1 **Experimental demonstration of prenatal programming of mitochondrial**

2 **aerobic metabolism lasting until adulthood**

3

4 Antoine Stier [a,b,c], Pat Monaghan [a] & Neil B. Metcalfe [a]

5

6 [a] Institute of Biodiversity, Animal Health and Comparative Medicine, University of Glasgow,

7 Glasgow, UK

8 [b] Department of Biology, University of Turku, Turku, Finland

9 [c] Univ Lyon, Université Claude Bernard Lyon 1, CNRS, ENTPE, UMR 5023 LEHNA, F-69622,

10 Villeurbanne, France

11

12

13 *Corresponding author: antoine.stier@gmail.com / amstie@utu.fi

14

15

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.

Introduction

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

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

3

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

4

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.

Material and Methods

Experimental design

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

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

6

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

7

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 -*

8

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

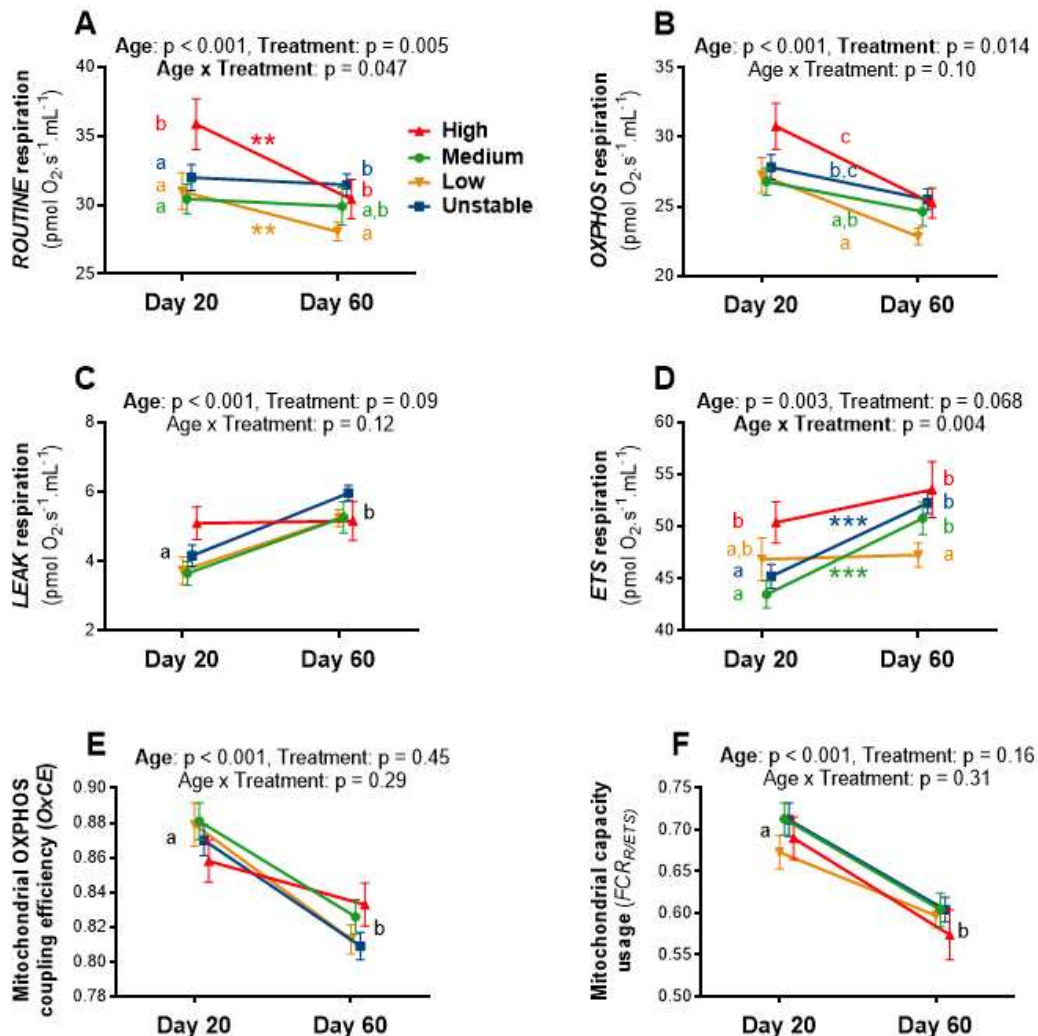### Effect of age and prenatal temperature regime on mitochondrial traits

Fig 1: **Effects of age and prenatal temperature regime on blood cells mitochondrial traits: (A)** *ROUTINE* **respiration: endogenous mitochondrial O₂ consumption, (B)** *OXPHOS* **respiration: mitochondrial O₂ consumption being linked to ATP synthesis (C)** *LEAK* **respiration: mitochondrial O₂ consumption being mostly linked to mitochondrial proton leak, (D)** *ETS* **respiration: maximal mitochondrial O₂ 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.

11

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:

12

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 χ^2 = 4.06, *p* = 0.044) and *LEAK* (Wald χ^2 = 6.27, *p* = 0.012),

284   but not significantly so for *OXPHOS* (Wald χ^2 = 2.15, *p* = 0.14) and *ETS* (Wald χ^2 = 3.29, *p* =

285   0.07). *OxCE* (Wald χ^2 = 2.40, *p* = 0.12) and *FCR$_{R/ETS}$* (Wald χ^2 = 0.53, *p* = 0.47) were not

286   significantly related to DNA damage levels (Fig 2A). Mitochondrial traits were not significantly

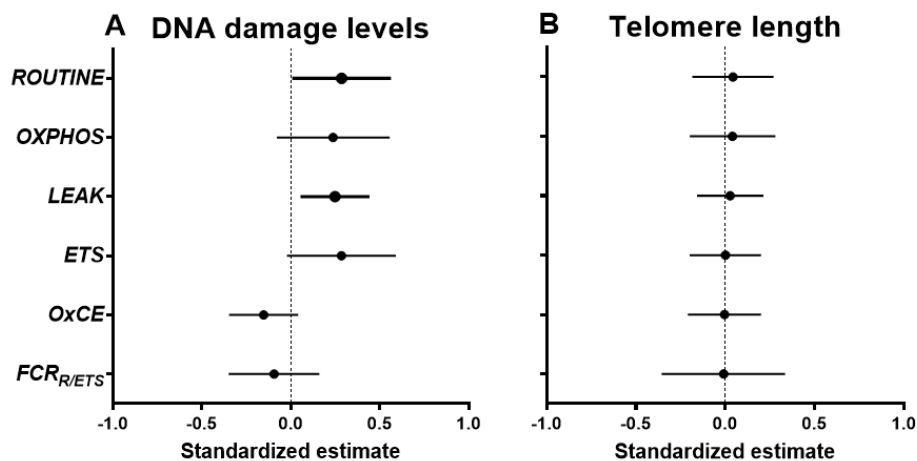287   related to telomere length (all Wald χ^2 < 0.14 and *p* > 0.71; Fig 2B).

288

Fig 2: Relationships between mitochondrial traits and (A) oxidative damage to DNA (8-OHdG), (B) 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.

13

Within-individual consistency in mitochondrial phenotype

300

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

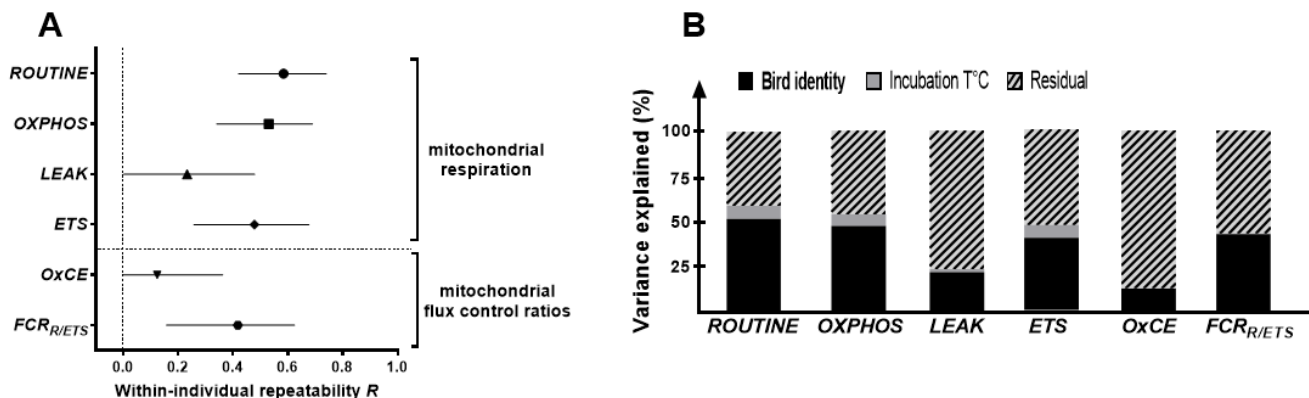312   mitochondrial flux ratios.

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).**

14

Discussion

313

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

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

16

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

17

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

427   **Acknowledgements**

428   We are grateful to two anonymous reviewers for their constructive feedback on an earlier

429   draft of this manuscript, to Karine Salin for help in setting up mitochondrial analysis of brain

430   samples, to Pierre Bize for kindly providing access to his O2k-oxygraph, to Norith Eckbo for the

431   graphic design of Fig. S2, to Franklin Lo, Becky Shaw and Katie Byrne for their help in collecting

19

440

441   **References**

442   1. Nicholls DG, Ferguson SJ. 2002 Bioenergetics. *Academic press*

443   2. Balaban RS, Nemoto S, Finkel T. 2005 Mitochondria, Oxidants, and Aging. *Cell* 120, 483–495.
444   (doi:10.1016/j.cell.2005.02.001)

445   3. Stier A, Bize P, Roussel D, Schull Q, Massemin S, Criscuolo F. 2014 Mitochondrial uncoupling as a
446   regulator of life-history trajectories in birds: an experimental study in the zebra finch. *Journal Of*
447   *Experimental Biology* 217, 3579–3589. (doi:10.1242/jeb.103945)

448   4. Delhaye J, Salamin N, Roulin A, Criscuolo F, Bize P, Christe P. 2016 Interspecific correlation
449   between red blood cell mitochondrial ROS production, cardiolipin content and longevity in birds. *Age*
450   *(Dordrecht, Netherlands)* 38, 433–443. (doi:10.1007/s11357-016-9940-z)

451   5. Hood WR *et al.* 2018 The Mitochondrial Contribution to Animal Performance, Adaptation, and Life-
452   History Variation. *Integrative and Comparative Biology* 58, 480–485. (doi:10.1093/icb/icy089)

453   6. Koch RE *et al.* 2021 Integrating Mitochondrial Aerobic Metabolism into Ecology and Evolution.
454   *Trends in Ecology & Evolution* 21, 1–12. (doi:10.1016/j.tree.2020.12.006)

455   7. Du SNN, Mahalingam S, Borowiec BG, Scott GR. 2016 Mitochondrial physiology and reactive
456   oxygen species production are altered by hypoxia acclimation in killifish (Fundulus heteroclitus).
457   *Journal Of Experimental Biology* 219, 1130–1138. (doi:10.1242/jeb.132860)

458   8. Pichaud N, m AE x000F6, Breton S, m FS x000F6, Rowinski P, Blier PU, Sandblom E. 2019 Cardiac
459   mitochondrial plasticity and thermal sensitivity in a fish inhabiting an artificially heated ecosystem.
460   *Scientific Reports* 9, 1–11. (doi:10.1038/s41598-019-54165-3)

461 9. Salin K, Villasevil EM, Anderson GJ, Auer SK, Selman C, Hartley RC, Mullen W, Chinopoulos C,
462 Metcalfe NB. 2018 Decreased mitochondrial metabolic requirements in fasting animals carry an
463 oxidative cost. *Funct Ecol* 32, 2149–2157. (doi:10.1111/1365-2435.13125)

464 10. Stier A, Bize P, Hsu B-Y, Ruuskanen S. 2019 Plastic but repeatable: rapid adjustments of
465 mitochondrial function and density during reproduction in a wild bird species. *Biology Letters* 15,
466 20190536. (doi:10.1098/rsbl.2019.0536)

467 11. Braganza A, Annarapu GK, Shiva S. 2020 Blood-based bioenergetics: An emerging translational
468 and clinical tool. *Molecular Aspects of Medicine* 71, 100835–12. (doi:10.1016/j.mam.2019.100835)

469 12. Gyllenhammer LE, Entringer S, Buss C, Wadhwa PD. 2020 Developmental programming of
470 mitochondrial biology: a conceptual framework and review. *Proceedings of the Royal Society of*
471 *London. Series B: Biological Sciences* 287, 20192713–10. (doi:10.1098/rspb.2019.2713)

472 13. Metcalfe N, Monaghan P. 2001 Compensation for a bad start: grow now, pay later? *Trends in*
473 *Ecology & Evolution* 16, 254–260.

474 14. Tarry-Adkins JL, Ozanne SE. 2017 Nutrition in early life and age-associated diseases. *Ageing*
475 *Research Reviews* 39, 96–105. (doi:10.1016/j.arr.2016.08.003)

476 15. Criscuolo F, Monaghan P, Nasir L, Metcalfe NB. 2008 Early nutrition and phenotypic
477 development: "catch-up" growth leads to elevated metabolic rate in adulthood. *Proceedings of the*
478 *Royal Society B: Biological Sciences* 275, 1565–1570. (doi:10.1098/rsbl.2006.0496)

479 16. Lee W-S, Monaghan P, Metcalfe NB. 2013 Experimental demonstration of the growth rate–
480 lifespan trade-off. *Proc Royal Soc B Biological Sci* 280, 20122370. (doi:10.1098/rspb.2012.2370)

481 17. Crowther NJ. 1998 Association between poor glucose tolerance and rapid post natal weight gain
482 in seven-year-old children. *Diabetologia* , 1–5.

483 18. Singhal A. 2010 Does Early Growth Affect Long-Term Risk Factors for Cardiovascular Disease? In
484 *Nestlé Nutrition Institute Workshop Series*, pp. 55–69. Nestlé Nutrition Institute Workshop Series.

485 19. Dillin A. 2002 Rates of Behavior and Aging Specified by Mitochondrial Function During
486 Development. *Science* 298, 2398–2401. (doi:10.1126/science.1077780)

487 20. Park KS, Kim SK, Kim MS, Cho EY, Lee JH, Lee K-U, Pak YK, Lee HK. 2003 Fetal and early postnatal
488 protein malnutrition cause long-term changes in rat liver and muscle mitochondria. *The Journal of*
489 *nutrition* 133, 3085–3090.

490 21. Pol M van de, Verhulst S. 2006 Age-Dependent Traits: A New Statistical Model to Separate
491 Within- and Between-Individual Effects. *Am Nat* 167, 766–773. (doi:10.1086/503331)

492 22. Stier A, Romestaing C, Schull Q, Lefol E, Robin J-P, ROUSSEL D, Bize P. 2017 How to measure
493 mitochondrial function in birds using red blood cells: a case study in the king penguin and
494 perspectives in ecology and evolution. *Methods in Ecology and Evolution* 8, 1172–1182.
495 (doi:10.1111/2041-210x.12724)

496 23. Beauchamp B, Harper M-E. 2016 In utero Undernutrition Programs Skeletal and Cardiac Muscle
497 Metabolism. *Frontiers in Physiology* 6, 1891–8. (doi:10.3389/fphys.2015.00401)

21

498   24. Krischek C, Janisch S, Naraballobh W, Brunner R, Wimmers K, Wicke M. 2015 Altered incubation
499   temperatures between embryonic Days 7 and 13 influence the weights and the mitochondrial
500   respiratory and enzyme activities in breast and leg muscles of broiler embryos. *Molecular*
501   *reproduction and development* 83, 71–78. (doi:10.1002/mrd.22596)

502   25. DuRant SE, Hopkins WA, Hepp GR, Walters JR. 2013 Ecological, evolutionary, and conservation
503   implications of incubation temperature-dependent phenotypes in birds. *Biological Reviews* 88, 499–
504   509. (doi:10.1111/brv.12015)

505   26. Nord A, Nilsson J-A. 2011 Incubation Temperature Affects Growth and Energy Metabolism in Blue
506   Tit Nestlings. *American Naturalist* 178, 639–651. (doi:10.1086/662172)

507   27. Ben-Ezra N, Burness G. 2016 Constant and Cycling Incubation Temperatures Have Long-Term
508   Effects on the Morphology and Metabolic Rate of Japanese Quail. *Physiological And Biochemical*
509   *Zoology* 90, 96–105. (doi:10.5061/dyad.76qt1)

510   28. Carvalho AV *et al.* 2020 Embryonic thermal manipulation has short and long-term effects on the
511   development and the physiology of the Japanese quail. *PLoS ONE* 15, e0227700-20.
512   (doi:10.1371/journal.pone.0227700)

513   29. Bratic A, Larsson N-G. 2013 The role of mitochondria in aging. *Journal of Clinical Investigation*
514   123, 951–957. (doi:10.1172/jci64125)

515   30. Sirsat SKG, Sirsat TS, Faber A, Duquaine A, Winnick S, Sotherland PR, Dzialowski EM. 2016
516   Development of endothermy and concomitant increases in cardiac and skeletal muscle mitochondrial
517   respiration in the precocial Pekin duck (Anas platyrhynchos domestica). *Journal Of Experimental*
518   *Biology* (doi:10.1242/jeb.132282)

519   31. Dawson NJ, Salmon P. 2020 Age-related increase in mitochondrial quantity may mitigate a
520   decline in mitochondrial quality in red blood cells from zebra finches (Taeniopygia guttata).
521   *Experimental Gerontology* 133, 110883. (doi:10.1016/j.exger.2020.110883)

522   32. Salin K, Villasevil EM, Anderson GJ, Lamarre SG, Melanson CA, McCarthy I, Selman C, Metcalfe NB.
523   2019 Differences in mitochondrial efficiency explain individual variation in growth performance.
524   *Proceedings of the Royal Society of London. Series B: Biological Sciences* 286, 20191466–8.
525   (doi:10.1098/rspb.2019.1466)

526   33. Stier A, Metcalfe NB, Monaghan P. 2020 Pace and stability of embryonic development affect
527   telomere dynamics: an experimental study in a precocial bird model. *Proceedings of the Royal Society*
528   *of London. Series B: Biological Sciences* 287, 20201378–9. (doi:10.1098/rspb.2020.1378)

529   34. Ottinger MA. 2001 Quail and other short-lived birds. *Experimental Gerontology* 36, 859–868.

530   35. Romao JM, Moraes TGV de, Teixeira RS de C, Buxade CC, Cardoso WM. 2009 INCUBATION OF
531   JAPANESE QUAIL EGGS AT DIFFERENT TEMPERATURES: HATCHABILITY, HATCH WEIGHT, HATCH TIME
532   AND EMBRYONIC MORTALITY. *Archives Vet Sci* 14, 155–162. (doi:10.5380/avs.v14i3.14887)

533   36. Jr FSO, Orcutt AB. 1976 Nesting and parental behavior in domestic common quail. *The Auk* 93,
534   135–141.

535   37. Karamercan MA, Weiss SL, Villarroel JPP, Guan Y, Werlin E, Figueredo R, Becker LB, Sims C. 2013
536   Can Peripheral Blood Mononuclear Cells Be Used as a Proxy for Mitochondrial Dysfunction in Vital
537   Organs During Hemorrhagic Shock and Resuscitation? *Shock* 40, 476–484.
538   (doi:10.1097/shk.0000000000000026)

539   38. Tyrrell DJ, Bharadwaj MS, Jorgensen MJ, Register TC, Molina AJA. 2016 Blood cell respirometry is
540   associated with skeletal and cardiac muscle bioenergetics_ Implications for a minimally invasive
541   biomarker of mitochondrial health. *Redox Biology* 10, 65–77. (doi:10.1016/j.redox.2016.09.009)

542   39. Tyrrell DJ *et al.* 2017 Blood-Based Bioenergetic Profiling Reflects Differences in Brain
543   Bioenergetics and Metabolism. *Oxidative Medicine and Cellular Longevity* 2017, 1–9.
544   (doi:10.1155/2017/7317251)

545   40. Hedges CP, Woodhead JST, Wang HW, Mitchell CJ, Cameron-Smith D, Hickey AJR, Merry TL. 2019
546   Peripheral blood mononuclear cells do not reflect skeletal muscle mitochondrial function or
547   adaptation to high-intensity interval training in healthy young men. *Journal of applied physiology*
548   *(Bethesda, Md. : 1985)* 126, 454–461. (doi:10.1152/japplphysiol.00777.2018)

549   41. Moorsel D van *et al.* 2016 Demonstration of a day-night rhythm in human skeletal muscle
550   oxidative capacity. *Molecular Metabolism* 5, 635–645. (doi:10.1016/j.molmet.2016.06.012)

551   42. Stoffel MA, Nakagawa S, Schielzeth H. 2017 rptR: repeatability estimation and variance
552   decomposition by generalized linear mixed-effects models. *Methods in Ecology and Evolution* 8,
553   1639–1644. (doi:10.1111/2041-210x.12797)

554   43. Short KR, Moller N, Bigelow ML, Coenen-Schimke J, Nair KS. 2008 Enhancement of Muscle
555   Mitochondrial Function by Growth Hormone. *Journal of Clinical Endocrinology & Metabolism* 93,
556   597–604. (doi:10.1210/jc.2007-1814)

557   44. Manoli I, Alesci S, Blackman MR, Su YA, Rennert OM, Chrousos GP. 2007 Mitochondria as key
558   components of the stress response. *Trends in Endocrinology & Metabolism* 18, 190–198.
559   (doi:10.1016/j.tem.2007.04.004)

560   45. McCurdy CE *et al.* 2016 Maternal obesity reduces oxidative capacity in fetal skeletal muscle of
561   Japanese macaques. *JCI Insight* 1. (doi:10.1172/jci.insight.86612ds1)

562   46. Abraham M, Collins CA, Flewelling S, Camazine M, Cahill A, Cade WT, Duncan JG. 2018
563   Mitochondrial inefficiency in infants born to overweight African- American mothers. *International
564   Journal of Obesity* 83, 1–11. (doi:10.1038/s41366-018-0051-z)

565   47. Galli GLJ, Crossley J, Elsey RM, Dzialowski EM, Shiels HA, II DAC. 2016 Developmental plasticity of
566   mitochondrial function in American alligators, Alligator mississippiensis. *AJP: Regulatory, Integrative
567   and Comparative Physiology* 311, R1164–R1172. (doi:10.1152/ajpregu.00107.2016)

568   48. McCaw BA, Stevenson TJ, Lancaster LT. 2020 Epigenetic Responses to Temperature and Climate.
569   *Integr Comp Biol* 60, 1469–1480. (doi:10.1093/icb/icaa049)

570   49. López-Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G. 2013 The hallmarks of aging. *Cell*
571   153, 1194–1217. (doi:10.1016/j.cell.2013.05.039)

23

572   50. Chung DJ, Healy TM, Schulte PM. 2018 Mitochondria, temperature, and the pace of life.
573   *Integrative and Comparative Biology* (doi:10.1093/icb/icy013/4990407)

574   51. Curran JE *et al.* 2007 Genetic determinants of mitochondrial content. *Human Molecular Genetics*
575   16, 1504–1514. (doi:10.1093/hmg/ddm101)

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}).
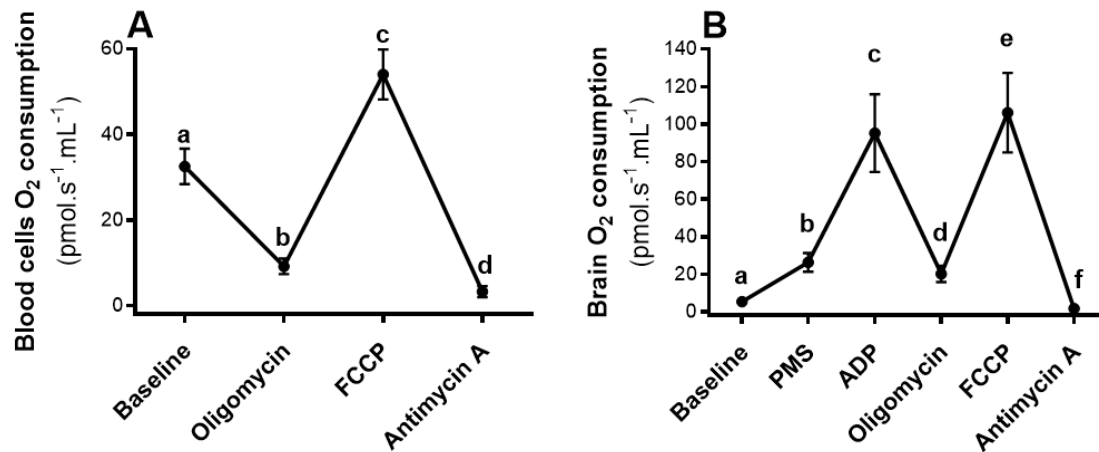

Fig S1: **Mitochondrial O$_2$ consumption responses during high-resolution respirometry assays in intact blood cells (A) and permeabilized brain samples (B) of adult Japanese quails.** Letters indicate significant differences according to GEE post-hoc tests. PMS stands for pyruvate+malate+succinate as 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).
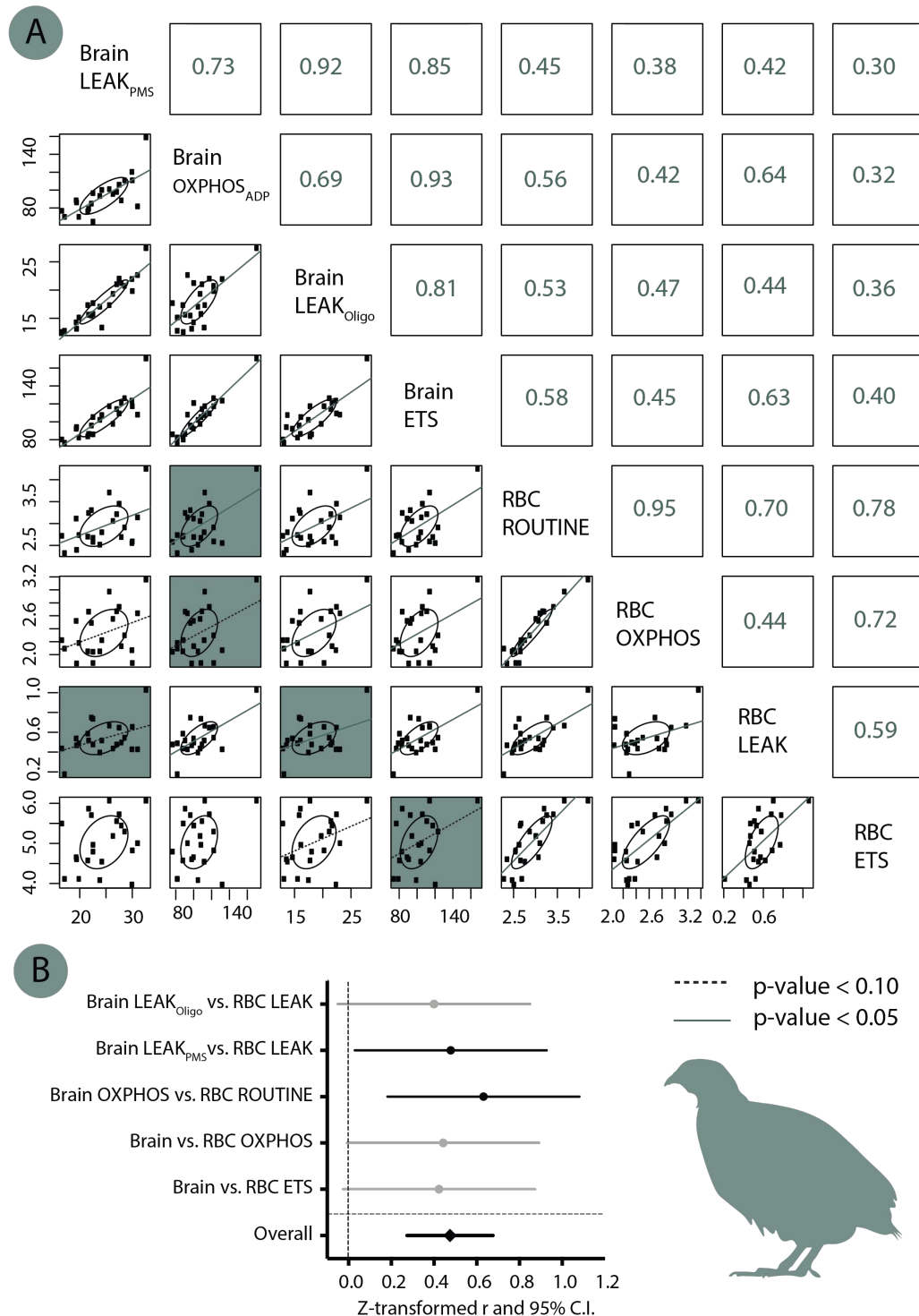
Fig S2: Relationships between brain and blood cell mitochondrial respiration rates. (A) Correlation matrix between the different mitochondrial parameters in the two tissues. Numbers in right part represent the *Pearson* coefficients of correlation *r*, and significance is indicated by the lines in the 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.

Table S1: **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 ≤ 0.05) are reported in bold and parameters presenting a non-significant trend (p ≤ 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)

Table S2: **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)