Aging Cell (2013) **12**, pp584–592 Doi: 10.1111/acel.12082

Low hydrogen peroxide production in mitochondria of the long-lived *Arctica islandica*: underlying mechanisms for slow aging

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Summary

The observation of an inverse relationship between lifespan and mitochondrial H_2O_2 production rate would represent strong evidence for the disputed oxidative stress theory of aging. Studies on this subject using invertebrates are surprisingly lacking, despite their significance in both taxonomic richness and biomass. Bivalve mollusks represent an interesting taxonomic group to challenge this relationship. They are exposed to environmental constraints such as microbial H_2S, anoxia/reoxygenation, and temperature variations known to elicit oxidative stress. Their mitochondrial electron transport system is also connected to an alternative oxidase that might improve their ability to modulate reactive oxygen species (ROS) yield. Here, we compared H_2O_2 production rates in isolated mantle mitochondria between the longest-living metazoan—the bivalve *Arctica islandica*—and two taxonomically related species of comparable size. In an attempt to test mechanisms previously proposed to account for a reduction of ROS production in long-lived species, we compared oxygen consumption of isolated mitochondria and enzymatic activity of different complexes of the electron transport system in the two species with the greatest difference in longevity. We found that *A. islandica* mitochondria produced significantly less H_2O_2 than those of the two short-lived species in nearly all conditions of mitochondrial respiration tested, including forward, reverse, and convergent electron flow. Alternative oxidase activity does not seem to explain these differences. However, our data suggest that reduced complex I and III activity can contribute to the lower ROS production of *A. islandica* mitochondria, in accordance with previous studies. We further propose that a lower complex II activity could also be involved.

Key words: alternative oxidase; *Arctica islandica*; complex I; complex II; mitochondria; reactive oxygen species.

Introduction

Senescence is progressive, endogenous, deleterious, irreversible, and ubiquitous in metazoan somatic tissues; however, the pace of this fundamental clock of cellular aging differs widely among species. The general hypothesis, stated as the 'oxidative stress theory of aging', suggests that the molecular basis of senescence resides in the accumulation of unrepaired damage inflicted by reactive oxygen species (ROS). Damage accrued to protein, lipid, and DNA would participate in the process, but particular emphasis is placed on damage to mtDNA (Kujoth *et al.*, 2005; Pamplona, 2011). Longevity differences would then depend on differences in baseline redox status. This concept has not been supported by comparative studies investigating antioxidant defenses (Hulbert *et al.*, 2007). However, there is evidence that the mitochondrial ROS production rate is lower in long-lived species (hereafter referred to the ROS–longevity relation; reviewed in Barja, 2004), and that these species possess macromolecules (such as lipids and proteins) with a better intrinsic resistance to oxidation (Hulbert *et al.*, 2007; Munro & Blier, 2012).

While the macromolecule resistance–longevity relationship is acquiring increasing experimental support, the same cannot be said of the ROS–longevity relation. Indeed, the promising early studies showing this relationship were confounded by the nonindependence of body size and nonindependence of phylogenetic distance between investigated species. More recently, Lambert *et al.* (2007) conducted similar experiments on 12 species and found that the ROS–longevity relationship was valid for heart mitochondria after correction for body mass and phylogenetic distances. However, this was true only when mitochondrial respiration was based on succinate, a substrate for complex II (CII), and not when complex I (CI) substrates were used. The use of succinate induces reverse electron flow (REF), and, as pointed out by Lambert *et al.* (2007), the relevance of this condition for determining *in vivo* metabolism is highly disputable. This is because in normal physiological conditions, mitochondria also oxidize CI substrates, such as pyruvate, glutamate, and malate, which induce forward electron flow (FEF). Even if early studies (reviewed in Barja, 2004) associated longevity with low mitochondrial ROS production during FEF for heart and brain, three recent ones did not. These studies rather found no clear association for any conditions/organs with the sole exception, again, of heart mitochondria provided with succinate (Brown *et al.*, 2009; Montgomery *et al.*, 2011, 2012). Furthermore, the only study examining the relationship in invertebrates (Sohal *et al.*, 1995) used flies of very different body sizes and only tested the condition of REF. Hence, additional studies are needed to establish if the prevalence of this relationship is restricted to vertebrate heart mitochondria during succinate oxidation.

Long-living bivalve mollusks are emerging longevity models (Abele *et al.*, 2009) that represent a particularly interesting group of invertebrates to challenge the ROS–longevity relation. Indeed, many species living in the intertidal zone face wide temperature variations and cycle through anoxia/reoxygenation as a function of the tide,

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Accepted for publication *24 March 2013*

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which exposes them to oxygen and temperature variations that can elicit strong oxidative stress in homeotherm mitochondria. Furthermore, bivalves are often exposed to high concentrations of microbial H_2S that are capable of partly inhibiting complex IV (cytochrome *c* oxidase, CIV) activity (Abele *et al.*, 2007), which could increase ROS production. This means that if the ROS production rate is associated with longevity in bivalves, then the relationship not only has a very remote origin in terms of evolutionary distance, but it also supersedes other physiological constraints imposed by the ecological niche, that is, it is not limited to species with constant body temperature that are exposed to a stable oxygen partial pressure and chemical environment. Among these organisms, the mud clam *Arctica islandica* is of particular interest because of its extraordinary longevity. This species has long been known to be by far the longest-living noncolonial organism, and its maximum reported longevity (MRL) has recently been extended by the finding of a 507-year-old individual (Butler *et al.*, 2013). Moreover, this species is known to undergo periods of burrowing during which it experiences near-complete anoxia without suffering from a burst of ROS production upon reoxygenation (Strahl *et al.*, 2011), as observed after ischemia/reperfusion in mammalian mitochondria.

It is tempting to suggest that the capacity to face such challenges is partly linked to the presence of an alternative oxidase (AOX) in bivalve ETS. AOX is a dimer located in the inner mitochondrial membrane that transfers electrons from reduced quinone (coenzyme Q) to oxygen, thus bypassing CIII, without participating in proton pumping (van Dongen *et al.*, 2011). Although AOX are cyanide- and antimycin-insensitive, they are specifically inhibited by salicylhydroxamic acid (SHAM) (reviewed in Lenaz & Genova, 2010). Their affinity for oxygen is much lower than CIV, and their recruitment requires a high degree of reduction of the Q-pool. Because of these last two characteristics, AOX is thought to represent a simple mechanism that protects the organism against conditions known to elicit high ROS production rates; this would be achieved at the expense of maintaining futile proton cycling (Abele *et al.*, 2007; Donaghy *et al.*, 2012). For instance, AOX mRNA levels have been found to be up-regulated after 12 and 24 h of hypoxia and during reoxygenation (Sussarellu *et al.*, 2013). A constitutively active AOX has been found for *A. islandica* (Tschischka *et al.*, 2000; Abele *et al.*, 2007), and the presence of this enzyme in all phyla possessing very long-lived species such as plants, fungi, and bivalves (Lenaz & Genova, 2010) support its possible involvement in longevity.

Homeotherms and insects lack an AOX, and the reduced ROS production rate observed for long-lived species has been proposed to reflect adjustments at the level of ETS component stoichiometry and their regulation. In these species, the major ROS sources include CI (Q-binding site or FeS clusters; Lambert & Brand, 2004a; Herrero & Barja, 2000) and CIII (outer and inner centers; Brand, 2010). Secondary sources include CII, glycerophosphate dehydrogenase, and ETF-dehydrogenase (reviewed in Brand, 2010). Some studies using substrates for complex I (CI; initiating forward electron flow, FEF) suggest that the rate of ROS production was lowered by maintaining a lower steady-state degree of electronic reduction of complexes I and III (Barja & Herrero, 1998; Gredilla *et al.*, 2001). Consequently, electrons remain a shorter time inside each complex, reducing the chances of deviating from their course to form superoxide anion ($O_2^{\cdot-}$). When a substrate for CII is used (initiating reverse electron flow, REF), a reduced CI content has been proposed to explain the reduced ROS production rate (Lambert *et al.*, 2010). Alternatively, maintaining a lower proton-motive force (Δp) across the inner membrane has been related to reduced ROS production in long-lived species (Lambert & Brand, 2004b; Lambert *et al.*, 2010).

The present study aims to compare H_2O_2 production rates of isolated mitochondria from *A. islandica* and two short-lived, similarly sized, and taxonomically related species, that is, *Mya arenaria* (MRL = 28 years) and *Spisula solidissima* (MRL = 37 years). We also investigated whether differences in H_2O_2 production between bivalves could be explained by the same adjustments in composition/regulation of the ETS proposed for homeotherms or whether it relies on AOX. For this purpose, we measured mitochondrial respiration using high-resolution respirometry and enzymatic activities in the two species having the largest difference in MRL (*A. islandica* and *M. arenaria*).

Results

Size, age, and normalization

Bivalves were chosen by size to have individuals at the onset of or within the period of slow asymptotic growth phase for each species (Table 1). Hydrogen peroxide production and oxygen consumption were measured in the same isolation buffer, and all measurements (including enzymatic activities) were carried out at 10 °C because it approximates the mean annual temperature for the three species. The results presented have been normalized by both citrate synthase and CIV activities because these have been suggested to represent optimal estimates of mitochondrial content (CS) and respiratory activity (CIV) (Hulbert *et al.*, 2006; Larsen *et al.*, 2012). Enzymatic activities are presented as moles of electrons transferred per minute for the ETS complexes while it is per mole of product formed for CS. Further details on the choice of individual bivalves and biomarkers are available in the online Supporting information.

H_2O_2 production rate of isolated mitochondria

The respiratory control ratios (RCR) of mitochondria were 1.8, 2.35, and 4.5, respectively, for *S. solidissima*, *M. arenaria,* and *A. islandica* when glutamate and malate were used as a substrate. As expected under the ROS–longevity relationship hypothesis, mitochondria from *A. islandica* produced much less H_2O_2 than those of the short-lived *M. arenaria* (MRL = 28 years) under all conditions tested. Figure 1 presents the results normalized by CS activity (A and C) and by CIV activity (B and D). The largest relative differences were found during succinate-driven state 3. The production of H_2O_2 was also significantly lower for *A. islandica* than for *S. solidissima* in all conditions tested, with the exception of succinate oxidation in the absence of ADP (state 2).

We found a significant interaction between species and conditions when data were normalized with CS ($F_{10,116} = 25.23$, $P < 0.001$). We thus performed *post hoc* tests (Tukey's HSD) to

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Table 1 Life history traits of the three species investigated

Species	Common name	Maximum reported longevity (y)	Maximum size (mm)*	Size of collected individuals (mm) Range	Mean ± SD	Growth rate (K; VBGF)†	References
Mya arenaria	Soft-shell clam	28	96	57–104	78.7 ± 10.4	5.111	(Brousseau, 1979; MacDonald & Thomas, 1980; Giguère *et al.*, 2007)
Spisula solidissima	Atlantic surfclam	37	130	119–136	125.0 ± 7.8	0.269	(Sephton & Bryan, 1990; Giguère *et al.*, 2005)
Arctica islandica	Ocean quahog	507	103	80–94	88.7 ± 4.5	0.052	(Roddick *et al.*, 2007; Butler *et al.*, 2013)

**M. arenaria* and *S. solidissima*: maximum size reported from fishery management reports for the sampled population; *A. islandica*: maximum size reported for a near-by Canadian population.

†For near-by populations. VBGF: Von Bertalanffy Growth Function.

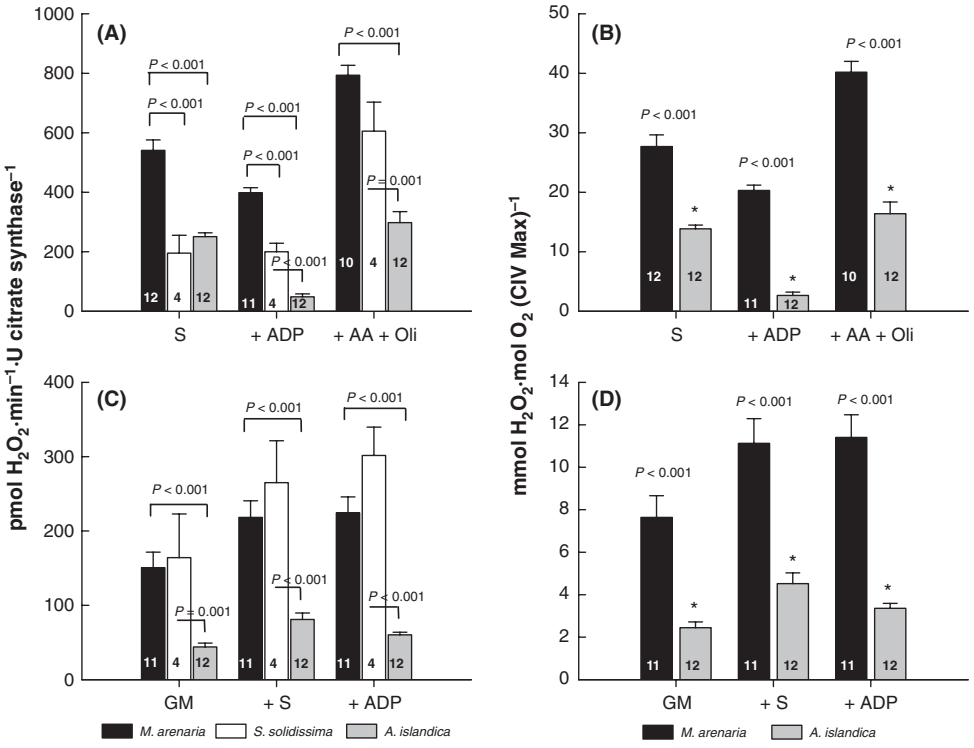

Fig. 1 Hydrogen peroxide production of isolated mantle mitochondria. Results for the sequential addition of substrates and inhibitors (from left to right) are normalized with citrate synthase activity (A and C) and with CIV activity (B and D). Substrates: G, glutamate; M, malate; S, succinate; inhibitors: AA, antimycin A; Oli, oligomycin. Species are presented in order of increasing MRL from left to right: *M. arenaria* = 28 years, *S. solidissima* = 37 years, and *A. islandica* = 507 years. Values are means ± SEM; sample size is indicated within bars. Significant differences are for Tukey's HSD (left panels) and *t*-test (right panels).

further refine the analysis. The two species having the higher RCR values (*M. arenaria* and *A. islandica*) showed a different pattern across conditions compared with *S. solidissima*. Indeed, in the presence of substrate alone, H_2O_2 production was higher ($P < 0.001$) with succinate than with glutamate + malate for these species but not for *S. solidissima*. Furthermore, the addition of ADP during succinate oxidation significantly ($P < 0.001$) reduced the rate of H_2O_2 production for the same two species but not for *S. solidissima*. More generally, the condition of REF (succinate + ADP + antimycin + oligomycin) resulted in significantly ($P < 0.001$) higher rates of H_2O_2 production than for any other conditions for *M. arenaria and S. solidissima.* It also resulted in the highest absolute value among conditions for *A. islandica* (Fig. 1A). In contrast to what was found during succinate-driven respiration, the addition of ADP during convergent electron flow (after state 2 was monitored) did not discernibly lower the rate of H_2O_2 production (Fig. 1C).

The parallel measurement of H_2O_2 production and of O_2 consumption in identical conditions of convergent electron flow allowed us to calculate the percent free-radical leak (FRL). We present these results as the percent of O_2 diverted toward the formation of $O_2^{\cdot-}$ instead of being reduced to water. Compared with the short-lived *M. arenaria*, the FRL of *A. islandica* was significantly lower in both states 2 and 3 (Fig. 2). Compared with *S. solidissima,* no differences were observed during state 2. However, addition of ADP to enter state 3 largely reduced the FRL of *A. islandica*, resulting in a difference that just failed to reach significance, most probably because of the small sample size of *S. solidissima* individuals.

Oxygen consumption and enzymatic activity

To test previous hypotheses on the mechanisms allowing the reduction of ROS production rate in long-lived species, we measured

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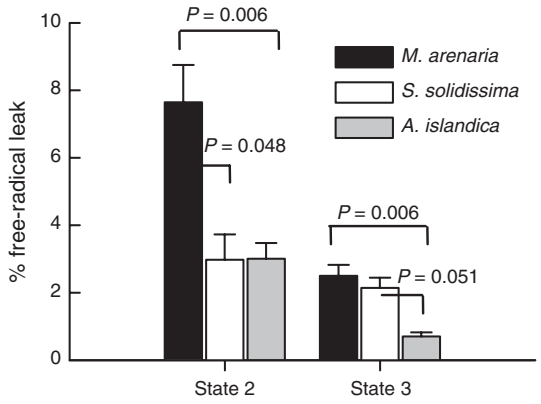

Fig. 2 Free-radical leak expressed as the percent of oxygen consumption diverted to the production of hydrogen peroxide. Measurements of H_2O_2 production and O_2 consumption were taken at 10 °C during convergent electron flow (glutamate + malate + succinate) in both state 2 and state 3. Values are means ± SEM for *M. arenaria* (n = 11), *S. solidissima* (n = 3), and *A. islandica* (n = 8). Significant differences were determined by Tukey's HSD.

the oxygen consumption of fresh mitochondrial isolates for the two species that showed the greatest differences in MRL and the most uniform differences in the rate of H_2O_2 production, that is, *M. arenaria* and *A. islandica*. Measurements were taken using high-resolution respirometry (Oroboros O2K, Oroboros Instruments, Innsbruck, Austria). The quality of the mitochondrial preparations was checked by the addition of cytochrome *c* during state 3 respiration. Results showed oxygen consumption increases of 11% for *M. arenaria* and 5% for *A. islandica* (Fig. 3A), which confirmed the intactness of the outer mitochondrial membrane (Kuznetsov *et al.*, 2008) and the quality of mitochondrial preparation.

Oxygen consumption during glutamate + malate–driven state 2 was higher for *M. arenaria*, but no differences were found between the two species after the addition of ADP to initiate state 3 (Fig. 3A). Accordingly, RCR were significantly higher for *A. islandica* (Fig. 3B). The addition of FCCP to uncouple mitochondria and initiate maximum ETS activity (ETSmax) slightly increased the respiration rate in both species, resulting in an uncoupling control ratio (UCR) slightly above a value of one. The addition of TMPD and ascorbate to measure maximal CIV activity (CIVmax) nearly doubled the rate of oxygen consumption. No differences were found between the two species for ETSmax and CIVmax (Fig. 3A). However, when ETSmax was divided by the activity of CIV, the ratio was lower for *M. arenaria* (Fig. 3B), suggesting a higher activity of either CI or CIII in *A. islandica*.

Results from enzymatic assays in frozen/thawed mitochondrial suspensions contrast with this last conclusion. Indeed, CI activity was much higher for *M. arenaria* compared with *A. islandica* when normalized both by CS activity (Fig. 4A) and by CIV activity (Fig. 4B). Moreover, when the NADH–cytochrome *c* oxidoreductase capacity (CI and III) was measured (an assay confusingly named ETS assay even though CIV is not involved), the relative differences between species were exactly the same as observed for CI alone. Therefore, direct enzymatic activity measurements suggest both a higher CI activity and a higher ratio of either CI/CIV or CIII/CIV in the short-lived *M. arenaria*. Surprisingly, CII activity was about seven times higher for *M. arenaria* than for *A. islandica*, representing a much larger difference between species than for CI (Fig. 4).

Activity of the alternative oxidase

The alternative oxidase (AOX) is known to possess a lower affinity for oxygen than CIV. Therefore, its activity can be evaluated by manipulating the oxygen partial pressure (PO_2) (Tschischka *et al.*, 2000). During convergent electron flow (glutamate + malate + succinate), increasing the PO_2 from low (8.6 kPa) to high (47.5 kPa, saturation being at 21.33 kPa) accordingly increased oxygen consumption for both species in state 2 (M.a.: $F_{1,11}$ = 22.83, P < 0.001; A.i.: $F_{1,7}$ = 12.55, P = 0.009) and also for *M. arenaria* in state 3 ($F_{1,11}$ = 11.12, P = 0.007; Fig. 3C). However, AOX recruitment capacities, estimated by the ratios of state 2 high/low and state 3 high/low, were similar for *A. islandica* and *M. arenaria* (Fig. 3D).

AOX capacities can also be estimated using its specific inhibitor, SHAM. As the enzyme requires a high degree of reduction of the Q-pool to be fully activated, we used a combination of CI and CII substrates. While maintaining a high PO_2, we added antimycin A to block electron flow through CIII and added SHAM thereafter. Figure 3C presents the absolute decrease in oxygen consumption observed after the addition of SHAM; this decrease gives an estimate of the maximal capacity of AOX (AOXmax). In contrast to the hypothesis of a higher recruitment of AOX in the long-lived species, AOXmax was significantly higher for *M. arenaria* (Fig 3C).

Discussion

Hydrogen peroxide production

This study was designed to compare *A. islandica* with two taxonomically related shorter-lived species of similar body size found in an overlapping distribution range while controlling for differences in temperature acclimation and feeding conditions. The general finding is a reduced H_2O_2 production rate in mitochondria of the long-lived *A. islandica* compared with the two short-lived species. More specifically, when compared with *M. arenaria*, the rate of H_2O_2 generation was lower for *A. islandica* in all conditions of mitochondrial metabolism tested, regardless of the use of a marker of mitochondrial content (CS) or respiratory capacity (CIV) (Larsen *et al.*, 2012) as a denominator. The FRL was also lower for the long-lived *A. islandica* in both states 2 and 3. This latter measure is especially important because respiration is coupled with ATP production in the presence of ADP. Although we have not measured the actual P/O ratio, we have good reasons to suggest that state 3 FRL differences between the two species would actually underestimate the difference in the ratio of H_2O_2 to ATP produced. This is because *M. arenaria* (i) has a lower RCR and higher state 2 oxygen consumption, suggesting a leakier membrane; (ii) has a higher CII activity, suggesting less proton pumping per O_2 consumed; and (iii) has equal or higher AOX activity, again suggesting equal or less proton pumping per O_2 consumed. Overall, it suggests an increased rate of futile proton cycling in the short-lived species resulting in a lower production of ATP for equal oxygen consumption. Therefore,

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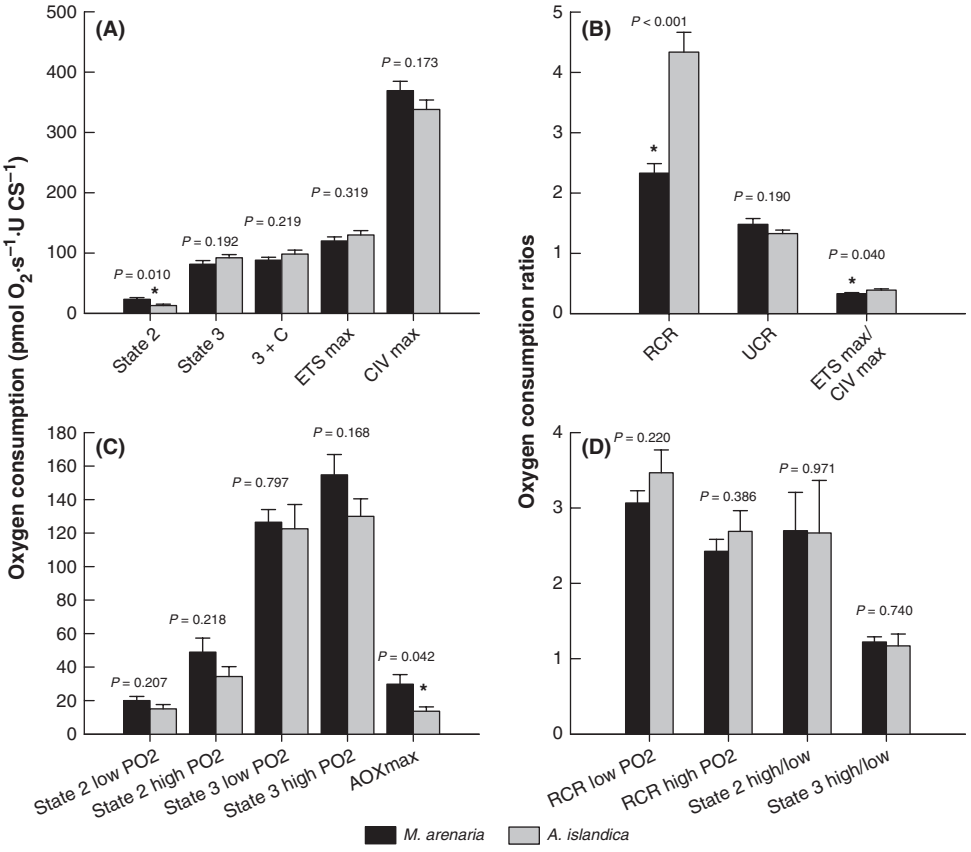

Fig. 3 Oxygen consumption of mantle mitochondria. Measurements were made at 10 °C and values normalized with citrate synthase activity (μmol min^{-1} mL^{-1}). Oxygen consumptions in FEF (glutamate + malate) are presented in (A) and ratios in (B). Conditions for the sequential addition of substrates and inhibitors were as follows: state 2: glutamate + malate; state 3: addition of ADP; 3 + C: addition of cytochrome *c*; ETSmax: addition of the uncoupler FCCP; CIVmax: presence of TMPD, ascorbate, cytochrome *c*, and antimycin A. RCR: respiratory control ratio, UCR: uncoupling control ratio. Oxygen consumption during convergent electron flow is presented in (C) and ratios in (D). State 2: glutamate + malate + succinate; state 3: addition of ADP. High (47.5 kPa) and low (8.6 kPa) PO_2 are, respectively, above and below PO_2 saturation. AOXmax: maximal activity of the AOX (see text); RCR: respiratory control ratio. Ratio of oxygen consumption in high/low PO_2 is presented for both state 2 and state 3. Values are means ± SEM. *A. islandica*, $n = 8$; *M. arenaria*, $n = 12$. *P*-values (Student's *t*-test) for comparison between species are indicated, and significant differences are denoted by asterisks.

mitochondria of *A. islandica* appear to be able to minimize the production of H_2O_2 for an equivalent amount of ATP provided to the cell. This is in accordance with a role for ROS flux toward mtDNA in setting the pace of senescence.

When compared with *S. solidissima*, the rate of H_2O_2 generation was lower for *A. islandica* in five of six conditions normalized with CS, including the most physiologically relevant ones, that is, forward and convergent electron flow. However, no differences were found for succinate-supported state 2 or FRL state 2. Failure to find differences during state 2 may be related to the quality of the mitochondrial isolates from this species. We found RCR values of 4.5, 2.35, and 1.8, respectively, for *A. islandica*, *M. arenaria*, and *S. solidissima.* These values are comparable or higher to what was found in previous studies for *A. islandica* and *M. arenaria* (Tschischka *et al.*, 2000; Philipp *et al.*, 2005). However, we have no reference for *S. solidissima,* and therefore, we are unable to confirm whether the low RCR value is a characteristic of the species or whether it is the sign of damaged mitochondria. However, adding cytochrome *c* during state 3 respiration is known to help restore the activity when mitochondria have been damaged by the isolation procedure (Kuznetsov *et al.*, 2008). A preliminary test with *S. solidissima* showed increases well in excess of the 15% limit, suggesting a partially broken outer membrane. This should have underestimated the H_2O_2 production rate for this species during succinate-supported state 2. Indeed, according to previous studies, H_2O_2 production during REF is highly sensitive to the proton gradient (Herrero & Barja, 1997; Lambert & Brand, 2004b). Therefore, a lower proton gradient of partially uncoupled mitochondria would explain the low H_2O_2 rates before ADP addition. Accordingly, the addition of ADP during succinate respiration decreased the production of H_2O_2 of the two other species in proportion to their RCR value, while no decrease was found for *S. solidissima*. Furthermore, a partially broken outer membrane would have overestimated O_2 consumption during convergent electron flow state 2 because of increased futile proton cycling, and thus underestimated FRL. We have no idea why our mitochondrial isolation protocol would have partially damaged the outer membrane for this species and not for the others; however, it should have led to an underestimation of the actual H_2O_2 production rate in *S. solidissima* for the two conditions where we found no significant difference with *A. islandica*.

Our general finding of a lower H_2O_2 production rate in *A. islandica* mitochondria is in line with previous studies. In a preliminary study not subjected to peer review, Buttemer *et al.* (2010) reported that isolated mitochondria of *A. islandica* produced less H_2O_2 than those of *M. arenaria* in states 2 and 4 (oligomycin). More recently, Ungvari *et al.* (2011) found lower rates of H_2O_2 and $O_2^{\cdot-}$ generation in tissue preparations of *A. islandica* when compared with the other quahog species, the hard-shell clam *Mercenaria mercenaria*. It seems therefore that the longest-living noncolonial animal, the mud clam *A. islandica*, is characterized by a reduced rate of ROS production when compared with shorter-lived species. This contrasts with a study of the longest-living rodent species, the naked mole-rat (*Heterocephalus glaber*). Indeed,

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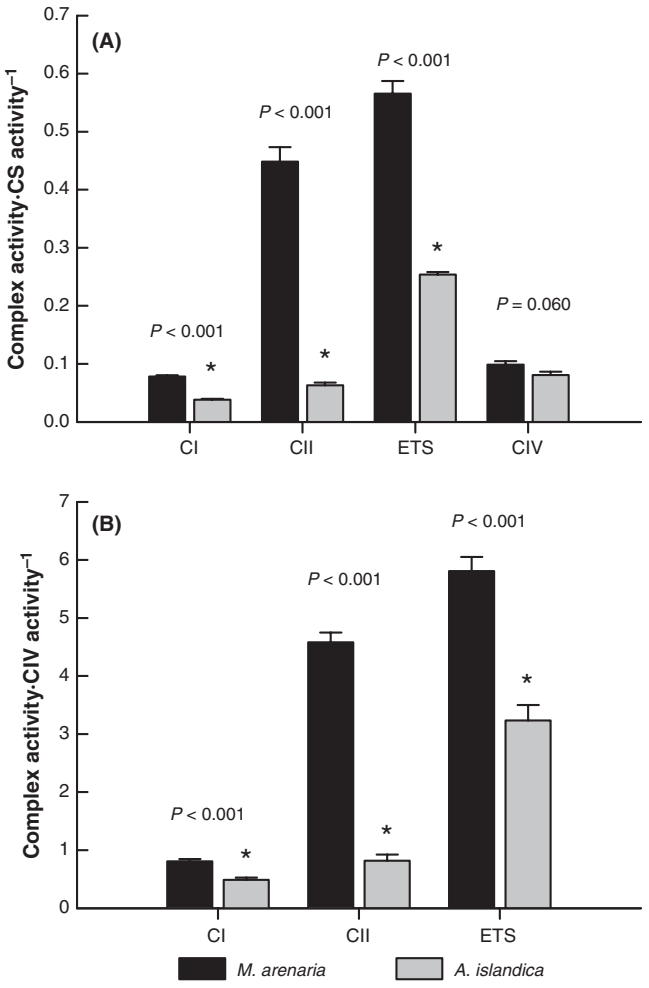

Fig. 4 Enzymatic activities of the ETS components normalized with citrate synthase (A) and complex IV (B) activity. Abbreviations are as follows: complex I (CI), complex II (CII), complex I to III segment (ETS), and complex IV (CIV). Activities of the ETS complexes are express as moles of electrons transferred, while that of the CS is per mole of product. Values are means ± SEM for six mitochondrial preparations measured in duplicate. Asterisks denote significant differences (Student's *t*-test) between species.

endothelial and smooth muscle cells of the carotid artery were found to produce comparable or higher rates of $O_2^{\cdot-}$ and H_2O_2 for the naked mole-rat compared with three shorter-lived rodents (Labinskyy *et al.*, 2006).

Concerning bivalves as a group, studies on *A. islandica* agree with that of Philipp *et al.* (2005), who found that H_2O_2 production of isolated mitochondria from *M. arenaria* was higher than in the longer-lived *L. elliptica*. Taken together, these first studies provide evidence to suggest that the ROS–longevity relationship could very well exist for bivalves.

Mechanisms for reduced hydrogen peroxide production rate

To explore possible mechanisms that allow a reduction of ROS production, we focused on the contrast between *A. islandica* and *M. arenaria* because the differences between them were significant in all conditions tested.

We first examined AOX, which is an additional complex of the ETS of many plants, fungi, and animals. It has been proposed that it acts as an 'electron overflow security', reducing ROS production whenever the Q-pool is in a highly reduced state in the presence of elevated PO_2 (Abele *et al.*, 2007). Such a mechanism may serve to avoid the burst of ROS production accompanying the reoxygenation of tissues with tidal exposure (*M. arenaria*) or upon emergence after burrowing in anoxic conditions (*A. islandica*; Strahl *et al.*, 2011). We found evidence for an active AOX electron pathway in both species using two complementary methods: (i) increasing the PO_2, we found increased oxygen consumption during both state 2 and state 3, which has been attributed to AOX recruitment (Tschischka *et al.*, 2000); and (ii) using antimycin A to inhibit CIII, we observed a significant residual O_2 consumption sensitive to the AOX-specific inhibitor (SHAM). However, AOX activity was either similar or higher in the short-lived *M. arenaria* compared with *A. islandica*. Hence, we must rule out the possibility that higher AOX activity could explain the reduced ROS production rate of *A. islandica*.

In vertebrates, a lower CI content has been suggested as a simple explanation for the reduced ROS production rate of long-lived species (Lambert *et al.*, 2010). CI content is also reduced in rat mitochondria after diet restriction treatments (Ayala *et al.*, 2007; Caro *et al.*, 2008). Complex I is known to be a major contributor to ROS production. When reverse electron flow is initiated by the combination of succinate and CIII inhibitors, the Q-pool is maximally reduced and electrons can only flow through CI. It is easily conceivable that this condition maintains CI in a state of maximal reduction. It is therefore reasonable to suggest a direct relationship between CI content and the ROS production rate during this condition. In this study, we found a lower enzymatic CI activity in the long-lived *A. islandica* compared with the short-lived *M. arenaria*. We conclude that this difference can contribute to the lower ROS production observed during REF in *A. islandica.* This could act in parallel with other mechanisms such as the maintenance of a lower proton-motive force in this species (Lambert & Brand, 2004b; Lambert *et al.*, 2010). However, REF requires the use of inhibitors and does not simulate *in vivo* conditions.

During FEF, no inhibitors are used and CI and/or CIII are not maintained in a state of maximal reduction, at least in presence of ADP. It is nonetheless conceivable that a lower catalytic capacity of these complexes can reduce ROS production, explaining the differences observed between our species for this condition. We found that the higher enzymatic activity of CI and CIII does not translate into higher ETSmax in fresh mitochondrial isolate, suggesting that these ETS components are not limiting the capacity of mitochondria to oxidize substrates such as NADH and coenzyme Q. However, higher enzymatic activity of these complexes may maintain a higher steady-state degree of electronic reduction at their ROS producing sites (Barja & Herrero, 1998; Barja, 2004). This would result in a higher ROS production rate for similar respiratory capacities. Notwithstanding, our data do not suggest that the maintenance of a lower proton gradient would reduce the rate of ROS production during FEF. Indeed, the addition of ADP during convergent electron flow had no detectable effect on ROS production rate. Furthermore, during preliminary experiments (data not

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shown), the addition of the uncoupler FCCP during convergent electron flow just slightly increased ROS production. This suggests that the rate of ROS production does not seem to be affected by the proton gradient when CI substrates are used.

Not only do our data generally support the involvement of CI content or activity in modulating ROS production between species, but they further suggest that CII activity may also be involved. Indeed, we found that CII activity is about seven times lower in *A. islandica* than in *M. arenaria*. This is a larger difference than the differences observed in the activity of CI, CIII or any other parameters measured in this study. When succinate is present, higher CII capacities should maintain higher degree of electronic reduction of the Q-pool and hence of the ROS production sites of CI and CIII, leading to higher rate of ROS production. Furthermore, a recent study provides compelling evidence for high direct ROS production by CII in rat skeletal muscle mitochondria (Quinlan *et al.*, 2012). A trend for a lower CII content was also found in long-lived strains of drosophila (Neretti *et al.*, 2009). More strikingly, a 40% or 80% methionine restriction in rats both increased longevity and decreased CII content to a greater extent than the decreases found for any other complexes of the ETS (Caro *et al.*, 2008). Although few in number, these studies clearly suggest that it may be worth investigating CII content in relation to longevity in future studies.

Conclusion and perspectives

The main finding of this study is that isolated mitochondria of the extraordinarily long-lived bivalve *Arctica islandica* are characterized by a reduced rate of ROS production when compared with two short-lived species. This difference does not appear to be linked to AOX capacity. However, a lower capacity of CI or CI and CIII may be involved, as previously proposed. Furthermore, we suggest that CII capacity or content should be measured by future studies because there is early evidence that it may be adjusted to modulate the overall ROS production of the ETS in long-lived species. We also showed that the state of mitochondrial respiration (substrate and inhibitor) influences the relative difference in ROS production found between species. In this regard, there is currently a lack of standardization between studies investigating the ROS–longevity relationship, and very few discussions have been focused in this direction. We suggest that the condition of convergent electron flow in the presence of ADP (state 3) should be included (among others) to compare species on the basis of a respiratory state that best simulates *in vivo* conditions.

Experimental procedures

Bivalve collection and care

Individuals of *A. islandica* and *S. solidissima* were collected in the Îles-de-la-Madeleine (47°22′N, 61°58′W, Québec, Canada). *M. arenaria* were collected in Bic (48°23′N, 68°40′W, Québec, Canada). All individuals were kept at the ISMER marine research station in Pointe-au-Père (Québec, Canada) for at least 1 month before dissection. The open flow-through two-tank system contained a 25-cm layer of sand that allowed normal positioning of bivalves in the sediments. Bivalves were fed a live microalgal diet consisting of *Nannochloropsis* sp., Isochrysis galbana, and *Pavlova lutheri* (12%, 44%, and 44% in cell numbers, respectively) provided at 1% body mass/d at 8 °C; quantities were adjusted for water temperature fluctuations between 12 °C (summer) and 3.5 °C (winter).

Isolation of mitochondria

After water temperature had stabilized at 12 °C for at least two weeks in the tanks, two individuals were brought to the laboratory each day for dissection. Bivalves were opened by inserting a knife blade in the siphon orifice. About 4 g of mantle tissue was removed, rinsed, and minced. Homogenization was achieved using three passes of a loose-fitting and one pass of a tighter-fitting precooled glass/Teflon pestle in 34 mL of ice-cold homogenization buffer (buffer compositions are available in the online Supporting information). The homogenate was centrifuged at 1500 *g* for 10 min to eliminate large cellular debris; this step was repeated with the supernatant to remove remaining debris. The final supernatant was considered free of unbroken cells or cell debris and was centrifuged at 10 500 *g* for 15 min. The final mitochondrial pellet was gently resuspended in 1.8 mL of respiration buffer. All steps were carried out at 4 °C. Hydrogen peroxide production measurements and oxygen consumption measurements were made using common buffer composition, substrate (and inhibitor) concentration, and experimental temperature (10 °C). Measurements were taken in parallel immediately after mitochondrial isolation. The remaining mitochondrial isolates were frozen at −80 °C for enzymatic assays of the ETS complexes, which were also carried out at 10 °C. Protein concentrations in mitochondrial isolates were determined using the bicinchoninic acid assay kit (SIGMA®, St-Louis, MO, USA).

Mitochondrial hydrogen peroxide production

The hydrogen peroxide production rate was determined in an aliquot of the fresh mitochondrial isolate using a thermostated fluorometer (Hitachi F2500) set at 10 °C. Mitochondria were incubated at 62–233 μg mL^{-1} of mitochondrial protein in respiration buffer containing 6 μM Amplex Red® (Invitrogen, Eugene, OR, USA), 2 U mL^{-1} horseradish peroxidase, and 50 U mL^{-1} superoxide dismutase. The increase in fluorescent resorufin resulting from the HRP-catalyzed oxidation of Amplex Red by H_2O_2 was monitored at excitation/emission values of 560/587 nm. Standard curves generated in the presence of the same substrates and inhibitors were used to calibrate the raw signal. Rates of H_2O_2 production were calculated by linear regression from the linear rate of fluorescence increase within the resorufin concentration range generated in the assay. When present, substrate and inhibitor concentrations were as follows: 10 mM succinate, 24 mM glutamate, 10 mM malate, 5 mM ADP, 1 μM antimycin A, and 2 μg mL^{-1} oligomycin. Results are presented as mean ± SEM and normalized by citrate synthase activity. See the online Supporting information for details of the sequential measurements, preliminary tests, and the rationale for using citrate synthase as the denominator.

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Oxygen consumption

Oxygen consumption of freshly isolated mitochondria was measured using two thermostated high-resolution oxygraphs (Oxygraph O2K, Oroboros Instruments, Innsbruck, Austria) operated simultaneously and held at 10 °C. Sequences were initiated by the addition of 0.5 mg mitochondrial protein per mL to the respiration buffer containing substrates (24 mM glutamate, 10 mM malate, and 10 mM succinate when present). Sequential injections of inhibitors (1 μM antimycin, 1 μM rotenone, and 1 μM SHAM), uncoupler (1.5 μM FCCP), ADP (5 mM), and cytochrome *c* (50 μM) were performed during the course of the sequences. The maximal activity of CIV (CIVmax) was measured after addition of ascorbate (2 mM) and tetramethyl-p-phenylenediamine dihydrochloride (TMPD, 0.5 mM). When measuring the AOX activity, chamber stoppers were temporarily raised and nitrogen or oxygen was introduced to achieve high (47.5 kPa) or low (8.6 kPa) PO_2. The oxygen solubility coefficient was taken from the study by Tschischka *et al.* (2000), who used the same respiration buffer, and adjusted to the experimental temperature of 10 °C according to Rasmussen & Rasmussen (2003). Appropriate instrumental background corrections were applied for each sequence because they covered a different range of PO_2. Chemical background and residual oxygen consumption were used to correct the respiration rates according to Gnaiger (2009). The oxygen consumption rates were normalized with citrate synthase activity (pmol O_2 s^{-1}.U CS activity^{-1}). Oxygen consumption measurements in *S. solidissima* were restricted to state 2 and 3 (presence of ADP) during forward (glutamate + malate) and convergent (glutamate + malate + succinate) electron flow to calculate the RCR and the FRL, respectively. See the online Supporting information for details about the sequences, preliminary experiments, and calculation of free-radical leak.

Enzymatic activity

Enzymatic activities of different ETS complexes and of citrate synthase were determined using a frozen aliquot of the mitochondrial isolate. Measurements were made in duplicate using a thermostated UV/VIS spectrophotometer (Ultrospec 2100 pro) held at 10 °C. CIV activity was determined by measuring the oxygen consumption rate using Clark-type electrodes (Hansatech® instruments, Norfolk, England). Activity of the ETS complexes is presented as moles of electrons transferred per unit time and was normalized with citrate synthase activity, defined as mole of product formed per unit time. See the online Supporting information for details of this calculation and assay conditions.

Statistical analysis

To compare species within experimental conditions, significant effects (alpha = 0.05) were determined by ANOVA (more than two species) or Student's *t*-test. Where ANOVA revealed a significant effect, Tukey–Kramer HSD was used to provide *P*-values for pair comparisons. Variable were $\log_{10}$ transformed if necessary. *P*-values for these comparisons are presented in Figs 1–4. To compare all experimental conditions of H_2O_2 production within species, repeated measurement with the same individuals was assessed using a general linear mixed model (GLMM) with species and condition as the fixed effects and individual nested in species and as the random effects. To compare oxygen consumption between high and low PO_2 for the same species, repeated measurements with the same individuals were assessed using paired Student's *t*-test comparisons with Bonferroni's correction. When the GLMM revealed a significant effect, Tukey–Kramer HSD was used to provide *P*-values for pair comparisons. *P*-values for comparison between conditions are presented in the text. In all cases, homogeneity of variance was estimated using the Brown–Forsythe test except when the GLMM procedure was used, in which case it was ascertained by visual examination of the residual distribution. All analyses were performed using the JMP v10.0 statistical package (SAS Institute Inc., Cary, NC, USA). Results are presented as means ± SEM. A Table listing *F*-ratio, df, and *P*-value for the ANOVA and GLMM that preceded Tukey–Kramer HSD is available online in the Supporting information section.

Acknowledgment

We are grateful to Véronique Desrosier for help in acquiring the H_2O_2 production data and to an anonymous reviewer who helped us further complement our literature review and position our study. Daniel Munro was supported by scholarship grants from NSERC and the FONCER program from Réseau Aquaculture Québec. This project was supported by an NSERC Discovery grant to P.U. Blier.

Author contributions

D. Munro and P. U. Blier designed experiment. D. Munro and F. Paquin carried out specimen collection and care. D. Munro acquired oxygen consumption and H_2O_2 data. F. Paquin acquired spectrofluorometric enzymology data. D. Munro and N. Pichaud analyzed data. D. Munro, N. Pichaud, and P. U. Blier wrote the first draft.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Data S1 Complementary experimental procedures.

Table S1 Parameters of statistical test (ANOVA and GLMM) that preceded the *post hoc* test used to produce *P*-values presented in the figures of the article.

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