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1 **Liver mitochondrial coupling efficiency and its relationship to adenine nucleotide**
2 **translocase content: A comparative study among crocodiles, birds and mammals**

3
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14
15 **Keywords:** Oxidative phosphorylation, Liver, Adenine nucleotide translocase (ANT), Proton
16 leak, Archosaurs

19 **Summary**

20 The primary objective of this study was to assess whether adenine nucleotide translocase (ANT)
21 content could be associated with phylogenetic disparities in mitochondrial coupling efficiency,
22 within liver mitochondria obtained from rats, crocodiles, and ducklings. Our measurements
23 included mitochondrial membrane conductance, ANT content, and oxidative phosphorylation
24 fluxes at various steady-state rates. We observed significant variations in ANT content in liver
25 mitochondria across the three species, and these variations correlated with interspecific
26 differences in mitochondrial oxidative phosphorylation activity and coupling efficiency. These
27 findings expand upon previous research by highlighting the pivotal role of ANT in modulating
28 mitochondrial efficiency on an interspecific scale.

29

30 **1. Introduction**

31 Thyroid hormone levels, temperature, and body size represent major determinants of the
32 mitochondrial oxidative phosphorylation activity and coupling efficiency, deeply ingrained in
33 phylogenetics. For instance, mitochondria from endothermic vertebrates demonstrate higher
34 mitochondrial proton conductance compared to ectotherms (Brand et al., 1991; Brookes et al.,
35 1998; Hulbert et al., 2002; Polymeropoulos et al., 2017). This proton conductance activity,
36 which does not generate ATP, can lower mitochondrial coupling efficiency, i.e., the ATP/O
37 ratio, and consequently increase the energy expenditure for mitochondrial ATP synthesis. In
38 conjunction with greater mitochondrial oxidative and enzyme activities (Bennett, 1972;
39 Akhmerov, 1986; Paital and Samanta, 2013; Wiens et al., 2017; see however Berner, 1999),
40 these findings suggest that mitochondria from endotherms convert energy from reduced
41 coenzymes into ATP at a higher energy cost than ectotherms. However, it was reported that the
42 contribution of proton leak to the resting metabolic rate of hepatocytes is as high in endotherms
43 as it is in ectotherms (Brand et al., 1991; Stuart et al., 2001; Hulbert et al., 2002), suggesting

44 similar mitochondrial coupling efficiency, and ultimately, no substantial difference in the cost
45 of mitochondrial ATP synthesis in cells. Furthermore, the proportion of cellular oxygen
46 consumption allocated to mitochondrial proton leak (Porter and Brand, 1995; Else et al., 2004a),
47 as well as the maximal effective coupling efficiency of mitochondria (Boël et al., 2019; Boël et
48 al., 2023; Barbe et al., 2023), remains similar in birds and mammals regardless of their size,
49 despite the negative correlation between mitochondrial proton conductance negatively and
50 body mass in these endothermic species (Porter and Brand, 1993; Brand et al., 2003). All these
51 data indicate that phylogenetic or allometric differences in the magnitude of mitochondrial
52 proton conductance may not necessarily lead to alterations in mitochondrial coupling
53 efficiency, i.e., in the energy cost of ATP production.

54 Mitochondrial coupling efficiency can also be influenced by other characteristics of the
55 mitochondrial function capable of altering the proton motive force, thus the proton leak activity
56 across the inner mitochondrial membrane. These characteristics may include, among others,
57 oxidative capacity (Nogueira et al., 2001; Roussel et al., 2018) or the abundance of
58 mitochondrial anion carrier proteins such as adenine nucleotide translocase (ANT) (Schönfeld,
59 1990; Cadenas et al., 2000; Brand et al., 2005; Bertholet et al., 2019). For instance, previous
60 research has indicated that mitochondrial contents of cytochrome-c oxidase (e.g., cytochrome
61 a) and ANT contribute to interspecific variations in mitochondrial function (Guderley et al.,
62 2005; Hulbert et al., 2006). Expanding upon these observations, we examined whether ANT
63 content could be linked to phylogenetical differences in mitochondrial coupling efficiency, i.e.,
64 the ATP/O ratio, in liver mitochondria isolated from rats (*Rattus norvegicus*), crocodiles
65 (*Crocodylus niloticus*), and ducklings (*Cairina moschata*). We assessed the capacity for
66 succinate oxidation in these mitochondria, measuring their membrane proton conductance and
67 ANT content. Additionally, we quantified oxygen consumption, ATP synthesis, and the
68 resulting coupling efficiency (ATP/O ratios) at both maximal and submaximal oxidative

69 phosphorylation rates. Our findings reveal that liver mitochondria from crocodiles display high
70 coupling efficiency and low inner membrane proton conductance compared to rat mitochondria.
71 Conversely, rats demonstrate high coupling efficiency and similar inner membrane proton
72 conductance when compared to duckling mitochondria. These interspecific variations in
73 mitochondrial coupling efficiency can be attributed to differences in mitochondrial ANT
74 content.

75

76 **2. Materials and methods**

77 *2.1. Animals*

78 In the present work, animals of similar body weight were used to limit confounding allometric
79 effects on liver mitochondrial bioenergetics as described in endotherms and ectotherms (Porter
80 and Brand, 1993; Brand et al., 2003; Roussel et al., 2015; Boël et al., 2023). Nile crocodiles of
81 approximately 12-week old (*Crocodylus niloticus*) were captive-bred juveniles kindly donated
82 by a crocodile farm (Pierrelatte, France). Crocodiles were chosen because their mitochondria
83 can be assayed at high temperature (Guderley and Seebacher, 2011). They were maintained at
84 28-30°C for at least 2 weeks prior to the experiments, and were fed on meat. Muscovy ducklings
85 of ~ 2-week old (*Cairina moschata*) were obtained at the age of 1 day from a commercial
86 stockbreeder (Ecllosion Grimaud Frères, Roussay, France), maintained at thermoneutrality for
87 at least 10 days, and fed *ad libitum*. Sprague-Dawley rats (~ 6-week old) were housed in cages
88 at ~25°C and fed *ad libitum*. All animals had access to water *ad libitum*.

89

90 *2.2. Mitochondrial isolation*

91 Liver mitochondria were isolated at 4°C by differential centrifugation as previously described
92 (Salin et al., 2010; Roussel et al., 2015). We focus our study on liver because that organ
93 performs identical physiological function and contributes to a significant part of the resting

94 metabolic rate in the three species. In addition, a large amount of mitochondria can be isolated
95 from this organ, which was necessary to perform the different biochemical protocols described
96 below. Liver was rapidly dissected, weighed, and homogenized with a Potter-Elvehjem
97 homogenizer (3 passages) in ice-cold isolation buffer (250 mM sucrose, 1 mM EGTA, 20 mM
98 Tris-base, and pH 7.3 at 4°C). For crocodile, liver samples from two individuals were pooled
99 for each mitochondrial preparation. The liver homogenate was centrifuged at 800×g for 10 min.
100 The supernatant was centrifuged at 1000×g for 10 min, filtered and re-centrifuged at 8,700×g
101 for 10 min to pellet mitochondria. The mitochondrial pellet was re-suspended in ice-cold
102 isolation buffer and re-centrifuged twice at 8,700×g for 10 min. The final pellet was re-
103 suspended in 100 µL isolated buffer and protein content of mitochondrial suspension was
104 assayed at 540 nm using the biuret method with bovine serum albumin used as standard. Of
105 note, the mitochondrial preparation from crocodile liver contained a dark pigment which
106 absorbs at 540 nm. Therefore, for each sample, the absorbance of the same volume of
107 mitochondria assayed in Biuret solution without copper sulfate (i.e. 0.6% K-Na-tartrate and 3%
108 NaOH) was used as a blank.

109

110 *2.3. Mitochondrial oxidative phosphorylation activity and efficiency*

111 Mitochondrial oxidative phosphorylation efficiency was assessed at 37°C by measuring the
112 rates of oxygen consumption and ATP synthesis in a respiratory buffer (120 mM KCl, 5 mM
113 KH₂PO₄, 1 mM EGTA, 2 mM MgCl₂, 0.3 % fatty acid-free bovine serum albumin, 3 mM
114 HEPES, pH 7.4) supplemented with 1.5 U/ml hexokinase, 20 mM glucose and 5 µM rotenone.
115 Respiration was initiated by adding succinate (5 mM), then mitochondrial ATP synthesis was
116 initiated by the addition of 500µM, 100 µM, 25 µM, 10µM or 5 µM ADP. Respiration rates
117 were measured in a thermostatically regulated glass chamber containing a Clarke-type oxygen
118 electrode (Rank Brothers Ltd, Cambridge, UK). After recording the phosphorylating respiration

119 rate for at least 2 min, four 300 μ L samples of mitochondrial suspension were withdrawn from
120 the suspension every 2 min and were quenched in 200 μ L perchloric acid solution consisting of
121 10% HClO₄ and 25 mM EDTA. After centrifugation (15,000 g, 5 min) and neutralization of the
122 resulting supernatant with a KOH solution (2 M KOH, 0.3 M MOPS), ATP production was
123 determined from the glucose-6-phosphate content of samples as described previously (Salin et
124 al., 2010; Roussel et al., 2015). To ensure that mitochondrial rates were specific to
125 mitochondrial ATP synthase activity, we determined oxygen consumption and ATP synthesis
126 rates in the presence of oligomycin (2 μ g/mL). These values were taken into account to calculate
127 the rate of mitochondrial ATP synthesis. The maximal activity of the electron transport system
128 was measured as the carbonyl-cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP)-induced
129 maximal respiration rate in mitochondria respiring on succinate (5 mM) in the presence of 5
130 μ M rotenone and 2 μ g/mL oligomycin.

131

132 *2.4. ANT content of mitochondria*

133 The ANT content was determined by titrating the rate of phosphorylating respiration with
134 increasing concentrations of carboxyatractyloside, an irreversible inhibitor of ANT activity.
135 Mitochondria (1.35 \pm 0.17 mg/mL for crocodile, 0.76 \pm 0.11 mg/mL for rat, and 0.50 \pm 0.01
136 mg/mL for ducklings) were incubated in respiratory buffer supplemented with 1.5 U/mL
137 hexokinase, 20 mM glucose, and energized with 5 mM succinate in the presence of 5 μ M
138 rotenone. After 3-5 minutes, 100 μ M ADP was added to initiate phosphorylating respiration.

139 The mitochondrial content of ANT protein was determined by the amount of
140 carboxyatractyloside required to completely inhibit the rate of phosphorylating respiration
141 (Schönfeld, 1990; Roussel et al., 2000).

142

143 *2.5. Mitochondrial membrane potential*

144 Mitochondrial respiration and membrane potential were measured at 37°C in respiratory buffer
145 supplemented with 5 µM rotenone, 2.5 µg/mL oligomycin, 60 ng/mL nigericin, 2 µM triphenyl-
146 methyl-phosphonium (TPMP⁺). The TPMP⁺ electrode was calibrated by four sequential 0.5 µM
147 additions of TPMP⁺, then 5 mM succinate was added to start the reaction. The kinetic response
148 of proton leak to proton-motive force was determined by titration of oxygen consumption with
149 malonate, up to 10 mM (Salin et al., 2010; Roussel et al., 2018). Membrane potentials were
150 calculated as previously described by Brand (1995), assuming a TPMP⁺-binding correction of
151 0.42, 0.54 and 0.66 (µL/mg of protein)⁻¹ for liver mitochondria of rats, ducklings and crocodiles,
152 respectively (Brookes et al., 1998; Hulbert et al., 2002; Brand et al., 2003).

153

154 *2.6. Statistical analysis*

155 Data were tested with analysis of variance (ANOVA) for independent values, followed by
156 protected least significance tests (Staview v4.5 statistical software). The relation between some
157 of the mitochondrial parameters and the mitochondrial content of ANT was compared using
158 linear regression. Data are reported as means ± S.E.M. with significance considered at $p < 0.05$.

159

160 **3. Results and Discussion**

161 Table 1 provides information on the body weight and liver mass of the animals. As
162 expected (Else and Hulbert, 1985), the liver mass relative to body weight was lower in
163 crocodiles than in rats or ducklings. Fig. 1A shows that the steady state rates of ADP-induced
164 phosphorylating oxygen consumption were significantly different between species, being the
165 lowest in crocodiles and the highest in ducklings. These disparities in the phosphorylating
166 respiration among species were associated with variations in the maximal respiratory capacity
167 of mitochondria, as determined by FCCP-induced maximal uncoupled respiration (Fig. 1B).
168 These findings align with comparative studies that have reported lower mitochondrial activity

169 in ectotherms compared to endotherms (Akhmerov, 1986; Hulbert et al., 2002; Guderley et al.,
170 2005; Paital and Samanta, 2013; Wiens et al., 2017; see however Berner, 1999). Thyroid
171 hormone level and membrane lipid composition are among mechanisms well known to cause
172 variation in metabolic rate and mitochondrial activity (Brookes et al., 1998; Nogueira et al.,
173 2002; Brand et al., 2003). Hence, a lower level of thyroid hormones and/or a lower proportion
174 of docosahexanoic acid (C22:6n-3) within membrane are mechanisms able to cause lower
175 mitochondrial activity in crocodiles compared to endotherms. Interestingly, these mechanisms
176 are, more generally, involved in the evolution of endothermy (Else et al., 2004b; Little and
177 Seebacher, 2014).

178 Fig. 2A reports the linear relationship between the rates of ATP synthesis and oxygen
179 consumption in liver mitochondria from crocodiles, rats, and ducklings. The maximal rates of
180 ATP synthesis and the corresponding oxygen consumption (i.e., the highest points on the right
181 side of the linear relationships in Fig. 2A) were lowest in crocodiles and highest in ducklings,
182 with the order being crocodile < rat < duckling. Crocodile mitochondria exhibited
183 approximately 2.5 and 3.5 times lower rates of ATP synthesis and oxygen consumption,
184 respectively, compared to rat and duckling mitochondria. The slopes of the linear relationships
185 did not show significant differences between species ($P/O_{\text{crocodile}} = 1.54 \pm 0.12$; $P/O_{\text{rat}} = 1.34 \pm$
186 0.06 ; $P/O_{\text{duckling}} = 1.36 \pm 0.04$; $p=0.18$), indicating that these relationships were parallel.
187 However, the rates of non-phosphorylating respiration (the points where the curves intersect the
188 x -axis) significantly varies among the three species (crocodile < rat < duckling; see Table 1 and
189 Fig. 2A). Consequently, the linear curves for crocodile mitochondria and duckling
190 mitochondria were notably shifted to the left and right, respectively, compared to the curve for
191 rat mitochondria (Fig. 2A). This suggests that at any given steady-state rate of ATP production,
192 crocodile liver mitochondria consumed less oxygen, whereas duckling mitochondria required
193 more oxygen than rat mitochondria. This difference is better illustrated in Fig. 1B, which shows

194 that for any given rate of oxygen uptake, the ATP/O of liver mitochondria was higher in
195 crocodiles and lower in ducklings compared to rats. Overall, crocodile liver mitochondria
196 exhibited greater efficiency, whereas duckling mitochondria displayed lower efficiency than rat
197 mitochondria (Fig. 2). In the present work, we used juveniles that were of the same body mass
198 but not of the same age due to growth rate differences between species. In juvenile brown trout,
199 it has been shown that individuals with a higher growth performance also exhibited a higher
200 coupling efficiency in their liver mitochondria (Salin et al., 2019). In common frog, an
201 intraspecies comparison of populations having large differences in body mass at the same age
202 found that frogs with high growth rates and large body sizes exhibited higher mitochondrial
203 coupling efficiency compared to the smaller frogs (Salin et al., 2012). In contrast, differences
204 in growth performance in broilers were not associated with mitochondrial efficiency variations
205 in skeletal muscle and liver (Bottje et al., 2002). In rat, values of thermodynamic efficiency of
206 liver mitochondrial oxidative phosphorylation did not seem to vary in 40- to 180-day-old rats
207 (Lionetti et al., 2004; Iossa et al., 1998). In endotherms. Altogether these studies suggest that
208 in the present study, the difference in stage of development between juveniles would not fully
209 explain differences in mitochondrial coupling efficiency between the three species.

210 As shown in Fig. 2B, the effective ATP/O ratio displays a non-linear relationship with
211 mitochondrial oxidative activity. It increases sharply as the phosphorylation rate rises before
212 leveling off and reaching a maximum. This non-linear relationship results from the fact that
213 proton leak pathways and ATP synthesis compete for the same driving force, known as the
214 proton motive force (Brand, 2005). Consequently, during the dynamic transition from the basal
215 non-phosphorylating state to the maximal phosphorylating state (Fig. 2), proton leak
216 significantly decreases as oxidative phosphorylation increases, leading to better mitochondrial
217 efficiency (ATP/O ratio). Therefore, there is no fixed value for the ATP/O ratio that can
218 describe effective coupling efficiency, as it closely depends on the degree of oxidative

219 phosphorylation activity. Nonetheless, this non-linear mitochondrial property can be quantified
220 by fitting the relationships between ATP/O ratios and the rates of oxygen consumption with a
221 mono-exponential function $[ATP/O = ATP/O_{max} \times (1 - e^{-mtEC \times (J_O - J_{O_{basal}})})]$; Mogensen and Sahlin,
222 2005], where J_O represents the phosphorylating oxygen consumption rate, $J_{O_{basal}}$ is the non-
223 phosphorylating oxygen consumption rate (the intersection point with the x -axis), and “mtEC”
224 is the gradient of the non-linear curve. This factor has been termed the mitochondrial efficiency
225 channeling (mtEC) factor because it describes how rapidly mitochondrial coupling efficiency
226 reaches its maximum. The mtEC factor value can then be used as a single mitochondrial
227 parameter to describe the overall variation in effective coupling efficiency with changes in
228 mitochondrial activity or power (Boël et al., 2019). In the present study, mtEC was highest in
229 crocodiles and lowest in ducklings (Fig. 2B inset). These results show that the increase in
230 effective ATP/O ratios was steeper, indicating better coupling in crocodiles than in ducklings,
231 with rat mitochondria falling in between.

232 Differences in mitochondrial proton conductance may contribute to variations in
233 mitochondrial coupling efficiency (Beavis and Lehninger, 1986; Brand et al., 1991; Nogueira
234 et al., 2002). This is supported by the fact that the shifts in the relationships depicted in Fig. 2
235 seem to be driven by differences in the rates of non-phosphorylating respiration, a respiratory
236 state characterized by the absence of ATP synthesis, a high proton motive force, and maximal
237 proton leakage (Brand and Nicholls, 2011). In the present study, the inner membrane proton
238 conductance of liver mitochondria was higher in rats than in crocodiles (Fig. 1S.A), but there
239 was no significant difference between rats and ducklings (Fig. 1S.B). At comparable potential
240 values, the permeability of the inner membrane to protons was approximately three times
241 greater in rats than in crocodiles. These results align with previous studies that have shown that
242 mitochondria from small reptile species (< 2-3 kg) exhibit lower proton conductance than
243 mitochondria from mammals of similar body weight (Brand et al., 1991; Brookes et al., 1998).

244 It is worth noting that these differences between reptiles and mammals are less pronounced
245 when comparing larger species (Hulbert et al., 2002; Polymeropoulos et al., 2017). Further
246 insight, the higher proton conductance in rat mitochondria was associated with a lower value
247 of effective ATP/O ratio at maximal phosphorylating activities (the highest points to the right
248 of the non-linear curve in Fig. 2B) than in crocodile mitochondria ($ATP/O_{Rat} = 1.14 \pm 0.05$
249 versus $ATP/O_{Crocodile} = 1.26 \pm 0.11$). Although the difference did not reach significance, this
250 result aligns with previous studies showing that agents that increase mitochondrial proton
251 conductance, such as thyroid hormones or mitochondrial uncouplers, also decrease the effective
252 coupling efficiency at maximal phosphorylating activity (Beavis and Lehninger, 1986;
253 Nogueira et al., 2001; Nogueira et al., 2002). Taken together, these observations suggest that
254 differences in membrane conductance to protons may partly explain the differences in
255 mitochondrial efficiency between crocodiles and rats. In contrast to ectothermic species,
256 mitochondrial proton conductance is less distinct between bird and mammal species (Brookes
257 et al., 1998; Brown et al., 2009; Polypopoulos et al., 2017). In the present study, the
258 mitochondrial inner membrane proton conductance was not different between rats and
259 ducklings (Fig. 1S.B). This result implies that difference in coupling efficiency between rats
260 and ducklings was mainly linked to a difference in the mitochondrial oxidative capacity. This
261 hypothesis is largely supported by previous studies showing that mitochondria with a lower
262 oxidative phosphorylation capacity often exhibit a higher coupling efficiency, such as in the
263 presence of low dose of cyanide or nitric oxide or following caloric restriction (Zangarelli et
264 al., 2006; Clerc et al., 2007; Roussel et al., 2018).

265 The mitochondrial coupling efficiency (ATP/O ratio) is not only negatively controlled
266 by proton leak, but also by its sensitivity to changes in phosphorylation reactions, which exert
267 roughly equal positive control. These reactions encompass several steps, including those
268 involving the ATP synthase, the phosphate carrier, and the ANT (Brand et al., 1993; Roussel et

269 al., 2004). ANT is a major transport protein located in the inner mitochondrial membrane,
270 supplying the cell with ATP during periods of high energy demand. Previous research has
271 reported a correlation between ANT content correlates and interspecific variations in
272 mitochondrial oxidative capacity in muscle mitochondria (Guderley et al., 2005). In the present
273 study, the ANT content in liver mitochondria differed significantly among the three species,
274 with the lowest content observed in crocodiles and the highest in ducklings (crocodile < rat <
275 duckling; see Fig. 3).

276 While ANT's primary role is to export ATP from the mitochondrial matrix to the cytosol
277 while importing cytosolic ADP when cellular energy needs are high, it can also catalyze proton
278 leakage in the resting state when cellular energy needs are low (Bertholet et al., 2019). In
279 addition, ANT can also facilitate fatty acid- or AMP-induced uncoupling in resting
280 mitochondria (Schönfeld, 1990; Roussel et al., 2000; Cadenas et al., 2000; [Bottje et al., 2009](#);
281 [Guderley and Seebacher, 2011](#)). It has also been reported that ANT may contribute from half
282 to two-thirds of the basal proton conductance in resting mitochondria (Brand et al., 2005). In
283 the present study, the basal non-phosphorylating state of respiration, measured in the presence
284 of oligomycin, was sensitive to CAT addition (Table 1). Based on these data, we can estimate
285 that ANT may contribute to more than half of the basal non-phosphorylating respiration rate in
286 rats, which was significantly higher than the values calculated in crocodiles and ducklings
287 (Table 1). The basal non-phosphorylating respiration serve as a measure of the maximal proton
288 leakage across the inner mitochondrial membrane. Hence, these data suggest that differences in
289 mitochondrial ANT content may drive part of the differences in coupling efficiency between
290 species. This hypothesis is reinforced by the fact that, when mitochondrial fluxes (ATP
291 synthesis and corresponding oxygen uptake) are normalized by mitochondrial ANT content,
292 the relationships between the rates of ATP synthesis and oxygen consumption from crocodiles,
293 rats and ducklings overlap (Fig. 4). [It must be kept in mind that the present results are based on](#)

294 succinate oxidation. Previous studies have shown that ANT is a major controlling step of the
295 oxygen-consumption flux in rat liver mitochondria respiring on succinate (Groen et al., 1982;
296 Kunz et al., 1988; Rossignol et al., 2000). In contrast, it appears that ANT becomes a minor
297 controlling step of the phosphorylating respiration in mitochondria respiring on
298 pyruvate/malate (Rossignol et al., 2000). Whether differences in mitochondrial ANT contents
299 between species would have mediated differences in coupling efficiency of mitochondria
300 respiring on NADH-linked substrates remain an open question that deserves further
301 experimental testing.

302 In conclusion, these results offer a comprehensive insight into the variations in oxidative
303 phosphorylation activity, membrane proton conductance, and coupling efficiency among the
304 major taxonomic groups. They also expand upon previous research by highlighting the
305 significant role of ANT in regulating mitochondrial efficiency across different. ~~The question of~~
306 ~~the evolutionary pressures influencing ANT and the precise mechanisms through which it~~
307 ~~influences differences in mitochondrial energetics among major taxonomic groups remains~~
308 ~~unanswered but presents an intriguing avenue for further exploration in this field.~~

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461 21-month-old rats: a mitochondria-mediated pathway. *FASEB J.* 20, 2439-2450.

463 **Figure Legends**

464 **Figure 1:** Mitochondrial oxidative capacity of liver mitochondria. (A) The rate of oxygen
465 consumption as a function of ADP concentration from crocodiles (grey diamonds), rats (white
466 circles), and ducklings (black triangles). (B) Maximal respiratory capacity measured in the
467 presence of FCCP from crocodiles (grey bar), rats (white bar), and ducklings (black bar). Values
468 represent means \pm s.e.m. from (n) independent mitochondrial preparations; (n) is provided
469 alongside the species name. Overall kinetics (A) or bars (B) with different superscript letters
470 are significantly different at $P<0.05$.

471

472 **Figure 2:** Mitochondrial oxidative phosphorylation activity (A) and efficiency (B) in liver
473 mitochondria from crocodiles (grey diamonds), rats (white circles), and ducklings (black
474 triangles). Inset of Panel B: Mitochondrial efficiency channel factor (mtEC; details in text).
475 Values represent means \pm s.e.m. from (n) independent mitochondrial preparations; (n) is
476 provided alongside the species name. Data with different superscript letters are significantly
477 different at $P<0.05$; In panels A, lower case and upper case letters compare rates of oxygen
478 consumption and ATP synthesis, respectively. † $P<0.05$, indicates significant overall
479 differences in kinetics (see text for details).

480

481 **Figure 3:** ANT content in liver mitochondria from crocodiles (grey diamonds), rats (white
482 circles), and ducklings (black triangles). (A) Phosphorylating respiration was titrated by
483 successive additions of carboxyatractyloside (CAT) until maximal inhibition of mitochondrial
484 respiration was achieved. ANT content was determined by the amount of CAT required for
485 complete inhibition of phosphorylating respiration. (B) ANT contents measured through CAT
486 titration. Data with different superscript letters are significantly different at $P<0.05$. Values

487 represent means \pm s.e.m. from (n) independent mitochondrial preparations; (n) is indicated
488 alongside the species name.

489

490 **Figure 4:** Mitochondrial oxidative phosphorylation efficiency in liver mitochondria from
491 crocodiles (grey diamonds), rats (white circles), and ducklings (black triangles). The rates of
492 ATP synthesis and oxygen consumption from Fig. 1A were normalized by the corresponding
493 ANT contents of mitochondria from Fig. 2B. Values represent means \pm s.e.m. from (n)
494 independent mitochondrial preparations; (n) is indicated alongside the species name. * and †
495 indicate that the maximal rates of ATP synthesis and oxygen consumption in rats were
496 significantly different from crocodiles and ducklings ($P < 0.05$).

497

498 **Supplemental Figure 1:** Basal proton conductance of liver mitochondria from crocodiles (grey
499 diamonds), rats (white circles), and ducklings (black triangles). Proton conductance was
500 assessed in medium containing rotenone, succinate, oligomycin and nigericin, and membrane
501 potential and respiration rate were titrated with malonate as described in the “Materials and
502 Methods” section. Proton leakage rate was calculated from respiration rate by assuming a H^+/O
503 ratio of 6 for succinate oxidation. Values represent means \pm s.e.m. from (n) independent
504 mitochondrial preparations; (n) is given alongside the species name.

505

506 Table 1: Body weight, liver mass and properties of liver mitochondria

	Crocodile (5)	Rat (6)	Duckling (6)
Body weight (g)	143 ± 13 ^a	194 ± 9 ^b	189 ± 14 ^b
Liver (g)	2.9 ± 0.3 ^a	7.4 ± 0.7 ^b	8.5 ± 0.9 ^b
Liver (g/100 BW)	2.0 ± 0.1 ^a	3.8 ± 0.3 ^b	4.6 ± 0.4 ^b
Mitochondrial oxygen consumption (nmol O/min.mg protein)			
Non-phosphorylating rate	10.6 ± 2.3 ^a	19.8 ± 2.7 ^b	30.1 ± 3.6 ^c
CAT-insensitive non-phosphorylating rate ¹	7.5 ± 1.6 ^a	7.9 ± 1.7 ^a	22.3 ± 2.4 ^b
CAT-sensitive respiration (% of non-phosphorylating state) ¹	15 ± 3 ^a	54 ± 7 ^b	21 ± 5 ^a

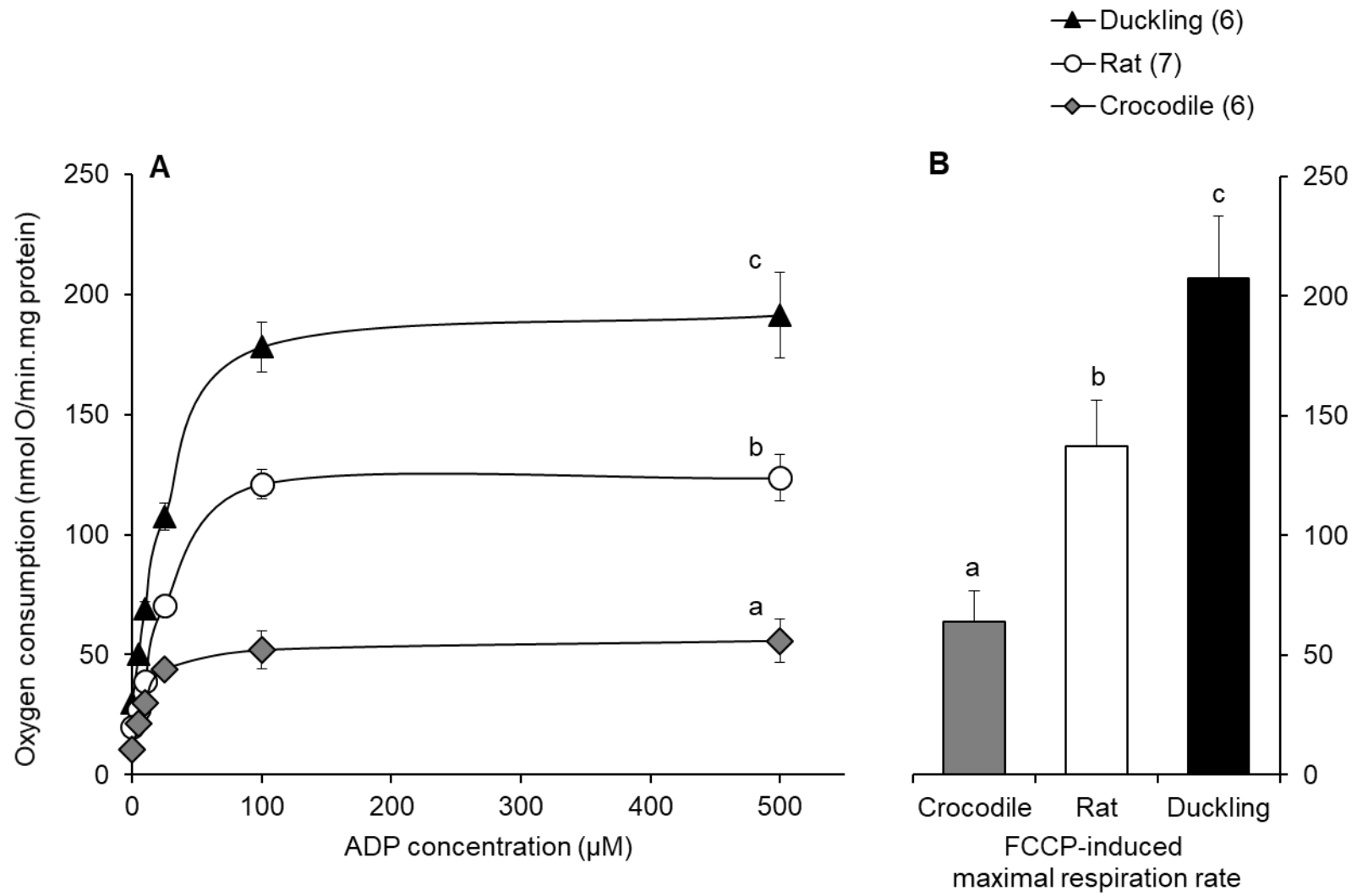
507 Values are means ± s.em. from (n) independent mitochondrial preparations; (n) is provided alongside the species name. Data with different
 508 superscript letters are significantly different at $P < 0.05$.

509

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511
512

Figure 1



513

Figure 2

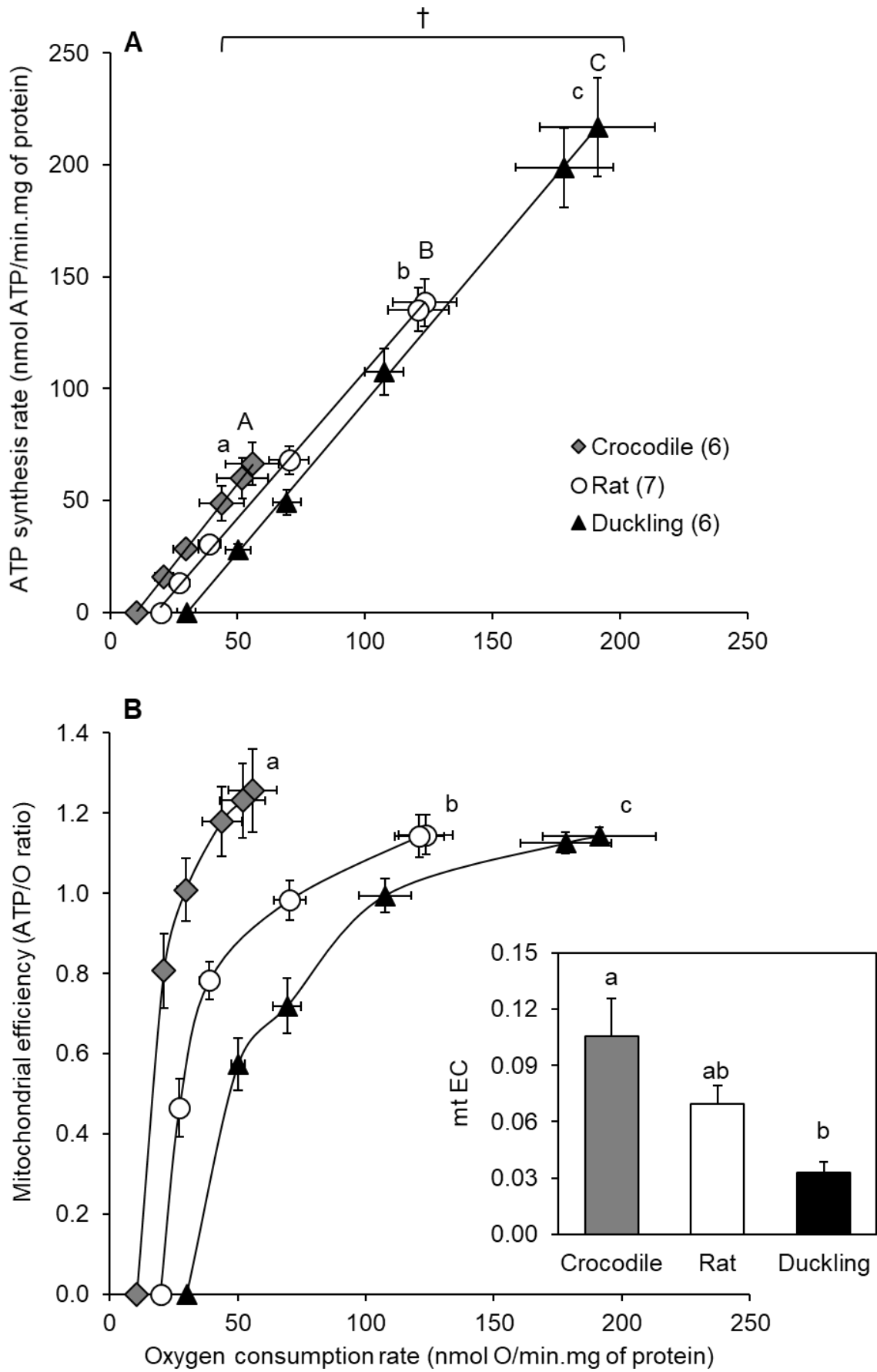


Figure 3

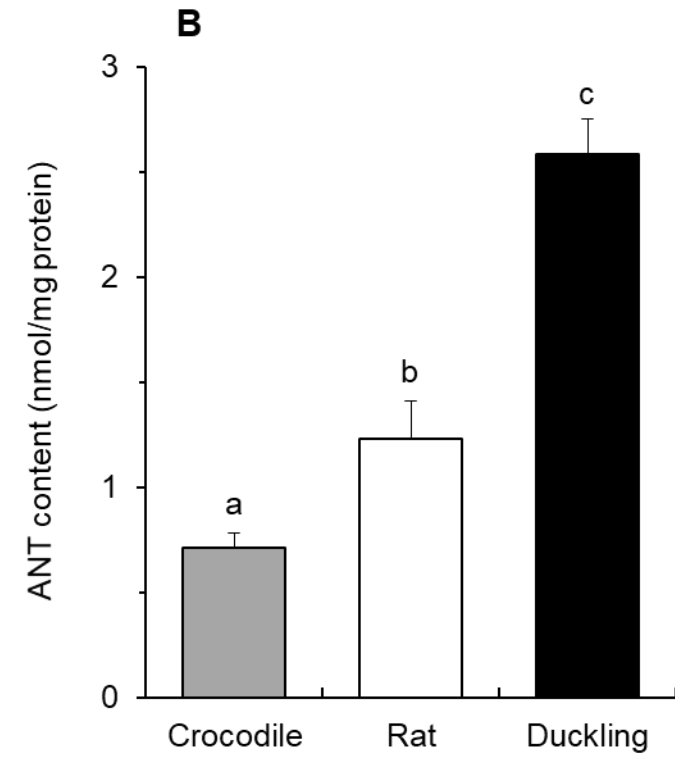
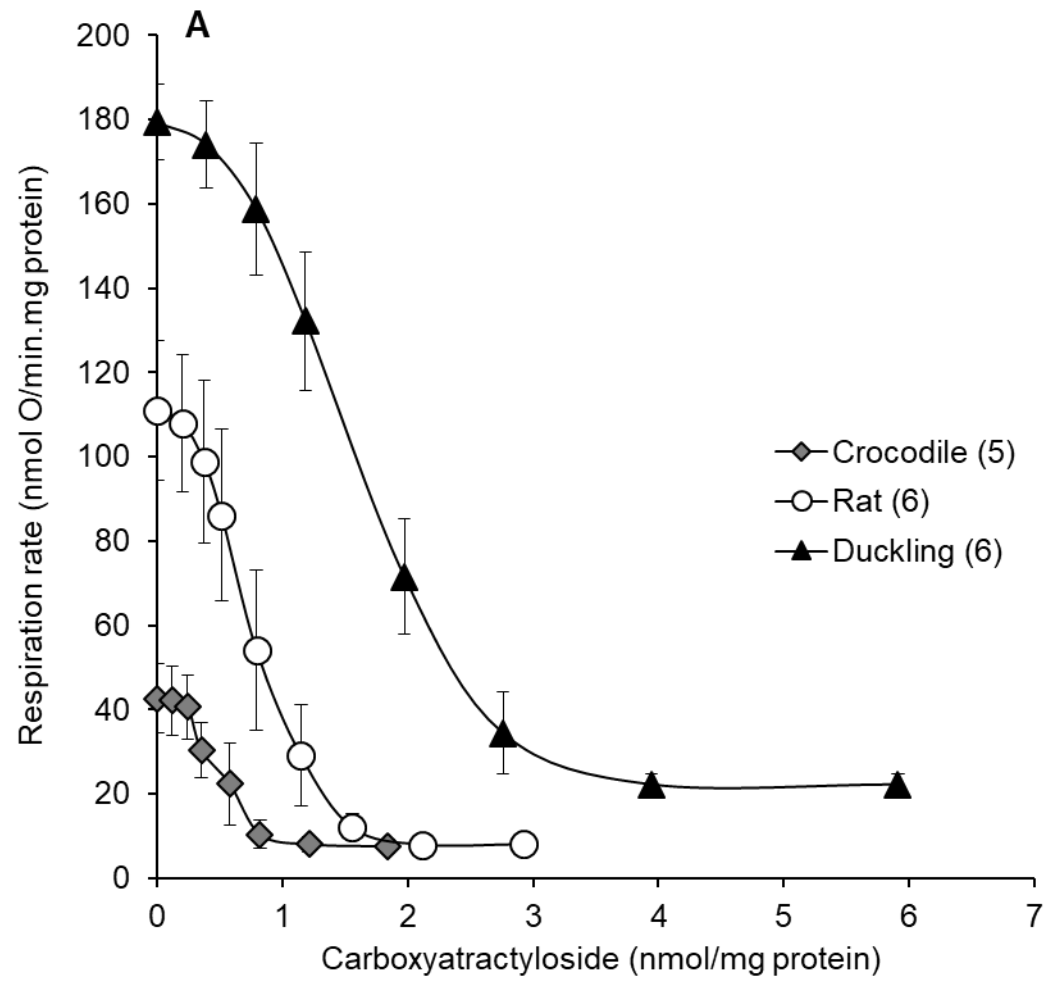
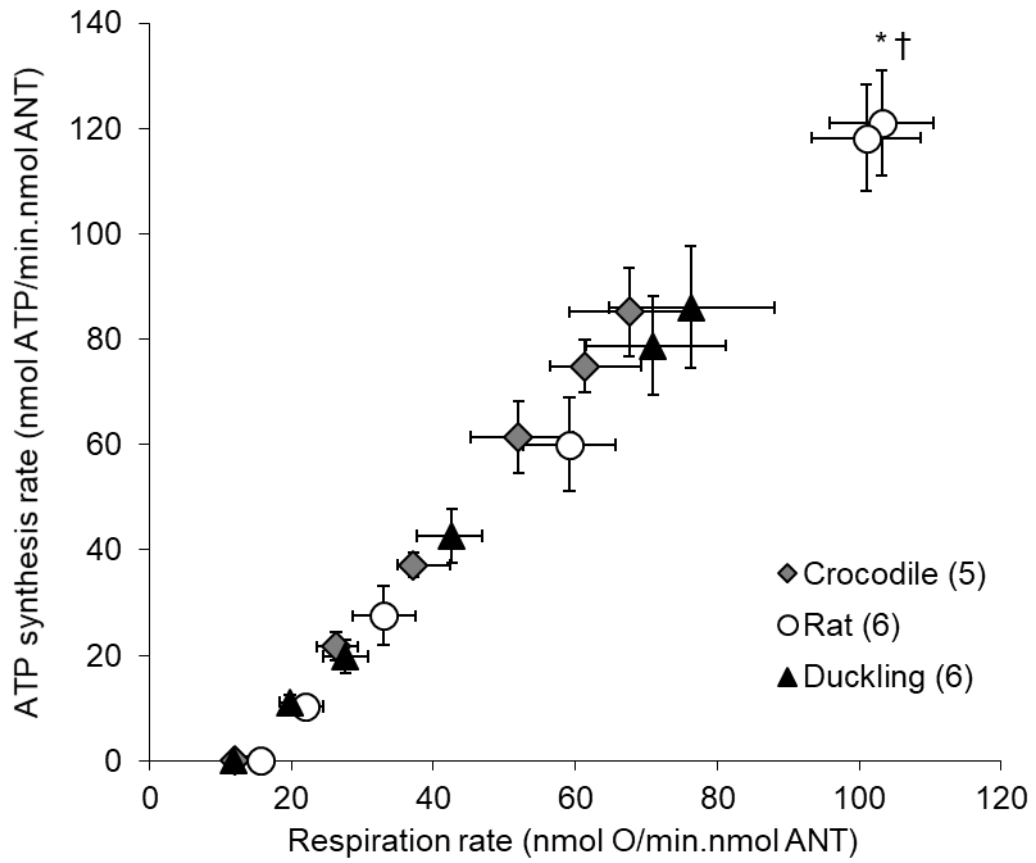


Figure 4



Supplemental Figure 1S

