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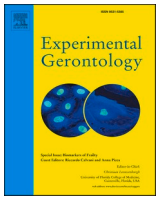

Review

Mitochondrial ROS production, oxidative stress and aging within and between species: Evidences and recent advances on this aging effector

José Gómez [a], Natàlia Mota-Martorell [b], Mariona Jové [b], Reinald Pamplona [b,*], Gustavo Barja [c,*]

[a] Department of Biology and Geology, Physics and Inorganic Chemistry, ESCET, Rey Juan Carlos University, E28933 Móstoles, Madrid, Spain
[b] Department of Experimental Medicine, University of Lleida (UdL), Lleida Biomedical Research Institute (IRBLleida), E25198 Lleida, Spain
[c] Department of Genetics, Physiology and Microbiology, Faculty of Biological Sciences, Complutense University of Madrid (UCM), E28040 Madrid, Spain

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ABSTRACT

Mitochondria play a wide diversity of roles in cell physiology and have a key functional implication in cell bioenergetics and biology of free radicals. As the main cellular source of oxygen radicals, mitochondria have been postulated as the mediators of the cellular decline associated with the biological aging. Recent evidences have shown that mitochondrial free radical production is a highly regulated mechanism contributing to the biological determination of longevity which is species-specific. This mitochondrial free radical generation rate induces a diversity of adaptive responses and derived molecular damage to cell components, highlighting mitochondrial DNA damage, with biological consequences that influence the rate of aging of a given animal species. In this review, we explore the idea that mitochondria play a fundamental role in the determination of animal longevity. Once the basic mechanisms are discerned, molecular approaches to counter aging may be designed and developed to prevent or reverse functional decline, and to modify longevity.

1. Introduction

It is generally considered that mitochondria play a fundamental role in biological aging. The mitochondrial free radical theory of aging (MFRTA), proposed just 50-years ago (Harman, 1972) as an update of the Harman's free radical theory of aging (Harman, 1956), posits that aging is driven largely by reactive oxygen species (ROS) produced in mitochondria and their derived biological effects at cell, tissue and organism level.

Mitochondrial reactive oxygen species production (mitROSp), in addition to their signaling role, is the cause of non-enzymatic chemical modifications (damage) within cells, both to mitochondria themselves and potentially to all kinds of macromolecules and cellular components (Halliwell and Gutteridge, 2007). The consequences of this injury cover and involve a wide spectrum of biological functions such as loss of proteostasis, genome instability, telomere dysfunction, cell senescence, epigenetic alterations, dysregulated nutrient sensing pathways, apoptotic cell death, and inflammation (Berry and Kaeberlein, 2021). As Berry and Kaeberlein (2021) point out, these interactions illustrate the importance of mitochondrial function for preserving youthful cellular, tissue, and organism health.

Mitochondria, however, are a dynamic system adapted to the different cellular metabolic profiles and bioenergetic demands due to the intrinsic different functions. These adaptations determine variations between tissues and cell types in mitochondrial morphology, activity, biogenesis, and proteome profile, among others characteristics (Kunz, 2003; Mootha et al., 2003; Benard et al., 2006; Johnson et al., 2007a; Johnson et al., 2007b; Fernández-Vizarra et al., 2011). Consistent with this idea, the mitochondrial interorgan differences are also expressed in the biology of free radicals. Table 1 offers an interorgan comparative approach on oxidative stress parameters in different tissues from male Wistar rats, confirming that oxidative stress is a tissue-specific trait. It seems that the biology of aging affects the different organs in a cell type-specific way, and it may be inferred that for an organism-species, its rate of aging integrates the rate of aging of the different cell types.

Recent evidence also demonstrated that mitochondrial free radical production is a highly regulated mechanism involved in the determination of longevity. Longevity is a species-specific biological trait. This mitROSp induces a diversity of adaptive responses and derived molecular damage to cell components, especially to mitochondrial DNA (mitDNA), with biological consequences which contribute to determine the rate of aging of a given animal species. In this review, we explore the idea that mitochondria biology of free radicals plays a fundamental role in the determination of animal longevity.

* Corresponding authors.
E-mail addresses: reinald.pamplona@udl.cat (R. Pamplona), gbarja@bio.ucm.es (G. Barja).

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Table 1
Oxidative stress parameters in different tissues from male Wistar rats: an interorgan comparative approach.

	Heart[a]	Brain	Liver[a]
Rate of mitochondrial H_2O_2 production with pyruvate/malate as substrates (nmol H_2O_2/min·mg protein)	1.0 ± 0.07	0.56 ± 0.05	0.23 ± 0.04
Maximum rate of H_2O_2 production with pyr/mal + rotenone (nmol H_2O_2/min·mg protein)	8.9 ± 1.0	3.2 ± 0.21	1.01 ± 0.1
Oxygen consumption (nmol O_2/min·mg protein) in the presence of pyr/mal (upper line, State 4; lower line, State 3)	31.7 ± 4.7 111.5 ± 15	9.4 ± 0.8 38.5 ± 2.6	11.9 ± 0.5 32.19 ± 2.32
Free radical leak (FRL, %)	1.7 ± 0.2	3.01 ± 0.2	0.95 ± 0.1
8-oxodG/10^5dG in mtDNA	5.4 ± 0.3	5.8 ± 0.5	3.5 ± 0.3
Mitochondrial DBI#	229.0 ± 1.2	213.6 ± 3.7	205.7 ± 1.1
Mitochondrial PI#	221.2 ± 0.9	214.8 ± 4.9	190.1 ± 1.5
Mitochondrial MDAL (μmol/mol lys)#	396 ± 13	711.2 ± 109.1	336 ± 14

[a] Values are mean ± SEM from 3 (for heart) or 4 (for liver) different experimental studies. # DBI, double bond index, refers to the density of double-bonds in the mitochondrial lipid membrane and is calculated as: DBI = [(1 × Σmol% monoenoic) + (2 × Σmol% dienoic) + (3 × Σmol% trienoic) + (4 × Σmol% tetraenoic) + (5 × Σmol% pentaenoic) + (6 × Σmol% hexaenoic)]; PI, peroxidation index, refers to the mitochondrial membrane susceptibility to lipid peroxidation and is calculated as: PI = [(0.025 × Σmol% monoenoic) + (1 × Σmol% dienoic) + (2 × Σmol% trienoic) + (4 × Σmol% tetraenoic) + (6 × Σmol% pentaenoic) + (8 × Σmol% hexaenoic)]; MDAL, malondialdehyde-lysine is an advanced lipoxidation end-products formed in proteins in the context of lipoxidation reactions. References used: Gredilla et al. (2001), Pamplona et al. (2002), Sanz et al. (2004), Sanz et al. (2005a, 2005b), Sorensen et al. (2006), Ayala et al. (2007), and Sanz et al. (2006).

2. Mitochondrial free radical production and longevity

This initial section describes a highly condensed summary of key points concerning the MFRTA (Son and Lee, 2021). MFRTA was first proposed in 1972 as a wear and tear and an antioxidant-based theory (Harman, 1972). The initial emphasis for proposing this theory was focused on the total cell and tissue antioxidants trying to decrease the aging rate simply by feeding antioxidants to humans. This was mainly due to their much easier manipulation compared to mitROSp and to the methodological difficulties to reach the necessary sensitivity to quantitatively measure mitROSp, which persisted during the following two decades. However, a huge number of experimental studies of antioxidant manipulation were mainly performed in mice and rats in the 1970's and 80's and lead to the highly consistent conclusion that increasing the total cell and tissue antioxidants, that in some cases increased survival, did not change species longevity, indicating that those antioxidants were not able to decrease the rate of mammalian aging. This was true both supplementing the diet with low molecular weight (natural or artificial) antioxidants or increasing antioxidant enzymes by genetic overexpression or hormetic manipulations (reviewed in Barja, 2004a, 2004b), except, very interestingly, in one case in which longevity increased after overexpression of catalase inside mitochondria but not when the researchers increased it inside peroxisomes or the nucleus (Schriner et al., 2005).

Surprisingly, when basal levels of total cell and tissue enzymatic and non-enzymatic antioxidants were compared between species, strongly negative correlations with longevity were observed in 21 out of 27 correlations from five different laboratories and not a single case of positive correlation with longevity was found (Pérez-Campo et al., 1998). It was then hypothesized that the evolutionary reason responsible for such result was that the rate of mitROSp was lower as the

Table 2
Summary of comparative studies on mitochondrial free radical generation in animal species differing in their longevities.

Animal species comparison	Longevity	Mitochondria from:	mitROSp in long-lived species	Reference
5 mammals	3.5–30 years	Liver	Lower	Sohal et al., 1989
6 mammals	3.5–30 years	Liver	Lower	Sohal et al., 1990
7 mammals	3.5–30 years	Heart, kidney	Lower	Ku et al., 1993
Mouse vs white-footed mouse	3.5, 8 years	Heart, brain	Lower	Ku and Sohal, 1993
Rat vs pigeon	4, 35 years	Heart, brain, kidney	Lower	Ku and Sohal, 1993
Rat vs pigeon	4, 35 years	Brain, liver, lung	Lower	Barja et al., 1994
5 flies	29–65 days	Flight muscle mitochondria	Lower	Sohal et al., 1995
Rat vs pigeon	4, 35 years	Heart	Lower	Herrero and Barja, 1997; Lambert et al., 2010
Mouse vs parakeet	3.5, 21 years	Heart	Lower	Herrero and Barja, 1998
Mouse vs canary	3.5, 24 years	Heart	Lower	Herrero and Barja, 1998
Mouse vs parakeet	3.5, 21 years	Brain	Lower	Barja, 1998
Mouse vs canary	3.5, 24 years	Brain	Lower	Barja, 1998
Rat vs pigeon	4, 35 years	Brain (nonsynaptic)	Lower	Barja and Herrero, 1998
Male Ames dwarf mouse vs wild type mouse	3.5, 4.9 years	Liver	Lower	Brown-Borg et al., 2001
Two non-flying mammals vs little brown bat	2, 8, 34 years	Heart, kidney, brain	Lower FRL	Brunet-Rossinni, 2004
Mouse vs the white-footed mouse	3.5, 8 years	Endothelial cells from aortas	lower	Csiszar et al., 2007
Mammals (10) and birds (2)	3.5–37.5 years	Heart	Lower	Lambert et al., 2007
Long-lived vs short-lived colubrid snakes	7–25+ years	Liver	Lower	Robert et al., 2007
Wild type fungus vs long-lived mutant	12, >400 days	Whole	Lower	Sellem et al., 2007
Mouse, rat, and human	3.5–120 years	Brain	Lower	Kudin et al., 2008
Tarantulas: male vs females	6 vs 21 years	hemolymph immune cells	Lower	Criscuolo et al., 2010
Three wild-type strains of *D. melanogaster*: Dahomey (D), Canton S (C), and Oregon R (O).	Males (days): D, 49; C, 53; O, 74. Females (days): D, 84; C, 81; O, 91.	Whole	Lower	Sanz et al., 2010
Cells with mitochondrial haplogroups associated with longevity	n.a.	fibroblasts	Lower	Chen et al., 2012
13 primates	20–120 years	fibroblasts	Lower	Csiszar et al., 2012
3 bivalves	28, 37, 507 years	Mantle tissue	Lower	Munro et al., 2013
		Whole	Lower	

(continued on next page)

Table 2 (*continued*)

Animal species comparison	Longevity	Mitochondria from:	mitROSp in long-lived species	Reference
Wild type and 29 mutant strains of *Caenorhabditis elegans* differing in their longevity.	20–60 days			Shen et al., 2014
C57Bl/6 mouse vs ICRFa long-lived mouse	n.a.	Liver, brain	Lower	Miwa et al., 2014

longevity of the species increased. Effectively, this idea was corroborated and confirmed in many different investigations. Table 2 shows a list of comparative studies on mitROSp in animal species differing in their longevities. The results listed strongly suggest that mitROSp contributes to increase the aging rate in mammals. We also studied bird species because they have a higher longevity than mammals of the same body size and metabolic rate. We observed that pigeons have less mitROSp than rats while their longevity is 30 years instead of 4 (Barja et al., 1994). The same was later observed in other two birds from other two different families, parakeets and canaries, which live up to 20 and 24 years and have smaller mitROSp than mice. It is important to note that in the three cases, these birds obtained their lower mitROSp without decreasing their rate of mitochondrial oxygen consumption and global metabolic rate. What they do is to decrease the percentage of electrons out of sequence which leak from the mitochondrial electron transfer chain (ETC) instead of reaching cytochrome oxidase to fully reduce O_2 to water: they lower the percentage free radical leak (FRL). This means that species can *actively* modulate their mitROSp and FRL, which converts MFRTA from a stochastic into a programmed aging theory. MitROSp is not a simple by-product of the respiratory chain because it is not a fixed percentage of the total electron flow. Instead, it can be actively modulated at species or even at individual level to contribute to modify longevity without affecting the mitochondrial O_2 consumption and the aerobic metabolic rate. That is possible because basal levels of mitROSp in the intact animal come from around 1 % or less of total ETC electron flow. In long-lived animals, gene expression of the mitochondrial subunits responsible for mitROSp is quantitatively modified according to instructions codified in the nucleus and controlled by molecules including transcriptional factors like FOXOs, in order to obtain a lower final ROS production value at mitochondria. The rate of mitROSp is programmed in each species to have an adequate value for its longevity which is in turn adapted to the ecological environment of the animal or species. Bats are long-lived mammals in spite of their small body size. Like birds, they also have a much higher longevity than expected for their body size and aerobic metabolic rate compared to most mammals of similar size. At least in one bat species a lower FRL was also observed than in equivalent sized mammals (Brunet-Rossinni and Austad, 2004). Therefore, this bat species, like birds, leak less ROS per unit of mitochondrial oxygen consumption, likely contributing to its extraordinary longevity.

3. DNA damage and longevity

Among cellular macromolecules, DNA modification is of paramount relevance for aging (Kowalska et al., 2020). The low rate of mitROSp of long-lived animals is reflected on their lower levels of the oxidatively modified and mutagenic 8-oxodG (8-oxo-7,8-dihydro-2′deoxyguanosine) in mitDNA (highly significant $P < 0.0001$ correlation with longevity) but not in genomic DNA (nDNA; Barja and Herrero, 2000). Both point mutations and large deletions in mitDNA increase with age, the latter being the most potentially damaging because mitDNA contains 13 genes of the ETC. In addition, knock-in mice that express a proof-reading-deficient version of mtDNA polymerase gamma show a threefold to fivefold increase in the levels of mtDNA point mutations, as well as increased amounts of deleted mtDNA in association with premature aging and reduced lifespan (Trifunovic et al., 2004). However, it is possible that the deletions cause aging acting as mtDNA fragments inserted inside nDNA (see below) instead of through decreases in mitochondrial ATP production.

After decades of discussion, it was found that the percentage of cells in old individuals with mitDNA deleted in homoplasmia in most vital tissues except in substantia nigra is only 2 % in old humans (Bender et al., 2006) whereas the threshold to stop cell respiration is around 70 % mitDNA mutated in homoplasmia. The presence of up to 5000 copies of each mitochondrial gene in each cell rules out mitDNA mutations as direct causes of aging. This left MFRTA without a mechanism by which mitDNA damage could contribute to aging. However, in 2010 two independent laboratories found that mitDNA fragments, most likely resulting from the mitDNA deletions, accumulate with age inserted in nDNA in somatic tissues in mice and rats (Caro et al., 2010) and yeast (Cheng and Ivessa, 2010), as well as in humans disseminated across all chromosome arms (Singh et al., 2017). This affords a mechanism by which mitROSp can modify many genes, 99,99 % of which are present inside the nucleus. This mechanism, differing from the case of mitDNA mutations, is not negatively affected by the high copy number of mitDNA genes per cell. On the contrary, the high abundance of mitDNA copies can supply a higher flux of mitochondrial DNA fragments to the nucleus. These fragments connect mitROSp to aging through deleterious modification of nuclear genes and thus their expression in old individuals which contribute to aging. Indeed, studies in yeast have shown that such accumulation of mitDNA fragments inside the nucleus shortens the chronological lifespan of yeast (Cheng and Ivessa, 2012). Moreover, studies in mice have demonstrated that rapamycin, the only known drug that increases mammalian longevity (Harrison et al., 2009; Zhang et al., 2021), decreases both mitROSp and mitDNA fragments accumulation in the nucleus to the same extent (Martínez-Cisuelo et al., 2016). In addition, mitDNA fragments not only insert in the nucleus of the same cell in which they are produced by mitochondria, but also enter tissue capillaries and disseminate through the vascular system. Inside vessels they are known to be part of the DAMPS (damage-associated molecular patterns) which promote inflammaging (Franceschi et al., 2018; Picca et al., 2018). Moreover, mitDNA fragments can exit the vascular system and insert in the nDNA of the cells situated in other organs far away from the cell in which they were originated, extending the damage and thus helping to coordinate the aging rate of different organs. Such rate is known to be somewhat different among organs and individuals but is kept controlled within rather well-defined ranges in different species.

4. Mitochondria as target of pro-longevity interventions

The role of mitROSp as aging effector is not limited to interspecies differences. Caloric restriction (CR), the best-known experimental manipulation that increases mammalian longevity (Weindruch and Sohal, 1997; Austad and Hoffman, 2021) by up to 40 %, also decreases mitROSp, FRL and 8-oxodG in mitochondrial DNA, and all these decreases occur again in the same quantitative amount: 30–40 % decreases. Interestingly, the decreases observed in all these parameters are quantitatively similar both in CR and across mammalian species, and agrees with the up to 1.4-fold increase in longevity induced by CR. All the other successful intra-species increases in longevity obtained up to now in mammals curiously share this 1.4-fold limit including those shown by protein restriction (PR), life extending drugs like rapamycin, or single gene mutant long-lived mice. In contrast, between the compared species, the differences in longevity reach up to 10-fold. The reason for this difference in longevity extension with the same quantitative change in oxidative damage-related parameters is unknown. However, there is now a likely explanation based on the Unified Theory of Aging (Barja, 2019) that posits that many different aging mechanisms

Table 3
Summary of comparative studies on mitochondrial complex I content in animal species differing in their longevities and experimental models under different pro-longevity interventions.

Animal species comparison/pro-longevity intervention	Longevity/animal species	Mitochondria from:	Complex I content in long-lived species/effect of intervention	Reference
Mouse vs parakeet	3.5, 21 years	Brain	Lower	Pamplona et al., 2005
Mouse vs canary	3.5, 24 years	Brain	Lower	Pamplona et al., 2005
Rat vs pigeon	4, 35 years	Heart	Lower	Lambert et al., 2010;
C57Bl/6 mouse vs ICRFa long-lived mouse	n.a.	Liver, brain	Lower	Miwa et al., 2014
8 mammals	3.5–46 years	Heart	Lower	Mota-Martorell et al., 2020
26 mammals	3–37 years	Brain, heart, liver, lung, kidney, skin	Lower	Lu et al., 2022
40 % protein restriction	Rat	Liver	Decreased	Ayala et al., 2007
80 % MetR	Rat	Liver, Heart	Decreased	Sanz et al., 2006
80 % MetR	Rat	Brain	Decreased	Naudí et al., 2007
25 % CR	Rat	Liver	Decreased	Gómez et al., 2007
8.5 % CR	Rat	Liver	Decreased	Gómez et al., 2007
EOD-DR	Mouse	Liver	Decreased	Caro et al., 2008a, 2008b
80 % MetR	Rat	Liver	Decreased	Caro et al., 2008a, 2008b
40 % MetR	Rat	Liver	Decreased	Caro et al., 2008a, 2008b
40 % MetR	Rat	Brain	Decreased	Caro et al., 2009a, 2009b
40 % MetR	Rat	kidney	Unmodified	Caro et al., 2009a, 2009b
40 % RESTAAS	Rat	Liver	Unmodified	Caro et al., 2009a, 2009b
2.5 % methionine supplementation	Rat	Liver	Unmodified	Gómez et al., 2009
2.5 % methionine supplementation	Rat	Heart	Unmodified	Gómez et al., 2009
40 % MetR	Rat	Heart	Decreased	Sanchez-Roman et al., 2011
40 % MetR at old age	Rat	Liver	Unmodified	Sanchez-Roman et al., 2012
40 % MetR	Rat	Heart	Decreased	Sanchez-Roman et al., 2014
40 % CR	Mouse	Liver	Decreased	Miwa et al., 2014
Cysteine supplementation	Rat	Liver	Unmodified	Gómez et al., 2015
40 % MetR	Pig	Liver	Decreased	Ying et al., 2015
Rapamycin	Mouse	Liver	Unmodified	Martínez-Cisuelo et al., 2016

CR, caloric restriction. EOD-DR, Every other day dietary restriction, MetR, methionine restriction; RESTAAS, restriction of amino acids; MetS, methionine supplementation.

exist in addition to mitROSp. Therefore, different species could vary a larger number of different aging effectors than a single species, resolving the paradox.

It is well known that PR increases rodent longevity. Furthermore, restriction of a single amino acid in the diet, methionine (MetR), increases longevity both in rats and mice (Fang et al., 2022). When rats were subjected to MetR for seven weeks, many studies showed that mitROSp, FRL, and 8-oxodG in mitDNA decrease and they do it to the same extent than in CR although the increase in longevity observed during CR is twice that in MetR. Interestingly, restricting all the other amino acids in the diet except methionine does not decrease any of these three oxidative damage-related parameters. These results indicate that the decreases in mitROSp, FRL, and 8-oxodG in mitDNA observed during CR are mainly due to restriction of a single molecule: methionine. The other half of the increase in longevity in CR over that observed in MetR must be due to restriction of other dietary compounds (for review see Pamplona and Barja, 2006; Sánchez-Roman and Barja, 2013).

If low methionine levels contribute to control mitROSp, 8-oxodG in mitDNA and longevity within species, it would be logical to expect that excessive levels of methionine would have the opposite effect. In most developed countries protein consumption currently exceeds the recommended daily allowance by two to three-fold. Long-term too high protein diets cause various detrimental effects on the body organs in part due to the higher level of methionine consumed and increase of oxidative stress (Żebrowska et al., 2019).

Excessive methionine dietary intake produces higher vascular organ damage than any other amino acid. This toxicity can be due to various mechanisms including increases in iron levels (Kumagai et al., 2002), or in methionine metabolites like S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), cysteine or homocysteine. Methionine can also induce damage by increasing liver lipid peroxidation and oxidative stress (Park et al., 2008), or by oxidation of methionine residues in proteins to methionine sulfoxide leading to their loss of biological function (Ciorba et al., 1997). Overexpression of methionine sulfoxide reductase, which repairs methionine sulfoxide in proteins, protects against oxidative stress and increases longevity in *Drosophila melanogaster* (Lim et al., 2012), and long-lived Ames dwarf mice have increased activity of the transulfuration pathway (Uthus and Brown-Borg, 2006).

There is evidence that methionine itself is responsible for some of the adverse effects produced by the excess of this amino acid in the diet (Troen et al., 2003). Direct addition of methionine to isolated mitochondria in vitro increases the rate of mitROSp in liver and kidney (Gómez et al., 2011). Therefore, part the effect of methionine supplementation on mitROSp can be due to direct interaction of methionine with mitochondria.

Several studies indicate that methionine is an important factor affecting rodent longevity. Furthermore, protein and methionine supplementation increase plasma levels of its metabolites such ass-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH) and homocysteine in rodents (Velez-Carrasco et al., 2008; Gómez et al., 2009) and homocysteine in humans (Verhoef et al., 2005). Based on the

following examples, it is plausible that some products of methionine metabolism are responsible for part of the detrimental effects of methionine on health: i) increased homocysteine levels in blood can induce both atherosclerosis and hypertension (Troen et al., 2003); ii) elevated plasma homocysteine levels may accelerate aging of the vascular system (Fau et al., 1988) and possibly in the brain (Algaidi et al., 2006); iii) cysteine, a product of methionine metabolism, can be responsible for part of the pro-aging effect of methionine on longevity; iv) plasma cysteine levels have been found to be related to fat mass increasing adiposity in humans (Elshorbagy et al., 2008). Furthermore, various beneficial changes induced by MetR including decreases in mitROSp, mitDNA damage, body weight gain, body fat, subcutaneous fat, visceral fat, and serum hormones associated with adiposity are reversed by cysteine dietary supplementation (Elshorbagy et al., 2011; Gómez et al., 2015).

5. Mitochondrial ROS production: the origin

Where in the mitochondrial ETC are ROS produced, and what sites of production are relevant for aging? During the last three decades of the 20th century it was widely believed that the relevant site of mitROSp was exclusively situated at Complex III (CxIII). This believe was due to various reasons including the small sensitivity of the spectrophotometric techniques used to measure mitROSp and to the frequent use of succinate as substrate especially in the presence of rotenone which avoided electrons to reach Complex I (CxI) trough reverse electron flow. When fluorometry increased the sensitivity for ROS detection, and substrates like pyruvate/malate or glutamate/malate were used to feed electrons to the respiratory chain at CxI through NADH (which feeds most of the electrons to the ETC in vivo), it became established (Herrero and Barja, 1997; Barja and Herrero, 1998; Genova et al., 2001; Kushnareva et al., 2002) that mitROSp are also produced at CxI. Interestingly, although the amount of ROS produced at CxIII can be higher than that coming from CxI, studies with highly selective ETC inhibitors localized the site responsible for the decrease in mitROSp in long-lived species (both in rodents and birds) exclusively at CxI (Herrero and Barja, 1997, 1998). Posterior investigations further localized that site at the hydrophilic domain of CxI (Miwa et al., 2014), and recently, agreeing with a previous suggestion not related to longevity (Kushnareva et al., 2002), it was further localized at the tip of that CxI domain as the N1a FeS cluster inside the polypeptide NDUFV2 which is situated in a lateral dead-end of the electron path within CxI (Mota-Martorell et al., 2020; Pamplona et al., 2021). Further studies are needed to clarify if NDUFV1, which is situated very near to NDUFV2 and contains the N3 FeS cluster plus the flavin which receives the electrons directly from matrix NADH is also involved in producing the ROS related to aging. A high NADH/NAD+ ratio will increase the degree of electronic reduction of the N1a FeS and N3 FeS clusters and FAD, and such degree is one of the main factors increasing their rate of ROS production. Therefore, the description of a positive effect of high NAD+ levels on longevity would work not only through histone deacetylation as it is commonly described (Schultz et al., 2019) but also through a decrease in NADH/NAD^+ ratio which will lower the degree of electronic reduction of N1a FeS cluster of CxI and thus its rate of mitROSp. In addition, Fes N1a and N3 and the flavin of complex I are redox centers with the most negative mid-point redox potential of all the ETC, which also strongly favors mitROSp.

6. Mitochondrial complex I content in long-lived species

The molecular mechanism responsible, at least partially, for the low mitROSp in long-lived animal species has been attributed to a lower steady-state degree of electronic reduction of CxI (Barja and Herrero, 1998; Barja, 2013). However, additional observations comparing mammals versus birds (short- vs long-lived species) demonstrated that a lower CxI content is also present in and may contribute to explain the low mitROSp of those long-lived species (Table 3). In line with this, pro-longevity nutritional interventions such as CR, PR and MetR also induced decreased CxI content concurrently with a lower mitROSp at CxI (Table 3).

Consistent with this concept, a recent work (Miwa et al., 2014) analyzing the mitochondrial proteome of long-living mice (resulting from a CR intervention) compared to a control group, to predict mammalian longevity, demonstrated that animals under CR activate an adaptive response characterized by a decreased content of mitochondrial ETC proteins and, specially, of hydrophilic arm subunits of CxI. This adaptation was associated with improved CxI assembly rates, and decreased mitROSp at CxI. On the contrary, when CxI maturation was suppressed, reduced mitochondrial oxidative metabolism, increased mitROSp and accelerated premature aging that shortened longevity was observed. This finding demonstrated that non-assembled N module of the CxI cause reduced electron transport and increased free radical generation; whereas when CxI is fully assembled, substrate is used for efficient electron transport and proton pumping resulting in a lower mitROSp associated with higher longevity (Miwa et al., 2014). Reinforcing this finding, incompletely assembled hydrophilic arm subunits can still use substrate and produce free radicals (Chen et al., 2005), but without electron transport and proton pumping, thus reducing the overall efficiency of the respiratory chain and increasing mitROSp. Consequently, to minimize the content of free catalytic CxI components, specially of the N module (NDUFV1 and NDUFV2 subunits), is a key trait of a long-lived mitochondrial phenotype in animal species. A recent work (Rodríguez-Nuevo et al., 2022) demonstrates that oocytes maintain ROS-free mitochondrial metabolism by suppressing CxI, suggesting that CxI down-regulation represents an evolutionary conserved strategy that allows longevity while maintain biological activity in long-lived oocytes, like in long-lived species.

7. Mitochondrial DNA damage and repair

If DNA damage is involved in aging, DNA repair should be also important. Interestingly, total cell and tissue (mainly nuclear) base excision repair (BER) has been observed to be, like total cell and tissue antioxidants, negatively correlated with species longevity in mammals (Page and Stuart, 2012). In addition, nuclear BER decreases or does not change in CR (Stuart et al., 2004). Although these results apparently rule out the role of BER in longevity determination, recent studies have found the opposite in the case of mitochondrial BER (Allkanjari and Baldock, 2021) which is positively correlated with longevity (Gredilla et al., 2020).

Both BER and antioxidants concentration can have different concentration inside and outside mitochondria in long-lived animals. This is already demonstrated for BER but is still almost unexplored for antioxidants, although there are already various evidences supporting it. First, it has been suggested that antioxidants are present at higher concentration inside mitochondria than in the rest of the cell based on indirect measurements comparing mitROSp and consumption in mammalian species with different longevities (Munro and Pamenter, 2019). Second, manganese superoxide dismutase (MnSOD), although it has been measured in total cells and tissues, is the mitochondrial form of the superoxide radical detoxifying enzyme and comes from this organelle exclusively. Interestingly, MnSOD is positively correlated with mammalian longevity, in contrast with the negative correlation found for all the other antioxidants mentioned above, which mainly come from the cell in general. Third, overexpressing catalase inside mitochondria but not inside peroxysome or the nucleus increased mouse longevity (Schriner et al., 2005) whereas a large number of studies showed that overexpression of all the other kinds of antioxidant enzymes, which do not act specifically at mitochondria, did not increase rodent longevity. Therefore, it is most important to clarify if other mitochondrial antioxidants also correlate positively with longevity, like in the case of MnSOD and mitBER. This would mean that these antioxidant and repair enzymes are present in long-lived animals at high levels inside mitochondria and

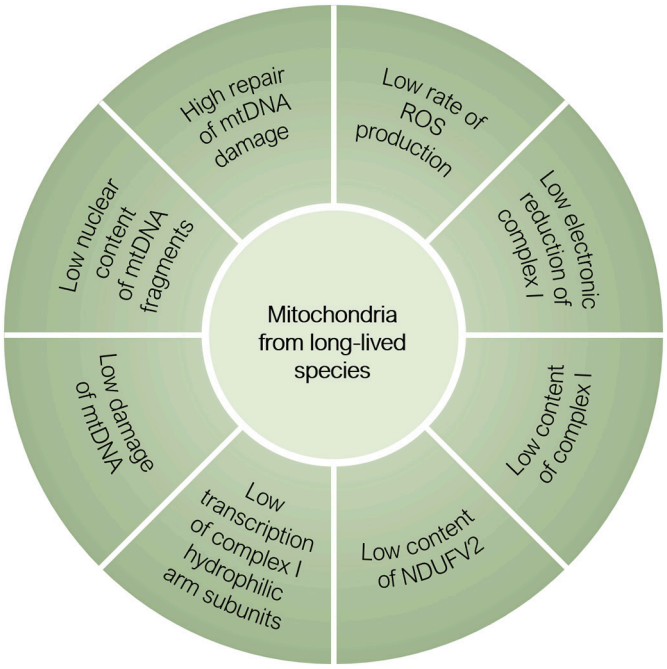

Fig. 1. Mitochondrial traits linked to the biology of free radicals associated with long-lived animal species.

at low levels in the rest of the cell where they are much less needed because mitochondria are the main site of a rather continuous ROS production. If this were the case, at least three mitochondrial parameters could contribute to aging: a low mitROSp, a high mitBER, and a high level of mitochondrial antioxidants. This would mean that cell compartmentation, which has been overlooked up to now, is a key factor for the aging process. Large differences in concentration of ROS, BER and antioxidants seem to occur across mitochondrial membranes, analogously to what happens for Na^{+}, K^{+} and Ca^{++} across the cell plasma membrane. For further details related to the contents of this section the reader is referred to the following reviews (Barja, 1999, 2004a, 2004b, 2013, 2019; Gredilla and Barja, 2005; Pamplona and Barja, 2006; Pamplona et al., 2021; Parkhitko et al., 2019; Sazanov, 2015).

8. Conclusion

The mitochondrial biology of free radicals shows traits that are species-specific and they are related to the animal longevity (Fig. 1). Thus, the rate of mitROSp is lower in long-lived animals (vertebrates and invertebrates). This mitROSp and FRL is actively regulated and converts MFRTA from a stochastic into a programmed aging theory. Thus, in long-lived animals, gene expression of the mitochondrial subunits responsible for mitROSp is quantitatively modified according to instructions codified in the nucleus. The rate of mitROSp is programmed in each species to have a value adequate for its longevity which is in turn adapted to the ecological environment of the animal or species. Consequently, decreases in CxI or its NDUFV1 polypeptide represent an evolutionary conserved strategy that allows longevity while maintain biological activity in long-lived species. mitDNA fragments connect mitROSp to aging through deleterious modification of nuclear gene expression in old individuals. Once the basic mechanisms are discerned, molecular approaches to counter aging may be designed and developed to prevent or reverse functional decline, and to modify longevity.

CRediT authorship contribution statement

G.B. and R.P. conceptualized the manuscript; J.G., R.P., and G.B., wrote the outline of the manuscript; J.G., N.M-M., M.J., R.P., and G.B., investigation; R.P. and G.B. wrote the manuscript, reviewed and edited the manuscript. All authors have participated, read and approved the final manuscript.

Declaration of competing interest

The authors declare no competing interests.

Data availability

No data was used for the research described in the article.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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