROS formation rates, compared to those of shorter-lived species, even after correcting for methodological and statistical caveats [9-11]. More recently, however, a critical assessment of the methodological approach suggested that a significant caveat in these studies had been downplayed. Indeed, all of these classical studies of ROS formation rate by mitochondria *ex vivo* relied on the enzyme horseradish peroxidase (HPx) to oxidize various fluorescent probes, by consuming the

ROS hydrogen peroxide (H_2O_2) in the process [12]. Contrary to H_2O_2, the HPx cannot cross the inner mitochondrial membrane; it was therefore assumed that a rapid H_2O_2 consumption by the HPx outside of the inner membrane would generate an important gradient, minimizing the time spent by H_2O_2 in the presence of matrix antioxidants, and thus avoiding any critical bias. Because the superoxide anion is rapidly converted to H_2O_2 by the superoxide dismutase, it was further assumed that the HPx/probe methods for measuring H_2O_2 would also include this other major initial ROS species. The correct term for the measurement obtained by this classical approach would therefore be *efflux* and not *production* of H_2O_2 since it was acknowledged that at least some H_2O_2 is consumed inside the matrix before reaching the detection system.

Matrix-based antioxidants capable of eliminating H_2O_2 comprise the two NADPH-dependent peroxidases (glutathione peroxidase, GPx, and peroxiredoxin, PRx), and catalase, with an assumed negligible contribution from cytochrome *c*. In a seminal study aiming to address the question of how much efflux are underestimating true ROS formation rate, Treberg and colleagues used the glutathione depleting agent 1-Chloro-2,4-dinitrobenzene (CDNB) to inhibit the GPx pathway [13]. They found that a significant proportion of H_2O_2 formed inside mitochondria is also consumed within them, thus remaining undetected by the HPx-based assay. Munro and colleagues [12] later validated that the thioredoxin reductase inhibitor auranofin (affecting PRx 3 and 5 in the thioredoxin-dependent pathway) had no major off-target effect on skeletal muscle mitochondria of rats, and used it in combination with CDNB to achieve a complete inhibition of matrix antioxidants, since these mitochondria are naturally almost entirely devoid of catalase. They showed that the proportion of H_2O_2 consumed inside the mitochondrion could reach up to 84% of the total production when using substrates for complex I such as malate, thus further invalidating the use of efflux for the purpose of measuring mitochondrial ROS formation rates. Using the double inhibition approach (CDNB + auranofin), the comparison between naked mole rats (NMR) and mice was revisited, given this species' relevance as a biogerontological model [14]. Measuring true rates of ROS formation didn't support a lower rate for NMR mitochondria as previously reported using efflux [9]. The study however revealed a much higher capacity of NMR mitochondria to consume H_2O_2. These findings cast doubts on the nature of the lower rate of H_2O_2 formation frequently found for mitochondria of long-lived species by the many previous "efflux studies", and raises the case for a misinterpretation of the results. Long-lived species used in these studies could potentially all be characterized by higher mitochondrial capacities for the endogenous removal of H_2O_2, leading to the wrong conclusion that they produce less H_2O_2 when efflux is measured [15].

If the aging process is partly dictated by the ability of the cell, or by mitochondria, to manage ROS, it is crucial to delineate which pathways are responsible for the reduction of ROS efflux, to define how mitochondrial traits can evolve to modulate longevity. That is, does selection pressure adapt the Electron Transport System (ETS) to reduce ROS production or the antioxidant mitochondrial enzymes to buffer these ROS? Answering this question can enlighten us on the evolvability and plasticity of longevity, and to this end, bivalve molluscs constitute an appropriate comparative biogerontological model [3]. Indeed, comparing the marine bivalve *Arctica islandica* (*A.I.*) with the evolutionarily related veneroid species of comparable sizes *Mya arenaria* (*M.A.*) and *Spisula solidissima* (*S.S.*) offers an impressive longevity gradient. Indeed, *A.I.* holds the record for the longest-lived metazoan species, with one individual reaching 507 years of age [16]: this is 18 times the maximum lifespan of the shortest-lived bivalve species in this model (*M.A.*: 28 y, *S.S.*: 37 y). We previously compared these bivalves and found lower rates of mitochondrial H_2O_2

efflux in *A.I.*, than in *M.A.* and *S.S.* in multiple substrate combinations [11]. Because catalase is present and significantly active in the tissues of these species, it is not possible to fully inhibit all matrix antioxidants and measure true ROS formation rates when using the double inhibition approach. Nevertheless, measuring mitochondrial H_2O_2 consumption capacity can address the question of whether the lower efflux found for this species can be ascribed to a greater capacity of *A.I.* mitochondria to consume H_2O_2. More importantly, it allows to continue testing if the capacity of matrix antioxidants better correlate to longevity than efflux did for the last 30 years of research. The aim of the present study was therefore to compare rates of mitochondrial H_2O_2 consumption in *A.I.* to those of the shorter-lived *S.S.* and *M.A. ex vivo* in a number of relevant substrate and inhibitor conditions.

2. Experimental procedures

2.1. Bivalve collection and mitochondrial isolation

Bivalve species were either sampled at low tide (*Mya arenaria*) in Le Bic 48°23'N, 68°40'W, Quebec, Canada) or collected by a professional diver (*Spisula solidissima*, *Arctica islandica*) in the Magdalen Islands, Quebec (47°22'N 61°58'W, Quebec, Canada). They were shipped to the UQAR-ISMER research station in Pointe-au-Père, Quebec, Canada and maintained in open flow-through tanks at 8 ºC with ample sediment layer for burrowing for at least a month before experiments. They were fed a live microalgal diet (*Nannochloropsis sp.*, *Isochrysis galbana*, *Pavlova lutheri*; 12% to 44% to 44% in cell numbers) at 1% body mass per day. Following our previous studies on these species, sexually mature adult individuals within the same narrow range of shell sizes were selected for the current experiment: all were estimated to be at or close to their middle age [11, 17, 18]. The average size (shell length) of individuals was 109 mm ± 4 for *A.I.*, 66 mm ± 3 for *M.A.* and 141 mm ± 10 for *S.S.* The estimated age of *A.I.* individuals was between 100 and 200 years, while maturity was reached approximately between 30 and 60 years (according to growth slopes in [19]). Estimated ages of *M.A.* of these sizes were between 8 and 9 years, and size at maturity is reached approximately at 41-42mm and max of 96 mm [20]. *S.S.* were between 10 and 25 years old, according to published growth slopes [21], are sexually mature at 4 years and at sizes between 80 and 90 mm in the area were they were sampled [22].

Bivalves were transported to the laboratory and kept on ice during all dissection steps. Mantle and gill mitochondrial isolation was carried out as previously described, where approximately 8 g of mantle, 4 g of gills were first excised from 2-4 individuals [17]. The tissue was then rinsed in the mitochondrial isolation buffer (400 mM Sucrose, 100 mM KCl, 70 mM HEPES, 6 mM EGTA, 3 mM EDTA, 10 mg/ml aprotinin, 1% [w/w] bovine serum albumin, pH 7.6), minced and homogenized using a glass-Teflon potter on ice. The homogenate was centrifuged at 4 ºC twice at 1,250 *g* for 10 min, the supernatant was centrifuged at 10,500 *g* and the pellet obtained was resuspended with a 0.5% BSA isolation buffer. After a final centrifugation at 10,500 *g*, the pellet was resuspended in a few drops of isolation buffer. Isolate volume was determined and protein concentration was immediately analyzed using the Biuret test, hence high-resolution respirometry and H_2O_2 consumption assays were carried out with known mitochondrial protein concentrations.

2.2. High-resolution respirometry

Mitochondrial respiratory function was evaluated to guarantee the quality of the mitochondrial preparations to be used for H_2O_2 consumption assays. Each isolate was analyzed through high-resolution respirometry at 10ºC using an Oxygraph-2K (Oroboros Instruments, Austria). Leak respiration ("state 2") was assessed using complex I substrates glutamate (G, 25 mM) and malate (M, 2 mM), after which saturating ADP (5 mM) was added to measure coupled respiration ("state 3"). The integrity of the outer mitochondrial membrane was assessed by the extent of the increase in respiration after addition of 10 µM cytochrome c [11]. The activity of cytochrome *c* oxidase (CIV) was also measured by adding electron donor TMPD (0.5 mM) and ascorbate (2 mM) to avoid the auto-oxidation of TMPD, after which calibration for chemical background was applied according to the manufacturer's instructions [23].

2.3. H2O2 consumption assay

The assay method for measuring the rate of H_2O_2 removal in the medium by isolated mitochondria is described in detail in [12]. Briefly, the assay was carried out in a 1 ml eppendorf kept afloat in a temperature-controlled bath. The remaining concentration of H_2O_2 in the eppendorf was assessed over ten minutes by sampling the eppendorf at time intervals and measuring the Amplex UltraRed fluorescence of each subsample in a 96 well plate, using the appropriate corrections for time of sampling and residual H_2O_2 formation rate in the plate.

In order to find the bivalve-specific combination of substrates and other effectors producing the fastest rates of H_2O_2 consumption, preliminary tests were made with glycerol-3-phosphate (G3P, 10 mM, feeding electrons into the Q-junction at the glycerophosphate dehydrogenase complex) and proline (Pro, 10 mM, feeding electrons into the Q-junction at proline dehydrogenase), in addition to the traditional substrates glutamate (G, 25 mM), malate (M, 2 mM), and succinate (S, 10 mM). The addition of ADP (5 mM, to stimulate coupled respiration) and auranofin (Aura, 2 µM, thioredoxin-reductase inhibitor) were also tested before data collection due to their possible effect on respiration; as well as to determine the specific combination of substrates allowing to separate the relative contribution of each pathway to H_2O_2 consumption (see results).

2.4. Statistical analysis

Significant differences among species for the rate of H_2O_2 consumption were detected ($p < 0.05$) first by ANOVA in each condition of respiratory substrate. If ANOVA was significant, a Tukey's HSD test was performed to assign group letters. An Arctan transformation was used to reach homoscedasticity in three cases: Aura GM + G3P for mantle mitochondria standardized to mg of proteins; GM + G3P + ADP and Aura GM + G3P for gill mitochondria standardized to CIV. When no transformations would satisfy homoscedasticity for the Tukey's HSD test, a Steel Dwass all pairs non-parametric test was used. Significant difference in the relative contribution of each pathway for the consumption of H_2O_2 among species was also detected first by ANOVA followed by Tukey's HSD test to assign group letters.

3. Results

We first analyzed mitochondrial respiration with complex I substrates glutamate and malate, and obtained mean respiratory control ratio (RCR) values of 2.29 (±0.25), 1.78 (±0.23), and 4.18 (±0.49) for mantle mitochondrial isolates of *M.A.*, *S.S.* and *A.I.*, respectively. These values

were similar to our previous study on this tissue [11]. In the gills, RCR values were 1.66 (±0.29) for *M.A.*, 1.62 (±0.23) for *S.S*, and 2.24 (±0.16) for *A.I.* The values following addition of cytochrome *c* during NADH-OXPHOS respiration for the three species studied, ranged from 11 to 20%, mirroring our previous results [11, 18] and were not suggestive that mitochondria were adversely damaged during isolation.

Mitochondrial consumption of H_2O_2 in the three species differed in the presence of GM, but the addition of G3P amplified the divergences, resulting in a drastically greater rate for *A.I.*, in both gill and mantle when standardizing the rates by mg of proteins (Fig. 1A and B). This pattern of a greater rate of consumption by *A.I.* mitochondria with G3P remained for gills when standardizing by CIV activity, but not uniformly in the mantle (Fig. 1C and D), where it only remained for the condition eliciting the highest rate of H_2O_2 removal (GM + G3P + ADP). The most remarkable differences were found for the mantle when standardizing by mg of protein. Indeed, in this condition, the rate for *A.I.* mitochondria was higher by up to a factor of 14 in the physiologically relevant respiratory state combining GM + G3P + ADP: 8.39 (±0.89) nmol $H_2O_2.min^{-1}.mg^{-1}$ for *A.I.*, 0.58 (±0.16) for *S.S.*), with most differences being statistically significant ($p< 0.05$) between the species (Fig. 1B). In the gills, the highest difference was found in the absence of substrates, where consumption by *A.I.* was 3 times higher than in *S.S.* (1.87 (0.27) vs 0.58 (0.07) nmol $H_2O_2.min^{-1}.mg^{-1}$, respectively). The relative importance of the three pathways for the matrix consumption of H_2O_2 was estimated in the hope of revealing a signature of longevity. Three major enzymatic pathways are present in the mitochondrial matrix that participate in removing exogenously added H_2O_2. Catalase does not require the presence of substrates to be fully active, and its relative contribution can therefore be obtained directly after addition of a spike of H_2O_2 in the medium. Addition of GM + G3P + ADP elicits the maximal rates of H_2O_2 consumption by mitochondria by further recruiting the activity of the GPx and PRx pathways. Auranofin is an inhibitor of the thioredoxin reductase that can be used to specifically inhibit the PRx pathway (see Fig. 1 in [12] for a complete scheme of H_2O_2 metabolism). Addition of auranofin in the presence of GM + G3P + ADP therefore reveals the combined maximal contribution of catalase and GPx. From there, the isolated contribution of the PRx and GPx pathways can be deduced, the results of which are presented in Fig. 2. It is apparent that catalase is the dominant pathway in bivalve gills, whereas it is rather GPx in the mantle. Comparing species revealed no difference for the relative use of the three consumers of H_2O_2 within each tissue, except for the greater use of PRx in the mantle of S.S.

4. Discussion

The present study is the second experimental comparison of mitochondrial capacities for the consumption of H_2O_2 between long-lived and short-lived species. As found previously for the comparison of the NMR and mice, we show here that the mitochondria of the long-lived species are removing exogenously added H_2O_2 faster than those of the short-lived species. More precisely, in two important bivalve tissues, mitochondria of *A.I.* were found to remove H_2O_2 at a rate 3-14 times faster than those of its shorter-lived counterparts *M.A.* and *S.S.*

In line with our results, a previous study found greater catalase activity in tissues of *A.I.* compared to *M.A.* and other related bivalves [24], which corresponds to our finding of the greatest difference in absolute capacities of the matrix antioxidants at the level of catalase for the mantle

(Fig. 2B). Moreover, *A.I.* has been shown to be particularly resistant to oxidative insult in *in vivo* studies using drugs eliciting oxidative stress at the mitochondrial level (rotenone and paraquat), and treatments damaging DNA, lipids and proteins (*tert*-butyl hydroperoxide); compared to shorter-lived species of bivalves [25, 26]. Again, this could be explained by greater matrix antioxidant defenses that have the capacity to intervene directly at the source of the stress. This is suggested by other studies addressing mitochondrial functions in bivalves during chronological aging [27, 28], which unfortunately do not offer the possibility for a direct comparison with our findings.

Very few comparative studies have attempted to make the case for a particular role for the modulation of mitochondrial antioxidants in longevity, this theoretical framework being much more developed in the context of pharmacological and/or genetic interventions *in vivo*. Results from these fields are line with our findings, with catalase targeted to mitochondria improving lifespan in a cellular murine model [7], and its genetic overexpression partially rescuing aging phenotypes in mice [29]. Shabalina and colleagues [4] also recently showed that the pharmacological matrix-bound antioxidant SkQ1 improves both the lifespan and healthspan of prematurely aging mtDNA mutator mice, potentially through the alleviation of ROS-induced damage.

The present study contrasts with early comparative studies of longevity (starting in the 1980s) that concluded on the absence of any relationship between longevity and antioxidants [30]. However, the theoretical framework considered in many of these studies did not distinguish the roles of mitochondrial antioxidants from that of cytosolic ones, resulting in indistinct measures of all cellular antioxidants. In the rare cases where mitochondrial-specific antioxidants were of interest, an incomplete assessment of the enzymatic machinery was unfortunately almost always performed. For instance, only GPx activity would be measured, which not only ignores other pathways, but fails to approximate the actual activity of the GSH-dependent pathway itself (for a discussion on pathway flux control, see [31]). To illustrate this point, consider that the actual activity of the GSH-dependent pathway is also dependent on the relative flux control of the glutathione reductase, the availability of GSH and potentially of the activity of the nicotinamide nucleotide transhydrogenase (NNT) transmembrane enzyme to reduce NADP+ back into NADPH, which is in turn further depends upon the protonmotive force in most species. The same type of functional dependency is found for the Trx-dependent pathway leading to the activity of the peroxiredoxins. Measuring protein or RNA abundance of one or two antioxidants has also been done, which is even more remote from assessing the activity of these pathways *in vivo* [31]. In support of these considerations, we herein found that adding glutamate + malate, further adding G3P and later ADP, all apparently contributed increasing the absolute difference between *A.I.* and other species for their capacity to remove H_2O_2. Therefore, if previous studies of longevity correctly addressed the question of a putative general relationship between longevity and antioxidants, unfortunately none are answering the question of the same relationship specifically with matrix bound-integral antioxidant pathways.

Our results have to be placed in perspective with the long series of studies comparing mitochondrial H_2O_2 efflux between long and short-lived species, which obtained some degree of correlation with longevity after correcting for methodological caveats [9]. In this series however, only the recent study on NMR actually separated production from consumption, finding no difference with the mice for production, but much higher capacity of buffering ROS in NMR

mitochondria. This raises the possibility that the association between longevity and low H_2O_2 efflux was the result of greater matrix consumption of H_2O_2 during these assays. Our group previously found a lower rate of H_2O_2 efflux in *A.I.* compared to the same two other short-lived species, which we wanted to further investigate. Unfortunately, because catalase was active well above a negligible threshold in all three species, it was not possible to measure true H_2O_2 formation rates with the method used in the NMR and mice study [12]. At this point in the field of comparative studies of mitochondrial ROS metabolism, it is not possible to reject that a downregulation of ROS formation plays a role in extending longevity. It is however important to consider that if a real cause to effect association to ROS is at play for influencing longevity, it may have less to do with ROS balance achieved by healthy mitochondria, but may rather relate to the capacity to face acute bouts of oxidative stress in cells and mitochondrial networks. An illustration of this is Ungvari and colleagues' demonstration of how *A.I.* better survives *in vivo* challenges with paraquat and rotenone that are used to elicit mitochondrial oxidative stress [26].

A putative evolutionary modulation of mitochondrial antioxidant enzymes to increase lifespan appears, however, as a more parsimonious hypothesis than the downregulation of ROS formation rate. Single-electron leaks to oxygen generating free radicals occur at sites that are co-encoded by the nuclear and mitochondrial genomes, and any change at these sites involves the co-evolution of the two genomes, which is suggested as a major evolutionary constraint. Furthermore, decreasing the basal rate of ROS formation to increase lifespan would directly affect not only the mitochondrial, but the whole cellular ROS-based communication, meaning that a large number of interacting sites and biomolecules would have to co-evolve in order to accommodate such a change. The clonal evolution with no possibility of recombination of mitochondrial DNA [32] make this co-adaptation of mito-nuclear genomes quite complex [33]. Genetic studies of the different northern Atlantic Ocean populations of *A.I.* are suggestive that such extensive modifications are not necessary to achieve major extension of longevity. Indeed, whereas maximal lifespans across these populations differ by a factor of five, their nuclear genetic background has been found quite uniform, possibly due to long-distance gamete dispersal in this species [28, 34]. On the other hand, developmental plasticity in a few genes encoding mitochondrial antioxidants would be a simpler explanation for the interpopulation differences in lifespan in this species. Increasing antioxidant defences at the mitochondrial level would also better explain this species' resistance to drugs eliciting mitochondrial oxidative stress [25, 26] than a reduction of primary ROS formation rate.

Interestingly, and irrespective of the hypothesis regarding longevity, comparing matrix H_2O_2 removal capacities across widely differing taxa do not reveal large differences for this trait. Rat skeletal muscle mitochondria had a maximum removal rate slightly above 5 nmol $H_2O_2.min^{-1}.mg^{-1}$ proteins [12], whereas *A.I.* mitochondria could reach a little more than 8 nmol $H_2O_2.min^{-1}.mg^{-1}$. On the other hand, an intriguing difference between bivalves and rodents is the type of substrate eliciting the greatest rate of H_2O_2 removal. Rat skeletal mitochondria reached their peak H_2O_2 consumption rate with either malate alone or succinate + rotenone + ADP, whereas the bivalves herein needed a combination of GM + G3P + ADP. Proline was also tested as a substrate, but did not increase the rate of H_2O_2 removal and is therefore not presented. It is as if the evolution of matrix-bound antioxidants can accommodate important metabolic changes (*e.g.* a change in the main substrate), but their absolute capacity must remain rather conserved (not differing by order of magnitude) across taxa of vastly differing general physiology. A conserved capacity for the defense against ROS at close proximity to the sites of production may suggest a strong

mitochondrial evolutionary requirement, modulated by a few factors, longevity perhaps being one of them. Of course, this suggestion is derived from the comparison of skeletal muscle mitochondria of two mammals in one case, and mantle/gill mitochondria of three bivalves in the other.

While the absence of a relationship between H_2O_2 buffering capacity and species lifespan could have been considered as a rebuttal of the role of ROS management in life history traits and particularly lifespan modulation, our results rather support its key position as a driver of these traits in a wide taxonomic range. Additional studies are however required to firmly establish the positive association between mitochondrial antioxidant capacities and longevity. Preferably, additional studies designed to minimize off-target effects of CDNB and auranofin on mitochondria of species of interest would address both questions at once, *i.e.* also testing for a negative relationship between longevity and ROS by actually measuring H_2O_2 formation rate (and not only efflux). A favorable starting point would be to revisit pairs of species that have been selected in previous comparative studies of mitochondrial H_2O_2 efflux, because they avoid pseudoreplication, and large differences in body sizes. Alternatively, statistical corrections can be applied [35]. Pursuing in that direction may uncover a much clearer relationship, possibly ending an old debate.

Study limitation

Because of a lack of sufficient resources (amount of mitochondrial isolate), the condition of Auranofin + GM + G3P + ADP was not measured in this study, that later revealed a limitation for the accuracy of the data in Fig. 2, which we had to circumvent by an indirect calculation. Briefly, the relative contribution of the Prx pathway is normally obtained by the difference in consumption rate seen after addition of auranofin to the condition eliciting the most important consumption rate (here GM + G3P + ADP). However, ADP eliminates most residual concomitant ROS formation rate occurring during the consumption measurements. Here, we had to first measure the decrease in consumption between GM + G3P and Aura + GM + G3P, and subsequently correct for the difference between GM + G3P and GM + G3P + ADP. This eliminates the contribution of ADP that would otherwise increase the apparent contribution of Prx to the overall consumption.

5. Conflict of Interest Statement

The authors declare no conflicts of interest.

6. Author Contributions

D.M. and E.R. collected the specimens and did the experiments. D.M. analyzed the data. All authors contributed to the writing of the manuscript.

7. Funding

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8. Data availability statement

The authors will make the raw data available upon request.

9. Figures

Figure 1. Rate of change in exogenously added H_2O_2 in the assay medium, expressed in mg of protein (A, B) or per Complex IV activity (C, D). Mitochondria isolated from gill (A, C) and mantle (B, D) from three bivalve species (*Arctica islandica*, *A.I.*; *Mya arenaria*, *M.A.* and *Spisula solidissima*, *S.S.*) were allowed to consume H_2O_2 in the presence of various combinations of respiratory substrates with or without the thioredoxin reductase inhibitor auranofin. CIV = complex IV, G = glutamate, M = malate, G3P = glycerol-3-phosphate, Aura = auranofin. Different lower-case letters inside panels indicate significant differences ($p< 0.05$) among the three species according to Tukey's HSD post-hoc test.

Figure 2. Contribution of the catalase, gluathione peroxidase (GPx) and peroxiredoxin (Prx) pathways in absolute values for gill (A) and mantle (B) tissues of three species of extremely long-lived (*Arctica islandica*, A.I.) and short-lived (*Mya arenaria*, M.A.; *Spisula solidissima*, S.S.) marine bivalves. Relative values are presented in (C) for gill and (D) for mantle. Different lower-case letters inside panels indicate significant differences ($p< 0.05$) among the three species for each antioxidant pathway, according to Tukey's HSD post-hoc test or Kruskal-Wallis with Bonferroni correction when homoscedasticity could not be reached.

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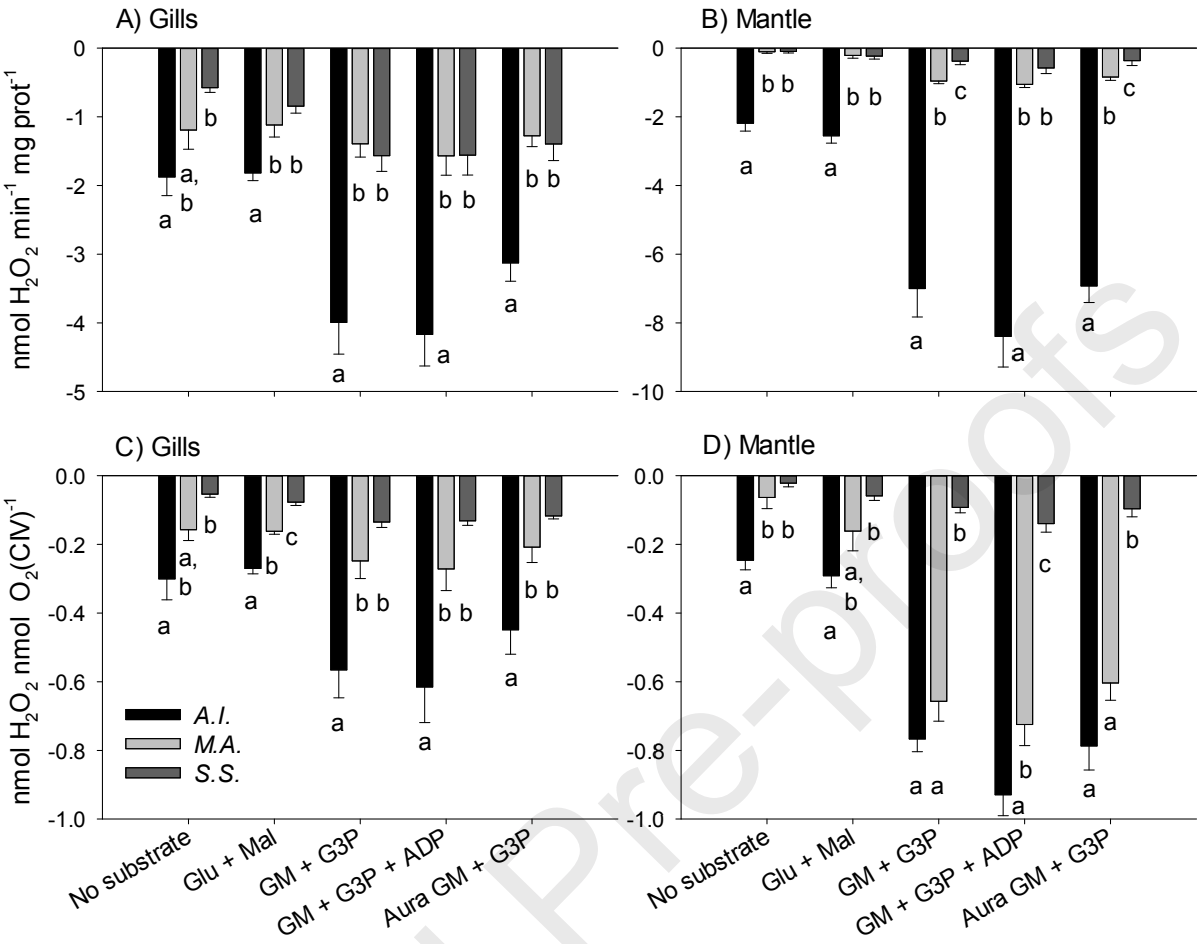

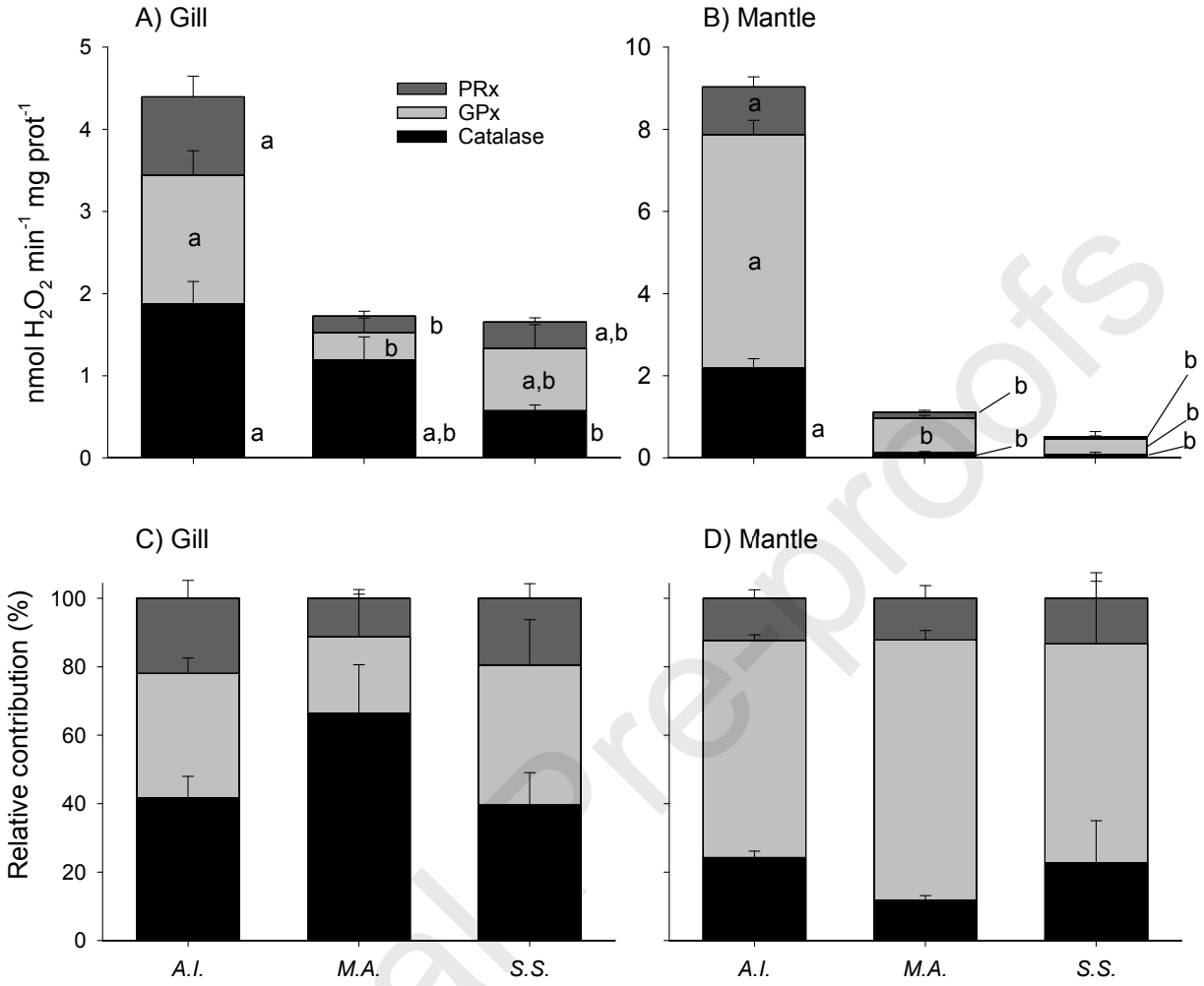

Highlights

- Mitochondria from the extremely long-lived marine bivalve *Arctica islandica* consumes 3-14 times more hydrogen peroxide than two shorter-lived counterparts.

- This finding supports previous results on mammals and points to consumption of reactive oxygen species (ROS), as a key determinant of a long-lived mitochondrial phenotype.

- Overall, these results support the mitochondrial oxidative stress theory of aging (MOSTA), although more work needs to be done to assess net production of hydrogen peroxide in these species.

Graphical abstract