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Mitochondrially encoded methionine is inversely related to longevity in mammals

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Summary

Methionine residues in proteins react readily with reactive oxygen species making them particularly sensitive to oxidation. However, because oxidized methionine can be reduced back in a catalyzed reaction, it has been suggested that methionine residues act as oxidant scavengers, protecting not only the proteins where they are located but also the surrounding macromolecules. To investigate whether methionine residues may be selected for or against animal longevity, we carried out a meta-examination of mitochondrial genomes from mammalian species. Our analyses unveiled a hitherto unnoticed observation: mitochondrially encoded polypeptides from short-lived species are enriched in methionine when compared with their long-lived counterparts. We show evidence suggesting that methionine addition to proteins in short-lived species, rather than methionine loss from proteins in long-lived species, is behind the reported difference in methionine usage. The inverse association between longevity and methionine, which persisted after correction for body mass and phylogenetic interdependence, was paralleled by the methionine codon AUA, but not by the codon AUG. Although nuclear encoded mitochondrial polypeptides exhibited higher methionine usage than nonmitochondrial proteins, correlation with longevity was only found within the group of those polypeptides located in the inner mitochondrial membrane. Based on these results, we propose that short-lived animals subjected to higher oxidative stress selectively accumulate methionine in their mitochondrially encoded proteins, which supports the role of oxidative damage in aging.

Key words: Aging; longevity; methionine; mitochondria; oxidative stress; reactive oxygen species.

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Introduction

The accumulation of oxygen in the primitive atmosphere opened the way for the appearance and spread of a respiratory chain based on O_2 as a terminal electron acceptor, an event that was required for the posterior development of large metazoans (Falkowski *et al.*, 2005). Although the origin of undifferentiated multicellularity may be rooted in an anoxygenic Earth (Aledo, 2008), there is no doubt that complex metazoans require an efficient metabolism. In this sense, aerobic respiration provides about an order of magnitude more energy for a given intake of food than fermentation (Catling *et al.*, 2005). However, this efficient metabolism did not come without a price, paid in terms of oxidative stress.

The mitochondrial respiratory chain consists of five multi-subunit complexes. Complexes I–IV mediate the transfer of reducing equivalent (electrons) from NADH or FADH to molecular oxygen. The energy that is released as the electrons traverse complexes I, III, and IV is used to pump protons out the mitochondrial matrix across the inner mitochondrial membrane (IMM), resulting in an electrochemical gradient. The potential energy stored in this gradient is coupled to the ATP synthesis by complex V. During this process, most of the oxygen consumed by the mitochondria is converted to the harmless by-product water at complex IV. However, a variable small percentage of this O_2 can accept electrons directly from complexes I and III, leading to the formation of potentially harmful reactive oxygen species (ROS) (Balaban *et al.*, 2005; Sanz *et al.*, 2006). Although cells have developed various enzymatic and nonenzymatic systems to control ROS (Rial & Zardoya, 2009), a fraction of ROS escapes the cellular defenses causing permanent or transient damage to proteins, lipids, and nucleic acids (Adelman *et al.*, 1988; Richter *et al.*, 1988; Cabiscol *et al.*, 2000; Barja, 2002). In this sense, there is a wide agreement that oxidative damage is one of the major factors responsible for age-related diseases (Maccarrone & Ullrich, 2004; Sorolla *et al.*, 2008; Terni *et al.*, 2009) and may contribute to the aging process (Barja, 2002; Pamplona & Barja, 2007). Indeed, a negative correlation between ROS generation and species longevity has been observed in mammals and birds (Barja, 2002; Lambert *et al.*, 2007).

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All amino acid residues are potential targets of oxidative damage. The sulfur-containing residues cysteine and methionine, however, are particularly sensitive to oxidation (Berlett & Stadtman, 1997). Interestingly, Moosmann & Behl (2008) recently reported a negative correlation between mitochondrially encoded cysteine and longevity. These authors propose that it is the detrimental capacity of cysteine thiyl radicals and their potential to initiate irreversible protein cross-linking that caused a selection against cysteine in mitochondrial DNA-encoded proteins in long-lived species. Therefore, oxidation of susceptible residues like cysteine and methionine may lead to dysfunctional proteins, which might contribute to organismal aging.

Recently, the reassignment of the AUA codon from isoleucine to methionine during mitochondrial evolution has been reinterpreted as an adaptive anti-oxidant event (Bender *et al.*, 2008). Protein-based methionine is readily oxidized by ROS to form methionine sulfoxide (MetO), which can be reduced back to methionine by methionine sulfoxide reductases (Msrs) at the low metabolic cost of one molecule of NADPH (Stadtman *et al.*, 2002). In so doing, it has been noted that one equivalent of ROS is destroyed for every methionine residue repaired (Levine *et al.*, 1996). Thus, because of the Msr system, methionine residues in proteins have been suggested to act as catalytic anti-oxidants by removing ROS (Luo & Levine, 2009). In this context, an enrichment of methionine in respiratory chain complexes may have provided anti-oxidant protection, imposing in this way a selection pressure (Bender *et al.*, 2008).

Taking into account all these precedents, and considering the roles of ROS in aging and age-related diseases, including the relationship between ROS generation and species lifespan, methionine content in mitochondrial proteins may then be associated with species longevity. To test this hypothesis, we used an evolutionary comparative approach and analyzed an extensive set of mitochondrial genomes from mammals. We found that mitochondrially encoded proteins from short-lived species are enriched in methionine when compared with their long-lived counterparts. Because mitochondrial proteins (and membrane proteins in particular) are enriched in methionine, thus suggesting a protective role for methionine, we favor the interpretation in which a more efficient accumulation of methionine occurred in the mitochondrial proteins of short-lived mammals subjected to high oxidative stress.

Results

Methionine usage in mtDNA-encoded proteins is related to longevity

As pointed out in the Introduction, if protein-based methionine fulfills an anti-oxidant role in the mitochondrion, then there may be an association between species maximum lifespan potential (MLSP) and methionine usage in mitochondrial proteins. The current work addresses the association of these two variables using a data set formed by 168 species of mammals, encompassing 24 different orders (see Supporting information). As observed in Fig. 1a, methionine usage and MLSP exhibited a marginally significant negative correlation ($n = 168$, $r = -0.122$, $P = 0.058$). However, species are part of a hierarchically structured phylogeny, raising the possibility that data from different species may not necessarily be statistically independent from one another (see Fig. S2 from Supporting information). Therefore, to correct for nonindependence of the individual-species data, phylogenetically independent contrasts were calculated according to Felsenstein's method (Felsenstein, 1985). Interestingly, the association between methionine content and longevity became more significant ($n = 167$, $r = -0.162$, $P = 0.019$) after phylogenetic correction (Fig. 1b).

The association of methionine enrichment with decreasing lifespan also remained significant after mathematical correction for body mass, a potentially confounding factor known to correlate with lifespan (Speakman, 2005; de Magalhães *et al.*, 2007). To reach this conclusion, we calculated the partial correlation coefficient between methionine abundance and longevity controlling for body mass either with ($n = 167$, $r = -0.165$, $P = 0.017$) or without ($n = 168$, $r = -0.263$, $P = 0.0003$) phylogenetic correction (Fig. 1d,c, respectively). The independence of the methionine-lifespan correlation from the variable body mass indicates that even in species with identical size, the shorter-lived animals exhibit an enrichment of methionine. In fact, the correlation using mass-corrected variables became much stronger (Fig. 1c).

Considering that ROS production is associated with metabolism, we investigated the relation between metabolic rate and methionine usage. Using a subset of 64 species for which we obtained basal metabolic rate (BMR) data, and correcting BMR for the effects of body mass using partial correlation analysis (de Magalhães *et al.*, 2007), we observed a borderline significant correlation between BMR residuals and methionine usage ($r = 0.192$, $P = 0.064$). This positive correlation suggests that methionine usage increases in species with high BMR for their body size.

Life history traits within the mammalian class are highly variable (Nabholz *et al.*, 2008). Thus, we next studied the relationship between MLSP and methionine content within those eutherian orders with 10 or more species present in our compilation. All the analyzed orders, Carnivora ($n = 41$), Primates ($n = 23$), Artiodactyla ($n = 20$), Cetacea ($n = 18$), and Rodentia ($n = 10$), showed a negative covariance between MLSP and methionine content. However, after correcting for the phylogenetic inertia, only Carnivora ($P = 0.004$), Primates ($P = 0.049$), and Rodentia ($P = 0.016$) exhibited statistically significant negative correlations between the variables of interest. Furthermore, within these orders, the link between high longevity and low methionine content remained significant after controlling for body mass (see Fig. S4 in Supporting information).

The AUA methionine codon, but not the AUG codon, correlates negatively with longevity

As mentioned in the Introduction, Bender and coworkers have suggested that the recoding of AUA from isoleucine to

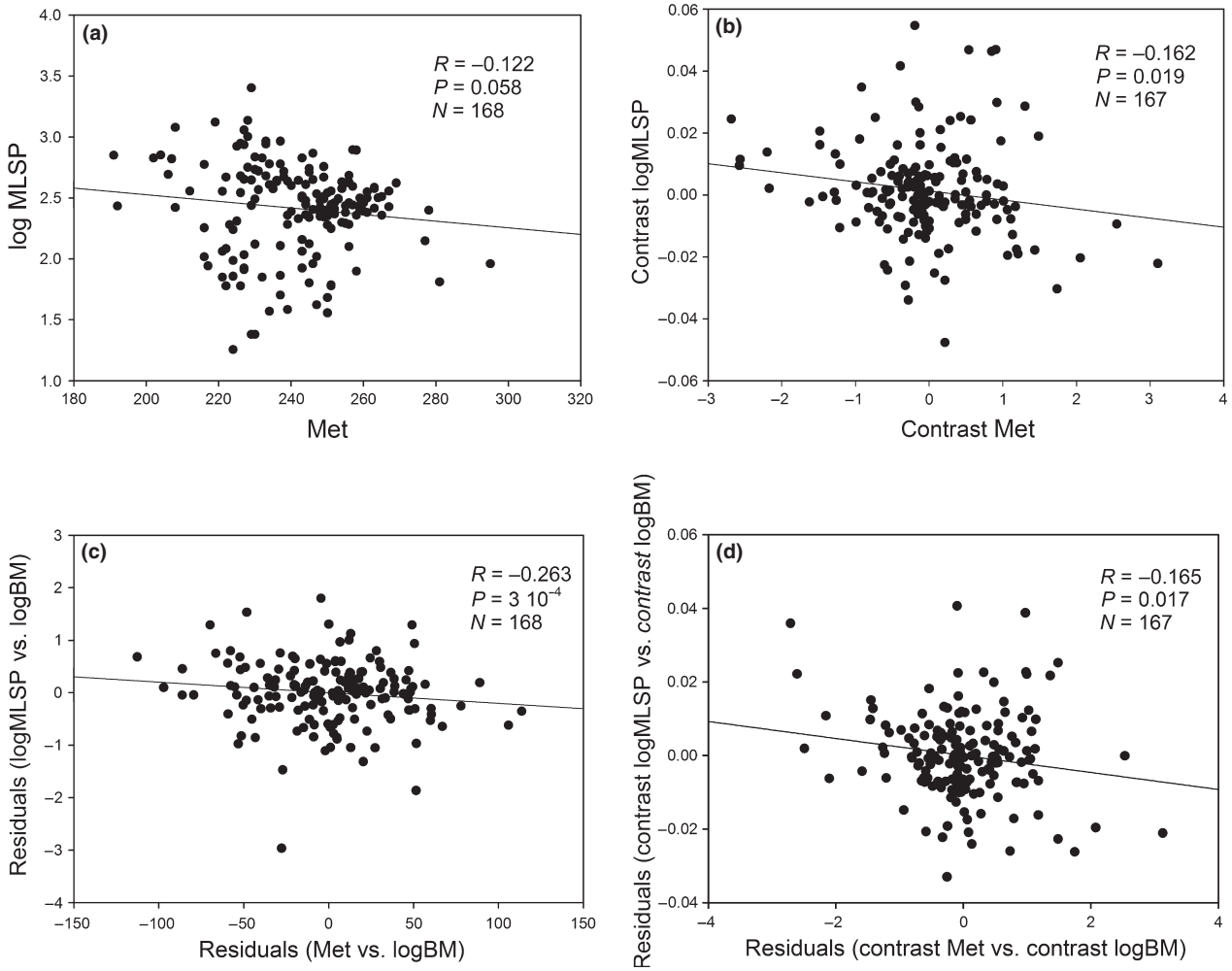

Fig. 1 Methionine usage in mitochondrially encoded proteins is negatively related to longevity. (a) Correlation of methionine abundance and logMLSP (n = 168, r = −0.122, P = 0.058). (b) Correlation of phylogenetically independent contrast values of methionine content and logMLSP (n = 167, r = −0.162, P = 0.019). (c) Partial correlation of methionine and longevity after correction for body mass (n = 168, r = −0.263, $P = 3 \times 10^{-4}$). (d) Partial correlation of methionine and longevity contrasts after correction for body mass contrasts (n = 167, r = −0.165, P = 0.017).

methionine may fulfill an adaptive anti-oxidant function. Therefore, we next investigated whether the two methionine codons, AUA and AUG, contributed equally to the above-reported link between methionine and longevity. The results are summarized in Table 1. Both types of codons are significantly correlated with longevity but, surprisingly, with opposite signs. At first glance, the positive relationship between AUG codons and MLSP may seem opposed to the idea that less longevity means more methionyl residues in proteins. However, while the number of AUA codons was strong and positively correlated with the number of methionine residues, AUG showed a slightly negative correlation with methionine usage in mtDNA-encoded proteins (see Fig. S5 from Supporting information).

Table 1 Only AUA abundance is negatively related to longevity

	Correlation between logMLSP and	
	AUA	AUG
Without correction	r = −0.214, P = 0.003, n = 168	r = +0.198, P = 0.006, n = 168
With correction	r = −0.195, P = 0.006, n = 167	r = +0.117, P = 0.066, n = 167

The number of AUA- and AUG-coded methionines in the 13 protein-coding mtDNA genes were computed and used either for direct correlation analysis (without corrections), or to calculate their corresponding contrasts previously to the correlation analysis (with correction). In this latter case, the contrasts of logMLSP were also computed using the phylogenetic tree showed in Fig. S1 (Supporting information).

The number of methionine-adding events is negatively correlated with longevity

As shown in the preceding sections, mtDNA-encoded proteins from short-lived mammals are enriched in methionyl residues when compared with those from long-lived animals. This difference could be brought about either by adding methionine in short-lived mammals or by losing methionine in long-lived ones. To investigate this issue, we developed a statistic method aimed

to discern between these two opposite alternatives. Briefly, for each species, we computed the number of methionine-adding and methionine-removing events, as detailed in Experimental procedures. The correlation between longevity and the number of adding or removing events was then assessed, and our results of such analyses are summarized in Fig. 2. Using uncorrected data, both the number of removing and adding events correlated negatively with MLSP. This is congruent with the observation that oxidative phosphorylation (OXPHOS) proteins from short-lived mammals exhibit evolutionary rates that are significantly higher than those from their long-lived counterparts (Galtier *et al.*, 2009), and thus this bias from molecular evolution rates must be addressed. Therefore, for each analysis, we corrected the number of removing and adding events by performing a partial correlation analysis using, respectively, the number of adding and removing events (see Experimental procedures). After this correction, we found a highly significant and negative correlation between longevity and the number of adding events ($r = -0.458$, $P = 2 \times 10^{-10}$, $n = 168$), which remained statistically significant after also controlling for phylogenetic inertia ($r = -0.167$, $P = 0.016$, $n = 167$). In contrast, when similar analyses were carried out to explore the relationship between the number of methionine-removing events and longevity, we observe a positive correlation that was not significant ($P = 0.190$) when phylogenetic relationship was accounted for (Fig. 2). Overall, these results suggest that methionine addition to proteins in short-lived species, rather than methionine loss from proteins in long-lived species, is responsible for the observed negative correlation between methionine usage and longevity.

Methionine usage in mitochondrial nDNA-encoded proteins

Because the majority of mitochondrial proteins are encoded by nuclear genes (Pagliarini *et al.*, 2008), we investigated whether the negative correlation between longevity and methionine usage in mtDNA-encoded proteins could be extended to those mitochondrial proteins that are encoded in the nucleus. To this end, we assembled four sets of sequences belonging to 10 mammalian species (see Experimental procedures). The first group was formed by the sequences of the 13 essential polypeptides that are encoded by mtDNA. A second assemblage encompassed the sequences of 243 polypeptides that are encoded by nuclear genes and are imported into the mitochondrion. Among the nDNA-encoded mitochondrial proteins, those localized in the IMM (52 polypeptides) formed the third subset. Finally, a comprehensive collection of 7326 highly conserved nonmitochondrial proteins were selected as the fourth (control) group.

We determined, separately in each of our protein groups, the methionine content and its relation with longevity (Table 2). Although methionine content and MLSP were only related within the group of inner mitochondrial proteins, nDNA-encoded mitochondrial proteins showed higher methionine usage than their nonmitochondrial counterparts. This higher methionine usage for mitochondrial proteins was statistically significant for both membrane and nonmembrane proteins. Besides, mitochondrial membrane proteins had a much higher methionine frequency than nonmembrane proteins, and this increase was much higher than in nonmitochondrial proteins (Fig. 3).

Re-evaluating the relationship between cysteine usage and longevity within the Mammalian class

Moosmann and Behl have reported that cysteine abundance is negatively correlated with lifespan in a wide range of animals covering mammals, birds, reptiles, amphibians, fishes, insects, crustaceans, and arachnids (Bender *et al.*, 2008). To put the methionine signature in perspective, we focused the cysteine analysis on the class Mammalia (Fig. 4). Although we found a highly significant negative relationship between longevity and cysteine usage ($n = 168$, $r = -0.476$, P-value $= 3 \times 10^{-11}$), this correlation vanished when data were corrected for the phylogenetic inertia using independent contrasts ($n = 167$, $r = 0.022$, P-value $= 0.389$). This result suggests a strong phylogenetic bias, which could be anticipated from the fact that the strong correlation between cysteine and longevity, observed using cross-species data, was not preserved when intra-order analyses were carried out (see Figs S6 and S7 in Supporting information).

On the other hand, Kitazoe and coworkers have noted a positive correlation between threonine abundance and longevity within the order Primates (Kitazoe *et al.*, 2008). Herein, we confirm and extend this observation using our sample of 168 mammalian species. After controlling for phylogenetic relatedness, a significant positive relationship remained between threonine usage and longevity ($n = 167$, $r = 0.181$, P-value $= 0.010$).

Of noteworthy, cysteine was the less abundant amino acid, exhibiting a small variation between species (the absolute difference in cysteine between the species with the higher number of cysteinyl residues and that with the lower number of cysteine was only 18). The variation of cysteine dramatically increased when invertebrate phyla were added to the analysis (results not shown). In contrast, threonine showed the highest absolute difference, followed by methionine (see Table S3 in Supporting information).

Discussion

Although ROS are generated in multiple compartments, the vast majority of cellular ROS production can be traced back to the mitochondrion. Not surprisingly, this organelle houses a variety of ROS scavenging systems (Balaban *et al.*, 2005). In this line, Bender and coworkers, based on previous works of Levine *et al.* (1996) and Stadtman *et al.* (2002), have put forward the idea that the higher methionine usage observed in mtDNA-encoded proteins may have an adaptive anti-oxidant function (Bender *et al.*, 2008). Herein, we tested this hypothesis by studying the relationship between methionine usage in mitochondrially

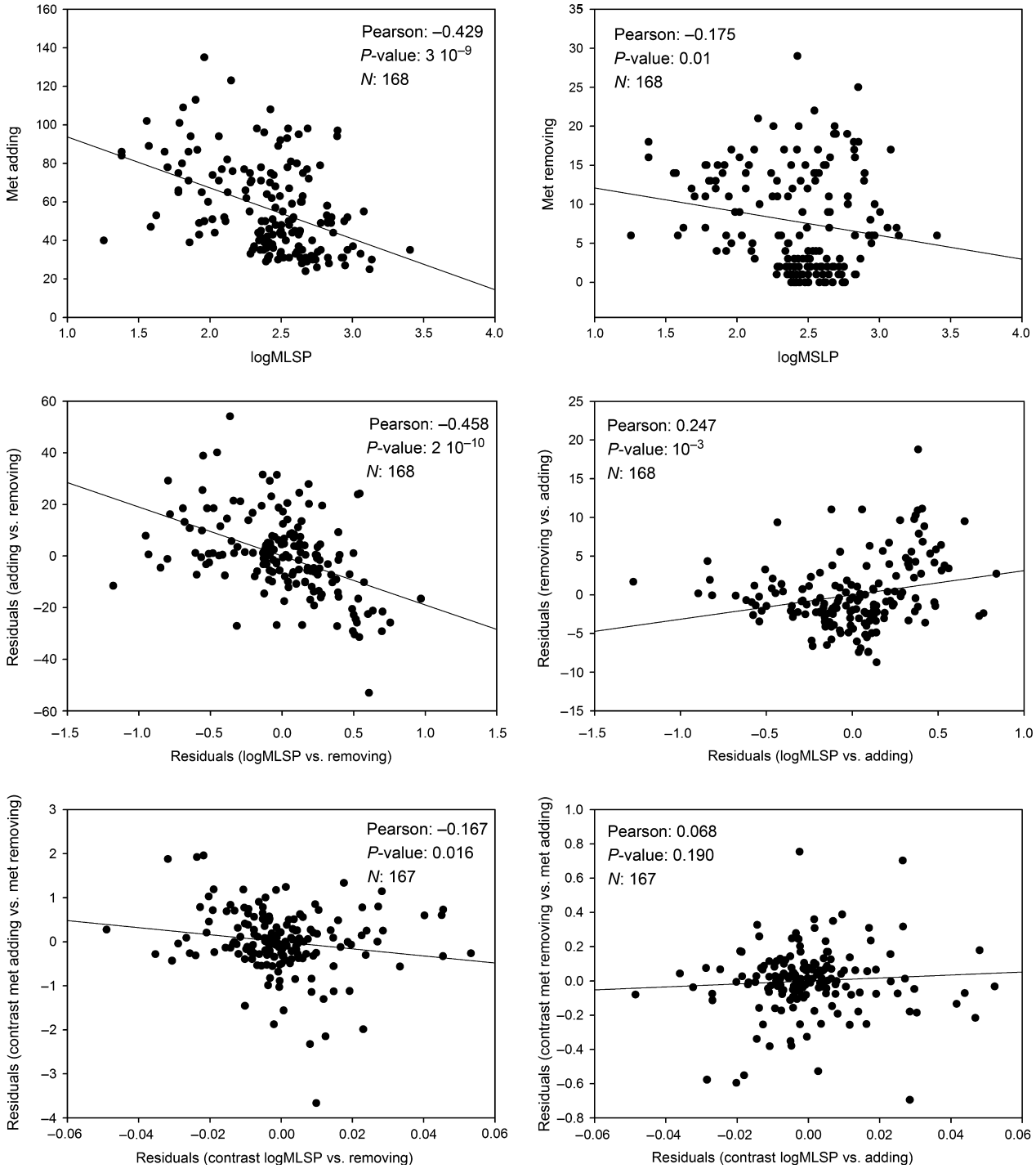

Fig. 2 Relationship between longevity and the number of methionine-adding or methionine-removing events computed for each species as described in the Experimental procedures. Short-lived species exhibited higher number of changes (both adding and removing), as indicated by the negative correlation with longevity (top plots). After correction for methionine-removing events, the partial correlation of methionine-adding events and logMLSP remained significant (middle left plot). However, short-lived mammals showed a lower number of removing events after correction for methionine-adding events as shown in the partial correlation of methionine-removing events and logMLSP (middle right plot). When phylogenetic inertia was also considered, adding methionine to mitochondrial proteins seemed to play a more relevant role than removing methionine from those proteins (bottom plots).

encoded proteins and MLSP. Despite a considerable scatter, which is not unexpected given that MLSP is a complex trait influenced by many variables (Samuels, 2004, 2005; Moosmann & Behl, 2008), we found a statistically robust negative correlation between methionine usage and MLSP (Fig. 1), which as far as we know is a novel finding.

Table 2 Correlation between longevity and methionine usage in different sets of proteins

	Without phylogenetic correction		With phylogenetic correction	
	Pearson's coefficient	*P*-value	Pearson's coefficient	*P*-value
Mitochondrial mtDNA encoded	−0.685	0.027**	−0.498	0.086*
IMM nDNA encoded	−0.661	0.019**	−0.561	0.058*
Mitochondrial nDNA encoded	−0.042	0.454	−0.299	0.217
Nonmitochondrial	0.176	0.314	0.024	0.476

For the 10 mammalian species indicated in Experimental procedures, the Pearson's correlation coefficients between methionine abundances and logMLSP were calculated before and after accounting for the phylogenetic inertia.

*Marginally significant ($P < 0.09$) and **significant at $P < 0.05$. The number of proteins analyzed within each category was as follows: 13 mtDNA-encoded proteins, 52 inner mitochondrial membrane (IMM) nDNA-encoded proteins, 243 mitochondrial nDNA-encoded proteins, and 7326 nonmitochondrial proteins.

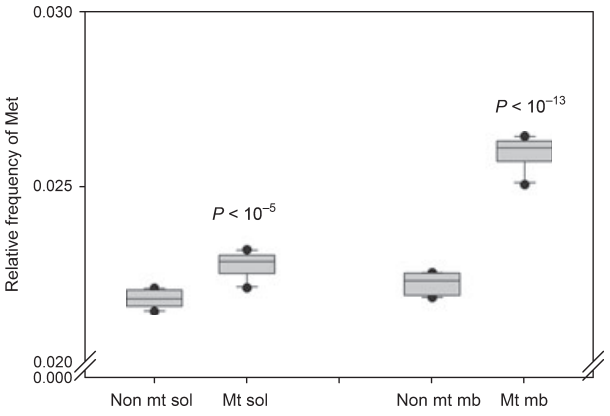

Fig. 3 Comparison of methionine usage distribution in different sets of nuclear encoded proteins: nDNA-encoded mitochondrial membrane proteins (Mt mb; $n = 135$), nDNA-encoded mitochondrial soluble proteins (Mt sol; $n = 108$), nonmitochondrial proteins membrane proteins (non mt mb; $n = 2744$), and nonmitochondrial soluble proteins (non mt sol; $n = 4582$). In the boxplots, the lines within the boxes represent medians and rectangles are the two central quartiles. In pairwise comparison, methionine usage in nDNA-encoded mithochondrial soluble proteins was significantly higher than for nonmitochondrial soluble proteins ($P < 10^{-5}$, *t*-test). When the comparison was focused on membrane proteins, again nDNA-encoded mitochondrial proteins exhibited a much higher methionine usage than their nonmitochondrial counterpart ($P < 10^{-13}$, *t*-test).

Further analyses within orders revealed that, with the exception of Cetartiodactyla, all the analyzed orders exhibited a negative relationship between methionine usage and longevity. Why Cetartiodactyla does not seem to follow the rule is an intriguing question for which we have no definitive explanation. Nevertheless, one is tempted to speculate that the causes may lie in specific metabolic adaptations to diet. Although the biochemical mechanisms remain largely unknown, it is well established that protein-rich diets translate into increased ROS production and

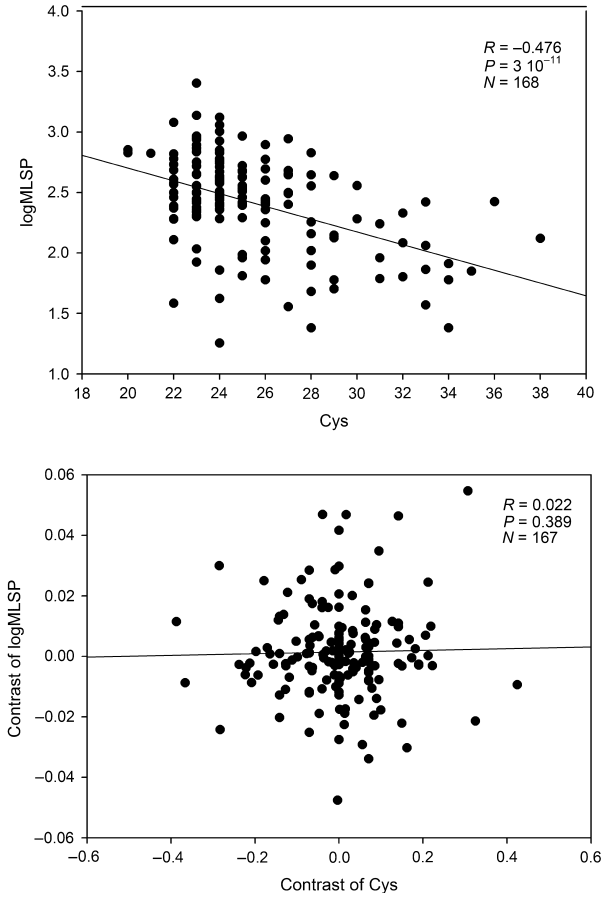

Fig. 4 Relationship between cysteine usage and longevity. The bivariate correlation between the number of cysteinyl residues and logMLSP was statistically significant (top panel), though the relationship between these variables vanished once the phylogenetic effect was accounted for (bottom panel).

ROS-related cellular damage (López-Torres & Barja, 2008). Thus, carnivorous, and to a less extent primates and rodents, may exhibit higher vulnerability than Artiodactyla, which are mainly herbivorous. With respect to Cetacea, this order includes some faunivory members. However, cetaceous, in addition to exhibit the lowest rates of mitochondrial protein evolution among mammals (Nabholz *et al.*, 2008), they possess specialized mitochondria adaptations because of their unusual oxygen requirements imposed by their diving style of life (da Fonseca *et al.*, 2008).

At any given moment, a mammalian mitochondrion houses between 1100 and 1500 different proteins (Meisinger *et al.*, 2008), including around 70 nuclear-encoded peptides forming part of the OXPHOS complexes, and many carriers working in the IMM. Although only marginally significant, longevity and methionine usage were negatively correlated within the set of nDNA-encoded inner mitochondrial proteins (Table 2). Methionine abundance in the general group of nDNA-encoded mitochondrial proteins, however, did not significantly correlate with longevity, suggesting that the role of scavenging protein may be circumscribed to a small subset of mitochondrial proteins

localized in the IMM where ROS are generated. In this respect, mtDNA-encoded proteins are particularly suited for this role given their higher methionine usage and faster evolution than proteins encoded by nuclear genes (Lane, 2005).

At first glance, our results could be interpreted as evidence suggesting that methionine residues are selected against in the mitochondrially encoded proteins of long-lived species. This is in line with the interpretation given to results from crude heart homogenates from eight mammalian species, in which a negative correlation between methionine content and longevity was observed (Ruiz *et al.*, 2005). These authors argued that the sensitivity of proteins to oxidative stress may increase as a function of the number of methionine residues, and consequently, a lower abundance of this amino acid in proteins from long-lived animals likely contributes to the superior longevity of these species.

Although interpreting our results as evidence of a pro-oxidant role for methionine is tempting, a growing body of evidence suggests an anti-oxidant role for methionine in proteins (Luo & Levine, 2009). Furthermore, having in consideration that mitochondria account for the bulk of ROS production and also are the main target for oxidative attacks (Adelman *et al.*, 1988; Richter *et al.*, 1988), an anti-oxidant role for protein methionine fits well with our observation that mitochondrial proteins exhibit much higher methionine usages than nonmitochondrial proteins (Fig. 3). Under this scenario, a negative correlation between methionine usage and longevity cannot be interpreted in terms of direct causation. Instead, we hypothesize that if methionine enrichment of mitochondrial proteins (and of mitochondrial membrane proteins, in particular) represents an adaptive response to oxidative stress, then those animals that exhibit higher rates of ROS generation (short-lived animals) might have been subjected to higher selective pressures to increase the methionine content of their mitochondrial proteins. In other words, if mitochondrial methionine residues serve as a ROS sink, then the proteins from animals subjected to high oxidative stress should accumulate methionine more effectively than their orthologous proteins from species exposed to lower oxidative stress.

To further explore whether methionine acts as a pro- or anti-oxidant, we developed a statistic analysis aimed to dissect the differential influence of adding and removing methionine on the reported differences in methionine content. Interestingly, we found that the number of adding events was negatively correlated with longevity while, after correcting for potential confounding factors, the number of removing events did not correlate with longevity (Fig. 2). Therefore, methionine addition, rather than methionine loss, is probably the mechanism determining the current differences in methionine usage among mammals with different longevities. Because the literature suggests that long-lived mammals exhibit a less effective purifying selection (Popadin *et al.*, 2007), it is unlikely that long-lived mammals avoid methionine addition events through a more effective purifying selection. Therefore, rather than long-lived mammals losing methionine, we think short-lived mammals gain methionine.

Bender and coworkers have put forward the idea that the recoding of AUA from isoleucine to methionine might have been selectively promoted by an anti-oxidant role of methionine residues. Bender *et al.* (2008) have also suggested that AUA encodes primarily protein surface methionine residues, which would be the ones expected to be redox-active and protective (Levine *et al.*, 1996). In line with this hypothesis, we have found that AUA, but not AUG, correlates positively with the number or methionine residues and negatively with longevity (Table 1 and Supporting information). These results further support our interpretation of an anti-oxidant role of methionine.

Evidence suggests that the rate of oxidative damage is inversely related to the lifespan of the organisms, and short-lived species produce higher levels of ROS (Adelman *et al.*, 1988; Richter *et al.*, 1988; Barja, 2002). Therefore, short-lived animals might have been forced to develop defenses that enable them to cope with this oxidative burden. This is reminiscent of the positive correlation between body size and the degree of mitochondrial coupling, once thought to be the cause of the higher oxidative stress in small mammals, but which has now been reinterpreted as an adaptive response known as the 'uncoupling to survive' hypothesis (Brand, 2000; Speakman *et al.*, 2004). Similarly, the analyses presented in this work support the view that short-lived mammals may have accumulated methionine in their mitochondrial proteins as an anti-oxidative strategy. In other words, in species with higher ROS generation rates, the selective pressure for methionine enrichment is stronger. Short-lived animals being subjected to a higher oxidative stress are under a higher selective pressure favoring the addition and accumulation of methionine into their proteins. Another line of evidence supporting our view comes from the observation that BMR residuals appear to be positively correlated with methionine usage: it seems that methionine usage increases in both species with high BMR (and presumably high ROS generation) and species with short lifespans (and again presumably high ROS generation).

Taken together, our hypothesis is that methionine enrichment is an adaptive process developed by short-lived species because of their high ROS mitochondrial production and metabolism. Thus, the methionine-mediated protection against ROS does not seem to be so critical in long-lived species, suggesting that such an adaptive anti-oxidant strategy is not causally involved in the evolution of mammalian lifespan. In this way, methionine enrichment is likely a secondary phenomenon to the evolution of lifespans, which is supported by the stronger relationship observed after phylongetic correction. Therefore, our results showing a protective role of methionine in short-lived species provide indirect but strong evidence for a role of ROS in aging.

Moosmann & Behl (2008) have previously reported a negative relationship between cysteine usage and longevity, suggesting that during the evolution of longevity, there may be adaptive pressures against mitochondrial residues that are easily oxidized. Together with our results, even though both cysteine and methionine are easily oxidized, it may be that cysteine is a pro-oxidant while methionine is an anti-oxidant. Nonetheless, when using our data set, cysteine fails to show a link with longevity within

the Mammalia class after correction for the effects of phylogeny (Fig. 4). Although we do not know the reason for this apparent discrepancy, it may lie in the fact that while Moosman and Behl extended their analysis on a wide range of taxa, we focused the analysis on mammalian species. To this respect, we noted that mammalian proteins exhibited an extremely low variability in cysteine usage, which may prevent our analysis to become statistically significant.

Conclusion

In this work, we show that mitochondrially encoded methionine usage inversely correlates with the maximum lifespan of mammalian species. This pattern of methionine usage seems to be the result of a gain of methionine in proteins from short-lived species, rather than a loss of this amino acid in proteins from long-lived animals. Methionine content is also higher in mitochondrial proteins and in particular in proteins from the IMM. These results support the view that methionine residues in proteins defend against oxidative stress and lead us to suggest that short-lived species, with higher metabolism and higher levels of oxidative stress, accumulate methionine in their mitochondrial proteins more effectively than their long-lived counterparts, to protect against oxidative damage. This adaptive increase of methionine in short-lived species indirectly supports a role of ROS in aging.

Experimental procedures

Sequence sources and calculations

Sequences for mitochondrial genomes and proteome analysis were obtained from the National Center for Biotechnology Information (NCBI) genome database (http://www.ncbi.nlm.nih.gov). Nuclear-encoded proteins were obtained from Ensembl (http://www.ensembl.org). The list of mitochondrial nDNA-encoded proteins was obtained from Gene Ontology annotations (Ashburner *et al.*, 2000). Proteomic amino acid contents were quantified by using customized Haskell code.

Inclusion criteria for the analyzed species

To investigate the relationship between methionine usage in mitocondrially encoded proteins and longevity, a collection of 168 species were assembled based on neutral selection criteria, completely unrelated to longevity or amino acid frequencies. Annotated complete mitochondrial sequences had to be available from NCBI genome database, and longevity information had to be given in a reliable source. The maximum longevity lifespan potentials were obtained from different sources: the AnAge database at http://genomics.senescence.info/species/, the Max Planck Institute for Demographic Research website (http://www.demogr.mpg.de/longevityrecords/) or from the compilation published by Welch *et al.* (2008). In those cases in which more than one numeric value for a given species was found, the AnAge record was preferred because data have been more recently updated (de Magalhães & Costa, 2009). Body mass and BMR data were also obtained from AnAge.

For those analyses involving nuclear encoded proteins, a data set encompassing 10 mammalian species with significantly different lifespans and whose genome has been sequenced at a high coverage was collected. This sample included four primates (*Pan troglodytes, Homo sapiens, Pongo pygmaeus*, and *Macaca mulatta*), three rodents (*Mus musculus*, *Rattus norvegicus*, and *Cavia porcellus*), along with *Canis familiaris, Bos Taurus*, and *Equus caballus*. We constructed an orthologs mapping of human proteins from InParanoid, a comprehensive database of eukaryotic orthologs (O'Brien *et al.*, 2005). A list of mitochondrial proteins was obtained from Gene Ontology annotations (Ashburner *et al.*, 2000) and compared against our mapping to weed out proteins that did not have any orthologs. This resulted in a list of 243 mitochondrial proteins and a subset of the list consisting of 52 mitochondrial proteins from the inner membrane. To select a group of conserved nonmitochondrial proteins and thus minimize errors, a stringent criterion was employed. Only those proteins sharing at least 50% identity (gaps were considered as mismatches) with the human sequence were considered as valid orthologs. In this way, 7326 proteins were included in this baseline group.

Methionine-adding and methionine-removing analyses

For each species, the 13 mtDNA-encoded protein sequences were concatenated and subjected to alignment using ClustalX 2.0.9 (free software available from the European Bioinformatics Institute; ftp://ftp.ebi.ac.uk/pub/software/clustalw2/). This procedure resulted in a 168 species × 3839 positions matrix. If in at least one species, but not in all the species, a methionine is found at a given position, this position is referred as relevant position. Then, we used customized Perl code to characterize each relevant position as a Removing Position or an Adding Position. To carry out this assignation, we reasoned in the following way: when for a given relevant position, most of the species exhibited a methionine, we assigned that position as a Removing Position, because it is more probable that a few species lose methionine than many species gain methionine at that position, and the reverse being true. Finally, for each of the 168 species, the numbers of adding and removing positions were computed as adding and removing events, respectively. Afterward, we addressed the relationship between these events and longevity, before and after correcting for phylogenetic inertia using Felsenstein's contrasts (Felsenstein, 1985). Because OXPHOS proteins from short-lived mammals exhibit faster molecular evolution rates (Galtier *et al.*, 2009), we corrected the number of removing and adding events by performing a partial correlation analysis using, respectively, the number of adding and removing events. In this way, the number of adding and removing events reflects a high ratio of, respectively, adding/removing events and removing/adding events.

Phylogenetic analyses

Phylogenetic reconstructions for both DNA and amino acid sequences were performed. The complete sequences of the two mitochondrial ribosomal RNA genes were concatenated for each species. Alignments were performed using ClustalX 2.0.9. Resampling with replacement (bootstrapping) was performed using the Seqboot program in the PHYLIP (http://evolution.genetics.washington.edu/phylip.html) package. The phylogeny was reconstructed using both maximum likelihood and parsimony methods. The results from the random datasets were summarized by constructing a majority rule consensus tree employing the Consense program from the above-mentioned package. The phylogenetic analyses were also carried out on the concatenated sequences of the 13 polypeptides encoded by the mitochondrial genome (See Supporting information for details).

Felsenstein's phylogenetically independent contrasts (Felsenstein, 1985) were calculated using the Contrast program from the PHYLIP package. Appropriate standardization was confirmed by means of a diagnostic test previously described (Abouheif, 1999).

Statistical analyses

To check whether our variables were phylogenetically autocorrelated, we used a test for serial independence originally proposed by von Neumann and coworkers (von Neumann, 1941; von Neumann *et al.*, 1941). For each of the 168 mammalian species, the values of each variable were ranked according to the structure of the tree. In other words, species were ordered from top to bottom according to how they are listed in Fig. S1. Afterward, the so-called *C*-statistic was computed as one minus the ratio between the sum of the successive squared differences and the sum of squares ($C = 1 - \frac{\sum_{i=1}^{n-1}(x_{i+1}-x_i)^2}{\sum_{i=1}^{n}(x_i-\bar{x})^2}$). When the observations are independent from each other, the *C*-statistic is normally distributed with mean 0. In contrast, if there is positive phylogenetic autocorrelation, then the observed *C*-statistic will be significantly different from zero, and the null hypothesis will be rejected. More details on the calculations can be found somewhere else (Abouheif, 1999).

Linear regression, Pearson correlation, partial correlations, and residuals analyses were carried out with SPSS. Differences between methionine usages in different proteomic subsets were evaluated by using a *t*-test.

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Author contributions

YL and JAPC compiled data; MRC carried out analyses; JPM designed the study and wrote the manuscript; JCA designed the study, performed analyses, and wrote the manuscript.

References

Abouheif E (1999) A method for testing the assumption of phylogenetic independence in comparative data. *Evol. Ecol. Res.* **1**, 895–909.

Adelman R, Saul R, Ames B (1988) Oxidative damage to DNA: relation to species metabolic rate and life span. *Proc. Natl. Acad. Sci. USA* **85**, 2706–2708.

Aledo JC (2008) An early and anaerobic scenario for the transition to undifferentiated multicellularity. *J. Mol. Evol.* **67**, 145–153.

Ashburner M, Ball C, Blake J, Botstein D, Butler H, Cherry J, Davis A, Dolinski K, Dwight S, Eppig J, Harris M, Hill D, Issel-Tarver L, Kasarskis A, Lewis S, Matese J, Richardson J, Ringwald M, Rubin G, Sherlock G (2000) Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat. Genet.* **25**, 25–29.

Balaban R, Nemoto S, Finkel T (2005) Mitochondria, oxidants, and aging. *Cell* **120**, 483–495.

Barja G (2002) Rate of generation of oxidative stress-related damage and animal longevity. *Free Radic. Biol. Med.* **33**, 1167–1172.

Bender A, Hajieva P, Moosmann B (2008) Adaptive antioxidant methionine accumulation in respiratory chain complexes explains the use of a deviant genetic code in mitochondria. *Proc. Natl. Acad. Sci. USA* **105**, 16496–16501.

Berlett B, Stadtman E (1997) Protein oxidation in aging, disease, and oxidative stress. *J. Biol. Chem.* **272**, 20313–20316.

Brand M (2000) Uncoupling to survive? The role of mitochondrial inefficiency in ageing. *Exp. Gerontol.* **35**, 811–820.

Cabiscol E, Piulats E, Echave P, Herrero E, Ros J (2000) Oxidative stress promotes specific protein damage in Saccharomyces cerevisiae. *J. Biol. Chem.* **275**, 27393–27398.

Catling D, Glein C, Zahnle K, McKay C (2005) Why O2 is required by complex life on habitable planets and the concept of planetary ''oxygenation time''. *Astrobiology* **5**, 415–438.

Falkowski P, Katz M, Milligan A, Fennel K, Cramer B, Aubry M, Berner R, Novacek M, Zapol W (2005) The rise of oxygen over the past 205 million years and the evolution of large placental mammals. *Science* **309**, 2202–2204.

Felsenstein J (1985) Phylogenies and the comparative method. *Am. Nat.* **125**, 1–15.

da Fonseca R, Johnson W, O'Brien S, Ramos M, Antunes A (2008) The adaptive evolution of the mammalian mitochondrial genome. *BMC Genomics* **9**, 119.

Galtier N, Blier P, Nabholz B (2009) Inverse relationship between longevity and evolutionary rate of mitochondrial proteins in mammals and birds. *Mitochondrion* **9**, 51–57.

Kitazoe Y, Kishino H, Hasegawa M, Nakajima N, Thorne J, Tanaka M (2008) Adaptive threonine increase in transmembrane regions of mitochondrial proteins in higher primates. *PLoS ONE* **3**, e3343.

Lambert A, Boysen H, Buckingham J, Yang T, Podlutsky A, Austad S, Kunz T, Buffenstein R, Brand M (2007) Low rates of hydrogen peroxide production by isolated heart mitochondria associate with long maximum lifespan in vertebrate homeotherms. *Aging Cell* **6**, 607–618.

Lane N (2005) *Power, Sex, Suicide: Mitochondria and the Meaning of Life*, New York: Oxford University Press.

Levine R, Mosoni L, Berlett B, Stadtman E (1996) Methionine residues as endogenous antioxidants in proteins. *Proc. Natl. Acad. Sci. USA* **93**, 15036–15040.

López-Torres M, Barja G (2008) Lowered methionine ingestion as responsible for the decrease in rodent mitochondrial oxidative stress in protein and dietary restriction possible implications for humans. *Biochim. Biophys. Acta* **1780**, 1337–1347.

Luo S, Levine R (2009) Methionine in proteins defends against oxidative stress. *FASEB J.* **23**, 464–472.

Maccarrone M, Ullrich V (2004) Redox regulation in disease and ageing. *Cell Death Differ.* **11**, 949–951.

de Magalhães JP, Costa J (2009) A database of vertebrate longevity records and their relation to other life-history traits. *J. Evol. Biol.* **22**, 1770–1774.

de Magalhães JP, Costa J, Church G (2007) An analysis of the relationship between metabolism, developmental schedules, and longevity using phylogenetic independent contrasts. *J. Gerontol. A Biol. Sci. Med. Sci.* **62**, 149–160.

Meisinger C, Sickmann A, Pfanner N (2008) The mitochondrial proteome: from inventory to function. *Cell* **134**, 22–24.

Moosmann B, Behl C (2008) Mitochondrially encoded cysteine predicts animal lifespan. *Aging Cell* **7**, 32–46.

Nabholz B, Glémin S, Galtier N (2008) Strong variations of mitochondrial mutation rate across mammals – the longevity hypothesis. *Mol. Biol. Evol.* **25**, 120–130.

von Neumann J (1941) Distribution of the ratio of the mean square successive difference to the variance. *Ann. Math. Stat.* **12**, 367–395.

von Neumann J, Kent RH, Bellinson HR, Hart BI (1941) The mean square successive difference. *Ann. Math. Stat.* **12**, 153–162.

O'Brien K, Remm M, Sonnhammer E (2005) Inparanoid: a comprehensive database of eukaryotic orthologs. *Nucleic Acids Res.* **33**, D476–D480.

Pagliarini D, Calvo S, Chang B, Sheth S, Vafai S, Ong S, Walford G, Sugiana C, Boneh A, Chen W, Hill D, Vidal M, Evans J, Thorburn D, Carr S, Mootha V (2008) A mitochondrial protein compendium elucidates complex I disease biology. *Cell* **134**, 112–123.

Pamplona R, Barja G (2007) Highly resistant macromolecular components and low rate of generation of endogenous damage: two key traits of longevity. *Ageing Res. Rev.* **6**, 189–210.

Popadin K, Polishchuk L, Mamirova L, Knorre D, Gunbin K (2007) Accumulation of slightly deleterious mutations in mitochondrial protein-coding genes of large versus small mammals. *Proc. Natl. Acad. Sci. USA* **104**, 13390–13395.

Rial E, Zardoya R (2009) Oxidative stress, thermogenesis and evolution of uncoupling proteins. *J. Biol.* **8**, 58.

Richter C, Park J, Ames B (1988) Normal oxidative damage to mitochondrial and nuclear DNA is extensive. *Proc. Natl. Acad. Sci. USA* **85**, 6465–6467.

Ruiz M, Ayala V, Portero-Otín M, Requena J, Barja G, Pamplona R (2005) Protein methionine content and MDA-lysine adducts are inversely related to maximum life span in the heart of mammals. *Mech. Ageing Dev.* **126**, 1106–1114.

Samuels D (2004) Mitochondrial DNA repeats constrain the life span of mammals. *Trends Genet.* **20**, 226–229.

Samuels D (2005) Life span is related to the free energy of mitochondrial DNA. *Mech. Ageing Dev.* **126**, 1123–1129.

Sanz A, Caro P, Ayala V, Portero-Otin M, Pamplona R, Barja G (2006) Methionine restriction decreases mitochondrial oxygen radical generation and leak as well as oxidative damage to mitochondrial DNA and proteins. *FASEB J.* **20**, 1064–1073.

Sorolla M, Reverter-Branchat G, Tamarit J, Ferrer I, Ros J, Cabiscol E (2008) Proteomic and oxidative stress analysis in human brain samples of Huntington disease. *Free Radic. Biol. Med.* **45**, 667–678.

Speakman J (2005) Correlations between physiology and lifespan – two widely ignored problems with comparative studies. *Aging Cell* **4**, 167–175.

Speakman J, Talbot D, Selman C, Snart S, McLaren J, Redman P, Krol E, Jackson D, Johnson M, Brand M (2004) Uncoupled and surviving: individual mice with high metabolism have greater mitochondrial uncoupling and live longer. *Aging Cell* **3**, 87–95.

Stadtman E, Moskovitz J, Berlett B, Levine R (2002) Cyclic oxidation and reduction of protein methionine residues is an important antioxidant mechanism. *Mol. Cell. Biochem.* **234–235**, 3–9.

Terni B, Boada J, Portero-Otin M, Pamplona R, Ferrer I (2009) Mitochondrial ATP-synthase in the entorhinal cortex is a target of oxidative stress at stages I/II of Alzheimer's disease pathology. *Brain Pathol.* **20**, 222–233.

Welch J, Bininda-Emonds O, Bromham L (2008) Correlates of substitution rate variation in mammalian protein-coding sequences. *BMC Evol. Biol.* **8**, 53.

Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1 Mammalian mitogenomic phylogenetic tree.

Fig. S2 Test for serial (phylogenetic) independence.

Fig. S3 Relationship between the contrasts of Met and logMLSP computed using the different trees described in Table S1.

Fig. S4 Relationship between methionine content and longevity in Carnivora, Primates, and Rodentia orders.

Fig. S5 Relationships among AUG, AUA, and Met.

Fig. S6 Cysteine usage data show a strong phylogenetic inertia.

Fig. S7 Intraorder correlation analyses.

Fig. S8 Raw data regarding the relationship between Ile, Leu, Ser, Thr, Met, and longevity.

Table S1 Robustness of the correlation between methionine usage and longevity.

Table S2 Distribution of methionine and methionine codons through the 13 mitochondrial protein-coding genes and their correlations with longevity.

Table S3 Amino acid usages and their correlations with logMLSP.

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