Testicular aging involves mitochondrial dysfunction as well as an increase in UCP2 levels and proton leak

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Abstract To address the possibility that mitochondria are involved in the age-related loss of testicular function, we characterized mitochondrial bioenergetics in rat testis. A peak of mitochondrial functionality was detected in adult animals, with a decrease in both young and older animals. In the latter group a decrease in mitochondrial function was matched with an increase in proton leak and expression and activity of uncoupling protein 2 (UCP2), suggesting that proton leak may be involved in managing age-dependent mitochondrial dysfunction.
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1. Introduction

Although men do not show a clear-cut cessation of reproductive capacity, increased life expectancy, in conjunction with a trend towards increased paternal age, have renewed interest in the issue of aging and male fertility [1].

In the past decade, several molecular mechanisms of aging have been proposed, including cumulative damage by reactive oxygen species (ROS) produced during aerobic metabolism [2], thus implicating mitochondria as a main target of free-radicals that culminate into the process of aging [3–6]. Mitochondria, beside being the major generators and ROS target in the animal cells, also play a role in signaling pathways leading to apoptosis [4,7]. Additionally, senescent mitochondria produce less ATP and may have a higher production of ROS [4]. On the other hand, there may be mitochondrial components with a protective role in the ageing process. Uncoupling proteins (UCPs) are mitochondrial H$^+$ carrier proteins able to dissipate the proton gradient across the inner mitochondrial membrane. Although this process may decrease the amount of ATP generated, some studies suggest a central role for UCPs in avoiding mitochondrial membrane hyperpolarization and ROS formation, thus potentially slowing the aging process [8–11].

Energy metabolism in the testis involves a unique network of reactions, involving several testis-specific enzymes [12,13]. A gradient in mitochondrial activity has also been described in the testis, with the stem cell spermatogonia presenting the least active mitochondria [14]. Although a role in aging has not yet been described, it has been demonstrated that UCP2 is the predominant UCP isoform present in the testes [15]. Also, variations in the expression levels of UCP2 in testis subjected to hyperthermia, suggest the involvement of these proteins in testicular physiology [16]. Since mitochondrial alterations have been associated with aging and fertility problems [4,6,17], mitochondrial respiration defects might contribute to age-related loss of reproductive potential. To better understand the contribution of mitochondria in the age-related decline observed in testicular function, we have characterized mitochondrial bioenergetics in the rat testis and discuss possible implications to testicular function and aging.

2. Materials and methods

2.1. Materials

All chemicals were obtained from Sigma, St. Louis, MO, unless otherwise described.

2.2. Animal groups

Male Wistar rats were kept under controlled conditions with free access to food and water. Rats were divided into three age groups taking in account the rat reproductive cycle: Group I (young, before full reproductive maturity): 21 days up to 2 months old ($n = 32$); Group II (adult, fully active): 2–5 months old ($n = 31$); and finally Group III (old, low litter production signaling breeding problems): 9–16 months old ($n = 12$).

2.3. Isolation of testicular mitochondria

Our aim was to study the effects of age in the testis as a whole and not individualized germ cell types, given the low yield in terms of functional mitochondrial protein. Testis mitochondria were prepared according to a modified methodology of Gazzotti et al. [18]. Following cervical dislocation the testis were immediately excised, weighed, decapsulated and minced in isolation medium containing 250 mM sucrose, 0.2 mM EGTA, 0.1 mM EDTA, 5 mM HEPES-KOH (pH 7.4) and 0.1% defatted BSA. The minced tissue was then homogenized with a Potter–Elvehjem homogenizer. The homogenate was centrifuged at $2500 \times g$ for 10 min (Sorvall RC-5c, Plus, SS 34 Rotor, 4–8 °C), and the supernatant fluid centrifuged at $10\,000 \times g$ for 10 min. The pellet (mitochondrial fraction) was resuspended and repelleted twice at $10\,000 \times g$ for 10 min. EGTA, EDTA and defatted BSA were omitted from the washing medium. Mitochondrial protein content was determined by the biuret method. To ensure that assay interpretation was not skewed due to distinct mitochondrial content, citrate synthase activity was also monitored, according to Coore et al. [19].

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2.4. Mitochondrial oxygen consumption

Oxygen consumption of isolated testis mitochondria was monitored polarographically with a Clark-type oxygen electrode [20] connected to a Kipp and Zonen flatbed recorder in a thermostated closed chamber with magnetic stirring, at 27 °C. The respiratory medium consisted of 65 mM KCl, 125 mM sucrose, 10 mM Tris, 20 μM EGTA, 2.5 mM KH_2PO_4, pH 7.4. Mitochondria were suspended at a concentration of 0.8 mg/ml in the medium and energized with both glutamate–malate (substrate for Complex I) and succinate (substrate for Complex II). When succinate was used, 3 μM rotenone was added to the medium to inhibit Complex I. ADP (25 μM) was added to induce state 3 respiration. Such a low concentration was chosen in order to avoid the saturation of the mitochondrial phosphorylative machinery, so that a physiological situation could be mimicked [21]. State 4 respiration was measured after ADP depletion (Fig. 1B). Respiratory control ratio (RCR) was determined as the ratio between mitochondrial respiration states 3 and 4. The ADP/O ratio was expressed by the ratio between the amount of ADP added and the oxygen consumed during state 3 [22].

2.5. Mitochondrial transmembrane electric potential

The mitochondrial transmembrane electric potential ($\Delta\Psi$) was estimated with a tetraphenyl phosphonium (TPP^+) electrode according to the equation of Kamo et al. [23]. A matrix volume of 1.1 μL/mg protein was assumed. Reactions were carried out, at 27 °C, in 1 ml of the reaction media supplemented with 2 μM TPP^+ and 0.8 mg mitochondria. Mitochondria were energized with 5 mM glutamate–malate (Complex I) and succinate plus 3 μM rotenone (Complex II) as discussed above (Fig. 1B).

2.6. Proton leak

In the presence of oligomycin (ATP synthase inhibitor) the respiration rate is proportional to the rate of protons leaking through the mitochondrial inner membrane. The kinetic response of the proton-conductance pathway to its driving force can therefore be measured as the relationship between respiration rate and mitochondrial membrane potential, when the potential changes by titration with electron-transport-chain inhibitors. Briefly, oxygen consumption and inner membrane potential were simultaneously recorded using a Clark-type oxygen electrode and a TPP^+ electrode, respectively. Testis mitochondria (0.8 mg) were incubated in 1 ml of reaction medium with 2 μM of oligomycin, 3 μM of rotenone and 80 ng/ml of nigericin (added to abolish ΔpH so that the total protomotive force can be equal to $\Delta\Psi$). The reaction was started with succinate. Oxygen consumption and electric potential were progressively inhibited with sequential additions of malonate up to a total of 2 mM. At the end of each experiment, valinomycin was added to dissipate the $\Delta\Psi$ [24]. To study UCP2 activation, Linoleic Acid (a known UCP2 activator) [25] was added before the additions of malonate, at a final concentration of 5 μM. In some experiments GDP, a known UCP2 inhibitor, was also employed (750 μM).

2.7. Western blot

Frozen mitochondrial pellets were homogenized in lysis buffer (1 mM urea, 10 mM Tris, 2% SDS, pH 7.5) and aliquots were run on 12% polyacrylamide gels (50 μg of protein per lane). After electrophoresis, separated proteins were transferred to polyvinylidene difluoride membranes, and blocked membranes (1 h in 5% non-fat milk solution, 37 °C) were incubated overnight at 4 °C with a mix of goat anti-UCP2 antibodies (1:500, Santa Cruz, SC 6526, 6525 and 6527) and a rabbit antibody against cytochrome oxidase subunit IV (COX IV- 1:1000, Cell Signaling # 4844). The proteins were detected separately, UCPs visualized with rabbit anti-goat IgG alkaline phosphatase-conjugated (1:2500, Sigma A4187) and COX IV with goat anti-rabbit IgG-AP (1:5000, Santa Cruz, SC 2007). COX was used as a loading control. The ECF detection system (GE Healthcare) and Versa Doc imaging system (Bio-Rad) were used. Densities from each band were obtained with Quantity One Software (Bio-Rad) [26].

2.8. Statistical analysis

All statistical analyses were done using SPSS (Statistical Package for the Social Sciences Program), version 16.00, software for Windows (SPSS Inc., Chicago, IL, USA). All variables were checked for normal distribution. Multiple comparisons were performed using Kruskall–Wallis test and statistical significance between three age groups was accessed using the Mann–Whitney U-test. For UCP experiments, statistical significance was accessed using t-test for independent samples and $P \leqslant 0.05$ was considered significant. Bivariate correlation (r) in different groups was evaluated by calculating the Spearman correlation coefficient with a two-tailed significance (p).

3. Results

3.1. Animal characterization

Older animals showed an increased testis and epididymus weight ($P \leqslant 0.001$) (Fig. 1A). However, there were no significant differences in citrate synthase activity, thus demonstrating similar purity in terms of mitochondria/mg protein in the different age groups (data not shown).

3.2. Mitochondrial bioenergetics

The transmembrane electrical potential ($\Delta\Psi$) is the main component of the electrochemical gradient accounting for more than 90% of the total mitochondrial proton motive force [27].

After substrate addition, the maximum $\Delta\Psi$ was increased in adult animals when compared with the young group ($P \leqslant 0.05$) (Table 1). The same pattern was observed for State 3 respiration in the presence of glutamate–malate ($P \leqslant 0.01$) (Table 1). In terms of membrane potential, depolarization following ADP addition was observed to be higher in the adult group when compared to the young ($P \leqslant 0.001$) and older group ($P \leqslant 0.001$) (Fig. 1D) suggesting that phosphorylation is more active in adult animals (also supported by an increase in state 3 respiration in this group). When succinate was used as substrate, a decrease in state 3 was observed in the older animals relatively to the young and adult groups (Table 1) and the phosphorylation lag phase was shortened in adult animals ($P \leqslant 0.05$), suggesting a faster phosphorylative activity (Table 1).

In the presence of glutamate–malate, ADP/O was increased in adult animals when compared to young and older rats (Fig. 1C). Additionally, correlations ($P \leqslant 0.05$) between age and ADP-induced depolarization, ADP/O and lag phase, using glutamate–malate as a substrate, were observed suggesting that ADP phosphorylation is affected by age.

After ADP depletion (state 4 respiration), again the adult group showed a higher value of oxygen consumption (Table 1). Older animals also had a higher RCR when compared to the young and adult animals groups ($P \leqslant 0.01$) when Complex II was called upon. However, when the substrate was glutamate–malate, the RCR of older animals was only significantly different from that of the young group ($P \leqslant 0.05$) (Table 1).

3.3. Proton leak and UCP2 activity

The titration curves demonstrate that mitochondria from older animals have a higher proton leak, when compared with mitochondria from the adult group. This can be inferred by the fact that more oxygen is consumed by mitochondria from older animals to generate the same value of $\Delta\Psi$ ($P \leqslant 0.01$) (Fig. 2A). Using linoleic acid (LA, 5 μM) as a specific UCP activator, older animals showed a higher stimulation of proton leak when compared with adult rats ($P \leqslant 0.01$) (Fig. 2C and D), suggesting that UCP2 levels may be up regulated in older animals. GDP, an inhibitor of UCP2 reverted LA effect (data not shown). Western blot analysis showed a higher UCP2/
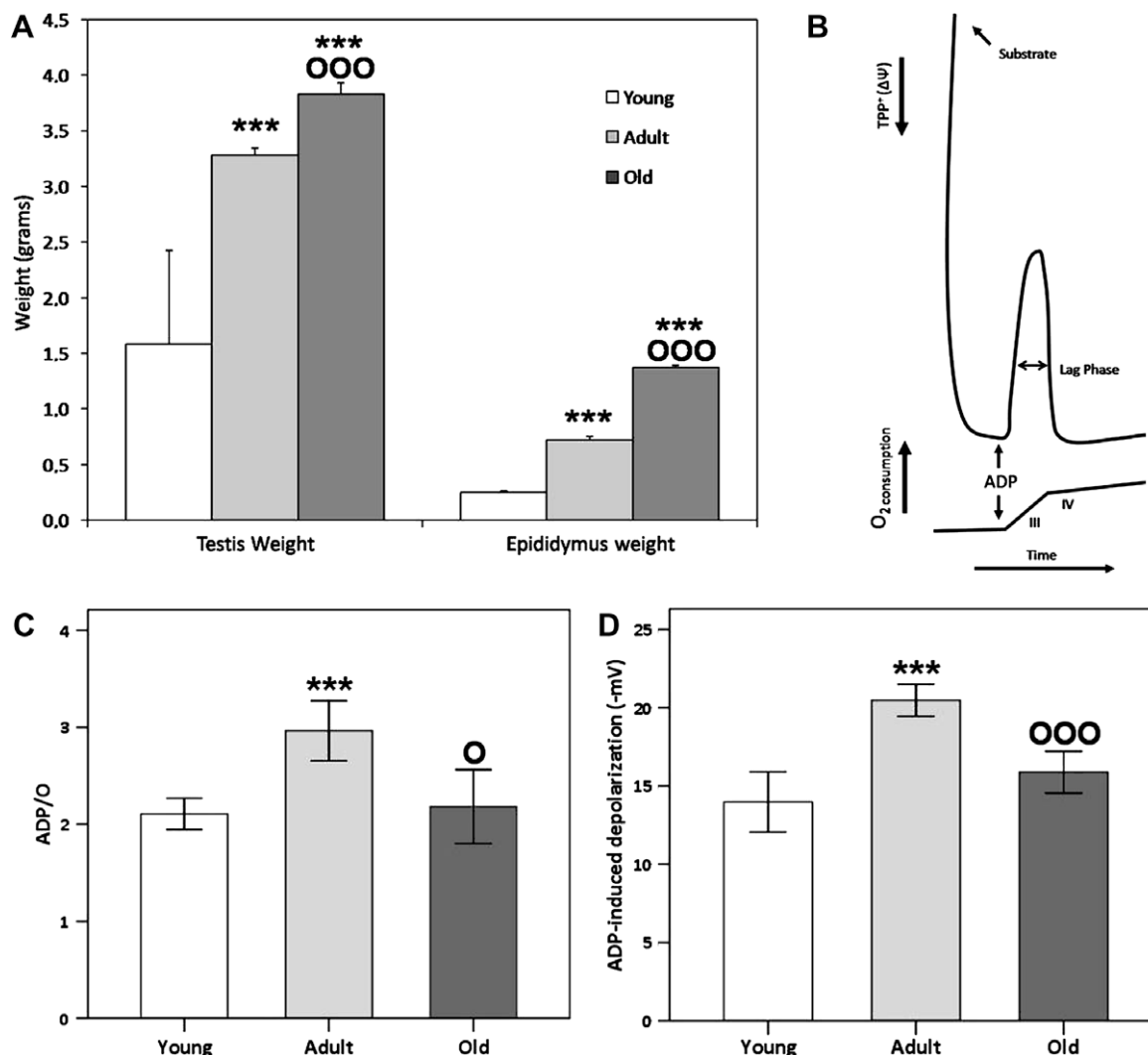
Fig. 1. (A) Testis and epididymus weight in the three age groups. (B) Upper recording: representative recording of changes in mitochondrial electric potential measured by a selective TPP$^+$; the lag phase corresponds to the time elapsed during ADP phosphorylation. Lower record: representative recording of changes in mitochondrial oxygen consumption measured using a Clark-type electrode, with state 3 respiration (after ADP addition) and state 4 respiration (after ADP depletion). (C) ADP/O ratios evaluated in the presence of glutamate + malate. (D) ADP-induced depolarization in the presence of glutamate–malate. Values statistically different from the young group: ***$P \leqslant 0.001$; and from the adult group: $^{\circ\circ\circ}P \leqslant 0.001$; $^{\circ}P \leqslant 0.05$. All bars show means ± S.E.M.

Table 1
Effects of age on testicular mitochondria respiratory function and transmembrane electrical potential parameters.

		Young group	Adult group	Older group
Complex I	State 3 (natmsO/min/mg prot)	19.83 ± 0.6	24.79 ± 1.38**	20.54 ± 1.42
	State 4 (natmsO/min/mg prot)	10.52 ± 0.49	12.77 ± 0.67**	9.48 ± 0.85$^{\circ\circ}$
	RCR	1.9 ± 0.92	2.04 ± 0.08	2.25 ± 0.14*
	$\Delta\Psi$ max (−mV)	200,01 ± 1.94	207.67 ± 1.6**	202.46 ± 2.46
	Repolarization $\Delta\Psi$ (−mV)	194.87 ± 1.62	198.59 ± 2.05	198.60 ± 2.42
	Lag phase(s)	28.19 ± 1.94	29.22 ± 1.99	35.00 ± 3.22
Complex II	State 3 (natmsO/min/mg prot)	31.85 ± 1.11	36.78 ± 1.95	26.44 ± 1.65*$^{,\circ\circ}$
	State 4 (natmsO/min/mg prot)	20.79 ± 0.91	24.75 ± 1.06*	13.35 ± 1.02***$^{,\circ\circ\circ}$
	RCR	1.52 ± 0.07	1.5 ± 0.053	1.92 ± 0.10**$^{,\circ\circ}$
	$\Delta\Psi$ max (−mV)	200.92 ± 1.72	206.72 ± 1.74*	207.5 ± 1.54*
	Repolarization $\Delta\Psi$ (−mV)	201.76 ± 1.59	203.64 ± 2.0	204.59 ± 2.2
	Lag phase(s)	43.56 ± 3.08	35.67 ± 3.41*	39.82 ± 3.03

Values are given as means ± S.E.M. *$P \leqslant 0.05$, **$P \leqslant 0.01$ compared to the young group; $^{\circ}P \leqslant 0.05$, $^{\circ\circ}P \leqslant 0.01$, $^{\circ\circ\circ}P \leqslant 0.001$ compared to the adult group; all values were determined as described in Section 2.
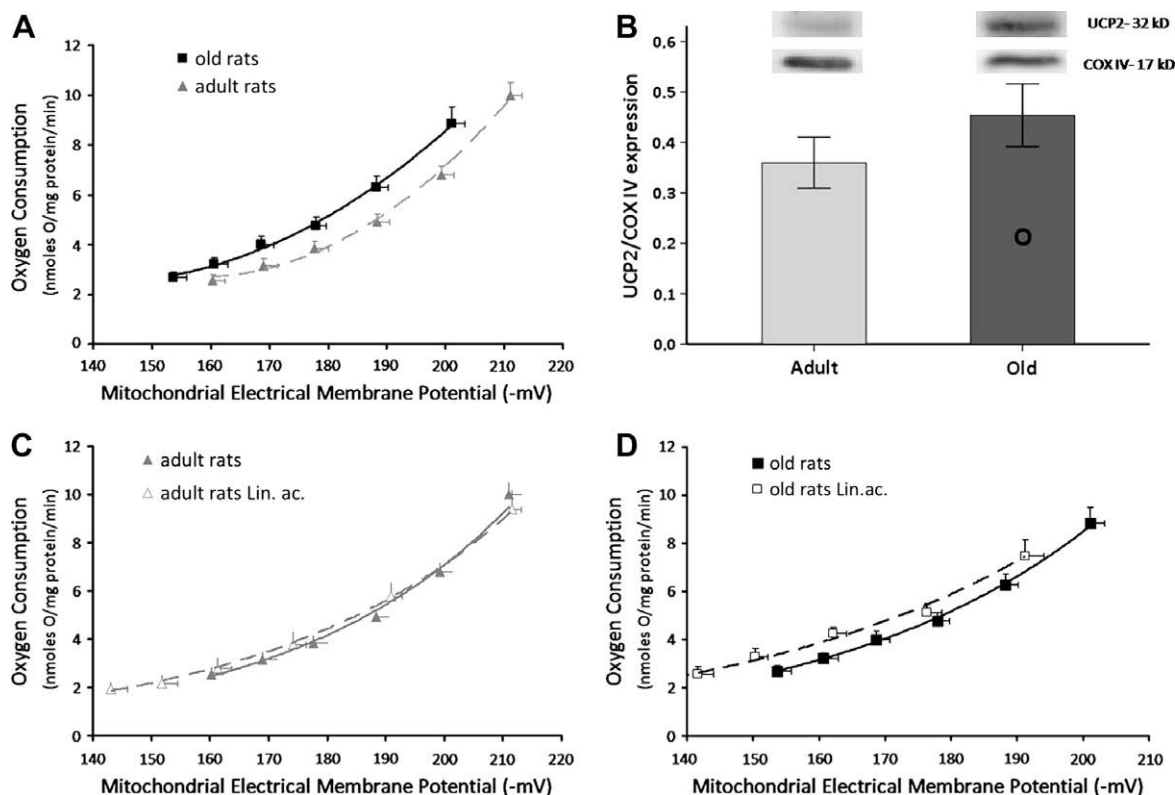
Fig. 2. UCP2-mediated proton leak. (A) Kinetic response of the proton leak to membrane potential in testis mitochondria (44 weeks old, $n = 7$; and 12 weeks old, $n = 17$). Data are expressed as means ± S.E.M. and differences between age groups are significant at $P \leqslant 0.01$. (B) UCP2 expression in testicular mitochondria, bars show means ± S.E.M. of UCP2 levels normalized with standard COX IV levels, $^{\circ}P \leqslant 0.05$. Representative Western blot data for both groups of animals are shown above the respective bars. (C, D) Simulation of proton leak using linoleic acid, in adult and old animal, respectively. The use of the UCP2 inhibitor GDP (750 μM) reverted the effect of linoleic acid (data not shown).

COX IV ratio in testicular mitochondria from older animals ($P \leqslant 0.05$) (Fig. 2B).

4. Discussion

Mammalian germ cells develop in a tubular environment that is mainly maintained by Sertoli cells, and have different preferred substrates to obtain energy, including mitochondrial ATP. Changes in the mitochondrial energy circuit may be related to testicular metabolism deregulation [12–14]. Therefore, we examined the effects of age on bioenergetic characteristics of testicular mitochondria isolated from rats of different ages.

Interestingly, testicular mitochondria seem to consume less oxygen to generate the same electrical membrane potential when compared to mitochondria from other tissues [27–29], which could constitute an adjustment of mammalian testis to low O_2 pressure in the seminiferous tubules [30], and stresses the importance of using testicular mitochondria as models when assessing the effect of compounds/procedures with possible impact on testicular physiology.

Mitochondrial respiratory and phosphorylative function were also correlated with the rat reproductive cycle, being depressed in both young and older animals and, as shown both by increase in state 3 respiration, ADP/O ratio, ADP-induced depolarization and $\Delta\Psi$, increased in adult animals. The lower respiratory function found in young animals could be explained by an increased spermatogonia/spermatocyte ratio

[31], which would mean that the less active spermatogonia mitochondria are more prevalent. In opposition, both adult and older animals should have lower spermatogonia/spermatocyte [31] ratios, thus containing more active mitochondria. However, it is clear that mitochondria from older animals are less functional, and this cannot be explained by the type of mitochondria, but rather by age-induced altered mitochondrial function. Previous studies observed that the activity of most oxidative phosphorylation complexes decrease with age [32], but the functional relevance of these findings in intact mitochondria had not been established. Although a declining mitochondrial function in older animals was observed, there was also evidence of the triggering of protective mechanisms, namely the increase in mitochondrial UCP2 content and function (as assessed by LA stimulation and GDP inhibition), which can promote proton leak, and attenuate ROS levels by causing a controlled decrease in mitochondrial membrane potential [8–11,16,33]. However, other mechanisms, such as the possible role of ANT, cannot be discarded. Further studies should also evaluate whether different types of testicular cells respond differently to aging at this level.

From the data obtained during proton leak measurements, several interesting questions can be raised. First, the older animals present a higher RCR and a slightly lower state 4 respiration. The results found for UCP2 protein levels and proton leak would predict that the older rats would have a decreased membrane potential and increased state 4 respiration. This is a very curious phenomenon that is not unique to our system. In

fact, Herlein et al. [34] recently demonstrated that heart and muscle mitochondria from diabetic animals were actually more coupled compared to controls despite an approximate 2–4 fold increase in uncoupling protein-3 content, which demonstrates that expression of UCPs, respiration and proton leak are not completely inter-connected. On the other hand, having a higher expression of UCP2 does not entirely correlate into increased proton leak, since different co-factors including superoxide anion, hydroxynonenal, and other alkenals or their analogues appear to be required for full UCP activity [35]. Also, it is important to note that titration assays for measuring proton leak were performed with oligomycin present in the media, which act to prevent proton leak through the ATP synthase and thus exclude this contribution from the overall proton backflux into the matrix. With the data obtained in the absence of oligomycin (states 3 and 4 rate measurements) and in its presence (proton leak titrations), it appears that ATP synthase-independent passive proton flux through the lipid membrane (which can be UCP-related) is clearly higher in the older animals.

A second important aspect regards the lack of effect of LA on the protein leak in the younger animals despite the presence of UCP2. Published data appears to suggest that both free fatty acid and superoxide anion cause UCP-mediated uncoupling [36]. In our case, we hypothesize that LA per se was not enough to induce visible uncoupling in the younger animals because (a) the amount of UCP was not enough for a visible effect on respiration/membrane potential or (b) additional co-factors such as superoxide anion are absent in the younger but present in the older animals. In fact, an attractive idea is that superoxide anion production by the respiratory chain is increased in the older animals, which can again explain the increased levels of UCP2 to counteract such effect.

Altogether, our results suggest that age-induced alterations in reproductive function may be caused by testicular mitochondrial dysfunction in agreement with the reported decreased fertility in aged individuals. This decrease is correlated with an increase in proton leak and UCP2 levels, which could possibly serve as a protective effect.

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