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. Environmental conditions experienced before birth have been suggested to programme postnatal mitochondrial biology, but the hypothesis that early-life conditions induce consistent and long-lasting differences in mitochondrial function among individuals remains mostly untested. 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 postnatal growth and at adulthood. Mitochondrial aerobic metabolism changed markedly across life stages, and part of these age-related changes were influenced by the prenatal experimental conditions. A high incubation temperature induced a consistent and long-lasting increase in mitochondrial aerobic metabolism, while unstable incubation conditions had a delayed effect, with an increased metabolism only being observed at adulthood. 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 underly among-individual variation in performance and health state.

Keywords: developmental programming, fetal programming, bioenergetics, mitochondria, Japanese quail.

40 **Introduction**

41 Mitochondria produce 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, O_2/food availability or reproductive stage;
49 [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
54 [6,12].

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

64 remains to be discovered about the importance of early-life conditions in determining
65 consistent and long-lasting differences in mitochondrial function between individuals.

66 Investigating the long-term programming of physiological function is more amenable
67 through longitudinal studies (*i.e.* measuring the same individuals over time). Indeed, while
68 avoiding issues linked to the selective disappearance of specific phenotypes [21], longitudinal
69 studies allow estimating 1) the transient, persistent or delayed nature of early-life effects at
70 the within-individual level, 2) within-individual changes through time (*e.g.* age-related
71 alteration) and how they could be affected by early-life conditions, and 3) if consistent among-
72 individual differences in mitochondrial function exist (*i.e.* within-individual consistency
73 through time) and to what extent they could be explained by early-life conditions. In recent
74 years, measuring mitochondrial function from blood cells has emerged as an opportunity to
75 conduct longitudinal studies of mitochondrial biology both in human and animal models
76 [10,11,22].

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

87 evaluation of the effect of manipulations on early-life mitochondrial function, and the
88 potential effects on age-related variation in mitochondrial function.

89 Oviparous species offer the opportunity to conduct experiments on the direct effects
90 of prenatal conditions on mitochondrial function, since the developmental conditions of the
91 embryo can be altered through direct manipulations of the eggs. For instance, it has been
92 shown that higher incubation temperature of chicken eggs led to higher oxidative capacity
93 (state 3 / *OXPHOS*) and mitochondrial proton leak (state 4 / *LEAK*) in developing bird embryos
94 [24]. However, this study did not control for treatment-induced differences in embryo
95 developmental stage and did not assess the persistence of the effects on mitochondrial
96 function postnatally and over the long term.

97 Here we investigate the potential prenatal programming of postnatal mitochondrial
98 function from early-life to adulthood by using manipulations of incubation temperature and
99 stability in an avian model. Both the absolute incubation temperature and the stability of
100 incubation temperature convey environmental information to the embryo that could be used
101 to adjust the postnatal phenotype to suit anticipated environmental conditions [25].
102 Mitochondrial function has been shown to exhibit changes according to age/life-stage in both
103 animal models and humans [26-28]. Therefore, a second aim was to characterize within-
104 individual changes in mitochondrial function between the peak of the growth phase and early-
105 adulthood, and to evaluate potential effects of the prenatal environment on such age-related
106 changes. Since it has been shown that individuals with more efficient mitochondria (*i.e.*
107 producing more ATP per unit of O_2 consumed) could grow faster [29], we hypothesize that
108 mitochondrial efficiency could be maximized during early life to support the costly process of
109 growth. Finally, our last objective was to characterize the extent to which among-individual
110 differences in mitochondrial function are consistent over time (*i.e.* within-individual

111 consistency), but also the extent to which such consistent among-individual differences could
112 originate from variation in the prenatal environment. This is conceptually important for
113 evaluating the potential for early-life conditions to have effects on mitochondrial function that
114 persist through adulthood.

115 ## Material and Methods

116 **Experimental design**

117 All procedures were conducted (as previously described in [30]) in 2016 in accordance
118 with UK regulations under the Home Office Project Licence 70/8335 granted to PM and the
119 Home Office Personal Licence ICB1D39E7 granted to AS. We used Japanese quail (*Coturnix*
120 *japonica*) as a model since successful artificial incubation has been widely used in this species
121 (*e.g.* [31]), they are precocial birds and can be reared successfully without the parent being
122 present, therefore avoiding any confounding effect linked to variation in parental care; in
123 addition, they reach sexual maturity and therefore adulthood very quickly (*ca.* 50 days [32])
124 making it possible to examine long term effects spanning life history stages within a
125 reasonable time. Japanese quail eggs were bought from Moonridge Farm (Devon, UK) and
126 delivered within 48 hours after collection. We used 164 eggs from which 107 chicks hatched,
127 6 died in the first 5 days and were excluded from the study, and 25 birds were not used for
128 mitochondrial function assessment due to logistical constraints, giving a sample size of 76
129 birds.

130 Eggs were incubated at 3 constant temperatures as described in more detail in [30]:
131 high (H) = 38.4°C, medium (M) = 37.7°C and low (L) = 37.0°C. Additionally a fourth group was
132 incubated under 'unstable' (U) temperature conditions, with an incubation temperature of
133 37.7°C but five incubation recesses of 30min during the day, leading to a daily average of
134 37.0°C, similar to the L group (*i.e.* its matched control for developmental speed [30]). Our
135 prenatal experimental treatments affected developmental speed in the predicted direction
136 (*i.e.* higher temperature leading to faster development) while having no significant effects on
137 hatching success or mass at hatching and during growth [30].

138 Animal husbandry rooms for post-hatching rearing were maintained at 21°C on a 14L:10D
139 cycle throughout the experiment. After hatching (Day 0) chicks were placed for 24h in a larger
140 incubator set at 37°C, before being placed into their respective enclosure within each room at Day
141 1 where an additional heat source was provided (Brinsea Comfort brooder 40, 42W), as well as *ad*
142 *libitum* food (Heygates starter crumbs, 22% protein) and water. The additional heat source was
143 removed at day 15 and chick food was switched to adult pellets (Heygates quail and partridge
144 pellets, 16% protein) at the same time (*i.e.* 5 days before the first blood sampling). Chicks were
145 maintained in mixed-sex groups until Day 25 when they can be sexed morphologically. Females
146 were then kept in groups in enclosures and males were placed by pairs in 0.8m^2 cages to avoid
147 female exhaustion due to male harassment and to limit male-male conflicts.
148
149 **Sampling procedures**
150 We used blood cells [22] to measure mitochondrial function of the same individuals at
151 both Day 20 (peak of the growth phase, see [30]) and Day 60 (early-adulthood). Blood samples
152 (*ca.* 200µL) were collected at Day 20 and Day 60 by venipuncture of the wing vein with a 26*G*
153 needle and collection using heparinised capillaries. Blood was centrifuged 10min at 3000*g* and 4°C
154 to separate plasma from packed blood cells, and 50µL of blood cells (*ca.* 150 to 200.10^6 cells) were
155 immediately re-suspended in 1mL of Mir05 buffer (0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-
156 lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM Hepes, 110 mM sucrose, free fatty acid
157 bovine serum albumin (1 g/L), pH 7.1) and kept on ice until analysis (< 1.5h after blood collection).
158
159 **High-resolution respirometry analysis of mitochondrial function in intact blood cells**
160 We followed the protocol described in detail for king penguins [22], with some minor
161 modifications. Briefly, samples were washed before the start of the mitochondrial

162 measurements by centrifugating the tubes to pellet the blood cells and discarding the
163 supernatant. Blood cells were then re-suspended in 1mL of respiratory buffer MiR05 pre-
164 equilibrated at 40.0°C in the chamber of the Oxygraph-2k (Oroboros Instruments, Innsbruck,
165 Austria), and transferred to the chamber already containing another 1mL of Mir05. After
166 closing the chamber, baseline O_2 consumption was recorded, followed by the inhibition of
167 ATP-synthesis using oligomycin (Oligo: 2.5µM), then by the stimulation of maximal uncoupled
168 respiration using a sequential titration of carbonyl cyanide-p-
169 trifluoromethoxyphenylhydrazone (FCCP: 0.5µM per step) until maximal stimulation was
170 reached, and finally by the inhibition of mitochondrial respiration using antimycin A (AA:
171 2.5µM). Mitochondrial responses of blood cells to this chemical titration is presented in Fig
172 S1A. We then calculated *ROUTINE* respiration (*i.e.* endogenous cellular respiration = *baseline*
173 - *AA*), *OXPHOS* respiration (*i.e.* O_2 consumption linked to ATP synthesis = *baseline* - *Oligo*),
174 *LEAK* respiration (*i.e.* O_2 consumption mostly linked to mitochondrial proton leak = *Oligo* - *AA*)
175 and *ETS* respiration (*i.e.* maximal O_2 consumption of the electron transport system = *FCCP* -
176 *AA*). We also calculated two mitochondrial *flux control ratios* (FCRs), namely the OXPHOS
177 coupling efficiency (*OxCE* calculated as *OXPHOS*/*ROUTINE*) indicating the proportion of
178 endogenous respiration being linked to ATP synthesis, and an index of mitochondrial capacity
179 usage ($FCR_{R/ETS}$ calculated as *ROUTINE*/*ETS*) indicating the proportion of maximal capacity
180 being used under endogenous cellular conditions [22]. To account for potential differences in
181 cell quantity between samples, we quantified the protein content of the cell suspension using
182 a BCA protein assay (ThermoScientific) following [22].

183 Some studies have shown that mitochondrial function measured in blood cells is
184 correlated to some extent to mitochondrial function in other tissues such as kidneys, heart,
185 skeletal muscles and brain (*e.g.* [22,33-35], while some other studies did not find any

186 significant relationship (*e.g.* [36]). Therefore, to evaluate the relevance of using blood cell
187 mitochondrial function in Japanese quails, we compared mitochondrial function between
188 blood cells and brain samples (see ESM) in a subsample of adult females (N = 22); and found
189 a moderate but overall significant correlation between these two tissues (meta-analytic r =
190 0.44, 95% C.I. = [0.27;0.59], $p < 0.001$; see ESM Fig. S2).
191
192 **Data analysis**
193 We used generalized estimated equations (GEEs) in *SPSS* 24.0 to investigate the effects of
194 age (*i.e.* repeated effect), prenatal treatment (*i.e.* low, medium, high or unstable incubation
195 temperature), sex and their interactions on mitochondrial parameters, with the associated post-
196 hoc tests (non-significant interactions were removed from the final models except the focal *age*
197 *x treatment* interaction). We included the cellular protein content as a covariate in the models
198 to account for potential variations in cell quantity between samples, and the time of day to
199 account for potential circadian variations in mitochondrial function [37]. To analyse within-
200 individual consistency in mitochondrial traits, we used the *RptR* package [38] in *R* 3.4.2 and
201 included bird identity as a random (*i.e.* focal) term, as well as age and cellular protein content
202 as fixed effects to account for changes in mitochondrial traits with age and quantity of cells per
203 sample. We re-ran the same analyses while including experimental treatment group as an
204 additional random term to investigate the proportion of within-individual consistency being
205 attributed to our prenatal treatments. Due to some failed laboratory assays (*e.g.* due to residual
206 inhibition of mitochondrial respiration in the Oroboros chambers), the final dataset includes n =
207 136 measurements from N = 76 individuals. A few (n = 8) *ETS* values being non-biological (*ETS* <
208 *ROUTINE*) were removed from the final dataset.

209 ## Results

210 ### Effect of age and prenatal temperature regime on mitochondrial respiration rates

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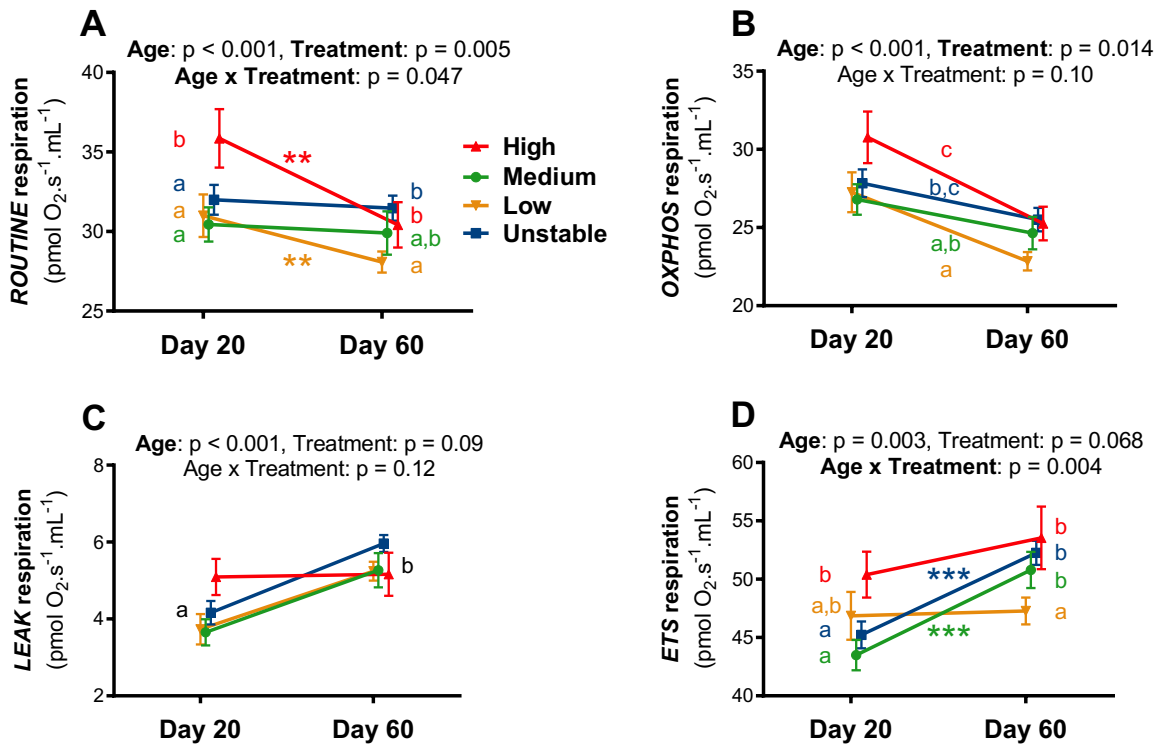

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222 **Fig 1: Effects of age and prenatal temperature regime on blood cells mitochondrial respiration rates: (A) *ROUTINE* respiration: endogenous mitochondrial O_2 consumption, (B) *OXPHOS* respiration: mitochondrial O_2 consumption being linked to ATP synthesis (C) *LEAK* respiration: mitochondrial O_2**

223 **consumption being mostly linked to mitochondrial proton leak, and (D) *ETS* respiration: maximal mitochondrial O_2 consumption induced by mitochondrial uncoupling.** Details of statistical tests are

224 given in Table S1, means are presented ± SE, letters indicate significant differences between groups according to GEE post-hoc tests, and within-group age effects are presented as * = $p < 0.05$, ** = $p <$

225 0.01 and *** = $p < 0.001$. Statistical models included blood cells protein content as a covariate to account for potential variations in blood cell quantity between samples.

226

227 Endogenous mitochondrial respiration (*ROUTINE*) was significantly affected by the age

228 of individuals and the prenatal experimental treatment, as well as by their interaction (Fig 1A,

229 Table S1A). Specifically, *ROUTINE* decreased overall with age, but in a treatment-specific

230 manner (H and L decreasing significantly, while M and U only showed a non-significant

231 decrease). Chicks from the high temperature group had a significantly higher *ROUTINE* at Day

232 20 than other groups. At adulthood (Day 60), birds from the high temperature and unstable

233 groups had higher *ROUTINE* than low temperature birds, while birds in the medium
234 temperature group were intermediate (Fig. 1A). *ROUTINE* respiration was significantly
235 affected by the time of day (*i.e.* decreasing through the daylight hours) but not by sex (Table
236 S1A).

237 Mitochondrial *OXPHOS* respiration decreased significantly with the age of individuals
238 (Fig 1B, Table S1B). It also significantly differed between treatment groups: birds from the high
239 temperature group had the higher *OXPHOS*, followed by unstable and medium temperature
240 ones, and finally by low temperature ones (see Fig 4B for significance). *OXPHOS* respiration
241 decreased significantly with the time of day, but was not significantly affected by sex (Table
242 S1B).

243 *LEAK* respiration increased significantly with age, but was not significantly affected by
244 treatment, time of day or sex (Fig 1C, Table S1C). *ETS* respiration increased overall with age,
245 but in a treatment-specific manner (significant age x treatment interaction, with M and U
246 increasing significantly while H and L only exhibited a non-significant increase; Fig 1D, Table
247 S1D). Chicks from the high temperature group had a significantly higher *ETS* at Day 20 than
248 those from the medium temperature and unstable groups, while low temperature ones had
249 an intermediate phenotype (Fig. 1D). At adulthood (Day 60), birds from the low temperature
250 group had lower *ETS* respiration than all other groups (no significant differences were
251 observed between H, M and U groups). *ETS* respiration was not significantly affected by the
252 time of day or sex (Table S1D).

253

254 **Effect of age and prenatal temperature regime on mitochondrial flux control ratios**

255 Mitochondrial OXPHOS coupling efficiency (*i.e. OxCE*) significantly decreased with age,
256 but was not significantly affected by the prenatal treatment, either as a main factor or in

interaction with age (Fig 2A, Table S2A). However, this parameter exhibited a sex difference, with females having slightly less efficient mitochondria than males (Table S2A, LS-mean ± SE: females = 0.836 ± 0.007 *vs.* males = 0.852 ± 0.005, p = 0.039), but there was no significant effect of the time of day (Table S2A).

The proportion of maximal respiration being used under endogenous cellular conditions (*i.e.* $FCR_{R/ETS}$) significantly decreased with age, but was not significantly affected by the prenatal treatment, either as a main factor or in interaction with age (Fig 2B), nor by the sex of individuals. However, this ratio decreased significantly with the time of day (Table S2B, p = 0.004).

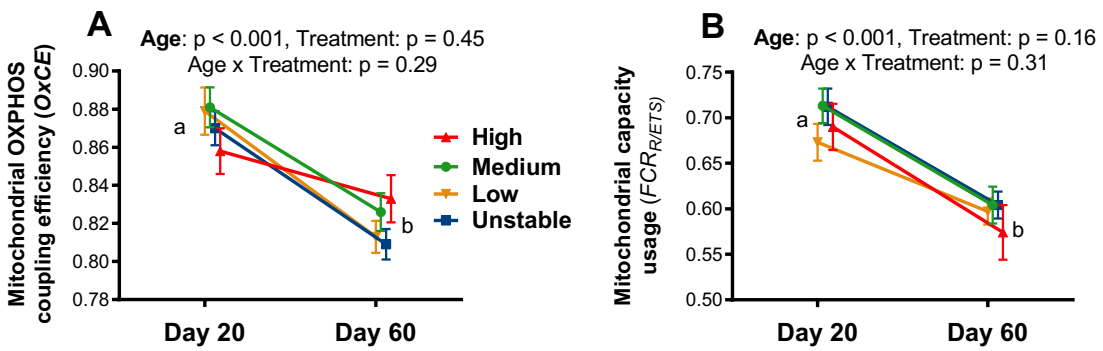

Fig 2: Effects of age and prenatal temperature regime on blood cells mitochondrial: (A) OXPHOS coupling efficiency (*OxCE*: proportion of endogenous respiration devoted to ATP synthesis) and (B) mitochondrial capacity usage ($FCR_{ROUTINE/ETS}$: proportion of maximal mitochondrial respiration being used under endogenous cellular conditions). Details of statistical tests are given in Table S2, means are presented ± SE and letters indicate significant differences.

Within-individual consistency in mitochondrial phenotype

All mitochondrial respiration rates exhibited a significant within-individual consistency through time between the peak of the growth phase (Day 20) and early adulthood (Day 60) (Fig 3A; *ROUTINE*: *R* = 0.58, p < 0.001; *OXPHOS*: *R* = 0.53, p < 0.001; *LEAK*: *R* = 0.23, p = 0.045; *ETS*: *R* = 0.48, p < 0.001). While mitochondrial OXPHOS coupling efficiency was not significantly repeatable (*OxCE*: *R* = 0.12, p = 0.17), the mitochondrial capacity usage was ($FCR_{R/ETS}$: *R* = 0.42,

281 p = 0.001; Fig. 3A). When looking at the variance in mitochondrial traits being explained
282 respectively by bird identity (*i.e.* intrinsic variation) and by incubation temperature (*i.e.*
283 extrinsic variation linked to our treatment), the contribution of the latter was relatively
284 modest (Fig 3B; all < 8%) compared to bird identity (all > 21%, except *OxCE*). Experimental
285 treatment thereby accounted for 7.9-17.5% of the within-individual consistency in
286 mitochondrial respiration rates, and less than 2% of the within-individual consistency in
287 mitochondrial flux ratios.

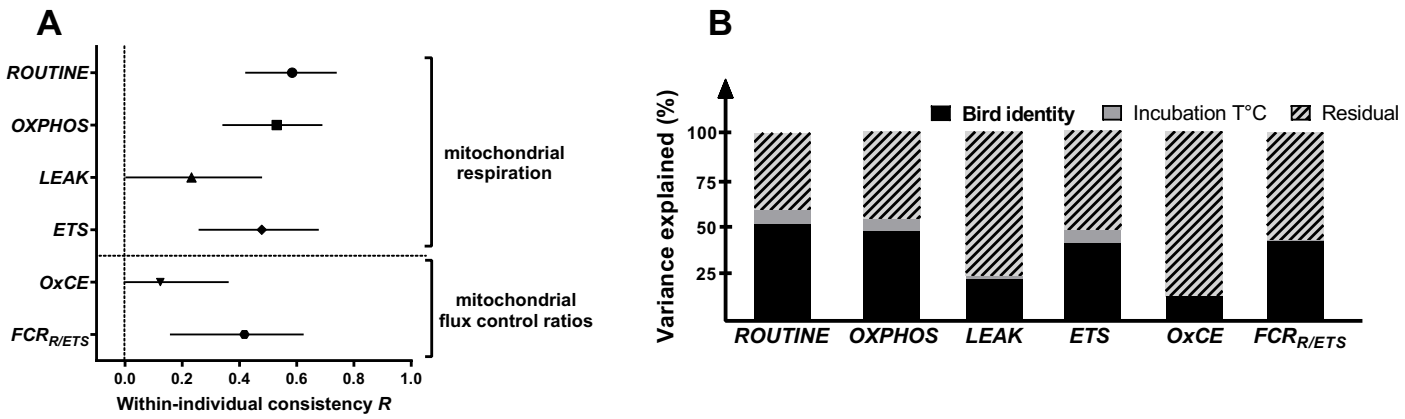

Fig 3: (A) Within-individual consistency of mitochondrial respiration rates and flux control ratios measured in intact blood cells at day 20 and 60 in Japanese quail. Age-adjusted repeatability (*i.e.* consistency) estimates *R* are presented along with their 95% C.I. The statistical model also contained 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)**.

288 ## Discussion

289 Our results demonstrate that variation in the prenatal environment can influence
290 mitochondrial function from post-natal life into adulthood. We found marked differences in
291 mitochondrial function between the peak of the postnatal growth phase and early adulthood,
292 and the pattern of this age-related change was influenced by the environmental temperature
293 regime experienced during prenatal development. Overall, our results suggest that prenatal
294 conditions can have both permanent and delayed effects on mitochondrial function, while
295 also influencing how mitochondrial function changes with age. The persistence of pre-natal
296 temperature effects on mitochondrial function into adulthood suggests that these are long
297 lasting and possibly permanent. We found significant within-individual consistency of
298 mitochondrial respiration rates across life-stages, with individuals showing consistent
299 relatively high or low values through time.

300

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

302 Mitochondrial parameters differed markedly between the peak of the growth phase
303 and early adulthood (6 parameters out of 6). The overall pattern suggests that oxidative
304 phosphorylation decreases with age, while both maximal mitochondrial capacity and proton
305 leak increase, leading to a higher mitochondrial coupling efficiency but also to a more intense
306 utilization of the total mitochondrial capacity during growth than at adulthood. These results
307 are in line with our hypothesis that mitochondrial efficiency should be maximized in early-life
308 to sustain the growth process, and also indicate that mitochondrial maximal capacity is more
309 intensively used during growth. Such effects could potentially be mediated by the known
310 impact of growth hormone on mitochondrial function [39], although this remains to be
311 properly tested.

312 **Prenatal programming of mitochondrial function by incubation temperature and stability**

313 Our results clearly show that the prenatal environment can affect mitochondrial
314 respiration rates (*i.e.* endogenous cellular respiration, oxidative phosphorylation and maximal
315 mitochondrial capacity) in the long-term since we found effects of incubation temperature
316 regime both during postnatal growth and at adulthood. Specifically, it seems that a high
317 incubation temperature increased subsequent mitochondrial metabolism at both life-stages
318 (*i.e.* persistent effect on *ROUTINE*, *OXPHOS* and *ETS* compared to low temperature). Instability
319 in incubation temperature also increased mitochondrial metabolism (compared to its
320 matched control for developmental speed, *i.e.* low temperature), but this was only observed
321 at adulthood (*i.e.* delayed effect on *ROUTINE* and *ETS*). This demonstrates that prenatal
322 environmental conditions can have relatively immediate effects (*i.e.* during postnatal growth)
323 as suggested by previous correlative studies in mammals [40,41], delayed effects as shown for
324 prenatal hypoxia in an experimental reptile model [42], and more importantly, persistent
325 effects lasting from early post-natal life to adulthood. Importantly, unlike previous studies in
326 mammals and reptiles, our results cannot be biased by differences in body mass between
327 experimental groups at the time of mitochondrial measurement [30]. Mitochondrial flux
328 control ratios were not affected by the prenatal treatments, suggesting that the differences
329 we observe in respiration rates between groups were relatively consistent across the different
330 mitochondrial respiration rates we measured.

331 Interestingly, age-related changes in mitochondrial function were also partly
332 influenced by the prenatal environment (*i.e.* age-related decrease in *ROUTINE* for H and L
333 groups only, as well as age-related increase in *ETS* for M and U groups only), suggesting that
334 the prenatal environment does not only affect how mitochondria work postnatally, but also
335 the way mitochondrial aerobic metabolism changes with age. Considering the importance of

336 mitochondria in the ageing process [26,43], such effects of the prenatal environment on both
337 mitochondrial aerobic metabolism and its age-related changes could have potential
338 consequences in influencing ageing trajectories. Even if this hypothesis remains to be properly
339 tested, our results on telomere length (a key hallmark of ageing [43,44]) using the same
340 experimental birds suggest that this might be the case. Indeed, both high temperature and
341 unstable incubation conditions led to increased aerobic metabolism (see above), while also
342 leading to shorter telomeres [30], a typical signature of premature ageing.

343 Although we found clear programming effects of mitochondrial aerobic metabolism by
344 prenatal environmental conditions, we have so far no information about their potential
345 adaptive or maladaptive value. In principle, high and unstable incubation temperatures
346 convey opposite information about environmental quality (*i.e.* high temperature = good
347 quality environment, unstable = poor quality environment; [25]), yet they were the two
348 experimental conditions leading to higher mitochondrial respiration rates (compared to their
349 respective controls) in the present study. Further studies investigating the adaptive role of
350 such variation in mitochondrial function (*e.g.* by testing individual performance under
351 contrasted postnatal environmental conditions) will be needed to conclude about the
352 potential adaptive value of prenatal programming through incubation temperature and
353 stability.

354

355 **Within-individual consistency in mitochondrial function and relative importance of prenatal**
356 **conditions**

357 Mitochondrial respiration rates and flux control ratios exhibited a significant within-
358 individual consistency over time (at the exception of *OxCE*), while they have been measured
359 at two very different life stages (peak of growth *vs.* early-adulthood) and that marked changes

360 in mitochondrial aerobic metabolism occur during postnatal development (see above). To the
361 best of our knowledge, this is the third demonstration that consistent among-individual
362 differences in mitochondrial aerobic metabolism exist (see [10] in wild adult passerine birds,
363 and [11] in adult humans), but the first to be conducted under well-controlled environmental
364 conditions (*i.e.* excluding bias linked to consistent individual differences in environmental
365 conditions) and across life-stages. These findings of a significant within-individual consistency
366 in mitochondrial function over time have important implications for the possibility for early-
367 life conditions to program mitochondrial function over the life course of individuals [12].
368 However, our experimental treatment only accounted for less than 20% of the within-
369 individual consistency observed in mitochondrial respiration rates, meaning that more than
370 80% of the observed within-individual consistency must be explained by other prenatal factors
371 (*e.g.* maternal transfer of nutrients, hormones) and/or by genetic differences between
372 individuals. To the best of our knowledge, there is no information published about the
373 heritability of mitochondrial function, but mtDNA copy number has been shown to be
374 significantly heritable (h^2 = 33%) in humans [45]. Therefore, estimating the relative importance
375 of genetic *vs.* environmental drivers of mitochondrial function appears now fundamental to
376 evaluate the magnitude to which early-life environment could program mitochondrial
377 function and its potential downstream effects such as disease risk and individual performance.
378

379 **Competing interests**

380 We declare having no competing interests

381

382 **Data availability**

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

384 **Author's contribution**

385 AS designed the study, conducted the experimental work, data analysis and wrote the
386 manuscript. NBM and PM had input on study design and data analysis, and commented on
387 the manuscript.

388

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399

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Electronic Supplementary Material (ESM)

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

18 **Correlation between blood cells and brain mitochondrial function**
19
20 In addition to blood samples (see main text and Fig. S1A for mitochondrial responses
21 of intact blood cells to a standard sequential substrate/inhibitor addition protocol), small brain
22 samples (*ca.* 10-15mg) were collected from a subsample of adult females (Day 90, N = 22)
23 following euthanasia by cervical dislocation and rapid dissection on ice (< 3min after
24 euthanasia). Brain samples were transferred in 1mL of Mir05 buffer and stored on ice until
25 analysis (< 1h after euthanasia). Brain samples were quickly blotted on absorbent paper and
26 weighed (± 0.01mg, Sartorius AC211S®). They were then homogenized during 1min using
27 micro-dissecting scissors and further diluted to $2mg.mL^{-1}$ in Mir05 (following Salin et al., 2016).
28 One mL of this preparation was then transferred to the Oxygraph-2k chamber already
29 containing 1mL of Mir05 equilibrated at 40°C, giving a final brain content of $1mg.mL^{-1}$ in the
30 chamber. Mitochondrial respiration was then assessed following a standard sequential
31 substrate/inhibitor addition protocol. First, baseline O_2 consumption was recorded (this
32 parameter is not used in further analysis), then substrates of complex I (pyruvate 5mM and
33 malate 2mM) and complex II (succinate 10mM) were added leading to a non-phosphorylating
34 state hereafter referred as $LEAK_{PMS}$ (*i.e.* equivalent to classical state 2), followed by the
35 addition of a saturating amount of ADP (2mM) to stimulate oxidative phosphorylation
36 (hereafter referred as $OXPHOS_{PMS+ADP}$; equivalent to classical state 3). ATP synthesis was then
37 inhibited with 2.5 µM of oligomycin leading a non-phosphorylating state hereafter referred as
38 $LEAK_{oligo}$ (*i.e.* equivalent to classical state 4), followed by the stimulation of maximal uncoupled
39 respiration (hereafter referred as *ETS*) using a sequential titration of FCCP (0.5µM per step)
40 until maximal stimulation was reached. Finally, we inhibited mitochondrial respiration using
41 antimycin A (2.5µM), and this residual non-mitochondrial O_2 consumption was subtracted
42 from the mitochondrial parameters described above. Average mitochondrial responses to this
43 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}).

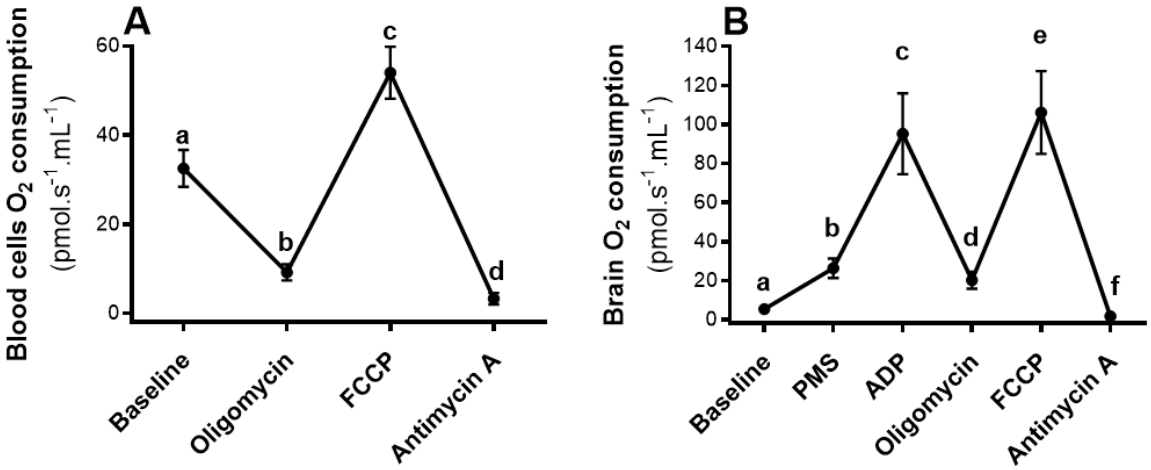

Fig S1: Mitochondrial O_2 consumption responses during high-resolution respirometry assays in intact blood cells (A) and permeabilized brain samples (B) of adult Japanese quails. Letters indicate significant differences according to GEE post-hoc tests. PMS stands for pyruvate+malate+succinate as substrates 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).

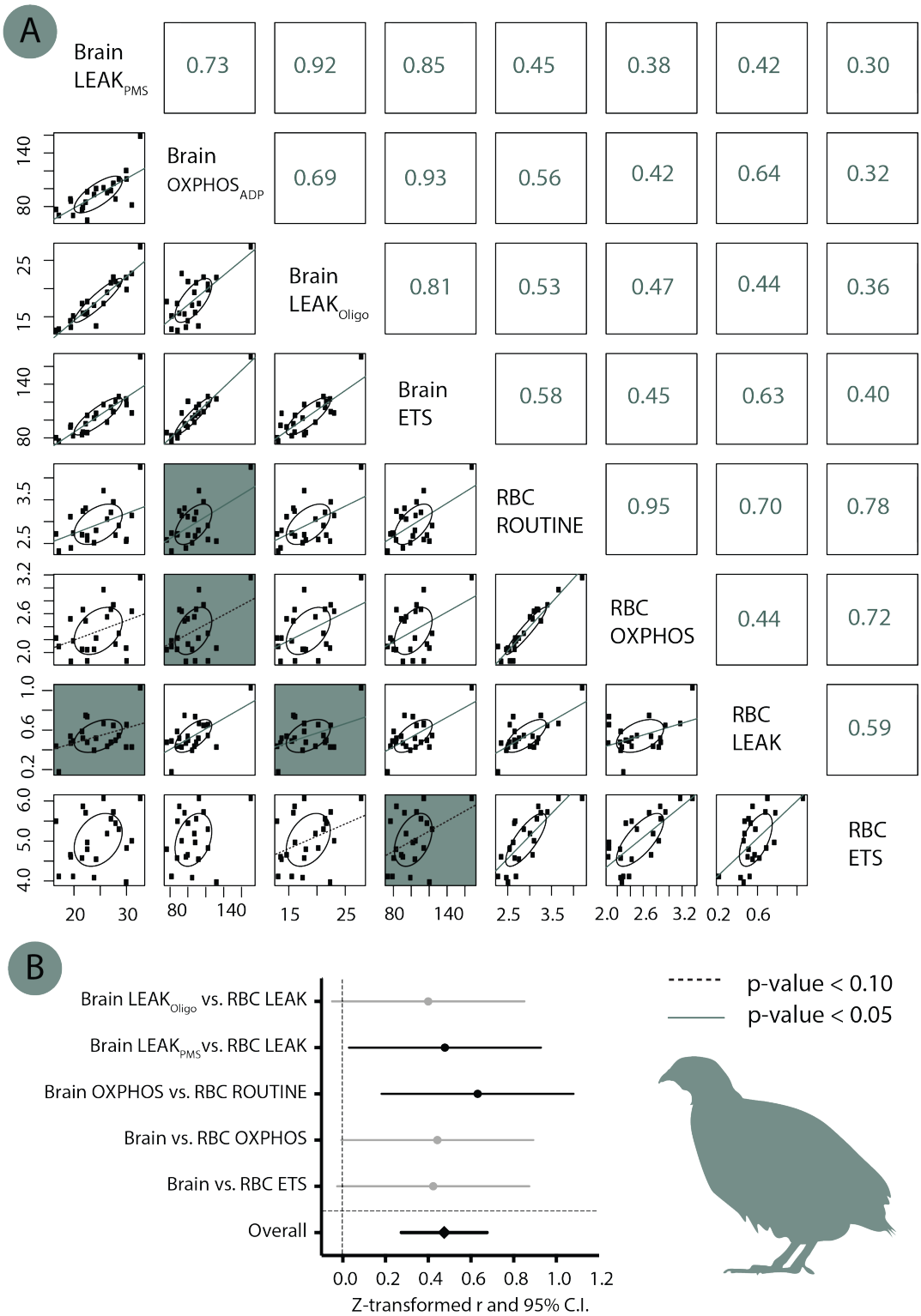

Fig S2: Relationships between brain and blood cell mitochondrial respiration rates. (A) Correlation matrix between the different mitochondrial parameters in the two tissues. Numbers in right part represent the *Pearson* coefficients of correlation *r*, and significance is indicated by the lines in the 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.

92 **Table S1**: **Summary of the statistical models (GEEs) testing the effect of age, prenatal treatment, sex**
93 **and the interaction between age and prenatal treatment on mitochondrial respiration rates of blood**
94 **cells while controlling for protein content (to correct for variations in cells number) and time of day.**
95 Significant parameters ($p \leq 0.05$) are reported in bold and parameters presenting a non-significant
96 trend ($p \leq 0.10$) in italic. Estimates are given for age = Day 20, prenatal treatment = medium
97 temperature and sex = females.
98

(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)

99
100

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)