Experimental demonstration of prenatal programming of mitochondrial aerobic metabolism lasting until adulthood

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Abstract

It is increasingly being postulated that among-individual variation in mitochondrial function underlies variation in individual performance (*e.g.* growth rate) and state of health. It has been suggested (but not adequately tested) that environmental conditions experienced before birth have been suggested to programme postnatal mitochondrial function, with persistent effects potentially lasting into adulthood. We tested this hypothesis in an avian model by experimentally manipulating prenatal conditions (incubation temperature and stability), then measuring mitochondrial aerobic metabolism in blood cells from the same individuals during the middle of the growth period and at adulthood. Mitochondrial aerobic metabolism changed markedly across life stages, and part of these age-related changes were influenced by the prenatal temperature conditions. A high incubation temperature induced a consistent and long-lasting increase in mitochondrial aerobic metabolism. Postnatal mitochondrial aerobic metabolism was positively associated with oxidative damage on DNA but not telomere length. While we detected significant within-individual consistency in mitochondrial aerobic metabolism across life-stages, the prenatal temperature regime only accounted for a relatively small proportion (<20%) of the consistent among-individual differences we observed. Our results demonstrate that prenatal conditions can program consistent and long-lasting differences in mitochondrial function, which could potentially underlie among-individual variation in performance and health state.

Keywords: developmental programming, telomeres bioenergetics, mitochondria, oxidative stress, Japanese quail.

40 **Introduction**

41 Mitochondria generate more than 90% of the cellular energy routinely required by
42 animal cells in the form of adenosine triphosphate (ATP), produced through oxidative
43 phosphorylation [1]. Mitochondria are also a major source of reactive oxygen species (ROS),
44 which are likely to be involved in the ageing process if produced in excess of the antioxidant
45 capacity [2]. Consequently, variations in mitochondrial function are likely to play an important
46 role in shaping the life and death of individuals, and to underlie phenotypic variation observed
47 both within and between species [3–6]. Importantly, while mitochondrial function is a very
48 plastic trait (*i.e.* influenced by tissue, temperature, oxygen/food availability or reproductive
49 stage; [7–10]), it has been shown in both human and animal models that adult individuals exhibit
50 some degree of within-individual consistency in mitochondrial traits through time [10,11].
51 Understanding the origin of such consistent among-individual differences in mitochondrial
52 function (*e.g.* genetics, early-life programming, permanent environmental effects) is key to
53 understand the role of mitochondria in shaping individual performance and health state [6,12].

54 Scientific evidence from both the biomedical and the ecological fields of research suggest
55 that conditions experienced during early-life have major and persistent effects on adult phenotype
56 and physiology [13,14], a phenomenon termed developmental or early-life programming. For
57 instance, accelerated postnatal growth has been associated with increased metabolic rate at
58 adulthood and reduced lifespan in animal models [15,16], as well as with increased risks of
59 developing various age-related pathologies [17,18]. The underlying molecular and physiological
60 mechanisms are probably numerous, but mitochondrial dysfunction has emerged as one potential
61 candidate linking early-life conditions to both immediate and delayed effects on phenotype and
62 health state[12,14,19,20]. However, much remains to be discovered about the importance of early-

63 life conditions in determining consistent and long-lasting differences in mitochondrial function
64 between individuals.

65 Investigating the long-term programming of physiological function is more amenable
66 through longitudinal studies (*i.e.* measuring the same individuals over time), especially to avoid
67 issues linked to the selective disappearance of specific phenotypes [21]. In recent years, measuring
68 mitochondrial function from blood cells has emerged as an opportunity to conduct longitudinal
69 studies of mitochondrial biology both in human and animal models [10,11,22].

70 To the best of our knowledge, only cross-sectional studies have been conducted to date in
71 the context of early-life effects on mitochondrial function [12]. These studies suggest for instance
72 that maternal food restriction during pregnancy can alter mitochondrial bioenergetics [12,23]. This
73 has been framed overall as the 'developmental programming of mitochondrial biology' hypothesis
74 [12]. However, none of these studies show an unequivocal direct and persistent effect of prenatal
75 conditions on mitochondrial function. For instance, early-life dietary manipulations usually result in
76 alterations of body mass (and body fat content) during growth and at adulthood, which could by
77 itself alter mitochondrial function independently of prenatal conditions. Additionally,
78 measurements of mitochondrial function are mostly conducted at a single time point, often months
79 or years after birth, so preventing evaluation of the effect of manipulations on early-life
80 mitochondrial function, and the potential effects on age-related variation in mitochondrial function.

81 Oviparous species offer the opportunity to conduct experiments on the direct effects of
82 prenatal conditions on mitochondrial function, since the developmental conditions of the embryo
83 can be altered through direct manipulations of the eggs. For instance, it has been shown that higher
84 incubation temperature of chicken eggs led to higher oxidative capacity (state 3 / *OXPHOS*) and
85 mitochondrial proton leak (state 4 / *LEAK*) in developing bird embryos [24]. However, this study did
86 not control for treatment-induced differences in embryo developmental stage and did not assess

87 the persistence of the effects on mitochondrial function postnatally and over the long term.

88 Here we investigate the potential prenatal programming of postnatal mitochondrial
89 function from early-life to adulthood by using manipulations of incubation temperature and stability
90 in an avian model [25]. Both the absolute incubation temperature and the stability of that
91 temperature could constrain pre- and postnatal development in non-adaptive ways (*e.g.* lower
92 and/or unstable incubation temperatures have been shown to slow postnatal growth and increase
93 metabolic rate; [26,27]). However, they can also convey environmental information that embryos
94 might use to adjust the postnatal phenotype to suit anticipated environmental conditions (*e.g.* a
95 higher incubation temperature limits the deleterious impact of post-hatching exposure to heat
96 stress [28]). Mitochondrial function has been shown to exhibit changes according to age/life-stage
97 in both animal models and humans [29–31]. Therefore, a second aim was to characterize within-
98 individual changes in mitochondrial function between the peak of the growth phase and early-
99 adulthood, and to evaluate potential effects of the prenatal environment on such age-related
100 changes. Since it has been shown that individuals with more efficient mitochondria (*i.e.* producing
101 more ATP per unit of O_2 consumed) could grow faster [32], we hypothesize that mitochondrial
102 efficiency could be maximized during early life to support the costly process of growth. Since we
103 previously showed that our incubation temperature manipulation led to differences in ageing
104 biomarkers (*i.e.* DNA damage and telomere length [33]), we also tested for potential relationships
105 between mitochondrial aerobic metabolism and those ageing markers. Finally, our last objective
106 was to characterize the extent to which among-individual differences in mitochondrial function are
107 consistent over time (*i.e.* within-individual consistency), but also the extent to which such consistent
108 among-individual differences could originate from variation in the prenatal environment. This is
109 conceptually important for evaluating the scope for permanent environmental effects linked to
110 early-life programming and heritable variation in mitochondrial aerobic metabolism.

111 ## Material and Methods

112 **Experimental design**

113 All procedures were conducted (as previously described for the same experiment in
114 [33]) in 2016 in accordance with UK regulations under the Home Office Project Licence
115 70/8335 granted to PM and the Home Office Personal Licence ICB1D39E7 granted to AS. We
116 used Japanese quail (*Coturnix japonica*) as a model since they are precocial birds and can be
117 reared successfully without the parent being present, therefore avoiding any confounding
118 effect linked to variation in parental care; in addition, they reach sexual maturity and
119 therefore adulthood very quickly (*ca.* 50 days [34]) making it possible to examine long term
120 effects spanning life history stages within a few months. Japanese quail eggs were bought
121 from Moonridge Farm (Devon, UK) and delivered within 48 hours after collection. The identity
122 of the parents was unfortunately unknown, but given that all the eggs were laid on the same
123 day, it is very unlikely that our study population contained full siblings. We used 164 eggs from
124 which 107 chicks hatched, 6 died in the first 5 days and were excluded from the study, and 25
125 birds were not used for mitochondrial function assessment due to logistical constraints, giving
126 a sample size of 76 birds.

127 Eggs were incubated at 3 constant temperatures as described in more detail in [33]:
128 high (H) = 38.4°C, medium (M) = 37.7°C and low (L) = 37.0°C. Additionally a fourth group was
129 incubated under 'unstable' (U) temperature conditions, with an incubation temperature of
130 37.7°C but five incubation recesses of 30min during the day, leading to a daily average of
131 37.0°C, similar to the L group (*i.e.* its matched control for developmental speed [33]).
132 Experimental temperature conditions were chosen based on existing literature [35] and pilot
133 experiments, so as to maximize differences in developmental speed and metabolism while
134 minimizing the risks of having differences in hatching success between groups (*i.e.* to avoid

135 the selective disappearance of embryos in some groups [33]). The conditions for the U group
136 were chosen based on the natural incubation recesses occurring when females leave the nest
137 to forage [36]. Temperature and humidity were checked daily within each incubator using a
138 digital thermo-hygrometer (R-Com DigiLog3) placed in the centre of the incubator, and did not
139 deviate by more than 0.2°C and 5% from their target values. Our prenatal experimental
140 treatments affected developmental speed in the predicted direction (*i.e.* lower temperature
141 leading to slower prenatal growth and metabolism, higher temperature leading to faster
142 development and metabolism, and unstable temperature treatment leading to growth similar
143 to low temperature), while having no significant effects on hatching success or mass at
144 hatching and during growth [33].

145 Animal husbandry rooms for post-hatching rearing were maintained at 21°C on a 14L:10D
146 cycle throughout the experiment. After hatching (Day 0) chicks were placed for 24h in a larger
147 incubator set at 37°C, before being placed into their respective enclosure within each room at Day
148 1 where an additional heat source was provided (Brinsea Comfort brooder 40, 42W), as well as *ad*
149 *libitum* food (Heygates starter crumbs, 22% protein) and water. The additional heat source was
150 removed at day 15 and chick food was switched to adult pellets (Heygates quail and partridge
151 pellets, 16% protein) at the same time (*i.e.* 5 days before the first blood sampling). Chicks were
152 maintained in mixed-sex groups until Day 25 when they can be sexed morphologically. Females
153 were then kept in groups in enclosures and males were placed by pairs in 0.8m^2 cages to avoid
154 female exhaustion due to male harassment and to limit male-male conflicts.

155

156 **Sampling procedures**

157 We used blood cells [22] to measure mitochondrial function of the same individuals at
158 both Day 20 (peak of the growth phase, see [33]) and Day 60 (early-adulthood). Blood samples

159 (*ca.* 200µL) were collected at Day 20 and Day 60 by venipuncture of the wing vein with a 26*G*
160 needle and collection using heparinised capillaries. Blood was centrifuged 10min at 3000*g* and 4°C
161 to separate plasma from packed blood cells, and 50µL of blood cells (*ca.* 150 to 200.10^6 cells) were
162 immediately re-suspended in 1mL of Mir05 buffer (0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-
163 lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM Hepes, 110 mM sucrose, free fatty acid
164 bovine serum albumin (1 g/L), pH 7.1) and kept on ice until analysis (< 1.5h after blood collection).
165

166 **High-resolution respirometry analysis of mitochondrial function in intact blood cells**

167 We followed the protocol described in detail for king penguins [22], with some minor
168 modifications. Briefly, samples were washed before the start of the mitochondrial
169 measurements by centrifugating the tubes to pellet the blood cells and discarding the
170 supernatant. Blood cells were then re-suspended in 1mL of respiratory buffer MiR05 pre-
171 equilibrated at 40.0°C in the chamber of the Oxygraph-2k (Oroboros Instruments, Innsbruck,
172 Austria), and transferred to the chamber already containing another 1mL of Mir05. After
173 closing the chamber, baseline O_2 consumption was recorded, followed by the inhibition of
174 ATP-synthesis using oligomycin (Oligo: 2.5µM), then by the stimulation of maximal uncoupled
175 respiration using a sequential titration of carbonyl cyanide-p-
176 trifluoromethoxyphenylhydrazone (FCCP: 0.5µM per step) until maximal stimulation was
177 reached, and finally by the inhibition of mitochondrial respiration using antimycin A (AA:
178 2.5µM). Mitochondrial responses of blood cells to this chemical titration are presented in Fig
179 S1A. We then calculated *ROUTINE* respiration (*i.e.* endogenous cellular respiration = *baseline*
180 *- AA*), *OXPHOS* respiration (*i.e.* O_2 consumption linked to ATP synthesis = *baseline - Oligo*),
181 *LEAK* respiration (*i.e.* O_2 consumption mostly linked to mitochondrial proton leak = *Oligo - AA*)
182 and *ETS* respiration (*i.e.* maximal O_2 consumption of the electron transport system = *FCCP -*

183 *AA*). We also calculated two mitochondrial *flux control ratios* (FCRs), namely the OXPHOS
184 coupling efficiency (*OxCE* calculated as *OXPHOS*/*ROUTINE*) indicating the proportion of
185 endogenous respiration being linked to ATP synthesis, and an index of mitochondrial capacity
186 usage ($FCR_{R/ETS}$ calculated as *ROUTINE*/*ETS*) indicating the proportion of maximal capacity
187 being used under endogenous cellular conditions [22]. To account for potential differences in
188 cell quantity between samples, we quantified the protein content of the cell suspension using
189 a BCA protein assay (ThermoScientific) following [22].

190 Some studies have shown that mitochondrial function measured in blood cells is
191 correlated to some extent to mitochondrial function in other tissues such as kidneys, heart,
192 skeletal muscles and brain (*e.g.* [22,37–39], while some other studies did not find any
193 significant relationship (*e.g.* [40]). Therefore, to evaluate the relevance of using blood cell
194 mitochondrial function in Japanese quails, we compared mitochondrial function between
195 blood cells and brain samples (see ESM) in a subsample of adult females (N = 22); and found
196 a moderate but overall significant correlation between these two tissues (meta-analytic r =
197 0.44, 95% C.I. = [0.27;0.59], $p < 0.001$; see ESM Fig. S2).

198 **Telomere length and DNA damage**

199 Telomere length and oxidative damage on DNA were measured for a proportion of
200 the individuals used here (n = 45 at day 20, n = 40 day 60) as part of another study [33]. Briefly,
201 DNA extracted from blood cells was used to measure absolute telomere length (using *in-gel*
202 terminal restriction fragment, TRF) and 8-hydroxydeoxyguanosine (8-OHdG), one of the
203 predominant forms of free radical-induced oxidative lesions in DNA (using a ELISA assay) [33].

204

205 **Data analysis**

206 We used generalized estimated equations (GEEs) in *SPSS* 24.0 to investigate the effects of

207 age (*i.e.* repeated effect), prenatal treatment (*i.e.* low, medium, high or unstable incubation
208 temperature), sex and their interactions on mitochondrial parameters, with the associated post-
209 hoc tests (non-significant interactions were removed from the final models except the focal *age*
210 *x treatment* interaction). We included the cellular protein content as a covariate in the models
211 to account for potential variations in cell quantity between samples, and the time of day to
212 account for potential circadian variations in mitochondrial function [41]. Relationships between
213 telomere length or DNA damage and mitochondrial traits were explored using GEEs, with either
214 telomere length or DNA damage as the dependent variable, age and treatment plus their
215 interaction as fixed effects, and one mitochondrial trait at a time (due to strong collinearity
216 between mitochondrial traits) as a covariate. Mitochondrial respiration rates were expressed
217 per quantity of blood cell protein for these analyses, and all continuous variables were z-
218 transformed to provide comparable estimates. To analyse within-individual consistency in
219 mitochondrial traits, we used the *RptR* package [42] in *R* 3.4.2, including bird identity as a
220 random (*i.e.* focal) term, as well as age and cellular protein content as fixed effects to account
221 for changes in mitochondrial traits with age and quantity of cells per sample. We re-ran the
222 same analyses while including experimental treatment group as an additional random term to
223 investigate the proportion of within-individual consistency being attributed to our prenatal
224 treatments. Due to some failed laboratory assays (*e.g.* due to residual inhibition of
225 mitochondrial respiration in the Oroboros chambers), the final dataset includes n = 136
226 mitochondrial [measurements from N = 76 individuals (L = 20, M = 20, H = 16 and U = 20)]. A
227 few (n = 8) *ETS* values being non-biological (*ETS* < *ROUTINE*) were removed from the final
228 dataset.

Results

Effect of age and prenatal temperature regime on mitochondrial traits

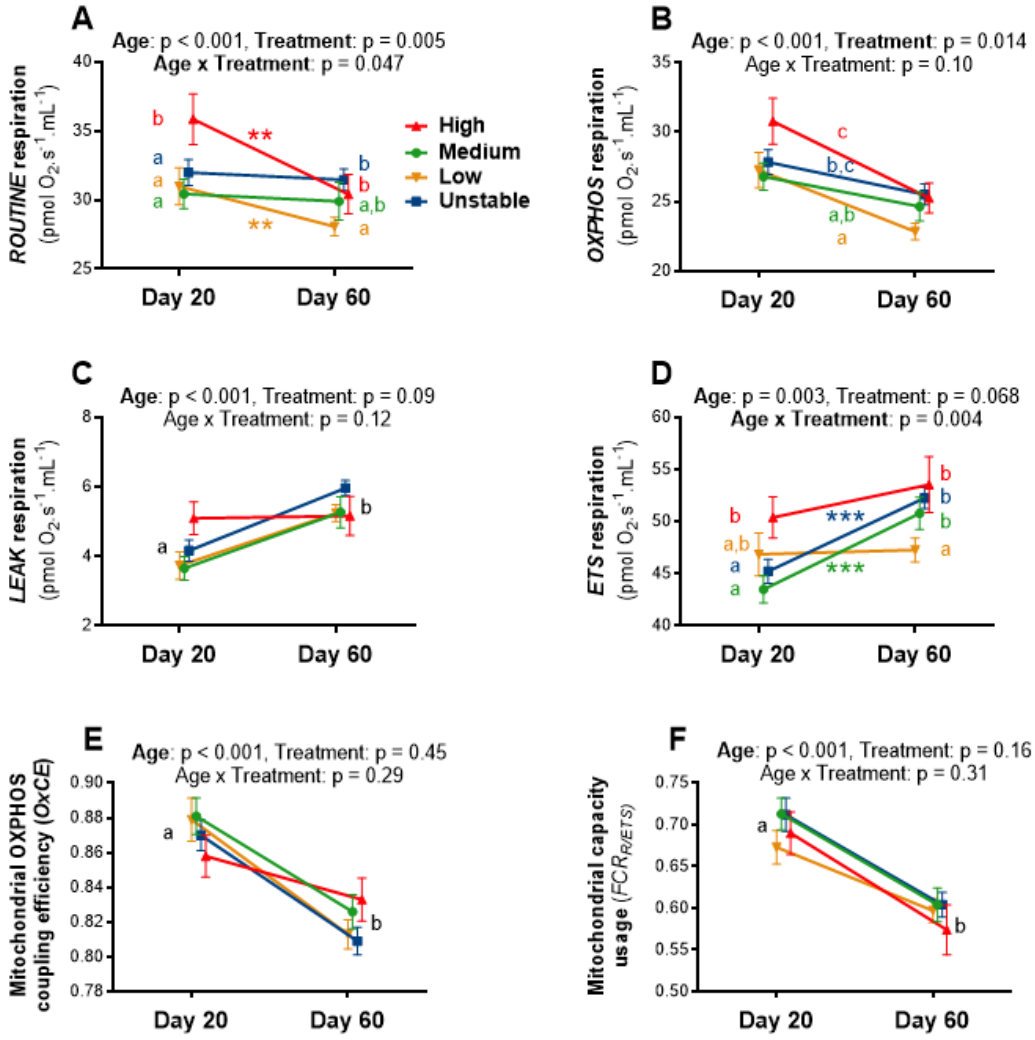

Fig 1: Effects of age and prenatal temperature regime on blood cells mitochondrial traits: (A) *ROUTINE* respiration: endogenous mitochondrial O_2 consumption, (B) *OXPHOS* respiration: mitochondrial O_2 consumption being linked to ATP synthesis (C) *LEAK* respiration: mitochondrial O_2 consumption being mostly linked to mitochondrial proton leak, (D) *ETS* respiration: maximal mitochondrial O_2 consumption induced by mitochondrial uncoupling, (E) OXPHOS coupling efficiency (*OxCE*): proportion of endogenous respiration devoted to ATP synthesis and (F) mitochondrial capacity usage ($FCR_{ROUTINE/ETS}$): proportion of maximal mitochondrial respiration being used under endogenous cellular conditions. Details of statistical tests are given in Tables S1 and S2, means are presented ± SE, letters indicate significant differences between groups according to GEE post-hoc tests, and within-group age effects are presented as * = p < 0.05, ** = p < 0.01 and *** = p < 0.001. Statistical models for A-D included blood cells protein content as a covariate to account for potential variations in blood cell quantity between samples.

252 Endogenous mitochondrial respiration (*ROUTINE*) was significantly affected by the age
253 of individuals and the prenatal experimental treatment, as well as by their interaction (Fig 1A,
254 Table S1A). Specifically, *ROUTINE* decreased overall with age, but in a treatment-specific
255 manner (H and L decreasing significantly, while M and U only showed a non-significant
256 decrease). Chicks from the high temperature group had a significantly higher *ROUTINE* at Day
257 20 than other groups. At adulthood (Day 60), birds from the high temperature and unstable
258 groups had higher *ROUTINE* than low temperature birds, while birds in the medium
259 temperature group were intermediate (Fig. 1A). Mitochondrial *OXPHOS* respiration decreased
260 significantly with the age of individuals (Fig 1B, Table S1B). It also significantly differed
261 between treatment groups: birds from the high temperature group had the higher *OXPHOS*,
262 followed by unstable and medium temperature ones, and finally by low temperature ones
263 (see Fig 4B for significance). *LEAK* respiration increased significantly with age, but was not
264 significantly affected by treatment (Fig 1C, Table S1C). *ETS* respiration increased overall with
265 age, but in a treatment-specific manner (significant age x treatment interaction, with M and
266 U increasing significantly while H and L only exhibited a non-significant increase; Fig 1D, Table
267 S1D). Chicks from the high temperature group had a significantly higher *ETS* at Day 20 than
268 those from the medium temperature and unstable groups, while low temperature ones had
269 an intermediate phenotype (Fig. 1D). At adulthood (Day 60), birds from the low temperature
270 group had lower *ETS* respiration than all other groups (no significant differences were
271 observed between H, M and U groups).

272 Mitochondrial OXPHOS coupling efficiency (*i.e. OxCE*) significantly decreased with age,
273 but was not significantly affected by the prenatal treatment, either as a main factor or in
274 interaction with age (Fig 1E, Table S2A). However, this parameter exhibited a sex difference,
275 with females having slightly less efficient mitochondria than males (Table S2A, LS-mean ± SE:

276 females = 0.836 ± 0.007 *vs.* males = 0.852 ± 0.005, p = 0.039). The proportion of maximal
277 respiration being used under endogenous cellular conditions (*i.e.* $FCR_{R/ETS}$) significantly
278 decreased with age, but was not significantly affected by the prenatal treatment, either as a
279 main factor or in interaction with age (Fig 1F, Table S2B).
280
281 **Relationships between telomere length/DNA damage and mitochondrial traits**
282 Mitochondrial respiration rates were positively related to DNA damage levels (Fig. 2A),
283 significantly so for *ROUTINE* (Wald $\chi^2 = 4.06$, $p = 0.044$) and *LEAK* (Wald $\chi^2 = 6.27$, $p = 0.012$),
284 but not significantly so for *OXPHOS* (Wald $\chi^2 = 2.15$, $p = 0.14$) and *ETS* (Wald $\chi^2 = 3.29$, $p =$
285 0.07). *OxCE* (Wald $\chi^2 = 2.40$, $p = 0.12$) and $FCR_{R/ETS}$ (Wald $\chi^2 = 0.53$, $p = 0.47$) were not
286 significantly related to DNA damage levels (Fig 2A). Mitochondrial traits were not significantly
287 related to telomere length (all Wald $\chi^2 < 0.14$ and $p > 0.71$; Fig 2B).
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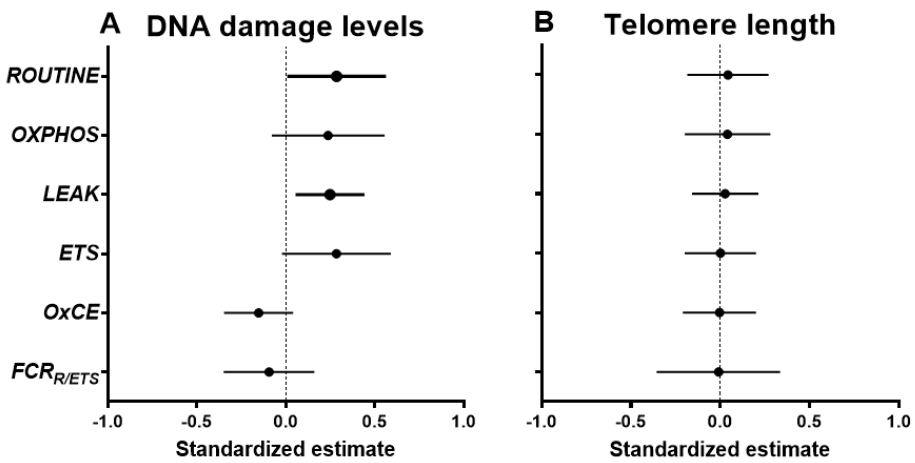

295 **Fig 2: Relationships between mitochondrial traits and (A) oxidative damage to DNA (8-OHdG), (B)**
296 **telomere length.** Effects are reported as standardized estimates from GEE models (based on z-transformed values) with 95% confidence intervals. Significant effects are shown in bold and details on statistics are provided in the main text.
297
298
299

300 **Within-individual consistency in mitochondrial phenotype**

301 All mitochondrial respiration rates exhibited a significant within-individual consistency
302 through time between the peak of the growth phase (Day 20) and early adulthood (Day 60)
303 (Fig 3A; *ROUTINE*: $R = 0.58$, $p < 0.001$; *OXPHOS*: $R = 0.53$, $p < 0.001$; *LEAK*: $R = 0.23$, $p = 0.045$;
304 *ETS*: $R = 0.48$, $p < 0.001$). While mitochondrial OXPHOS coupling efficiency was not significantly
305 repeatable (*OxCE*: $R = 0.12$, $p = 0.17$), the mitochondrial capacity usage was ($FCR_{R/ETS}$: $R = 0.42$,
306 $p = 0.001$; Fig. 3A). When looking at the variance in mitochondrial traits being explained
307 respectively by bird identity (*i.e.* intrinsic variation) and by incubation temperature (*i.e.*
308 extrinsic variation linked to our treatment), the contribution of the latter was relatively
309 modest (Fig 3B; all < 8%) compared to bird identity (all > 21%, except *OxCE*). Experimental
310 treatment thereby accounted for 7.9-17.5% of the within-individual consistency in
311 mitochondrial respiration rates, and less than 2% of the within-individual consistency in
312 mitochondrial flux ratios.

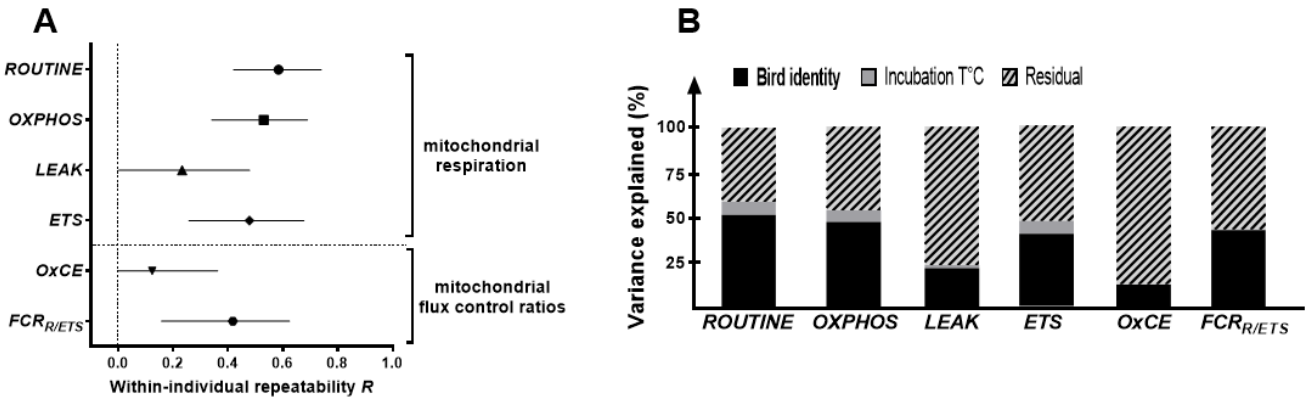

Fig 3: (A) Within-individual consistency of mitochondrial respiration rates and flux control ratios measured in intact blood cells at day 20 and 60 in Japanese quail. Age-adjusted repeatability (*i.e.* consistency) estimates *R* are presented along with their 95% C.I. The statistical model also contained cellular protein content as a covariate to control for potential variations linked to differences in cell quantity between samples. **(B) Respective variance in mitochondrial traits being explained by bird identity (*i.e.* intrinsic variation), our experimental treatment (incubation temperature, *i.e.* extrinsic variation) and other non-investigated factors as well as measurement error (*i.e.* residual)**.

313 ## Discussion

314 Our results demonstrate that variation in the prenatal environment can influence
315 mitochondrial function from post-natal life into adulthood, with a high incubation
316 temperature leading to higher postnatal mitochondrial aerobic metabolism. We found
317 marked differences in mitochondrial function between the peak of the postnatal growth
318 phase and early adulthood, but the pattern of this age-related change was partly influenced
319 by the prenatal temperature. Overall, our results suggest that prenatal conditions can affect
320 how mitochondria work, but also how mitochondrial function changes with age. The
321 persistence of pre-natal temperature effects on mitochondrial function into adulthood
322 suggests that these are long lasting. Higher mitochondrial aerobic metabolism was overall
323 associated with higher levels of oxidative damage on DNA, but not with shorter telomeres.
324 We found significant within-individual consistency of mitochondrial respiration rates across
325 life-stages, with individuals showing consistent relatively high or low values through time.
326

327 **Age-related variation in mitochondrial function**

328 Mitochondrial parameters differed markedly between the peak of the growth phase
329 and early adulthood (6 parameters out of 6). The overall pattern suggests that oxidative
330 phosphorylation decreases with age, while both maximal mitochondrial capacity and proton
331 leak increase, leading to a higher mitochondrial coupling efficiency but also to a more intense
332 utilization of the total mitochondrial capacity during growth than at adulthood. These results
333 are in line with our hypothesis that mitochondrial efficiency should be maximized in early-life
334 to sustain the growth process, and also indicate that mitochondrial maximal capacity is more
335 intensively used during growth. Such effects could potentially be mediated by the known

336 impact of growth hormone on mitochondrial function [43], although this remains to be
337 properly tested.
338
339 **Prenatal programming of mitochondrial function by incubation temperature and stability**
340 Our results clearly show that the prenatal environment can affect mitochondrial
341 respiration rates (*i.e.* endogenous cellular respiration, oxidative phosphorylation and maximal
342 mitochondrial capacity) in the long-term since we found effects of incubation temperature
343 regime both during postnatal growth and at adulthood. Specifically, it seems that a high
344 incubation temperature increased subsequent mitochondrial metabolism at both life-stages
345 (*i.e.* persistent effect on *ROUTINE*, *OXPHOS* and *ETS* compared to low temperature).
346 Mitochondrial aerobic metabolism of individuals in the unstable incubation treatment did not
347 differ from those in the medium temperature group (which shared the same incubation
348 temperature 90% of the time [33]), but was in some cases (*i.e. OXPHOS*, *ROUTINE* and *ETS* at
349 day 60) higher than the low temperature group (*i.e.* the group matched for daily average
350 incubation temperature and developmental speed [33]). This suggests that the temperature
351 experienced during the majority of the prenatal development is a more likely driver of
352 mitochondrial programming than average incubation temperature or developmental speed.
353 While instability in incubation temperature is sufficient to slow down embryo growth and elicit
354 a prenatal increase in glucocorticoid levels [33], it does not seem sufficient to affect
355 mitochondrial aerobic metabolism despite the documented effects of glucocorticoids on
356 mitochondrial biology [44]. Our results demonstrate that prenatal environmental conditions
357 can have relatively immediate effects (*i.e.* during postnatal growth) as suggested by previous
358 correlative studies in mammals [45,46], and more importantly persistent effects lasting from
359 early post-natal life to adulthood. Importantly, unlike previous studies in mammals and

360 reptiles [47], our results cannot be biased by differences in body mass between experimental
361 groups at the time of mitochondrial measurement [33]. Mitochondrial flux control ratios were
362 not affected by the prenatal treatments, suggesting that the differences we observe in
363 respiration rates between groups were relatively consistent across the different mitochondrial
364 respiration rates we measured and might be linked to changes in mitochondrial density. The
365 mechanism(s) by which incubation temperature programmes postnatal mitochondrial aerobic
366 metabolism on the long-term remain to be investigated, but modifications of the epigenome
367 could be a key candidate mechanism [48].

368 Interestingly, age-related changes in mitochondrial function were also partly
369 influenced by the prenatal environment (*i.e.* age-related decrease in *ROUTINE* for H and L
370 groups only, as well as age-related increase in *ETS* for M and U groups only), suggesting that
371 the prenatal environment does not only affect how mitochondria work postnatally, but also
372 the way mitochondrial aerobic metabolism changes with age. Considering the importance of
373 mitochondria in the ageing process [29,49], such effects of the prenatal environment on both
374 mitochondrial aerobic metabolism and its age-related changes could have potential
375 consequences in influencing ageing trajectories. We partly tested this hypothesis by
376 investigating the relationships between two biomarkers of ageing and mitochondrial traits,
377 and found only mixed evidence since high mitochondrial aerobic metabolism was associated
378 with higher levels of DNA damage, but not with shorter telomeres.

379 Although we found clear programming effects of mitochondrial aerobic metabolism by
380 prenatal environmental conditions, we have so far no information about their potential
381 adaptive or maladaptive value. Further studies investigating the adaptive role of such variation
382 in mitochondrial function (*e.g.* by testing individual performance under contrasted postnatal
383 environmental conditions) will be needed to determine the potential adaptive value of

384 prenatal programming through incubation temperature and stability. It is possible that the
385 increased aerobic metabolism programmed by high prenatal temperature provides
386 immediate benefits (*e.g.* higher competitivity and reproductive success) but at the expense of
387 long-term performance and survival (*i.e.* faster 'pace of life') as suggested by the positive
388 relationship found between mitochondrial respiration rates and oxidative damage on DNA.
389 Our results therefore pave the way for further research on the implication of mitochondrial
390 aerobic metabolism in the 'pace of life' syndrome [50].
391
392 **Within-individual consistency in mitochondrial function and relative importance of prenatal**
393 **conditions**
394 Mitochondrial respiration rates and flux control ratios exhibited a significant within-
395 individual consistency over time (at the exception of *OxCE*), despite being measured at two
396 different life stages (peak of growth *vs.* early-adulthood) over a period when there are marked
397 changes in mitochondrial aerobic metabolism (see above). To the best of our knowledge, this
398 is the third demonstration that consistent among-individual differences in mitochondrial
399 aerobic metabolism exist (see [10] in wild adult passerine birds, and [11] in adult humans), but
400 the first to be conducted under well-controlled environmental conditions (*i.e.* excluding bias
401 linked to consistent individual differences in environmental conditions) and across life-stages.
402 These findings of a significant within-individual consistency in mitochondrial function over
403 time have important implications for the possibility for early-life conditions to programme
404 mitochondrial function over the life course of individuals [12]. Our experimental treatment
405 only accounted for less than 20% of the within-individual consistency observed in
406 mitochondrial respiration rates, meaning that more than 80% of the observed within-
407 individual consistency must be explained by other prenatal factors (*e.g.* maternal transfer of

408 nutrients, hormones) and/or by genetic differences between individuals. To the best of our
409 knowledge, there is no information published about the heritability of mitochondrial function,
410 but mtDNA copy number, one proxy of mitochondrial density, has been shown to be
411 significantly heritable (h^2 = 33%) in humans [51]. Therefore, estimating the relative importance
412 of genetic *vs.* environmental drivers of mitochondrial function appears now fundamental to
413 evaluate the magnitude to which early-life environment could programme mitochondrial
414 function and its potential downstream effects such as disease risk and individual performance.
415

416 **Competing interests**

417 We declare having no competing interests
418

419 **Data availability**

420 Datasets used in this manuscript are available at: https://doi.org/10.6084/m9.figshare.16708483.v1
421

422 **Author's contribution**

423 AS designed the study, conducted the experimental work, data analysis and wrote the
424 manuscript. NBM and PM had input on study design and data analysis, and commented on
425 the manuscript.
426

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576

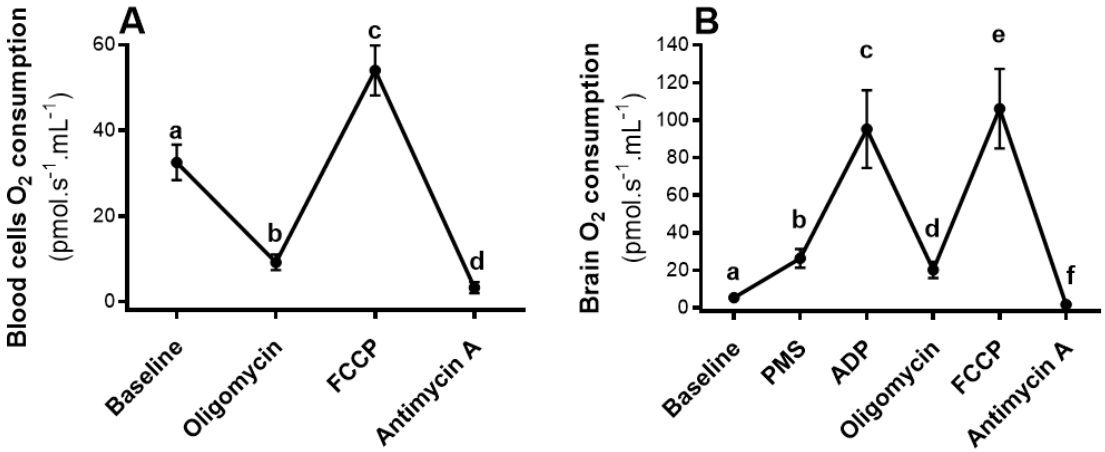
Electronic Supplementary Material (ESM)

Stier *et al.* (2021): **Experimental demonstration of prenatal programming of mitochondrial aerobic metabolism lasting until adulthood**

Correlation between blood cells and brain mitochondrial function

In addition to blood samples (see main text and Fig. S1A for mitochondrial responses of intact blood cells to a standard sequential substrate/inhibitor addition protocol), small brain samples (*ca.* 10-15mg) were collected from a subsample of adult females (Day 90, N = 22) following euthanasia by cervical dislocation and rapid dissection on ice (< 3min after euthanasia). Brain samples were transferred in 1mL of Mir05 buffer and stored on ice until analysis (< 1h after euthanasia). Brain samples were quickly blotted on absorbent paper and weighed (± 0.01mg, Sartorius AC211S®). They were then homogenized during 1min using micro-dissecting scissors and further diluted to $2mg.mL^{-1}$ in Mir05 (following Salin et al., 2016). One mL of this preparation was then transferred to the Oxygraph-2k chamber already containing 1mL of Mir05 equilibrated at 40°C, giving a final brain content of $1mg.mL^{-1}$ in the chamber. Mitochondrial respiration was then assessed following a standard sequential substrate/inhibitor addition protocol. First, baseline O_2 consumption was recorded (this parameter is not used in further analysis), then substrates of complex I (pyruvate 5mM and malate 2mM) and complex II (succinate 10mM) were added leading to a non-phosphorylating state hereafter referred as $LEAK_{PMS}$ (*i.e.* equivalent to classical state 2), followed by the addition of a saturating amount of ADP (2mM) to stimulate oxidative phosphorylation (hereafter referred as $OXPHOS_{PMS+ADP}$; equivalent to classical state 3). ATP synthesis was then inhibited with 2.5 µM of oligomycin leading a non-phosphorylating state hereafter referred as $LEAK_{oligo}$ (*i.e.* equivalent to classical state 4), followed by the stimulation of maximal uncoupled respiration (hereafter referred as *ETS*) using a sequential titration of FCCP (0.5µM per step) until maximal stimulation was reached. Finally, we inhibited mitochondrial respiration using antimycin A (2.5µM), and this residual non-mitochondrial O_2 consumption was subtracted from the mitochondrial parameters described above. Average mitochondrial responses to this chemical titration is presented in Fig S1B. We verified mitochondrial integrity by adding 20µM

of cytochrome c in preliminary experiments, as well as that the mechanical permeabilization we used was able to fully permeabilize brain samples by testing an additive effect of chemical permeabilization on *OXPHOS* respiration using digitonin (5-25ng.mL^{-1}).



<u>Fig S1</u>: Mitochondrial O_2 consumption responses during high-resolution respirometry assays in intact blood cells (A) and permeabilized brain samples (B) of adult Japanese quails. Letters indicate significant differences according to GEE post-hoc tests. PMS stands for pyruvate+malate+succinate as substrates to fuel brain mitochondrial respiration. A direct comparison of respiration rates between tissues is unfortunately not possible due to methodological constraints (*i.e.* intact cells *vs.* permeabilized tissue).

Relationships between blood cell and brain mitochondrial function were analyzed using *Pearson* coefficients of correlation *r,* and a meta-analysis on the Z-transformed *r* for the five main focal correlations (*i.e.* between blood cells and brain parameters measuring overall the same mitochondrial responses, see Fig. S2) using OpenMEE software (Wallace et al., 2017). For investigating potential relationships between brain and blood cell mitochondrial functions, blood cell respiration rates were normalized directly by the cellular protein content, and therefore are expressed as pmol O_2.s^{-1}.mg protein^{-1}.

Mitochondrial respiration rates were highly correlated within-tissues (Fig S2A), and weakly to moderately correlated between tissues (Fig S2A & S2B). While only two of the focal correlations out of five were significant, the overall meta-analytic correlation (*Zr*) was significant ($p < 0.001$) and of moderate effect size (Fig 2B).

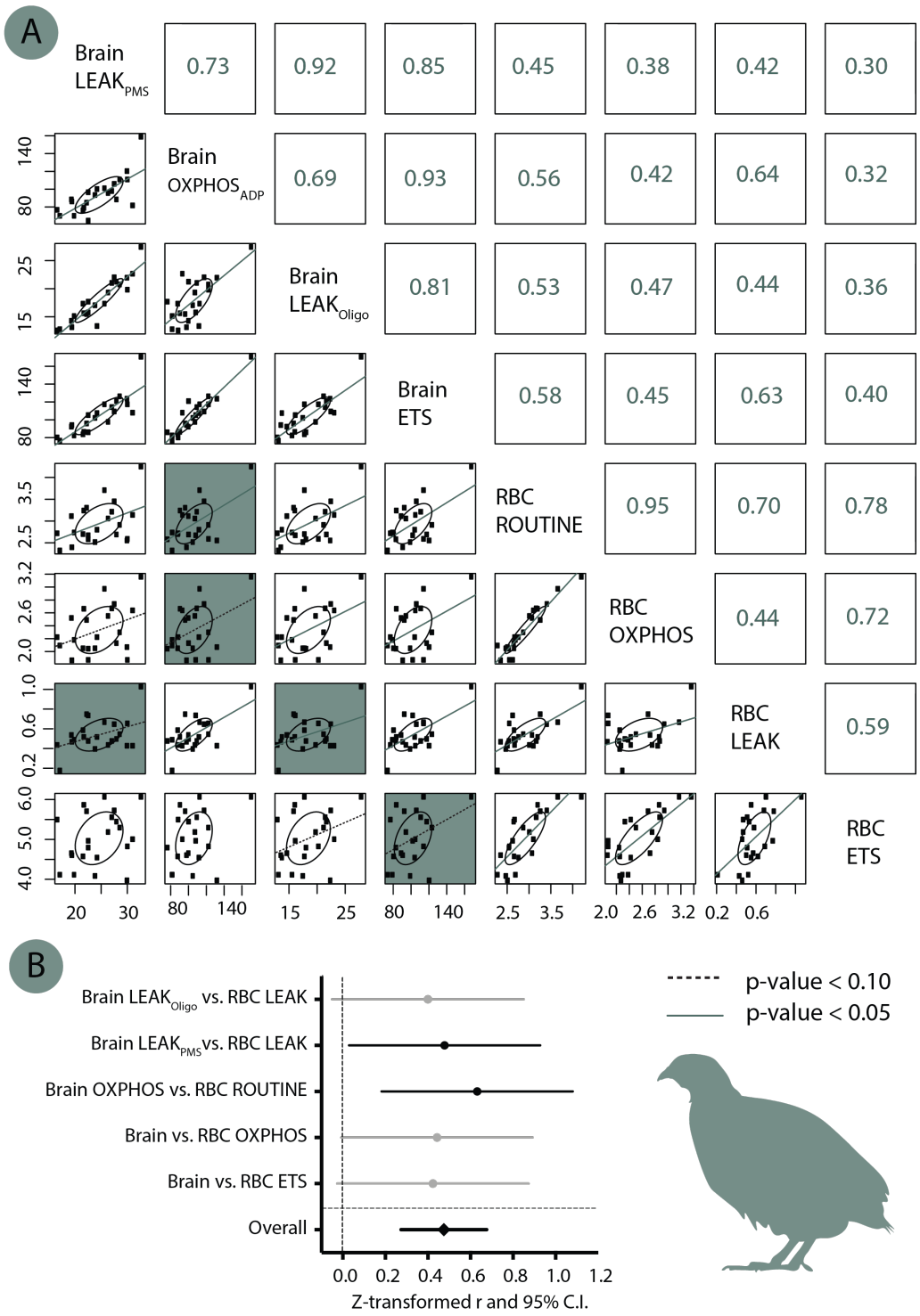


Fig S2: Relationships between brain and blood cell mitochondrial respiration rates. (A) Correlation matrix between the different mitochondrial parameters in the two tissues. Numbers in right part represent the *Pearson* coefficients of correlation *r*, and significance is indicated by the lines in the scatter plot (solid line: $p < 0.05$, dashed line: $p < 0.10$, no line $p > 0.10$). The 5 focal correlations we were the most interested in between the two tissues (being analysed separately in panel B) are highlighted with a blue background. **(B) Meta-analysis of the focal between-tissues correlations.** Z-transformed *r* are presented along with their 95% confidence intervals. Significant parameters are shown in black and non-significant ones in grey.

<u>Table S1</u>: Summary of the statistical models (GEEs) testing the effect of age, prenatal treatment, sex and the interaction between age and prenatal treatment on mitochondrial respiration rates of blood cells while controlling for protein content (to correct for variations in cells number) and time of day. Significant parameters ($p \leq 0.05$) are reported in bold and parameters presenting a non-significant trend ($p \leq 0.10$) in italic. Estimates are given for age = Day 20, prenatal treatment = medium temperature and sex = females.

(A) *ROUTINE* respiration		**Estimate ± SE**	**p-value (χ^2)**
	Intercept	18.39 ± 7.53	**0.007** (7.31)
Repeated effect	**Age**	1.74 ± 0.99	**< 0.001** (16.85)
Fixed effects & covariates	**Prenatal treatment**	-1.80 ± 1.30	**0.005** (12.76)
	Sex	0.28 ± 0.97	0.77 (0.08)
	Time of day	-9.90 ± 3.07	**0.001** (10.42)
	RBC protein content	1.67 ± 0.70	**0.017** (5.70)
	Age x Prenatal treatment	0.35 ± 1.44	**0.047** (7.96)
(B) *OXPHOS* respiration		**Estimate ± SE**	**p-value (χ^2)**
	Intercept	11.98 ± 6.48	**0.023** (5.15)
Repeated effect	**Age**	3.56 ± 0.88	**< 0.001** (44.54)
Fixed effects & covariates	**Prenatal treatment**	-1.07 ± 1.07	**0.014** (10.66)
	Sex	-0.08 ± 0.88	0.93 (0.01)
	Time of day	-9.40 ± 2.62	**< 0.001** (12.85)
	RBC protein content	1.70 ± 0.58	**0.004** (8.45)
	Age x Prenatal treatment	0.18 ± 1.33	*0.10* (6.20)

(C) *LEAK* respiration		**Estimate ± SE**	**p-value (χ^2)**
	Intercept	6.41 ± 2.79	**0.039** (4.27)
Repeated effect	**Age**	-1.82 ± 0.42	**< 0.001** (13.33)
Fixed effects & covariates	*Prenatal treatment*	-0.73 ± 0.50	*0.09* (10.66)
	Sex	0.36 ± 0.30	0.23 (1.43)
	Time of day	-0.50 ± 0.99	0.61 (0.26)
	RBC protein content	-0.030 ± 0.26	0.91 (0.01)
	Age x Prenatal treatment	0.18 ± 1.33	0.12 (5.82)
(D) *ETS* respiration		**Estimate ± SE**	**p-value (χ^2)**
	Intercept	38.07 ± 10.32	**< 0.001** (12.52)
Repeated effect	**Age**	-6.37 ± 1.49	**0.003** (8.95)
Fixed effects & covariates	*Prenatal treatment*	-1.53 ± 1.77	*0.07* (7.14)
	Sex	-0.53 ± 1.32	0.69 (0.16)
	Time of day	5.27 ± 4.23	0.21 (1.56)
	RBC protein content	1.08 ± 0.92	0.24 (1.37)
	Age x Prenatal treatment	-0.06 ± 2.10	**0.004** (13.25)

<u>Table S2</u>: Summary of the statistical models (GEEs) testing the effect of age, prenatal treatment, sex and the interaction between age and prenatal treatment on mitochondrial flux control ratios while controlling for the time of day. Significant parameters ($p \leq 0.05$) are reported in bold. Estimates are given for age = Day 20, prenatal treatment = medium temperature and sex = females.

(A) Coupling efficiency (*OxCE*)		**Estimate ± SE**	**p-value (χ^2)**
	Intercept	0.831 ± 0.016	**< 0.001** (104.37)
Repeated effect	**Age**	0.061 ± 0.011	**< 0.001 (60.94)**
Fixed effects & covariates	Prenatal treatment	0.020 ± 0.013	0.45 (2.66)
	Sex	-0.016 ± 0.008	**0.039 (4.26)**
	Time of day	-0.033 ± 0.026	0.21 (1.57)
	Age x Prenatal treatment	-0.006 ± 0.017	0.29 (3.77)
(B) Mitochondrial capacity usage ($FCR_{R/ETS}$)		**Estimate ± SE**	**p-value (χ^2)**
	Intercept	0.716 ± 0.028	**< 0.001** (765.11)
Repeated effect	**Age**	0.110 ± 0.018	**< 0.001 (76.65)**
Fixed effects & covariates	Prenatal treatment	-0.001 ± 0.023	0.16 (5.19)
	Sex	-0.010 ± 0.014	0.49 (0.48)
	Time of day	-0.212 ± 0.051	**< 0.001 (17.05)**
	Age x Prenatal treatment	0.002 ± 0.031	0.31 (3.55)