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MAXIMUM LIFE SPAN IN VERTEBRATES: RELATIONSHIP WITH LIVER ANTIOXIDANT ENZYMES, GLUTATHIONE SYSTEM, ASCORBATE, URATE, SENSITIVITY TO PEROXIDATION, TRUE MALONDIALDEHYDE, IN VIVO H_2O_2, AND BASAL AND MAXIMUM AEROBIC CAPACITY

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SUMMARY

In order to help clarify whether free radicals are implicated or not in the evolution of maximum life span (MLSP) of animals, a comprehensive study was performed in the liver of various vertebrate species Strongly significant negative correlations against MLSP were found for hepatic catalase, Se-dependent and -independent glutathione peroxidases, and GSH, whereas superoxide dismutase, glutathione reductase, ascorbate, uric acid, GSSG/GSH, in vitro peroxidation (TBA-RS), and in vivo steady-state H_2O_2 concentration in the liver did not correlate with MLSP Superoxide dismutase, catalase, glutathione peroxidase, and GSH results were in agreement with those independently reported by other authors, whereas the rest of our data are reported for the first time Potential limitations arising from the use of animals of different vertebrate Classes were counterbalanced by the possibility to study animals with very different MLSPs and life energy potentials Furthermore, the results agreed with previous data obtained using only mammals Since liver GSSG/GSH, peroxidation, and specially H_2O_2 concentration were similar in species with widely different MLSPs, it is suggested that the decrease in enzymatic H_2O_2 detoxifying capacity of longevous species represents an evolutionary co-adaptation with a smaller in vivo rate of free radical generation We propose the possibility that maximum longevity was increased during vertebrate evolution by lowering the rate of free radical recycling in the tissues

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INTRODUCTION

The basic causes of aging continue to be mainly unknown Among current proposals, the free radical theory of aging [1,2] is currently raising general interest Previous works have shown that free radicals are relevant in relation to degenerative diseases [3,4] and that induction of endogenous cellular antioxidants increases the mean life span of invertebrate [5,6] and vertebrate [7,8] animals Nevertheless, very few studies have investigated the possible relationship between free radicals and the maximum life span (MLSP) of different animal species [9,10]

Metabolic rate is the only factor clearly related to date to species-specific MLSP As first described by Rubner [11,12] and afterwards known as Pearl's rate of living theory [13], the higher the specific metabolic rate of mammals (cal/g per h), the shorter their MLSP This means that in many mammals [11,12] the total amount of energy processed per gram of tissue during their whole life span is roughly the same (approx 240 kcal/g, roughly equivalent to 50 10_2/g) This amount has been subsequently called 'absolute metabolic scope' [14] or 'life energy potential' (LEP) [15] and it is calculated as the specific metabolic rate multiplied by the MLSP Nevertheless, this 'Rubner's rule' has many exceptions [15] like primates, men [11,12], cats and bats, and other mammals Similarly, even though Rubner's rule still holds true among birds, they also live much longer than mammals of similar size or metabolic rate [16] These observations show that other factors in addition to metabolic rate are important contributors to the determination of MLSP in various species or groups of species It has been previously suggested that these additional factors could be the levels of endogenous antioxidants in the tissues [15,17] Thus, a positive relationship has been found in some mammals between MLSP and the ratio liver superoxide dismutase (SOD)/oxygen consumption of the whole animal at rest (Vo_2) [17] Similar relationships have been obtained for other low molecular weight antioxidants (mainly in plasma) when divided by the metabolic rate of the animal It was then proposed that antioxidants are important determinants of maximum longevity [9,15,18,19] Nevertheless, a strongly negative relationship between MLSP and other single antioxidants like catalase (CAT) [9], glutathione peroxidase (GPx) [20,21], reduced glutathione (GSH) [10,15], and ascorbate [9,22] has been described in the mammalian liver

Since this important problem was not clarified, we simultaneously measured the antioxidant enzymes SOD, CAT, total and Se-dependent GPx, and glutathione reductase (GR), the endogenous antioxidants GSH, ascorbic and uric acid, the estimators of oxidative stress GSSG/GSH ratio, in vivo and in vitro peroxidation by the thiobarbituric acid (TBA) test, malondialdehyde (MDA) determination by high

performance liquid chromatography (HPLC), and the in vivo steady state concentration of H_2O_2 — the main $OH^{\bullet}$ generating species in living tissues — in the liver of various vertebrate species strongly differing in MLSP and LEP, using the same assay methods Basal and maximum aerobic capacities were also studied measuring Vo_2 of the animal at rest and the cytochrome oxidase (COX) activity of the liver tissue An emphasis in the comprehensive approach was used because a compensatory balance among the different antioxidants exists in the tissues of both invertebrates [5,6] and vertebrates [7,8] Particular attention was also given to criteria for selection of species to enter the study (see Materials and Methods) The use of animals from different vertebrate Classes could potentially complicate the interpretation of the results but offered the possibility to study animals with widely varied LEPs thus showing differences in maximum longevity which can not be explained by their metabolic rates

MATERIALS AND METHODS

Selection of animal species MLSP and LEP

Animal species were selected to enter the study only after fulfilling the following requirements (1) they belonged to the different vertebrate Classes, (2) their MLSP was reasonably well known in large populations of individuals maintained under optimal conditions for scientific experiments — OF1 mouse, Wistar rat, Dunkin-Hartley guinea pig [23,24], *R perezi* frog [7,25,26], *Xenopus laevis* toad [26,27] — or economic reasons — *Columba livia* common pigeon [24,28], *Serinus canarius* canary [24,28], *Salmo trutta fario* trout [24,29] — , (3) their maximum longevity had been independently described two or more times, obtaining close MLSP values, (4) biomarkers or signs of senility at ages close to the MLSPs had been described, (5) the differences of both MLSP and LEP among species were large, and reached one (MLSP) or two (LEP) orders of magnitude between extreme values, the MLSP was small in mammals, intermediate in poikilothermic animals, and high in birds, whereas the LEP was low in poikilotherms, higher in mammals, and still much higher in birds (Table I), the large variation in MLSP allowed a meaningful statistical analysis, and the variation in LEP was necessary to study species whose MLSP can not be explained on the basis of their metabolic rates, (6) young but true adults in good state of health were available in our area, adults had reached growth saturation and sexual maturity, and had an age previous to their physiological aging decline and equal to around one-fourth their MLSP (5–7 years in the pigeon, 4–5 years in the canary, 4 years in *X laevis*, 3 years in the trout, 2 years in the frog, 15 months in the guinea pig, 9 months in the rat, and 6 months in mice) Since no reptilian species was available which could fulfill all these requirements, this was the only vertebrate Class not included in the study

Guinea pigs and mice were obtained from Iffa-Creddo, Lyon, France, rats from the Centre d'Elevage Raymond Janvier (CERS, Le Genest-Laval, France), *X laevis*

TABLE I

MAXIMUN LIFE SPAN, O_2 CONSUMPTION AND LIFE ENERGY POTENTIAL

Species	MLSP (years)	Vo_2 (μl O_2/g per h)	LEP (l O_2/g)
Pigeon (*C livia*)	35	1518 ± 97	465
Canary (*S Canarius*)	24	5812 ± 21	1222
Toad (*X laevis*)	15	47 ± 4	6 2
Trout (*S trutta*)	13	226	26
Frog (*R perezi*)	7	87 ± 5	5 3
Guinea pig	8	681 ± 27	48
Rat (Wistar)	4	790 ± 45	28
Mouse (OF 1)	3 5	2500	77

Life energy potential (LEP) = MLSP (years) × Vo_2(l O_2/g per year) Vo_2 values are are means ± S E M from 6 to 8 animals

from Blades Biological (Surrey, UK), trout from the Jucar fish farm (Cuenca, Spain), canaries and pigeons from Spanish commercial breeders, and the frogs were raised at our laboratory Previous studies and a revision of the literature showed, at least in rats and frogs, that small or no changes in the studied parameters occurred during aging in the liver [7,30] Male animals were used in every species All animals except trout (which were sacrificed at the fish farm) were acclimated in separated facilities in the laboratory at 20 ± 2°C (amphibia) or 22 ± 1°C (homeotherms), 12 12 light/dark cycle, and food ad libitum during at least one week before being sacrificed Animals were decapitated and liver samples were immediately frozen at −25°C until they were used, between 1 and 5 weeks afterwards Statistical analysis did not reveal any correlation of biochemical values with the time that samples remained frozen All the biochemical and respiratory parameters (except for trout Vo_2) were measured in our laboratory using the same analytical methods for every animal species

Enzyme activities

Liver samples were homogenized in 50 mM phosphate buffer (pH 7 4) SOD was measured after 24 h of dialysis following the enzyme inhibition of the rate of NADH oxidation at 340 nm in the presence of EDTA, $MnCl_2$, and mercaptoethanol [31] CAT was measured following H_2O_2 disappearance at 240 nm [32] Total GPx (cumene hydroperoxide as substrate) [33] and Se-dependent GPx (H_2O_2 as substrate) [34] were measured following NADPH oxidation at 340 nm in the presence of excess GR, GSH, and the corresponding peroxide GR was assayed following NADPH oxidation at 340 nm in the presence of GSSG [35] COX was measured following the rate of oxidation of cytochrome *c* at 550 nm [36] All the enzymatic reactions were performed at 25°C It was checked that concentrations of all

substrates (which were always the same) were saturating for the Se and non-Se GPx, GR, and COX reactions in all the species studied Protein concentrations were measured as described by Lowry et al [37]

Low molecular weight antioxidants

Liver samples were homogenized in 5% trichloroacetic acid with 0 01 N HCl for glutathione assays Total glutathione was measured by the spectrophotometric recycling assay [38] in the presence of 5,5′-dithiobis(2-nitrobenzoic acid), NADPH and GR at 412 nm GSSG was assayed by the same method after derivatization of GSH with 2-vinylpyridine [39] GSH values were obtained after subtracting GSSG from total glutathione A different portion of liver tissue was homogenized in 50 mM perchloric acid for simultaneous analysis of ascorbic and uric acid by ion pair HPLC with UV detection at 280 nm [40]

Oxidative stress

Three different estimators of tissue oxidative stress were used GSSG/GSH ratio, TBA-RS (TBA-reactive substances), and true MDA MDA was measured by ion-pair HPLC with UV detection at 267 nm [41] TBA-RS were measured colorimetrically by a method specially adapted to tissue extracts [42] Peroxidation was stimulated in vitro by incubating supernatants of liver samples with 0 4 mM ascorbate and 0 05 mM $FeSO_4$ for 0, 30, 60, or 90 min at 25°C previously to performing the TBA assay Production of TBA-RS represents tissue sensitivity to free radicals, whereas MDA values are steady-state concentrations of one of the products from the lipid peroxidation process normally present in the tissue in vivo

H_2O_2 concentration in vivo

Intraperitoneal injection with 3-amino-1,2,4-triazole (AT) irreversibly inactivates CAT in vivo Purified CAT is not inactivated by AT unless H_2O_2 is present in the medium because AT only reacts with the CAT reaction intermediate compound I (CAT — H_2O_2) forming an inactive covalent complex [44–46] It has been reported in various tissues that no H_2O_2-independent CAT inactivation occurs after a single AT treatment in vivo [47–49] Thus, the rate of inactivation of CAT in vivo is taken as an indirect estimation of tissue H_2O_2 concentration in the intact animal [47–50] Groups of male rats, frogs and pigeons were intraperitoneally injected with physiological saline or 1 mg/g of AT in saline and sacrificed 0 (saline), 2 or 6 h (AT groups) after treatment Injections were performed at 09 00 h in the three species The organs were immediately processed after sacrifice for CAT determination The concentration of AT/ml of saline was 50 mg/ml in all the species

Oxygen consumption at rest

The Vo_2 of all the animal species was measured at the laboratory, except for trout values which were taken from the literature [43] Trout (3 kg body wt) were

sacrificed in situ at the fish farm in order to avoid changes due to stress during transport and maintenance at the laboratory The Vo_2 of the animals was individually measured during the light phase between 09 00 h and 14 00 h, at 25°C, inside closed circuit-type respirometers, 1–4 days before sacrifice Respirometers of different volumes (250 ml for amphibians, 4 1 for the canary and mice, and 20 7 1 for rats, guinea pigs and pigeons) were used depending upon the Vo_2 of each animal species The minimum Po_2 at the end of each measurement period was always higher than 100 mmHg A thin film of water was present at the bottom of the animal compartment during the Vo_2 measurements in amphibians The space inside the respirometer was large enough to avoid stress but small enough to prevent the performance of spontaneous exercise Thus, the measurements represent the basal or standard metabolic rate of the animals at rest An oxygen electrode connected to an external O_2 meter, and 2–50 ml of 10% KOH (depending on respirometer volume) as CO_2 absorber were also included in separate compartments inside the respirometer An air pump recirculated the air in the chamber at a rate of 3 l/min, bubbling it through the KOH solution Air samples were taken from the respirometer at different time intervals during the measurements for CO_2 analysis Mean CO_2 concentration during the measurements varied between 0 5 and 0 75% The variations of oxygen tension inside the chambers during all the measurements were minimum Po_2 = 104–121 and mean Po_2 = 113 ± 5 mmHg (141 mmHg was the mean Po_2 at the laboratory during the measurements) The values were expressed in μl O_2/g per h in STP conditions (273°K and 1 atm of pressure)

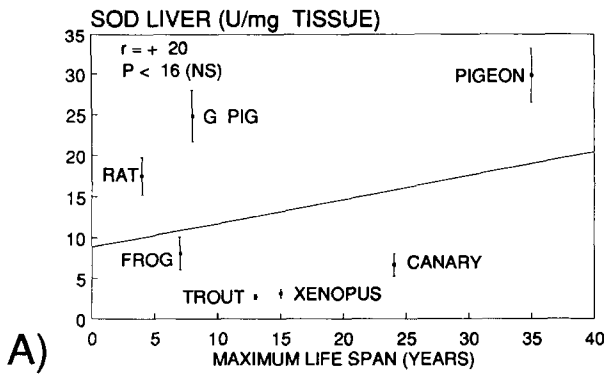
Statistical analysis

The relationship between free radical-related parameters and MLSP was studied by least squares regression analysis and the Pearson correlation coefficient and the statistical significance of the goodness of the linear fit were obtained Differences among species in liver H_2O_2 concentration at different times after injection with AT were subjected to analysis of variance After the ANOVA, the Fisher's least significant difference (LSD) test was used when necessary to analyze significance between paired groups The 0 05 level was selected as the minimal statistical significance in all the analyses

RESULTS

Antioxidant enzymes

The correlation between hepatic O_2^- and H_2O_2 detoxifying enzymes against the maximum life span of the vertebrate species considered is shown in Figs 1 and 2 SOD did not correlate significantly with maximum longevity (Fig 1A), whereas CAT activity showed a very significant negative correlation (Fig 1B) Total GPx — an enzyme activity representing the capacity for decomposition of both inorganic and organic hydroperoxides — showed also a very significant negative correlation



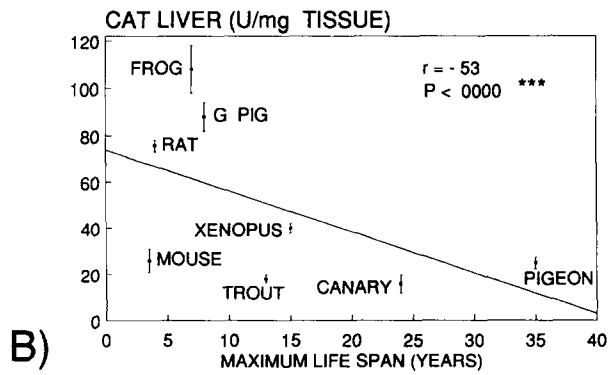


Fig 1 Correlation of liver SOD (A) and CAT (B) activities against MLSP in different vertebrate species CAT units are μmol H_2O_2 decomposed/min r = correlation coefficient, asterisks represent the statistical significance of the correlation, ***$P < 0.001$, NS, non-significant correlation $n = 6–8$ animals/species, regression lines were obtained from entering the 50 (SOD) or 57 (CAT) individual values

with MLSP, and the same was true for Se-dependent GPx — an H_2O_2 decomposing enzyme — (Fig 2) GR, on the contrary, did not show a relationship with MLSP (Fig 3A)

Low molecular weight antioxidants

GSH showed again a very significant negative association with MLSP (Fig 3B) On the other hand, ascorbate (which was measured by HPLC) did not show any relationship to maximum longevity (Fig 4A) Two of the species included in the study are not capable of synthesizing ascorbate, and their tissue levels reflect ascorbate concentration in the diet Ascorbate is thus a vitamin (vitamin C) for guinea pigs and fish [51], whereas amphibians [51], including *R perezi* liver [7], are known to synthesize ascorbic acid We then repeated the regression analysis exclusively using the

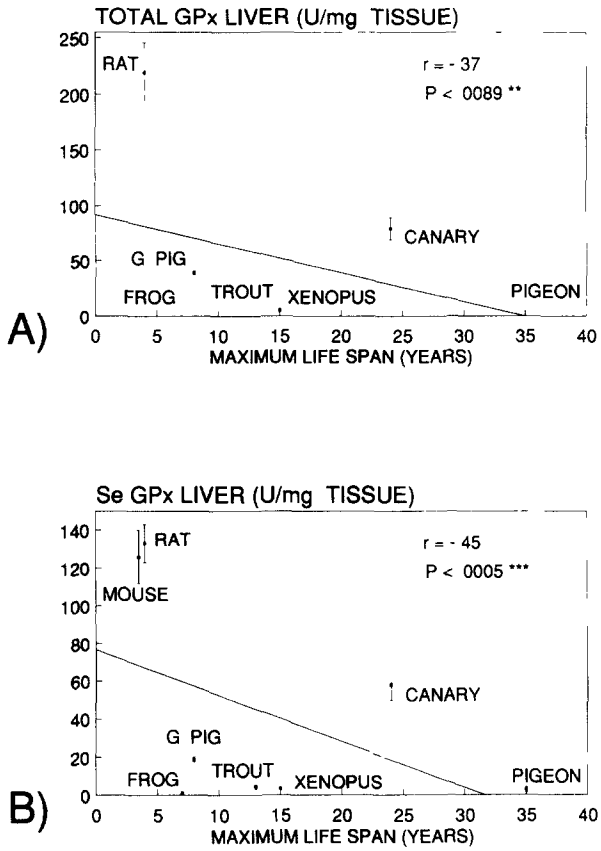
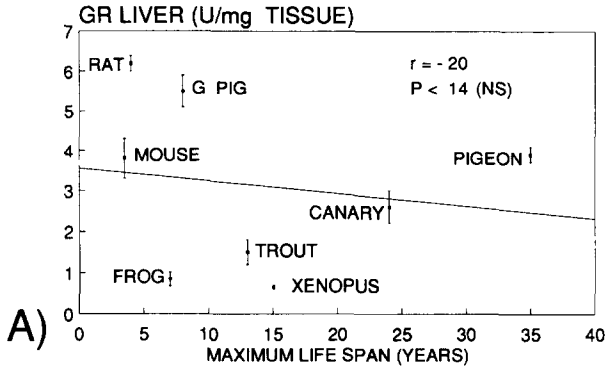


Fig 2 Correlation of liver total (A, Se plus non-Se-dependent) and Se-dependent GPx (B) activities against MLSP in different vertebrate species GPx units are nmol NADPH oxidized/min r = correlation coefficient, asterisks represent the statistical significance of the correlation, $^{**}P < 0.01$, $^{***}P < 0.001$ n = 6–8 animals/species, regression lines were obtained from entering the 50 (total GPx) or 56 (Se GPx) individual values

six species which synthesize ascorbate The results showed again a lack of correlation with MLSP ($r = +0.21$, $P < 0.20$) As it is shown in Fig 4B, the levels of uric acid — measured by HPLC — did not show a significant association with MLSP

Five of the species used were homeothermic and three were poikilothermic Since poikilothermic species show a smaller concentration of tissue proteins than that of homeotherms, we considered it important to use the total values (referred to tissue weight) in the regression studies of antioxidants constituted by amino acids (Figs 1–3) As it is shown in Table II, when the parameters were referred to protein the same result was obtained strongly significant negative associations between antioxidants and maximum longevity The statistical significance of these correlations was higher for all the considered parameters when they were referred to tissue weight



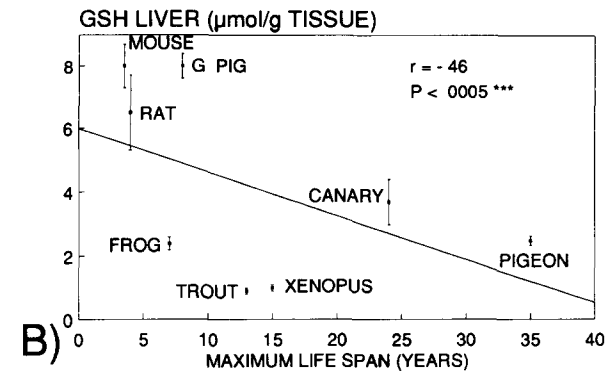


Fig 3 Correlation of liver GR activity (A) and GSH concentration (B) against MLSP in different vertebrate species GR units are nmol NADPH oxidized/min r = correlation coefficient, asterisks represent the statistical significance of the correlation, ***$P < 0.001$, NS, non-significant correlation $n = 6–8$ animals/species, regression lines were obtained from entering the 57 (GR) or 55 (GSH) individual values

(Figs 1–3, Fig 5) instead of to protein (Table II) Furthermore, parameters which did not correlate with MLSP on a tissue basis (SOD-Fig 1A, GR-Fig 3A, COX-Fig 8) continued to show a lack of association with maximum longevity on a protein basis (Table II)

Correlations per aerobic capacity or against LEP

Even though no positive correlations were found for antioxidants versus MLSP [15], it was proposed that the relevant correlations would be those between MLSP and antioxidants referred to basal oxygen consumption of the whole animal, or those between true antioxidant levels and LEP [15] In order to test this possibility we applied this kind of analysis to our data and we added a third correlation antioxidant/COX vs MLSP COX, an aerobic index of the tissue, would be more

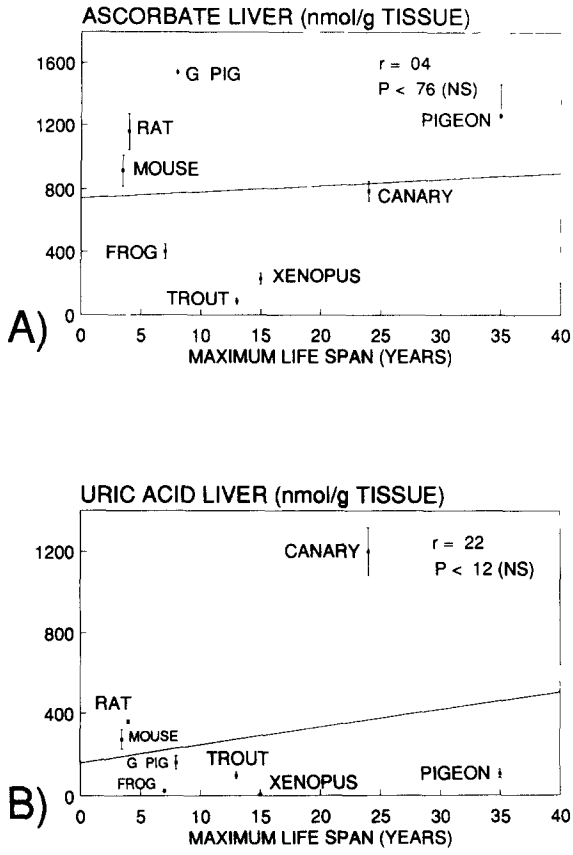
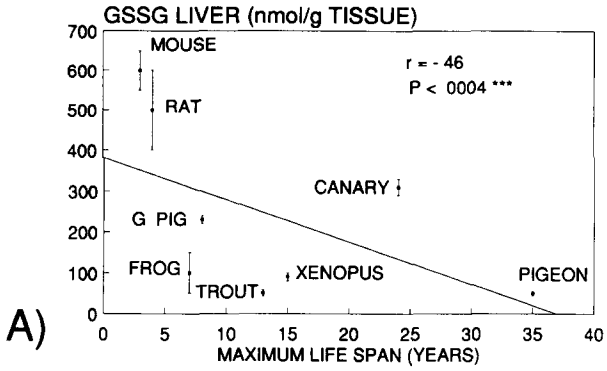


Fig 4 Correlation of liver ascorbic (A) and uric acid (B) concentrations against MLSP in different vertebrate species Both antioxidants were measured by HPLC r = correlation coefficient, NS, non-significant correlation n = 6–8 animals/species, regression lines were obtained from entering the 52 individual values

representative of liver aerobic capacity than the VO_2 of the whole animal The results obtained, presented in Tables III (reference to VO_2 or COX, vs MLSP) and IV (direct correlation against LEP) did not show the appearance of new positive correlations in any of the 24 additional regressions performed except between uric acid and LEP The general trend in all these regressions was to show again either no correlation or strongly significant negative correlations with MLSP or LEP New negative correlations against MLSP were observed for SOD/VO_2, GR/VO_2, and uric acid/VO_2, (Table III) When species not synthesizing ascorbate (trout and guinea pig) were excluded, no significant correlations were found for ascorbate in any of the three comparisons

Oxidative stress

The major part of liver glutathione exists in its reduced form, GSH The oxidized



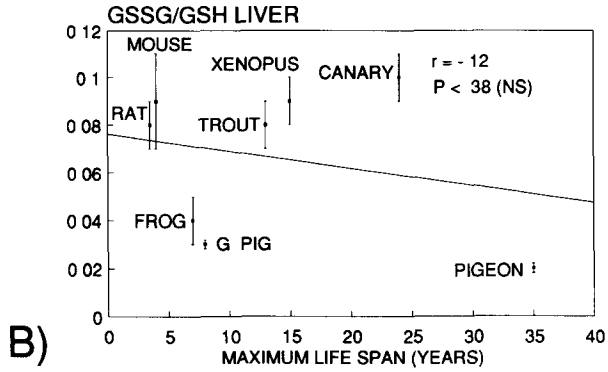


Fig 5 Correlation of liver GSSG (A) and GSSG/GSH ratio (B) against MLSP in different vertebrate species r = correlation coefficient, asterisks represent the statistical significance of the correlation, $^{***}P < 0\,001$, NS, non-significant correlation $n = 6$–8 animals/species, regression lines were obtained from entering the 55 individual values

TABLE II

SIGNIFICANCE OF CORRELATION BETWEEN MAXIMUM LIFE SPAN AND ANTIOXIDANT ENZYMES, OR GLUTATHIONE, WHEN EXPRESSED ON A PROTEIN BASIS

Parameter	r-value	P-value
SOD	−0 14	0 33 (NS)
CAT	−0 39	0 002**
Total GPx	−0 34	0 015*
Se GPx	−0 38	0 003**
GR	−0 17	0 20 (NS)
GSH	−0 46	0 0004***
GSSG	+0 40	0 002**

NS, non-significant correlation *Statistically significant correlation $^{*}P < 0\,05$, $^{**}P < 0\,01$, $^{***}P < 0\,001$

TABLE III

SIGNIFICANCE OF CORRELATION BETWEEN MAXIMUM LIFE SPAN AND ENZYMATIC OR NON-ENZYMATIC ANTIOXIDANTS, GSSG OR MDA (HPLC) WHEN EXPRESSED ON A Vo_2, OR CYTOCHROME OXIDASE BASIS

Parameter	*r*-value	*P*-value
SOD/Vo_2	−0 31	0 03*
CAT/Vo_2	−0 20	0 13 NS)
Total GPx/Vo_2	−0 55	0 0000***
Se GPx/Vo_2	−0 50	0 0001***
GR/Vo_2	−0 31	0 018**
GSH/Vo_2	−0 37	0 005**
GSSG/Vo_2	−0 21	0 12 (NS)
ASCORBATE Vo_2	−0 18	0 20 (NS)
URIC ACID/Vo_2	−0 36	0 008**
MDA/Vo_2	+0 04	0 83 (NS)
SOD/COX	−0 19	0 18 (NS)
CAT/COX	−0 19	0 16 (NS)
TOTAL GPx/COX	−0 27	0 05*
Se GPx/COX	−0 42	0 001**
GR/COX	−0 11	0 43 (NS)
GSH/COX	−0 42	0 001**
GSSG/COX	−0 30	0 028*
ASCORBATE/COX	−0 24	0 09*
URIC ACID/COX	−0 004	0 98 (NS)
MDA/COX	+0 07	0 67 (NS)

NS, non-significant correlation *Statistically significant correlation $*P < 0\,05$, $**P < 0\,01$ $***P < 0\,001$

TABLE IV

SIGNIFICANCE OF CORRELATION BETWEEN LIFE ENERGY POTENTIAL (LEP) AND ENZYMATIC OR NON-ENZYMATIC ANTIOXIDANTS, GSSG, OR MDA (HPLC)

Parameter	*r*-value	*P*-value
SOD	−0 06	0 67 (NS)
CAT	−0 48	0 0002***
TOTAL GPx	+0 039	0 79 (NS)
Se GPx	+0 026	0 85 (NS)
GR	−0 06	0 65 (NS)
GSH	−0 10	0 45 (NS)
GSSG	+0 046	0 74 (NS)
ASCORBATE	+0 099	0 49 (NS)
URIC ACID	+0 84	0 0000***
MDA	+0 62	0 0000***

*Statistically significant correlation $*P < 0\,05$ $**P < 0\,01$, $***P < 0\,001$
NS, non-non-significant correlation

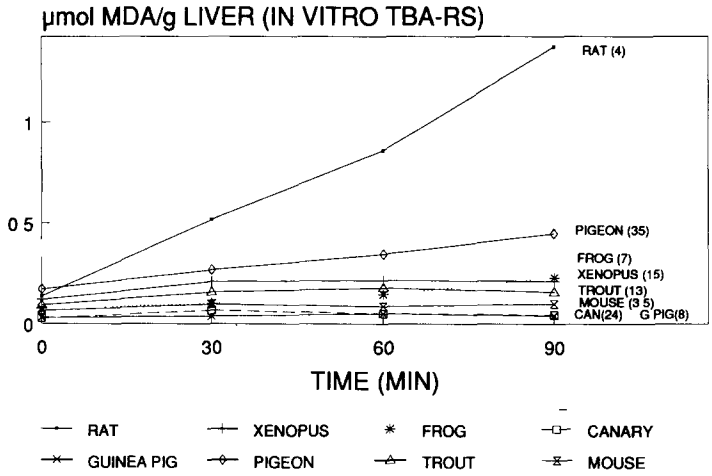


Fig 6 Time-course of in vitro lipid peroxidation (ascorbate-Fe^{2+}-stimulated) in liver homogenates of vertebrate species showing widely different MLSP Peroxidation was estimated as TBA-reactive substances and expressed as MDA equivalents/g tissue Numbers in parentheses show the MLSP of each animal species considered $n = 6–8$ in each species/time point

thiol form, GSSG, similarly to GSH, showed a strongly significant negative correlation against MLSP (Fig 5A) On the other hand, the GSSG/GSH ratio, a commonly used index of hepatic redox state, did not show any association with MLSP (Fig 5B) The sensitivity of liver tissue to peroxidation was kinetically studied by measuring the accumulation of thiobarbituric acid reactive substances after incubation of homogenates in the presence of a free radical generator system (Fig 6) Even though maximum stimulation of peroxidation was observed in the rat — a shortly lived spe-

TABLE V

SIGNIFICANCE OF CORRELATION BETWEEN MAXIMUM LIFE SPAN AND IN VITRO PEROXIDATION (ASCORBATE-Fe^{2+}) IN THE LIVER AT DIFFERENT INCUBATION TIMES OR PEROXIDATION RATE

Parameter	*r*-value	*P*-value
TBA value (nmol MDA/g tissue)		
0 min	+0 15	0 29 (NS)
30 min	+0 08	0 60 (NS)
60 min	+0 02	0 89 (NS)
90 min	−0 02	0 89 (NS)
Peroxidation rate (nmol MDA/min/g tissue)	−0 22	0 59 (NS)

NS, non-significant correlation

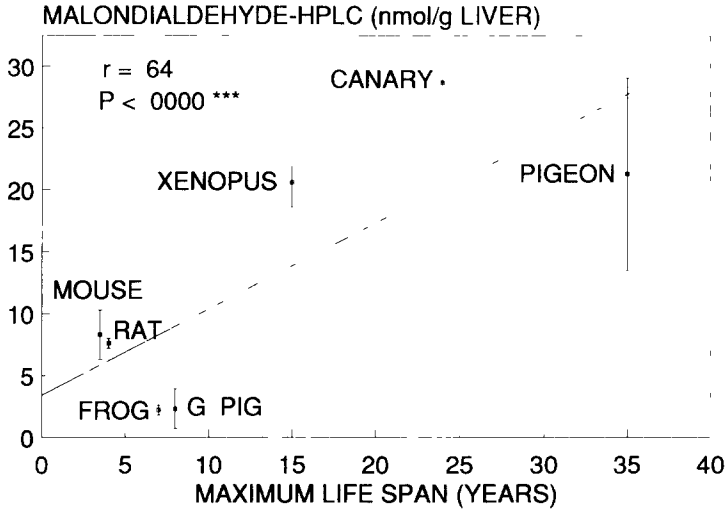


Fig 7 Correlation of liver true MDA (measured by high performance liquid chromatography) against MLSP in different vertebrate species r = correlation coefficient, asterisks represent the statistical significance of the correlation, $^{***}P < 0.001$ $n = 4$–7 animals/species, regression line was obtained from entering the 38 individual values

cies — the mouse showed a low rate of TBA-RS production, and the pigeon (the most longevous species studied) was second in intensity of peroxidation Thus, sensitivity to in vitro peroxidation did not seem to be related to MLSP across species This was corroborated by further statistical analyses Thus, the TBA values did not show significant correlation against MLSP at any of the four periods of incubation used (Table V) Furthermore, the peroxidation rate (rate of accumulation of TBA-RS per unit time) again did not correlated with MLSP (Table V) Measuring MDA by HPLC was considered complementary to the TBA test since this test is capable of detecting many different substrates and intermediates of the peroxidation process, whereas HPLC offers a total specificity for MDA In contrast to the TBA assay, true MDA showed a strongly positive association with MLSP (Fig 7)

Aerobic capacity

In order to get an idea about basal and maximum rates of oxygen utilization, the oxygen consumption of the whole animal at rest (at 25°C in unrestrained animals) and the hepatic COX activity were measured COX activity did not significantly correlate with MLSP (Fig 8) and tended to increase both in short-lived mammals and long-lived birds Similarly, Fig 9 shows the combination of a low metabolic rate and an intermediate MLSP in poikilotherms and the simultaneously high metabolic rate and small MLSP of mammals (rat and mouse), whereas birds show the unique combination of a high aerobic metabolism and a very high MLSP These $\dot{V}O_2$ and MLSP trends are not exclusive of the animal species shown, since they are typical in species of small body size from these vertebrate Classes

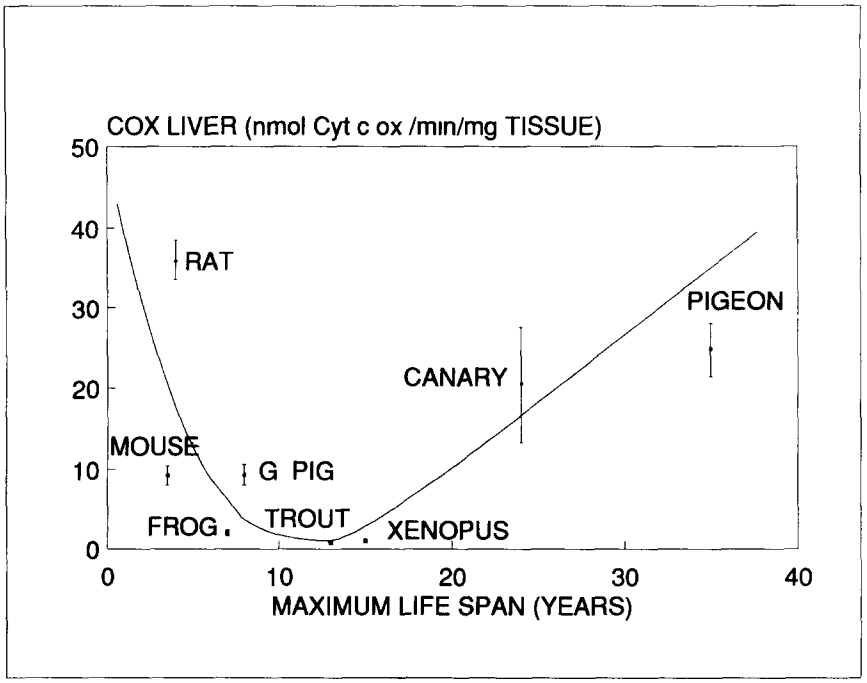


Fig 8 Liver COX and MLSP in different vertebrate species No significant linear correlation was found between COX and MLSP $r = +0\ 16$, $P < 0\ 22$, $n = 6–8$ animals/species The curve line was drawn by hand

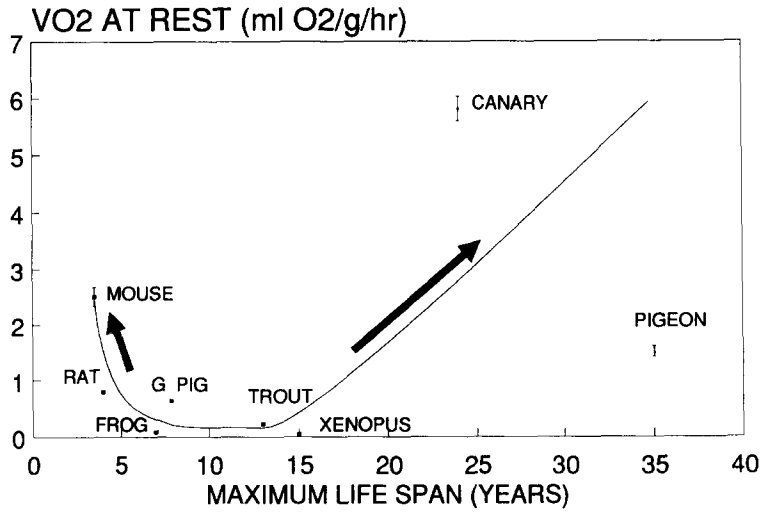


Fig 9 Simultaneous evolutionary change of metabolic rate (Vo_2) at rest and MLSP from poikilothermic (trout, frog and toad) to homeothermic (mammals and birds) vertebrates of small body size In mammals the increase in Vo_2 occurs with a reduction in maximum longevity whereas both metabolic rate and MLSP simultaneously and acutely increase in birds The curve line was drawn by hand $n = 6–8$ animals/species

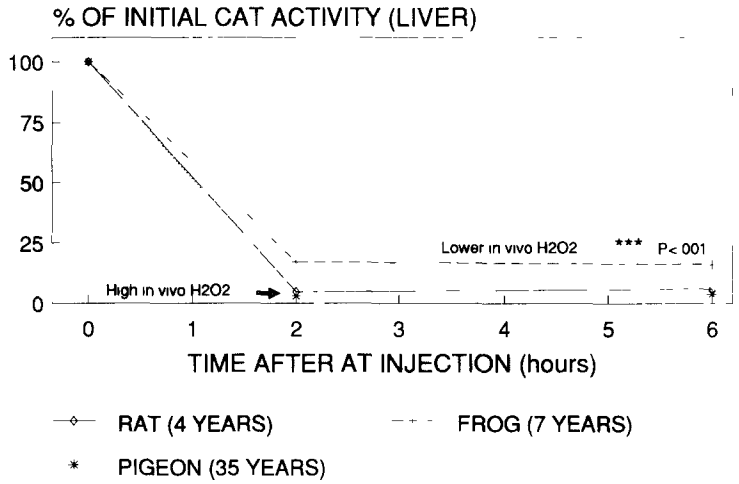


Fig 10 Rate of disappearance of liver CAT activity after a single i p injection with the catalase inhibitor aminotriazole (AT, 1 mg/g) in three species showing widely different MLSP The rate of inactivation increases with the steady-state liver in vivo H_2O_2 concentration (see materials and methods for further explanation) Inactivation of frog CAT was lower than that of rat and pigeon CAT both at 2 and 6 h after the AT injection $n = 3$ animals/time point in each species, each value was assayed in triplicate

H_2O_2 concentration in vivo in liver tissue

At the end of the study, the negative correlations of various liver antioxidants against maximum longevity decided us to indirectly estimate the in vivo steady-state H_2O_2 concentrations in liver tissue We wondered if longevous species were showing low levels of these antioxidants (specially the H_2O_2 detoxifying enzymes CAT and both GPx) because their rates of H_2O_2 generation in the liver were also much lower We tested this using a shortly-lived species (the rat), the most longevous species of this study (the pigeon), and a species of intermediate MLSP (the frog) The rate of inactivation of CAT activity after a single injection with the CAT inhibitor aminotriazole indirectly estimates in vivo steady-state H_2O_2 concentrations The percentage of residual catalase activity 2 and 6 h after aminotriazole injection was significantly higher ($P < 0\ 001$) in the frog than in the two homeotherm species (rat and pigeon) (Fig 10) Thus, liver H_2O_2 concentration in vivo seems to be lower in species showing a low rate of oxygen consumption at rest But the estimated in vivo levels of H_2O_2 in the liver of two homeotherms (rat and pigeon) showing widely different MLSPs (4 and 35 years) were similar

DISCUSSION

Previous studies from one laboratory have suggested that an increase in antioxidants, specially SOD, can explain why animals like man and other primates have MLSPs much longer than predicted by Rubner's metabolic rule [9,15,17,19] This

was originally based [17] in the observation that, even though liver, brain, and heart SOD did not correlate with MLSP in mammals, the ratio between SOD activity and the total VO_2 of the animal (SOD/VO_2) positively correlated with the MLSP in these tissues The rationale for using this ratio was that it would give an idea of the relationship between free radical detoxification and free radical generation in the tissues in vivo Nevertheless, we think that the rate of free radical generation in a particular tissue can not be represented by the VO_2 of the whole animal Furthermore, mammalian VO_2 is strongly and inversely correlated with MLSP as it was first shown by Rubner and Pearl [11–13] It is then not surprising that dividing a parameter not correlated (SOD) with MLSP by another parameter strongly and inversely correlated (VO_2) with MLSP resulted in a ratio positively correlated with maximum longevity In these comparisons the high SOD/VO_2 values of man and other longevous primates are not due to the possession of exceptionally high SOD activities, but rather to their low metabolic rates (which are typical for their relatively big size) The same problem affects the described positive correlations of ascorbate/VO_2, α-tocopherol/VO_2 and carotenoids/VO_2 in plasma versus mammalian MLSP [9,15] In many of these cases there are additional complications these substances were measured in plasma instead of in tissues, data for a particular antioxidant (α-tocopherol) in different species were gathered from many different works, and the antioxidants considered are diet-dependent (α-tocopherol and carotenoids), this makes it difficult for them to be responsible for an intrinsic, species-specific trait such as MLSP

It is interesting that in our work we have not found a significant correlation between SOD absolute values and vertebrate MLSP, in agreement with the mammalian results cited above [17] Furthermore, significant negative correlations of the absolute values of hydroperoxide detoxifying enzymes CAT and Se and non-Se-GPx, and GSH in vertebrate liver versus MLSP have been found In agreement with this, strongly negative correlations between mammalian MLSP and absolute values of CAT [9], GPx [20,21], and GSH [10,15] have been described by different authors in the liver of mammals The negative character of these correlations against MLSP was so strong that it was maintained even after dividing these antioxidants by the VO_2 of each species [9] The most longevous species of those works, *Homo sapiens*, showed the smallest levels of liver CAT, GPx, and GSH, and minimal levels of ascorbate among mammals No correlation with MLSP was found in our case for GR, ascorbate, and uric acid No previous data for GR in any tissue or for uric acid in liver have been reported, but liver ascorbate correlated inversely with MLSP in mammals [9,22] It is interesting that liver GSH-transferase, which is also a detoxifying enzyme activity, also showed a very strong inverse correlation with MLSP in mammals [15,52] Thus, the results available are greatly consistent showing that hepatic hydroperoxide enzymes and GSH are negatively correlated with MLSP, whereas SOD, GR and uric acid do not correlate with MLSP in the liver In spite of the use of very different sets of mammalian or vertebrate species, the wide concor-

dance between these independent works means that an increase in liver antioxidants can not be considered as a cause of a high MLSP during mammalian and vertebrate evolution Potential limitations arising from the use of animals of different vertebrate Classes in our work are counterbalanced by the possibility to study animals with very different life energy potentials and by the concordance with the results obtained by other authors using only mammals

Even though GSH negatively correlated with MLSP, the same was true for GSSG This, together with the absence of association between GR and MLSP, logically resulted in a lack of correlation between GSSG/GSH and maximum longevity In agreement with this, hepatic peroxidation (TBA-RS) was not correlated with MLSP at any of the four incubation times studied The positive correlation of MDA against MLSP contrasts with the TBA-RS results Nevertheless, it should be remembered that the specificity of the HPLC assay for MDA can be considered a limitation in relation to in vitro TBA values (which show minimal interference from substances not related to peroxidation at high incubation times) because MDA is only one of a high number of products of lipid peroxidation Many of these important products (e g , organic hydroperoxides), different from MDA, are detected by the TBA test In this work, GSSG/GSH, MDA (HPLC), and TBA-RS (liver) are reported for the first time as a function of MLSP

The constancy of liver oxidative stress — estimated as GSSG/GSH and TBA-RS values — in species with very different longevities is concordant with the H_2O_2 results Previous works related to MLSP have focused on the free radical decomposition capacity of the tissues — antioxidants — or on the free radical generation rate by mitochondria in vitro [53,54] However, no data about the in vivo concentrations or in vivo rates of generation of active oxygen species in tissues of animals with different MLSP have been previously reported It is known that the most reactive free radical present in tissues, OH , is formed from longer-lived species, such as H_2O_2, by a site-specific mechanism at the target macromolecule [55] H_2O_2 has not been directly measured in vertebrate tissues due to its instability in tissue homogenates, which contain hydroperoxide detoxifying enzymes such as GPx Nevertheless, the rate of disappearance of CAT activity after a single AT injection indirectly estimates relative in vivo H_2O_2 levels among different groups of animals [46,48–50], and has been even used to calculate absolute H_2O_2 concentrations in the rat brain tissue at different P_{O_2} [48] This method has previously showed its effectiveness in the brain [48], liver [7,46,56], lung [8,57], heart [50,57], and kidney [7,49] of rats [46,48,49], guinea pigs [56], mice [50] and *R perezi* frogs [7,8,57] In our study, the estimated basal hepatic H_2O_2 concentrations at the steady state were similar in two species with extremely different longevities (4 years in the rat and 35 years in the pigeon) Thus, the mean H_2O_2 concentration in the liver under basal conditions does not seem to be an important factor in relation to MLSP The somewhat lower H_2O_2 levels of the frog (a species with an intermediate MLSP, 7 years) can be explained by its lower total metabolic rate and higher CAT activity in relation to rats and pigeons The fact that this low level of H_2O_2 in the frog liver does not give rise to

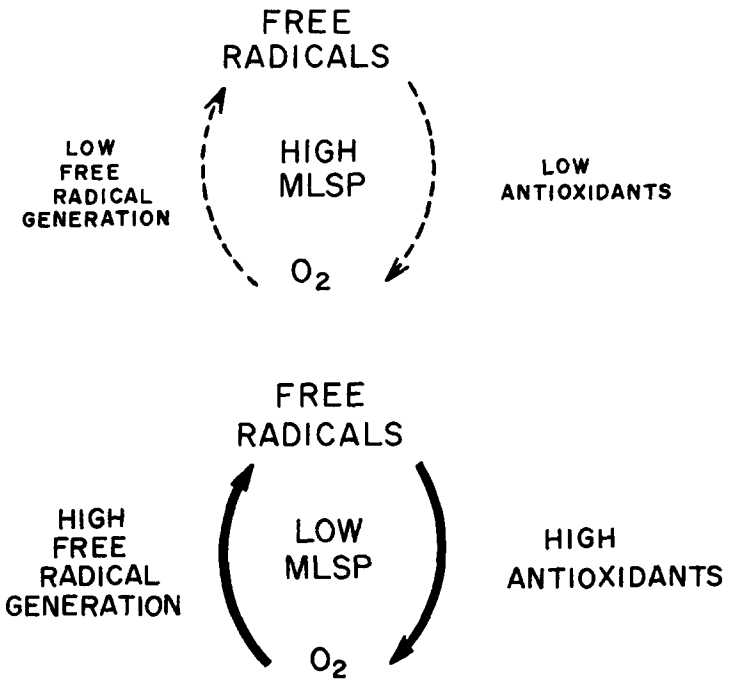


Fig 11 It is suggested that longevous species (A) show low levels of antioxidants in their tissues because their in vivo rates of generation of free radicals at rest are also much lower than in short-lived species (B) This low pace of free radical recycling can be a longevity determinant since it would reduce the local free radical concentration at sites of free radical generation (e g , mitochondria)

a very long MLSP again further supports the view that the mean concentration of H_2O_2 in the liver under basal conditions is not involved in determining the MLSP of vertebrates This is consistent with recent experiments showing that transgenic [58] or mutant [59] *D melanogaster* flies showing very high or low catalase activities do not experience a change in MLSP

The results obtained in this work show that longevous species combine low levels of antioxidants with similar estimated H_2O_2 concentrations in the liver in relation to short-lived animals This strongly suggests that the rate of H_2O_2 generation in vivo is also much lower in long-lived than in short-lived species This agrees with the previous description of strong negative correlations between $O_2^{\cdot-}$ [53] and H_2O_2 [54] generation by liver mitochondria in vitro and the MLSP of mammals We thus suggest that the low enzymatic H_2O_2 detoxifying capacity is an evolutionary co-adaptation with a small rate of generation of oxygen radicals in longevous species, short-lived species simultaneously showing high H_2O_2 generation and scavenging (Fig 11) It should be remembered that regulatory systems 'abhor V_{max}' and stable metabolic systems show a VO_2/VO_{2max} in the 10–30% region [60] Free radical formation and decomposition is of cyclic nature [61] since both SOD and CAT produce O_2 from $O_2^{\cdot-}$ or H_2O_2 Thus, it is possible that animal species with a simultaneous

high rate of free radical generation and scavenging suffer a higher cellular damage when a burst of free radical generation occurs in the cell, whereas the low pace of free radical recycling in longevous organisms would avoid the saturation of the antioxidant systems (Fig 11) Furthermore, a high rate of free radical recycling would increase local concentrations of active oxygen species near their sites of generation (e g , mitochondria) This would increase the likelihood of hydroxyl radical production near critical targets at macromolecules (e g , mitochondrial DNA) by site-specific mechanisms Thus, concentrations of active oxygen species would be higher near cellular sites of free radical generation in species with short — than in species with high MLSP even though mean cellular hydrogen peroxide concentrations at the steady-state would still be similar in both cases This would make compatible all the above mentioned comparative data with the recent demonstration that very longevous species show a smaller degree of oxidative DNA damage (tissue 8-OH-deoxyguanosine and thymine and thymidine glycol urinary output) than animals exhibiting a short MLSP [3,19] We thus think that it is important to develop methods to measure true in vivo concentrations of active oxygen species at different cellular sites in stressful metabolic conditions in animals with different MLSP and LEP

During evolution from poikilotherms to homeothermic vertebrates of small body size two different trends appeared In mammals the metabolic rate increased and the MLSP — following the observation of Rubner — decreased Nevertheless, in birds both the metabolic rate and the MLSP strongly increased These MLSP and VO_2 trends are not a particularity of the species used in our study since bird species generally show MLSPs around four times greater than that of mammals of similar metabolic rate [16] In the species of this study, the LEP of birds (pigeon and canary) was 16 times higher than that of mammals of equal size (rat and mouse) Thus, other still unknown longevity determinants, in addition to a low VO_2, are very important in determining MLSP This can also be the case of primates and man where the VO_2 value is similar to that expected for their relatively large body size Primates and birds are the only well described cases of large groups of animals which exhibit much higher MLSPs than predicted from their metabolic rates In primates the inverse relationship between VO_2 and MLSP is quantitatively intensified by those unknown longevity determinants, but in birds their strength is so high that very high VO_2 values can coexist with long MLSPs Birds thus appear as a very interesting model for the study of longevity, due to their unique combination of a high oxygen consumption and a high maximum longevity Only further research will tell us if a low rate of free radical recycling, as we suggest here, is a factor responsible for the extraordinarily high maximum longevity of animals like birds, primates and man

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