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1 **Mitochondrial threshold for H₂O₂ release in skeletal muscle of mammals**

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14

15 **Running title:** A universal threshold for H₂O₂ release in mammals

16

17

18 **Keywords:** Allometry; Bioenergetics; Oxidative phosphorylation; Skeletal muscle; Radical
19 oxygen species.

20

21 **Abstract**

22 The aim of the study was to evaluate the interplay between mitochondrial respiration and
23 H₂O₂ release during the transition from basal non-phosphorylating to maximal
24 phosphorylating states. We conducted a large scale comparative study of mitochondrial
25 oxygen consumption, H₂O₂ release and electron leak (% H₂O₂/O) in skeletal muscle
26 mitochondria isolated from mammal species ranging from 7 g to 500 kg. Mitochondrial fluxes
27 were measured at different steady state rates in presence of pyruvate, malate, and succinate as
28 respiratory substrates. Every species exhibited a burst of H₂O₂ release from skeletal muscle
29 mitochondria at a low rate of oxidative phosphorylation, essentially once the activity of
30 mitochondrial oxidative phosphorylation reached 26% of the maximal respiration. This
31 threshold for ROS generation thus appears as a general characteristic of skeletal muscle
32 mitochondria in mammals. These findings may have implications in situations promoting
33 succinate accumulation within mitochondria, such as ischemia or hypoxia.

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38 **1. Introduction**

39 In cells, ROS are continuously produced as a byproduct of the mitochondrial oxidative
40 phosphorylation process and more generally of aerobic metabolism (Kowaltowski et al., 2009;
41 Murphy, 2009). Because of its relationship with oxygen consumption, the link between ROS
42 production and metabolic rate has been extensively studied especially in the light of the free
43 radical theory of aging (Müller et al., 2007). Body mass dependence of respiration rates has
44 been largely reported in muscle and liver mitochondria from mammals and birds (Drabkin,
45 1950; Porter and Brand, 1993; 1995; Brand et al., 2003; Boël et al., 2019). An allometry of
46 mitochondrial oxidative activity is consistent with the lower activity and/or content of
47 mitochondrial oxidative enzymes reported in several tissues of large mammals compared with
48 small species (Drabkin, 1950; Fried and Tipton, 1953; Kunkel et al., 1956; Emmett and
49 Hochachka, 1981; Phillips et al., 2012). Consequently, all studies found a negative correlation
50 between the specific oxygen consumption and body mass at all levels of organization, from
51 whole body to cell and mitochondria. However, regarding mitochondrial ROS production and
52 body mass, similar pattern is often observed in different taxa (Sohal et al., 1989; 1990; Ku et
53 al., 1993; Sohal and Weindruch, 1996; Csiszar et al., 2012; Roussel et al., 2015), but not
54 systematically so (e.g. depending on the substrate used) (Lambert et al., 2007; Csiszar et al.,
55 2012; Montgomery et al., 2012). One part of the discrepancies between studies could be
56 explained by the fact that mitochondrial generation of ROS is a complex process which
57 results in the sum of rates from up to eleven mitochondrial sites involved in substrate
58 oxidation and electron transport (Brand, 2016).

59 Notwithstanding such a complex picture of mitochondrial ROS production, the main
60 driver of ROS generation and electron leak (the ratio ROS/O) remains the reduction state of
61 the electron donors in the mitochondrial electron transport system (Quinlan et al., 2013). It
62 has been clearly shown that the ROS generation is non-linearly sensitive to the redox state of
63 respiratory chain intermediates but also to the proton motive force value and so the energetic
64 state of mitochondria (Korshunov et al., 1997; Votyakova and Reynolds, 2001; Starkov and
65 Fiskum, 2003; Kikusato and Toyomizu, 2013; Cortassa et al., 2014; Quinlan et al., 2012;
66 Goncalves et al., 2015; Treberg et al., 2018). For instance, when mitochondria increase their
67 activity from basal respiration to maximal phosphorylating respiration, there is a 10%
68 decrease in membrane potential but a 10 fold reduction in ROS generation (Korshunov et al.,
69 1997; Starkov and Fiskum, 2003; Kikusato and Toyomizu, 2013). Thereby, ROS generation is
70 maximal at the non-phosphorylating state when the high value of the proton motive force
71 restricts the respiration rate and corresponding electron flux, leading to increased electron leak

72 and ROS formation (Treberg et al., 2018). In contrast, ROS generation decreases as the
73 intensity of phosphorylating activity increases, reaching a minimum at maximal
74 phosphorylating activity, due to a higher oxidized state of electron transporter intermediates
75 (Starkov and Fiskum, 2003; Barja, 2007; Quinlan et al., 2012; Goncalves et al., 2015; Roussel
76 et al., 2019).

77 So far, previous works on mitochondrial ROS production have essentially reported on
78 basal non-phosphorylating and maximal phosphorylating states. Such double assessment is
79 useful to compare species, populations or individuals facing environment variations but does
80 not fully reflect the interplay between oxygen consumption and ROS formation particularly at
81 intermediate activity levels. Indeed, mitochondria working in the cells are never in a basal
82 non-phosphorylating state and are only rarely running ATP synthesis at full maximal capacity.
83 ROS generation remains low until a threshold value of mitochondrial activity is reached, at
84 which point this production abruptly increases (Roussel et al., 2019). For instance,
85 mitochondrial oxidative phosphorylation activity of skeletal muscles in ducklings had to be
86 reduced by 60-70% before mitochondrial ROS production sharply increased (Roussel et al.,
87 2019). At some point, such a threshold value defines the frontier between two contrasted
88 functional states of mitochondria: i) one state characterized by a high ATP production and a
89 moderate ROS production, and ii) another state characterized by a low ATP production and a
90 high ROS production.

91 On the whole, the variation of mitochondrial thresholds for H₂O₂ generation is
92 globally unknown in animals. Therefore, the aim of the present study was to i) evaluate the
93 interplay between mitochondrial respiration and H₂O₂ release or electron leak (%H₂O₂/O)
94 during the transition from basal non-phosphorylating (LEAK) to maximal oxidative
95 phosphorylating (OXPHOS) states in different mammals' skeletal muscle mitochondria, ii)
96 estimate the range of variation of the resulting threshold values, i.e. the mitochondrial activity
97 level at which H₂O₂ release from mitochondria start to increase dramatically and iii) assess
98 the allometry of these parameters in mammal species ranging from 7 g to 500 kg.

99

100 **2. Materials and methods**

101 *2.1. Animals and tissue sampling*

102 All experiments were conducted in accordance with animal care guidelines and were
103 approved by the Ethics Committee of Lyon and the Ministère de la Recherche et de
104 l'Enseignement Supérieur. All animals used in the present study were males. African pygmy
105 mice (*Mus minutoides*) and house mice (*Mus musculus*) were obtained from laboratories

106 (respectively ISEM, Montpellier, France and LBBE, Lyon, France) and killed by cervical
107 dislocation. Syrian hamsters (*Mesocricetus auratus*) and black rats (*Rattus rattus*) were
108 obtained from laboratories (Chronobiotron, Strasbourg, France for *M. auratus*; Jardin
109 Zoologique de la Citadelle, Besançon, France for *R. rattus*) and were killed under isoflurane-
110 induced general anesthesia. Fresh tissues for bovines (*Bos taurus*) and sheep (*Ovis aries*)
111 were obtained from a local slaughter house (Cibevial, Corbas, France). Fresh tissues for all
112 other mammals were obtained from pest control (nutria - *Myocastor coypus* and muskrat -
113 *Ondatra zibethicus*), local farmers (rabbits – *Oryctolagus cuniculus*) or Fondation Pierre
114 Vérot at Saint-André-de-Corcy, France (boar – *Sus scrofa*).

115

116 2.2 Mitochondrial isolation

117 Fresh muscle tissue from every mammal studied was used for mitochondrial extraction.
118 Mitochondria were isolated from the hind-limb muscles for the two smallest species (*M.*
119 *minutoides* and *M. musculus*), from a mixture of gastrocnemius and quadriceps muscles for
120 medium-size species (*M. auratus*, *R. rattus*, *M. coypus*, *O. zibethicus*, *O. cuniculus*), and from
121 a sampling of the fore-leg muscles for the largest species (*O. aries*, *S. scrofa*, *B. taurus*). For
122 pygmy mice, skeletal muscle samples from three individuals were used for each
123 mitochondrial preparation. Muscle mitochondria were isolated in an ice-cold isolation buffer
124 (100 mM sucrose, 50 mM KCl, 5 mM EDTA, 50 mM Tris-base, and pH 7.4). Briefly, the
125 mitochondrial isolation procedure involved Potter homogenization, protease digestion and
126 differential centrifugations, with all steps at 4°C as described previously (Boël et al., 2019).
127 Finally, skeletal muscle mitochondrial populations were pelleted at 8,700×g (10 min) and the
128 protein concentration of the mitochondrial suspension was determined by a Biuret method
129 with bovine serum albumin as standard.

130

131 2.3 Mitochondrial oxygen consumption rate

132 Mitochondrial oxygen consumption rates were determined at 37°C in a respiratory buffer (120
133 mM KCl, 5 mM KH₂PO₄, 1 mM EGTA, 2 mM MgCl₂, 0.3% fatty acid-free bovine serum
134 albumin, 1.6 U/mL hexokinase, 20 mM glucose, and 3 mM HEPES, pH 7.4). Mitochondria
135 were energized by adding pyruvate/malate (5/2.5 mM) followed by 5 mM succinate and the
136 rates of basal respiration recorded. Active phosphorylating respiration was initiated by the
137 addition of 4, 10, 20, 100 and 500 μM ADP (Boël et al., 2019). The rate of oxygen
138 consumption was recorded in a closed glass cell fitted with a Clark oxygen electrode (Rank
139 Brothers Ltd, Cambridge, UK). Respiratory control ratio (RCR) was calculated as the ratio

140 between the rates of ADP (500 μ M)-induced phosphorylating respiration to basal non-
141 phosphorylating respiration.

142

143 *2.4 Mitochondrial reactive oxygen species production*

144 The rate of H₂O₂ released by isolated mitochondria was measured in the respiratory buffer at
145 37°C using a fluorometer (SFM-25, Kontron Instrument, Augsburg, Germany) at excitation
146 and emission wavelengths of 560 nm and 584 nm, respectively. The respiratory buffer was
147 supplemented with 5 U/mL horseradish peroxidase and 1 μ M Amplex Red fluorescent dye.
148 Respiratory substrates were successively added with 5 mM pyruvate/2.5 mM malate followed
149 by 5 mM succinate and the rates of H₂O₂ generation recorded. Thereafter, H₂O₂ generation
150 was titrated with the sequential addition of ADP at final concentrations of 4, 10, 20, 100, and
151 500 μ M. The fluorescent signal was finally calibrated using a standard curve obtained after
152 successive addition of H₂O₂ (up to 35 pmoles). Electron leak (%H₂O₂/O) was calculated as
153 the fraction (%) of the total electron flow that reduces oxygen into H₂O₂ instead of reaching
154 cytochrome-c oxidase to reduce oxygen into water (Rey et al., 2013).

155

156 *2.5. Statistical analyses*

157 The individual breakpoint values of oxygen consumption for significant H₂O₂ release have
158 been determined using the package ‘segmented’ of R core Team. Subsequently, the threshold
159 values for mitochondrial H₂O₂ release representing the percentage maximal oxidative
160 phosphorylation rate at which H₂O₂ release started to increase sharply were determined
161 individually. Following the determination of these parameters, all statistical analyses were
162 conducted on data corrected by the Phylogenetic Independent Contrast model in order to get
163 them phylogenetically independent. From the mammal phylogenetic super-tree proposed by
164 Fritz et al. (2009), a phylogenetic tree with the ten species chosen in the present study was
165 created. Thereafter, the data were corrected by the PIC function that applies the Phylogenetic
166 Independent Contrast method described by Felsenstein (1985), assuming that the evolution
167 model for life-history traits follows Brownian motion. After transforming body mass and
168 H₂O₂ release by the common logarithm (log₁₀), the relationship between these two variables
169 was analyzed with the linear model, with log₁₀(body mass) as a fixed factor. The same
170 statistical analyses were made to investigate the impact of body mass on oxygen consumption
171 rate, electron leak, or threshold value. Fits were performed on log₁₀-transformed data with the
172 linear model, with log₁₀(body mass) as fixed factor. For all these allometric relationships,
173 normality and homoscedasticity criteria for the model’s residues were checked by the

174 Shapiro-wilk normality test coupled to the Plot Diagnostics for a lm object. Statistical
175 analyses were done with the R Core Team (R Core Team, 2018) and the packages ‘phytools’,
176 ‘ape’, ‘multcomp’, ‘mvtnorm’, ‘survival’, ‘TH.data’, and ‘MASS’.

177

178 3. Results

179 3.1. Mitochondrial bioenergetics parameters

180 In the present protocol, mitochondria were energized with pyruvate/malate and the
181 resulting rates of oxygen consumption and H₂O₂ release were recorded before succinate was
182 added in the respiratory chamber. The basal non-phosphorylating respiration rate of skeletal
183 muscle mitochondria respiring on pyruvate/malate was significantly decreased with an
184 increasing body mass of mammals ($F_{(1,8)} = 10.38$; $p < 0.05$; Fig. 1). In contrast, the rate of
185 H₂O₂ release from mitochondria respiring on pyruvate/malate ($F_{(1,8)} = 2.55$; $p = 0.15$; Fig. 2)
186 and the corresponding % H₂O₂/O ($2.30 \times \text{mass}^{0.013}$; $r^2 = 0.011$; $F_{(1,8)} = 0.23$; $p = 0.64$; data not
187 shown) were not significantly related to body mass.

188 Table 1 describes several bioenergetics parameters of mitochondria isolated from the
189 skeletal muscle of the ten mammalian species and respiring on pyruvate/malate/succinate in
190 both basal and ADP-induced active phosphorylating state. The rates of mitochondrial oxygen
191 consumption significantly decreased with increasing body mass in both the basal state ($J_O =$
192 $157 \times \text{mass}^{-0.12}$; $r^2 = 0.85$; $F_{(1,8)} = 13.94$, $p < 0.01$) and in the active state, i.e. at maximum rates
193 of ATP synthesis ($J_O = 761 \times \text{mass}^{-0.10}$; $r^2 = 0.67$; $F_{(1,8)} = 7.77$, $p < 0.05$). The rate of H₂O₂
194 release was also related to body mass but only in the active state ($J_{H_2O_2} = 660 \times \text{mass}^{-0.091}$; $r^2 =$
195 0.33 ; $F_{(1,8)} = 6.38$, $p < 0.05$); the variation in the basal state failed to reach significance ($J_{H_2O_2}$
196 $= 4786 \times \text{mass}^{-0.073}$; $r^2 = 0.50$; $F_{(1,8)} = 4.61$, $p = 0.06$). The electron leak (%ROS/O) was not
197 correlated with body mass in either the basal or active states (%H₂O₂/O = $3.06 \times \text{mass}^{0.048}$; r^2
198 $= 0.31$; $F_{(1,8)} = 0.11$; $p = 0.74$ and % H₂O₂/O = $0.09 \times \text{mass}^{0.007}$; $r^2 = 0.003$; $F_{(1,8)} = 0.92$; $p =$
199 0.37 , in basal and active states respectively).

200

201 3.2. Relationships between H₂O₂ generation and oxygen consumption

202 Mitochondrial H₂O₂ release and oxygen consumption at different mitochondrial
203 phosphorylating steady-state rates are presented in Fig. 2. H₂O₂ generation and electron leak
204 (%H₂O₂/O) decreased non-linearly as mitochondrial oxidative phosphorylation activity
205 increased. From the maximum oxidative phosphorylation state to the basal state (from right to
206 left in Fig 2), the rate of H₂O₂ release and corresponding % H₂O₂/O increased slowly at high

207 oxygen consumption, up to a phosphorylating steady-state rate below which a sharp increase
208 in these parameters occurred. The rate of oxygen consumption at which H₂O₂ release start to
209 increase sharply (i.e. the breaking point of the non-linear curve shown in Fig. 2) was
210 determined for each individual of the ten mammal groups (see “Materials and Methods”
211 section for more details). Fig. 3 shows that the oxygen consumption rate at the breakpoint is
212 negatively correlated with body mass ($F_{(1,8)} = 30.87$; $p < 0.05$), but the correlation was not
213 quite significant when data were corrected for phylogenetic relatedness of species ($F_{(1,8)} =$
214 4.84 ; $p = 0.059$).

215

216 *3.3. Threshold values of mitochondrial ROS generation*

217 The above result indicates that small mammals trigger a sharp H₂O₂ release at higher oxygen
218 consumption rate than larger species. However, the respiratory activities of mitochondria from
219 small mammals were also significantly higher than in larger species (Table 1). To test whether
220 the higher mitochondrial oxidative activity of small mammals was responsible for the shift,
221 we expressed the oxygen consumption rate at the inflexion point as a percentage of maximal
222 oxidative rate. This fractional value defines a threshold in mitochondrial H₂O₂ release for
223 each of the ten mammal species, i.e. the percentage maximal oxidative rate at which H₂O₂
224 release starts to increase sharply (Roussel et al., 2019). Fig. 3 indicates that threshold values
225 were independent of body mass ($F_{(1,8)} = 0.0002$, $p = 0.99$). Regardless of the species studied,
226 the oxidative phosphorylation activity had to decrease by approximately 74% before a major
227 increase in H₂O₂ release occurred in skeletal muscle mitochondria.

228

229 **4. Discussion**

230 We followed the entire non-linear dynamic relationship between the intensity of oxidative
231 phosphorylation (i.e. from basal non-phosphorylating to maximal phosphorylating rates) and
232 mitochondrial H₂O₂ release or electron leak (%H₂O₂ /O) in ten mammal species. The present
233 results show a clear threshold for mitochondrial H₂O₂ release and electron leak (%H₂O₂ /O),
234 which remain low until a low level of oxidative phosphorylation activity is reached. Hence,
235 the activity of mitochondrial oxidative phosphorylation must reach 26% of the maximal
236 respiration before an H₂O₂ release burst occurs in skeletal muscle of mammals. The threshold
237 values for H₂O₂ release in mammals are slightly lower than those recently published in birds
238 (Roussel et al., 2019). Compared with the value of 26% in skeletal muscle mitochondria from
239 mammals (present study), the threshold values for H₂O₂ release in skeletal muscle
240 mitochondria from ducklings average 35% of the maximal oxidative phosphorylation activity,

241 ranging from 25% to 45% depending on nutritional status and muscle phenotypes (Roussel et
242 al., 2019). It has been clearly shown that the ROS generation is non-linearly sensitive to the
243 redox state of respiratory chain intermediates, which remain the main driver of ROS
244 generation (Quinlan et al., 2013). Thus, the kinetics of ROS release could be well explained
245 by the rate of electron leak to generate ROS, which decreases non-linearly during the
246 transition from basal to maximal phosphorylating activity due to an enhanced oxidized state
247 of electron transport chain intermediates (Quinlan et al., 2013; Starkov and Fiskum, 2003;
248 Cortassa et al., 2014). These studies further suggest that the non-linear relationships between
249 H₂O₂ release and mitochondrial activity might be a general feature of mammal and avian
250 mitochondrial functioning, i.e. a low release of H₂O₂ followed by a sharp increase once a
251 threshold of oxidative phosphorylation inactivity is reached.

252 Previous studies have reported a negative relationship between body mass and
253 mitochondrial ROS production in several mammalian tissues, including kidney, heart and
254 liver (Sohal et al., 1990; Ku et al., 1993; Lambert et al., 2007). The present data also show
255 that the rates of H₂O₂ release during basal non-phosphorylating respiration and maximal
256 phosphorylating respiration in skeletal muscle mitochondria respiring on
257 pyruvate/malate/succinate were on the whole negatively correlated with body mass of
258 mammals. Yet, mitochondrial respiration and H₂O₂ emission may vary according to the
259 muscle phenotypes, being respectively high and low in slow twitch/oxidative muscle and
260 conversely in fast twitch/glycolytic muscle (Anderson and Neuffer, 2006; Picard et al., 2008).
261 In the present study, we assumed that collected muscles were predominantly constituted of
262 mixed glycolytic/oxidative muscle fibers and representative of the whole musculature across
263 the ten species. Nevertheless, it has been reported that for a given skeletal muscle, the
264 activities of oxidative and glycolytic enzymes negatively and positively correlate to body
265 mass in mammals, respectively (Emmett and Hochachka, 1981). If the metabolic
266 (oxidative/glycolytic) and contractile (slow/fast twitch) phenotypes are related each other
267 across mammalian species, then we should expected the mitochondrial oxidative activity and
268 H₂O₂ emission in small mammals to be respectively greater (which was the case in the present
269 study) and lower (which was not the case in the present study) than in large mammals.
270 Although, we cannot exclude differences in the composition of skeletal muscle fiber types
271 between mammalian species, we suggest that this characteristic was not the main driver of the
272 negative relationship between body mass and mitochondrial H₂O₂ release reported here.

273 Further insight, it is important to keep in mind that we did not directly measure
274 mitochondrial ROS production, but the rate of H₂O₂ release from mitochondria, which is a

275 function of ROS producing and consuming processes within mitochondria. Allometry studies
276 on mitochondrial antioxidant systems are scarce, but there is some data suggesting no strong
277 relationship between antioxidant enzyme activities of different tissues and body mass in
278 mammals (Tolmasoff et al., 1980; Sohal et al., 1990; Stuart et al., 2013), including
279 mitochondrial superoxide dismutase (Page et al., 2010). Another study on four species (camel,
280 goat, rat and mouse) suggests that the activity of mitochondrial glutathione peroxidase might
281 be negatively related to body mass (Al-Otaiba et al., 2010). Although this result has to be
282 confirm with a larger number of species, it would be associated with a higher H₂O₂ release
283 from mitochondria of large mammals, which is not the case (present study) (Sohal et al.,
284 1990; Ku et al., 1993; Lambert et al., 2007). If mitochondrial antioxidant systems were the
285 main determinant, the rate of H₂O₂ release from mitochondria respiring on pyruvate/malate
286 should be as significantly correlated with body mass as in the presence of succinate, which
287 was not the case (present study). Even though, we cannot completely rule out the possible
288 influence of interspecific differences in the matrix H₂O₂ consuming processes, these processes
289 were not the main driver of the negative allometric relation reported in muscle mitochondria
290 respiring on pyruvate/malate/succinate (Table 1).

291 Of note, these interspecific correlations between body mass and mitochondrial ROS
292 release are essentially reported when mitochondria are respiring on succinate (Lambert et al.,
293 2007). Succinate oxidation generates ROS production both with a reverse electron transfer at
294 the quinol site of complex I and with a forward reaction at the flavin site of complex II
295 (Quinlan et al., 2012b). Interestingly, these two sites account for half of the total rate of H₂O₂
296 production in skeletal muscle mitochondria during rest (Goncalves et al., 2015). In the present
297 study, the addition of succinate in mitochondria respiring on pyruvate/malate resulted in a
298 drastic increase in H₂O₂ release, which was highly sensitive to the addition of ADP (Table 1).
299 Such high dependence of H₂O₂ release upon mitochondrial activity (basal non-
300 phosphorylating state *versus* active phosphorylating state) suggests that the reverse electron
301 transfer through complex I would be involved in the differences in the H₂O₂ release between
302 species (Zoccaroto et al., 2009; Goncalves et al., 2015). Indeed, the endergonic nature of the
303 reversed electron transport makes ROS generation by this process highly sensitive to proton
304 motive force (Korshunov et al., 1997; Votyakova and Reynolds, 2001; Starkov and Fiskum,
305 2003), and so to proton motive force consuming pathways such as ATP synthesis. These
306 observations thus suggest that the low level of H₂O₂ production in the larger mammals might
307 result from a low content of complex I (Lambert et al., 2010). The allometry of ROS
308 production in mammals might also be explained by changes in complex II level and/or

309 activity. This is suggested by an earlier study reporting a negative relationship between the
310 activity of succinate oxidase and body mass in several tissues of mammals (Fried and Tipton,
311 1953). Nevertheless, the ROS generation from complex II reaches a maximum at a low
312 concentration of succinate (<400 μ M), becoming very low at high concentrations (Quinlan et
313 al., 2012b), such as the 5 mM used in the present study. We thus postulate that the low level
314 of H₂O₂ production in mitochondria from the larger mammals would result mainly from a
315 lower activity of the reversed electron transport through complex I, a hypothesis that remains
316 to be investigated thoroughly.

317 The quinol site in complex I, which is active during reverse electron transport,
318 produces a significant rate of ROS in resting skeletal muscle, heart and cell lines from several
319 other tissues (Goncalves et al., 2015; Brand et al., 2016; Wong et al., 2019). This ROS
320 generation by the reverse electron transport in complex I is exacerbated with increasing
321 concentrations of succinate (Zoccaroto et al., 2009). Hence, this mechanism appears to be of
322 pathological and physiological importance in situations promoting succinate accumulation
323 within mitochondria, such as ischemia or hypoxia (Hochachka et al., 1975; Starkov, 2008;
324 Ralph et al., 2011). Succinate accumulates in several tissues of mammals, including heart,
325 liver, kidney and brain during ischemia (Chouchani et al., 2013), or during breath-hold dives
326 (Hochachka et al., 1975). During reperfusion, its rapid oxidation leads to a burst of ROS
327 production and ischemia-reperfusion injury (Dröse, 2013; Chouchani et al., 2016). In turn, the
328 ROS release from mitochondria could also serve as a signal, which may regulate cell
329 functions (Guzy and Schumaker, 2006; Stowe and Camara, 2009). For instance, ROS play a
330 vital role in the regulation of muscle metabolism and adaptation towards exercise (Morales-
331 Alamo and Calbet, 2016). ROS signaling may also be involved in the up-regulation of
332 antioxidant pathways and repair mechanisms of cellular damage reported in diving
333 vertebrates, limiting the oxidative stress associated with repeated prolonged apnea and re-
334 oxygenation cycles (Filho et al., 2002; Rey et al., 2016). Another interesting perspective is the
335 differential oxygen sensitivity of these two mitochondrial processes, i.e. the oxygen
336 respiration and the H₂O₂ release (Hoffman et al., 2007; Treberg et al., 2018). This oxygen
337 sensitivity of mitochondrial ROS generation varies between the respiratory substrates and the
338 ROS-generating sites within mitochondria (Hoffman and Brookes, 2009). For instance, it has
339 been shown that complex I remains the main source of ROS generation at low concentration
340 of oxygen (Hoffman and Brookes, 2009). Whether the oxygen sensitivity differs between
341 tissues and across mammalian species remain an open question.

342

343

344

345

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353

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356 acquisition and analysis: D.R., M.B., Y.V.; interpretation of data and validation: D.R., M.B.,
357 Y.V.

358

359

360 **Competing interests.** The authors declare that they have no competing financial interests.

361

362

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364

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517

518 **Table 1: Properties of muscle mitochondria isolated from mammals of different mass.**

Species (n)	Body weight (g)	Respiration rate		H ₂ O ₂ release		Electron leak	
		(nmol O ₂ .min ⁻¹ .mg protein ⁻¹)		(pmol H ₂ O ₂ .min ⁻¹ .mg protein ⁻¹)		(%H ₂ O ₂ /O)	
		Basal state	Active state	Basal state	Active state	Basal state	Active state
Pygmy mouse (10) (<i>M. minutoides</i>)	7.2 ± 0.4	141 ± 8	832 ± 49	5,062 ± 357	1,035 ± 76	3.69 ± 0.32	0.13 ± 0.02
House mouse (5) (<i>M. musculus</i>)	18.8 ± 0.6	98 ± 4	518 ± 16	4,598 ± 728	643 ± 178	4.75 ± 0.86	0.12 ± 0.03
Golden hamster (8) (<i>M. auratus</i>)	105.5 ± 2.6	95 ± 6	418 ± 14	4,067 ± 290	483 ± 24	4.29 ± 0.17	0.12 ± 0.01
Black rat (8) (<i>R. rattus</i>)	194.4 ± 7.2	88 ± 10	487 ± 50	2,452 ± 212	216 ± 34	2.92 ± 0.27	0.04 ± 0.00
Muskrat (1) (<i>O. zibethicus</i>)	1,600	85	443	2,091	206	2.5	0.05
Rabbit (6) (<i>O. cuniculus</i>)	3,200 ± 301	43 ± 3	218 ± 9	2,041 ± 190	209 ± 27	4.84 ± 0.49	0.10 ± 0.01
Nutria (3) (<i>M. coypus</i>)	5,150 ± 760	51 ± 2	222 ± 25	2,254 ± 418	181 ± 30	4.42 ± 0.83	0.08 ± 0.01
Sheep (6) (<i>O. aries</i>)	34,217 ± 1,633	42 ± 7	334 ± 60	1,834 ± 173	311 ± 92	4.97 ± 0.70	0.08 ± 0.01
Boar (5) (<i>S. scrofa</i>)	79,800 ± 4,420	52 ± 7	211 ± 18	3,422 ± 357	460 ± 36	6.94 ± 0.80	0.23 ± 0.03
Bovine (4) (<i>B. Taurus</i>)	486,300 ± 25,367	36 ± 5	260 ± 39	2,112 ± 226	242 ± 63	6.21 ± 0.85	0.09 ± 0.01

519 Values are means \pm SEM [number of independent mitochondrial preparations (n) in parentheses alongside the name of animals]. Basal state of
520 respiration was initiated by the addition of pyruvate/malate/succinate (5/2.5/5 mM) as respiratory substrates. Active state of respiration was
521 measured following the addition of 500 μ M ADP

522 **Figure legends**

523 **Figure 1:** Body mass dependence of basal respiration rate (J_O) and H_2O_2 release ($J_{H_2O_2}$) in
524 skeletal muscle mitochondria respiring on pyruvate/malate. Skeletal muscle mitochondria
525 were isolated from ten mammal species. Red symbols are two species taken to cover the
526 whole range of body mass (bovine with the highest mass and pygmy mice with the lowest
527 mass). Values are mean \pm s.d. from (n) independent mitochondrial preparations; (n) is given
528 alongside the name of the species

529

530 **Figure 2:** Relationships between oxidative phosphorylation activity and H_2O_2 release (A) or
531 electron leak (B). Mitochondria were isolated from skeletal muscle of ten mammal species
532 and were respiring on pyruvate/malate/succinate. Red symbols illustrate the relationships for
533 two species taken to cover the whole range of body mass (bovine with the highest mass and
534 pygmy mice with the lowest mass). Values are mean \pm s.d. from (n) independent
535 mitochondrial preparations; (n) is given alongside the name of the species

536

537 **Figure 3:** Body mass dependence of threshold H_2O_2 production values. Data are expressed as
538 the oxygen consumption rate at the breaking point of H_2O_2 release or as a percentage of
539 maximal oxidative phosphorylation activity (see text for more details) in skeletal muscle
540 mitochondria isolated from ten mammal species. A mixture of pyruvate/malate/succinate was
541 used as respiratory substrates. Red symbols are two species taken to cover the whole range of
542 body mass (bovine with the highest mass and pygmy mice with the lowest mass). Values are
543 mean \pm s.d. from (n) independent mitochondrial preparations; (n) is given in Fig. 2 alongside
544 the name of the species.

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