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1 **Developmental plasticity of mitochondrial aerobic metabolism,**
2 **growth and survival by prenatal glucocorticoids and thyroid**
3 **hormones: an experimental test in wild great tits**

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16 **Abstract**

17 Developmental plasticity is partly mediated by transgenerational effects, including those
18 mediated by the maternal endocrine system. Glucocorticoid and thyroid hormones may play
19 central roles in developmental programming through their action on metabolism and growth.
20 However, the mechanisms by which they affect growth and development remain understudied.
21 One hypothesis is that maternal hormones directly affect the production and availability of
22 energy-carrying molecules (*e.g.* ATP) by their action on mitochondrial function. To test this
23 hypothesis, we experimentally increased glucocorticoid and thyroid hormones in wild great tit
24 eggs (*Parus major*) to investigate their impact on offspring mitochondrial aerobic metabolism
25 (measured in blood cells), and subsequent growth and survival. We show that prenatal
26 glucocorticoid supplementation affected offspring cellular aerobic metabolism by decreasing
27 mitochondrial density, maximal mitochondrial respiration and oxidative phosphorylation, while
28 increasing the proportion of the maximum capacity being used under endogenous conditions.
29 Prenatal glucocorticoid supplementation only had mild effects on offspring body mass, size
30 and condition during the rearing period, but led to a sex-specific (females only) decrease in
31 body mass a few months after fledging. Contrary to our expectations, thyroid hormones
32 supplementation did not affect offspring growth or mitochondrial metabolism. Recapture
33 probabilities as juveniles or adults were not significantly affected by prenatal hormonal
34 treatments. Our results demonstrate that prenatal glucocorticoids can affect post-natal
35 mitochondrial density and aerobic metabolism. The weak effects on growth and apparent
36 survival suggest that nestlings were mostly able to compensate for the transient decrease in
37 mitochondrial aerobic metabolism induced by prenatal glucocorticoids.

38

39

40 **Keywords:** Cellular metabolism, corticosterone, prenatal programming, avian development,
41 thyroid hormones, *Parus major*

42 Introduction

43 Genetic inheritance has long dominated evolutionary thinking (Pigliucci, 2007). Yet,
44 recent advances in evolutionary biology are calling for an extension of this framework and are
45 emphasizing the role of complementary mechanisms (*e.g.*, epigenetic status; transmission of
46 substances such as hormones or RNA; transmission of nutrients) (Bonduriansky and Day,
47 2009; Forsman, 2015; Laland et al., 2015; Müller, 2017; Pigliucci, 2007). Developmental
48 plasticity, in particular, occurs when environmental conditions during ontogenesis create
49 anatomical, physiological and behavioral changes in individual phenotypes that remain
50 through life (Piersma and Gils, 2011). This plasticity can be a direct response to prevailing
51 environmental conditions, but also the consequence of parental effects, which can themselves
52 be a response to current environmental conditions (Proulx and Teotónio, 2017; Uller, 2008).
53 In this case, offspring's phenotype is not only determined by its own environment and
54 genotype, and the interactions between the two, but also by the environment and
55 characteristics of its parents, a phenomenon referred to as intergenerational, or
56 transgenerational plasticity (Marshall and Uller, 2007). Maternal effects, in particular,
57 represent a major pathway in transgenerational developmental plasticity. They rely on diverse
58 mechanisms, such as nutrient transfer or maternally-inherited epigenetic modifications
59 (Alfaradhi and Ozanne, 2011; Laland et al., 2015; Myatt, 2006).

60 The endocrine system, in particular, is a key mediator of maternal effects on
61 developmental plasticity (Dufty et al., 2002; Fowden and Forhead, 2009; Groothuis et al.,
62 2005). Hormone transfer from mother to offspring can have important effects on offspring traits
63 including on the development and growth of juveniles (Groothuis et al., 2019; Meylan et al.,
64 2012). This is particularly true during the initial stages of development when offspring rely on
65 maternally-transferred hormones, before starting their own endogenous hormone production
66 with a fully developed endocrine system (Darras, 2019; McNabb, 2006; Schwabl, 1999).
67 Variation in hormone levels promote developmental plasticity through changes in gene
68 expression, modifying a wide array of physiological, behavioral and morphological traits (*e.g.*
69 begging behavior, immune function; (Groothuis et al., 2005)) including metabolic rates (*e.g.*,
70 through transcription factors, cell signaling, growth factor) (Dufty et al., 2002; Meylan et al.,
71 2012).

72 Whereas the effects of maternal androgens (*e.g.*, testosterone, 5 α -
73 dihydrotestosterone, andostenedione) on offspring development have been well studied
74 (Groothuis et al., 2005; Podmokła et al., 2018), less is known on the effects of thyroid
75 hormones (THs). Yet, THs are central growth regulators, and coordinate maturation and
76 differentiation as transcription factors (Darras, 2019; Ruuskanen and Hsu, 2018). Thus,
77 variation in THs during critical periods may have marked effects on offspring development

78 (e.g., neurotrophic signals, cerebellar-mediated motor function, retinal layer) (Darras, 2019;
79 Ruuskanen and Hsu, 2018), and are also known to affect offspring behavior via early-life
80 imprinting (Bett et al., 2016; Yamaguchi et al., 2012). THs modulate metabolism associated
81 with (i) medium to long-term changes in the basal energy expenditure of the organism (Harper
82 and Seifert, 2008; Kim, 2008) and (ii) modulation of the activity of downstream regulatory
83 hormones and growth factors such as insulin, glucagon and catecholamines ((Grøntved et
84 al., 2015; Pucci et al., 2000; Sinha et al., 2018).

85 Glucocorticoid hormones (GCs) are other well-known regulators of metabolic (Rose et
86 al., 2010) and developmental processes (Miyazawa and Aulehla, 2018; Rieger, 1992).
87 Prenatal GC play a role in offspring developmental plasticity (Seckl, 2004), and GC-mediated
88 maternal effects potentially lead to long-lasting changes in offspring phenotype and
89 metabolism (e.g., neurodevelopmental and cardio-metabolic effects; (Aghajafari et al., 2002;
90 Eberle et al., 2021). GC have been shown to modulate the expression of up to 10% of the
91 genome (Le et al., 2005; Xavier et al., 2016). As direct regulators of metabolic processes, GCs
92 also enable the organism to accommodate changes in energetic demands through a variety
93 of mechanisms (ranging from appetite to glycogenolysis and lipolysis regulation; (Rose et al.,
94 2010; Sapolsky et al., 2000). The impact of GC on metabolism is often investigated from the
95 point of view of individual responses to stress (*i.e.*, as the consequence of stress-induced
96 changes in GC levels; (Crespi et al., 2013), though GCs primarily play a role in regulating body
97 homeostasis (MacDougall-Shackleton et al., 2019).

98 At the same time, a growing body of evidence is pointing towards mitochondrial
99 function (which central role is to transduce energy acquired from nutrients into ATP) as the
100 central link between the endocrine system, metabolism, and growth (Koch et al., 2021; Picard
101 et al., 2014; Salin et al., 2019). Specifically, TH have been shown to modulate mitochondrial
102 activity both directly (Cioffi et al., 2013; Noli et al., 2020), and indirectly by up-regulating
103 mitochondrial biogenesis (Weitzel and Iwen, 2011). Short and long-term exposure to low
104 physiological amounts of GC also enhance mitochondrial function (as measured through
105 membrane potential, proton leak, ATP production, or maximal mitochondrial capacity), while
106 chronic exposure to high levels of corticosterone may decrease it (Casagrande et al., 2020;
107 Manoli et al., 2007; Picard et al., 2014). Thus, we may expect the impact of maternal effects
108 on offspring phenotype (e.g. growth) to be mediated by the action of prenatal maternal
109 hormones on mitochondrial function. There is growing evidence that despite flexibility in
110 mitochondrial function, stable inter-individual differences through time exist (e.g. (Braganza et
111 al., 2020; Stier et al., 2019; Stier et al., 2022). Inter-individual differences might arise from
112 developmental plasticity (Gyllenhammer et al., 2020; Stier et al., 2022). Yet, to the best of our

113 knowledge, very little is known on the impact of prenatal hormones in shaping offspring
114 mitochondrial function (but see (Davies et al., 2021; Grilo et al., 2021)).

115 The purpose of our study was to investigate the effects of prenatal exposure to
116 elevated levels of TH and GC hormones on offspring mitochondrial aerobic metabolism,
117 growth and survival throughout postnatal development. We aimed at mimicking an increase
118 in maternal TH and GC hormonal levels deposited in the eggs by experimentally injecting eggs
119 of wild great tit (*Parus major*) before the onset of incubation with physiological doses of THs
120 and/or GC, or with saline solution (control), in a controlled full factorial (2x2) study design. We
121 assessed differences between individuals hatching from treated and control eggs in terms of
122 embryonic development duration, body size, body mass, body condition (body mass adjusted
123 for size), as well as changes in blood cell mitochondrial density and respiration. We evaluated
124 effects on offspring from hatching (day 2) through fledging (day 14), with an intermediate
125 measure performed at day 7 (see Fig.1 for the experimental timeline and sample size). We
126 also recaptured a fraction of the birds as juveniles (ca. 9 to 20 weeks after fledging) and as
127 adults (ca. 15 to 18 months after fledging) and tested for the consequences of elevated
128 prenatal hormone levels on short-term (fledging), medium-term (first autumn after fledging)
129 and long-term (second autumn after fledging) survival (using catching probability as a proxy).

130 As THs are known to stimulate mitochondrial aerobic metabolism and biogenesis while
131 potentially decreasing the efficiency at which nutrients are converted to ATP (Cioffi et al.,
132 2013), we expected nestlings hatched from eggs supplemented with THs to exhibit a higher
133 mitochondrial density and higher mitochondrial respiration rates, but a potentially higher
134 proton leak leading to less efficient mitochondria (Fig. 2). We predicted that such a higher
135 metabolic capacity could boost embryo development and early post-hatching growth and
136 survival, while the lower mitochondrial efficiency might impair body condition and performance
137 later during postnatal development (Salin et al., 2019) leading to a decrease in survival
138 prospects especially after fledging (but see (Hsu et al., 2019; Hsu et al., 2020; Hsu et al., 2021;
139 Ruuskanen et al., 2016; Sarraude et al., 2020), for the contrasted effects of prenatal THs on
140 growth in avian species). Since physiological amounts of GC have been suggested to enhance
141 mitochondrial density and aerobic metabolism (including ATP production, (Manoli et al., 2007),
142 we expected nestlings hatched from eggs supplemented with GC to exhibit a higher
143 mitochondrial density and higher mitochondrial respiration rate, as well as a higher efficiency
144 to produce ATP (Fig. 2, but see (Casagrande et al., 2020) for somewhat opposite effects of
145 high GC levels at the postnatal stage). Thus, we expected these individuals to have a faster
146 growth (both pre- and postnatal) leading to an increase in survival prospects on the short-term
147 (i.e. fledging and/or first autumn) but potential long-term costs (Hausmann et al., 2012;
148 Metcalfe and Monaghan, 2001). Finally, we tested if GC and TH hormones had interactions,

149 such as synergistic effects, affecting offspring mitochondrial function, growth and survival
150 (Brown et al., 2014). For instance, it has been shown that postnatal supplementation with THs
151 and GC has synergistic effects on growth (Khangembam et al., 2017). Yet, directional
152 predictions about the effects of prenatal hormones are very difficult to make considering 1. the
153 likely environmental-dependence of their cost-benefit balance, 2. the existence of non-linear
154 dose-responses and 3. the fact that embryos are not passive receivers of maternal hormones
155 but can manipulate such signals (Groothuis et al., 2019).

156

157

159 **Material and Methods**160 *Field site and population monitoring*

161 The study was conducted in a population of wild great tits (*Parus major*) breeding in artificial
162 nest boxes (n = 374) on Ruissalo island, Finland (60°26.055' N, 22°10.391' E). The data was
163 collected during the 2019 breeding season (April to July), and during the autumns of 2019 and 2020
164 (October to November). Nest boxes were checked every 5 days during the breeding season to
165 monitor occupation. We also recorded date of laying the first egg (laying date), incubation onset,
166 clutch size, hatching date (\pm 24h), developmental duration (\pm 24h) (*i.e.* time between incubation
167 onset and hatching), brood size, and fledging success.

168

169 *Experimental manipulation of glucocorticoids and thyroid hormones*

170 To manipulate the prenatal hormonal environment that offspring were exposed to, nests
171 were randomly divided into 4 groups, and eggs either received i) an injection of control isotonic saline
172 solution (CO, 2 μ L NaCl), ii) an injection elevating TH (a mixture of 0.325 ng T4 and 0.041 ng T3 per
173 yolk), iii) an injection elevating corticosterone (CORT) (0.202 ng per yolk), or iv) an injection elevating
174 both CORT and TH hormones (*i.e.* 0.325 ng of T4 + 0.041 ng of T3 + 0.202 ng of CORT). Our
175 objective was to increase yolk hormones content by 2 standard deviations (SD) while remaining in
176 their natural physiological range, as recommended by Podmokła and al. (2018). Based on the
177 literature and hormonal measurements from the same population, average TH content in great tits
178 are expected to be mean \pm SD : T3 = 0.053 \pm 0.020 ng/yolk and T4 = 0.458 \pm 0.162 ng/yolk
179 (Ruuskanen et al., 2018), while average CORT is expected to be mean \pm SD: 0.215 \pm 0.101 ng/yolk
180 (based on the averages for great tits from (Groothuis and Schwabl, 2008; Lessells et al., 2016;
181 Montesana et al., 2019) Groothuis & Schwabl, 2008; Montesana et al., 2019; Lessells et al., 2016,
182 calculated using an average yolk mass of 315 mg as in Lessells et al. 2016).

183 Hormone solutions were prepared using crystal T4 (L-thyroxine 98% HPCL, CAS number 51-
184 48-9, Sigma-Aldrich), T3 (3,3',5-triiodo-L-thyronine, >95% HPCL, CAS number 6893-02-3, Sigma-
185 Aldrich) and CORT (Corticosterone VETRANAL®, HPCL, CAS number 50-22-6, Sigma-Aldrich)
186 dissolved in 0.1M NaOH (TH) or 99% EtOH (CORT), and diluted in 0.9% NaCl to the targeted
187 concentrations. We followed the injection procedure as described in (Hsu et al., 2019; Sarraude et
188 al., 2020). We prepared the corresponding hormone solutions for each experimental group (CO, TH,
189 CORT or CORT + TH), so that each egg was injected only once with 2 μ l of the corresponding
190 hormone solution and all eggs in one nest received the same hormonal mix. Egg injections started
191 on the day the 5th egg was laid, and every day later on until the last egg was laid. This protocol
192 ensured injections were done before the incubation onset, meanwhile minimizing nest-disturbance
193 (*i.e.* we avoided visiting the nest every day) and allowing to closely monitor the onset of incubation,
194 given that great tits can start incubation well before clutch completion. When no new eggs were

195 observed for two consecutive days, the clutch was considered complete. Hatching was monitored
196 daily starting 2 days prior to the estimated hatch date. Hatching was considered as “day 0”.

197 Nestlings were individually marked (nail-clipping at day 2, metal ring at day 7), weighed with
198 an electronic scale (body mass $\pm 0.1\text{g}$) at 2, 7, 14 days old, and measured with a metal ruler (wing
199 length $\pm 1\text{mm}$) at 7 and 14 days old (see Fig. 2 for a timeline of the study). Nestlings fledge around
200 18-20 days old. When recaptured in the following autumns (see below), body mass and wing length
201 were measured. We also blood sampled individuals ($\sim 30\text{-}75\mu\text{L}$ from the brachial vein using
202 heparinized capillaries) at 7 and 14 days old and as juveniles in the following autumn. Blood samples
203 were used to measure mitochondrial DNA copy number (*mtDNA_{cn}*, an index of mitochondrial
204 density, see below) and evaluate mitochondrial aerobic metabolism in 7- and 14-days old nestlings
205 (Fig. 2). The use of blood samples has the advantage of being minimally invasive, allowing the
206 longitudinal sampling of the individuals (Koch et al., 2021; Stier et al., 2017).

207 We recaptured nestlings from the experiment as juveniles the following autumn (in 2019, *i.e.*
208 between 9 and 20 weeks after fledging). For this, we used mist-nests with playback at 7 feeding
209 stations in the study plots (3h / feeding station on 3 separate days over 2 months summing up to a
210 total of 100 hours of mist-netting). If a bird was recaptured several times during this period, only the
211 measurements from the first capture were used for body mass, body size and blood sample.
212 Nestlings were also recaptured as adults (*i.e.* between 15 and 18 months after fledging) using a
213 similar method (6 feeding stations, a total of 95 hours of mist-netting) in autumn 2020. In addition,
214 we included recapture data from a mist-netting site (Ruissalo botanical garden; 3 km from the study
215 plots) where mist-netting was conducted regularly throughout the year every 1 or two weeks (4h per
216 session). Data collected from the 2019 recapture sessions were used to analyze juvenile body mass,
217 size and condition, mitochondrial DNA copy number, and for estimating recapture probability a few
218 months after fledging (*i.e.* used here as a proxy of medium-term apparent survival). Data collected
219 from autumn 2020 trapping sessions and continuous mist-netting were used as a proxy of long-term
220 survival (*i.e.* recapture probability during and after the first winter experienced by juveniles).

221 In total, the experiment included 60 great tit nests resulting in 468 injected eggs ($n_{\text{CO(eggs/nests)}} = 108/13$, $n_{\text{TH}} = 118/16$, $n_{\text{CORT}} = 111/14$, $n_{\text{CORT} + \text{TH}} = 131/17$) and 267 chicks being monitored
222 ($n_{\text{CO(nestlings/nests)}} = 60/12$, $n_{\text{TH}} = 75/15$, $n_{\text{CORT}} = 58/13$, $n_{\text{CORT} + \text{TH}} = 74/13$). 112 juveniles were caught in
223 the autumn of 2019 ($n_{\text{CO(juveniles/nests)}} = 25/10$, $n_{\text{TH}} = 22/9$, $n_{\text{CORT}} = 28/10$, $n_{\text{CORT} + \text{TH}} = 37/10$), and 30
224 adults in the autumn of 2020 ($n_{\text{CO(adults/nests)}} = 6/5$, $n_{\text{TH}} = 6/5$, $n_{\text{CORT}} = 6/5$, $n_{\text{CORT} + \text{TH}} = 12/8$).

226

227 *Mitochondrial DNA copy number*

228 We randomly selected 2 nestlings per nest ($n = 104$ individuals) and estimated *mtDNA_{cn}* on
229 the same individuals at day 7, day 14 and as juveniles (autumn 2019) when samples were available
230 (respectively sample-sizes at day 7/ day 14 / juveniles: $n_{\text{CO}} = 26/27/9$, $n_{\text{CORT}} = 23/21/10$, $n_{\text{TH}} =$
231 $29/24/7$, $n_{\text{CORT} + \text{TH}} = 25/23/11$, resulting in 235 samples in total). Genomic DNA was extracted from

232 5 μ L of frozen blood samples using a salt extraction procedure adapted from (Aljanabi and Martinez,
233 1997). DNA quantity and purity were estimated using a *NanoDrop* spectrophotometer. Samples were
234 re-extracted if needed ([DNA] < 50ng/ μ L, 260/280 ratio < 1.80 or 260/230 < 2). DNA integrity of 48
235 randomly selected samples were evaluated and deemed satisfactory using gel electrophoresis (100
236 ng of DNA, Midori Green staining, 0.8 % agarose gel at 100 mV for 60 min). Samples meeting our
237 quality checks were then diluted at 1.2 ng/ μ L in sterile H₂O and stored at -80°C until qPCR assays.
238 *mtDNAcn* was quantified using real-time quantitative PCR (qPCR) assays as previously described
239 for other passerine species (Stier et al., 2019; Stier et al., 2020) and great tits (Hsu et al., 2021; Stier
240 et al., 2021). This technique estimates the relative mtDNAcn by determining the ratio of mtDNA
241 repeat copy number to a nuclear singly copy gene (SCG). qPCR reactions were performed in a total
242 volume of 12 μ L including 6ng of DNA sample, primers at a final concentration of 300nM and 6 μ L of
243 SensiFAST™ SYBR® Lo-ROX Kit (Bioline). We used Recombination Activating Gene 1 (RAG1) as
244 a single-copy control gene (SCG) verified using a BLAST analysis on the great tit genome. The gene
245 RAG1 was amplified using the primers RAG1 forward (5'-TCG GCT AAA CAG AGG TGT AAA G-3')
246 and RAG1 reverse (5'-CAG CTT GGT GCT GAG ATG TAT-3'). For *mtDNAcn*, we used cytochrome
247 oxidase subunit 2 (COI2) as a specific mitochondrial gene after verifying that it was not duplicated
248 as a pseudo-gene in the nuclear genome using a BLAST analysis on the great tit genome. We used
249 the primers sequences COI2 forward (5' – CAAAGATATCGGCACCCTCTAC-3') and COI2 reverse
250 (3'-GCCTAGTTCTGCACGGATAAG-5'). Samples were run in triplicates. qPCR conditions were 3
251 min at 95°C (polymerase activation), followed by 40 cycles of 10s at 95°C, 15s at 58°C, 10s at 72°C
252 (DNA denaturation, primers annealing, DNA extension and fluorescence reading). The melting curve
253 program was 15s at 95°C, 1min at 58°C, 0.1°C/s increase to 95°C, and then hold 15s at 95°C. A
254 DNA sample being a pool of DNA from 10 adult individuals was used as a reference sample (*i.e.*
255 ratio = 1.0 for *mtDNAcn*) and was included in triplicates in every plate. qPCR efficiencies of control
256 and mitochondrial genes were 91.4 \pm 0.003% and 104.5 \pm 0.005%, respectively. Repeatability of
257 mtDNAcn measurements estimated with samples-triplicates was high R = 0.921 (CI_{95%} = [0.907;
258 0.934], n = 1287). We also calculated the inter-plate repeatability of *mtDNAcn* measurements using
259 samples being measured on different plates: R = 0.867 (CI_{95%} = [0.822, 0.916], n = 211). All the
260 qPCR assays (n = 10 plates) were performed on a 384-QuantStudio™ 12K Flex Real-Time PCR
261 System (Thermo Fisher).

262 *Molecular sexing*

263 Nestlings were molecularly sexed using a qPCR approach adapted from (Chang et al., 2008;
264 Ellegren and Fridolfsson, 1997), using blood samples when available (2 nestlings per brood). Forward
265 and reverse sexing primers were 5'- CACTACAGGGAAACTGTAC-3' (2987F) and 5'-
266 CCCCTTCAGGTTCTTTAAAA -3' (3112R), respectively. qPCR reactions were performed in a total
267 volume of 12 μ L including 6ng of DNA, primers at a final concentration of 800nM and 6 μ L of
268 SensiFAST™ SYBR® Lo-ROX Kit (Bioline). qPCR conditions were: 3 min at 95°C, followed by 40

269 cycles of 45 s at 95°C, 60 s at 52°C and 60s at 72°C, then followed by a melting curve analysis
270 (95°C 60s, 45°C 50s, increase to 95°C at 0.1°C/s, 95°C 30s). Samples were run in duplicates in a
271 single plate and 6 adults of known sex were included as positive controls.

272

273 *Mitochondrial respiration*

274 Mitochondrial respiration was analyzed using high-resolution respirometry (Oroboros
275 Instruments, Innsbruck, Austria) at 40°C, adapted from the protocol described in (Stier et al., 2019)
276 (protocol modifications: mitochondrial respiration rates were estimated using 30µL of fresh blood
277 when available, suspended in Mir05 buffer). We analyzed 4 mitochondrial respiration rates: 1) the
278 endogenous cellular respiration rate before permeabilization (*ROUTINE*), 2) the maximum
279 respiration rate fueled with exogenous substrates of complex I and II, as well as ADP (*CI + II*), 3) the
280 respiration rate contributing to proton leak (*LEAK*, *i.e.*, not producing ATP but dissipating heat), 4)
281 the respiration rate supporting ATP synthesis through oxidative phosphorylation (*OXPHOS*). We
282 also calculated 2 mitochondrial flux ratios (FCRs): 1) *OXPHOS* coupling efficiency: $OxCE = (1 - LEAK) / CI + II$,
283 and 2) the proportion of maximal respiration capacity being used under endogenous cellular
284 condition (*i.e.*, $FCR_{ROUTINE} / CI + II$). The former provides an index of mitochondrial efficiency in
285 producing ATP, whereas the latter reflects the cellular control of mitochondrial respiration by
286 endogenous ADP/ATP turnover and substrate availability. Due to the logistical constraints of
287 respirometry measurements (*i.e.*, the need to work on freshly collected samples, > 2 h of processing
288 per 2 samples), the analysis of mitochondrial respiration was limited to 1 nestling per nest (repeated
289 measurements from same individuals at day 7 and day 14), summing up to 89 samples from 48
290 individuals (respectively sample-sizes at day 7/day 14: $n_{CO} = 11/11$, $n_{CORT} = 11/10$, $n_{TH} = 14/12$, $n_{CORT} + TH = 10/10$). Mitochondrial respiration rates were not analyzed from juveniles due to logistical
291 constraints. The technical repeatability of mitochondrial respiration measurements was high:
292 *ROUTINE* : $R = 0.989$ ($CI_{95\%} = [0.957, 0.997]$); *CI + II*: $R = 0.992$ ($CI_{95\%} = [0.968, 0.998]$); *LEAK*: R
293 $= 0.982$ ($CI_{95\%} = [0.929, 0.995]$) ; *OXPHOS*: $R = 0.992$ ($CI_{95\%} = [0.968, 0.998]$) based on $n = 9$
294 duplicates.
295

296 *Statistical analyses*

297 Statistical analyses were conducted using *R* v. 4.0.2 (R core team, 2020). To test for
298 the effects of prenatal hormones on bird development, mitochondrial function and survival, we
299 treated CORT and TH treatments (as separate 2-level factors: CORT yes/no and TH yes/no)
300 and their interactions as fixed factors. Non-significant terms were dropped (starting with
301 interactions) in a backward-stepwise procedure to obtain the lowest Akaike Information
302 Criterion (AIC) value. The effects of CORT and TH treatments on survival metrics (hatching
303 success, fledging success and recapture probabilities in autumns 2019 and 2020) were
304 evaluated using generalized linear mixed models (GLMM), with logistic binary distributions of
305 the dependent variables (survival: 0 = dead / 1 = alive). Nest box ID was considered as a
306 random intercept to account for the non-independence of nestlings reared in same conditions,
307 except for the recapture probability as adults since we did not re-capture enough individuals
308 per nest. We tested the effects of CORT and TH treatments on developmental time (incubation
309 time per nest) using a linear model (LM).

310 The effect of CORT and TH treatments on growth metrics were analyzed in two steps.
311 We first tested treatment effects on postnatal body mass growth (day 2, day 7, day 14) using
312 a linear mixed model (LMM) with nest box ID and bird ID as random intercepts, to account for
313 repeated measures on individual offspring and non-independence of nestlings reared in same
314 conditions. To test for differences in body mass gain, we also tested the effects of CORT and
315 TH treatments at each age (day 7, day 14 and in juveniles – Autumn 2019) on body mass,
316 while controlling for the previous body mass as a covariate in separate LMMs with nest box
317 ID specified as random intercept. We analyzed body size (using the wing length as a response
318 variable) and body condition (*i.e.*, body mass controlled for the wing length) at each age using
319 LMMs with nest box ID specified as random intercept.

320 *mtDNAcn* data distribution did not fulfill the criteria of normality according to a Cullen
321 and Frey plot ('fitdistrplus' package, (Delignette-Muller and Dutang, 2015), therefore we
322 evaluated the effects of CORT and TH treatments on *mtDNAcn* using a GLMM (gamma error
323 distribution, log link). We included nest box ID as a random intercept and bird ID as a repeated
324 factor to account for the non-independency of measures from a same individual. All
325 mitochondria respiration rates (recorded at day 7 and day 14; including *ROUTINE*, *LEAK*,
326 *OXPHOS*, *CI+II*) were tested with LMMs. We analyzed mitochondrial respiration rates at both
327 the cellular level (*i.e.*, respiration measurements expressed relative to cell number) that
328 indicates respiration properties per unit of cells, and at the mitochondrial level (*i.e.*, respiration
329 measurements controlled for mitochondrial density by inclusion of *mtDNAcn* as a covariate),
330 which indicates the respiration rate per unit of mitochondria. For models including repeated
331 measures across time (body mass, *mtDNAcn*, mitochondrial respiration measurements), we
332 initially included CORT, TH, age and all interactions as fixed factors and removed non-

333 significant interactions following a backward-stepwise procedure to obtain the lowest AIC
334 value.

335 We also preliminary included nestling sex as a fixed factor in our models to investigate
336 sex-specific effects on growth metrics and *mtDNAcn*. However, nestling sex never had a
337 significant effect on morphometric traits and we decided to remove sex from the associated
338 models to increase sample-sizes (only 2 nestlings per nests were molecularly sexed through
339 real-time qPCR, while for growth we collected morphometrics measurements for the whole
340 brood). For juveniles, all individuals were morphologically sexed and thus we also included
341 sex, as well as its interaction with CORT and TH treatments.

342 In all models, hatching date and brood size at day 2 (both proxies of environmental
343 conditions) were included as covariates (not scaled, except in the *mtDNAcn* model due to
344 convergence issue) when applicable as they are known to correlate with development,
345 physiology and survival. Normality and homoscedasticity of the residuals were visually
346 inspected (QQ plots). All models were performed using the 'lme4' package (Bates et al., 2015).
347 Results from type III anova tables with *F*-values (or χ^2 for GLMM) and *p*-values (*i.e.* testing
348 the main effect of each factor and interaction) calculated based on Satterwhaite's method are
349 presented in the text, and model estimates (with associated 95% C.I. and *p*-values) are
350 reported in Tables. The package 'emmeans' was used for conducting multiple post-hoc
351 comparisons (adjusted with Tukey Honest Significant Differences correction) and estimating
352 least-square means (lsmean) \pm SE as well as standardized effect-sizes (Lenth et al., 2018).
353 Results are given as means \pm SE. Values were considered as statistically significant for $p <$
354 0.05.

355

356 **Results**

357 *Prenatal hormonal effects on hatching, fledging success and developmental time*

358 Hatching success (CO = 55.6%, CORT = 53.4%, TH = 62.7%, CORT+TH = 58.6%)
359 and fledging success (CO = 90%, CORT = 89.8%, TH = 75.7%, CORT+TH = 74.4%) were not
360 significantly affected by the prenatal hormonal manipulation (GLMMs, all $\chi^2 < 2.5$, all $p > 0.11$).
361 Developmental time was significantly increased (+ 7%) by a prenatal CORT supplementation
362 (LM, CORT vs. non-CORT: lsmean \pm SE: 12.8 \pm 0.2 vs. 12.0 \pm 0.2 days, $F_{1,49} = 6.27$, $p = 0.015$),
363 but significantly decreased (- 5%) by a prenatal TH supplementation (TH vs. non-TH: lsmean
364 \pm SE: 12.1 \pm 0.2 vs. 12.7 \pm 0.2 days; $F_{1,49} = 4.26$, $p = 0.044$). However, there was no significant
365 CORT x TH interaction ($F_{1,49} = 2.24$, $p = 0.14$).

366

367 *Prenatal hormonal effects on mitochondrial density*

368 We found a significant effect of the prenatal CORT supplementation in interaction with
369 age on mitochondrial density (overall test for Age x CORT: $\chi^2 = 8.65$, $p = 0.013$, Fig. 3a).

370 Mitochondrial density was significantly influenced by age ($\chi^2 = 451.7$, $p < 0.001$), decreasing
371 from day 7 to day 14 (Tukey HSD post-hoc: $p < 0.001$) and from day 14 to the juvenile stage
372 (Tukey HSD post-hoc: $p < 0.001$; see Table 1 for estimates of final model). While prenatal
373 CORT did not significantly affect mitochondrial density at day 7 (Tukey HSD post-hoc: $p =$
374 0.29) or in juveniles (Tukey HSD post-hoc: $p = 0.92$), it significantly decreased mitochondrial
375 density by 27 % at day 14 (Tukey HSD post-hoc: $p = 0.006$, Fig. 3a). We found no significant
376 evidence for an effect of prenatal TH supplementation on mitochondrial density ($\chi^2 = 0.003$, p
377 $= 0.96$, Fig. 3b), nor for an interaction between prenatal TH and CORT ($\chi^2 = 0.006$, $p = 0.81$).
378 Brood size was negatively related to mitochondrial density ($\chi^2 = 4.31$, $p = 0.036$), while
379 hatching date was not significantly related to mitochondrial density ($\chi^2 = 1.50$, $p = 0.22$, Table
380 1).

381

382 *Prenatal hormonal effects on mitochondrial aerobic metabolism*

383 Prenatal CORT supplementation significantly decreased all mitochondrial respiration
384 rates measured at the cellular level (LMMs: *ROUTINE*: -15.8%, *LEAK*: -16.4%, *OXPHOS*: -
385 22.9%, *CI+II*: - 21.7%; all $F > 4.2$, all $p < 0.05$; Fig. 4), in a similar way at both day 7 and day
386 14 (LMMs, Age x CORT interactions not statistically significant; all $F < 0.71$; all $p > 0.41$). Yet,
387 all cellular respiration rates were positively associated with mitochondrial density (LMMs, all p
388 < 0.001 , Table 2). Controlling for mitochondrial density decreased the influence of prenatal
389 CORT on respiration rates (*i.e.* respiration at the mitochondrial level), as evidenced by smaller
390 effect sizes when correcting for mitochondrial density (Fig. 4; *ROUTINE*: -6.5% $F = 1.41$, $p =$
391 0.24 ; *LEAK*: -9.8%, $F = 2.29$, $p = 0.14$; *OXPHOS*: -14.2%, $F = 4.77$, $p = 0.037$; *CI+II*: -13.3%,
392 $F = 4.72$, $p = 0.037$; Table 2). Interestingly, nestlings from CORT-supplemented eggs had a
393 significantly higher (+7.9%) usage of their mitochondrial maximal capacity (higher
394 $FCR_{ROUTINE/CI+II}$, $F = 4.79$, $p = 0.034$, Fig. 4, Table 3), but we found no significant effect of
395 prenatal CORT on *OXPHOS* coupling efficiency (*OxCE*, $F = 1.32$, $p = 0.26$, Fig. 4, Table 3).

396 Contrary to prenatal CORT, there was no significant effect of the prenatal TH
397 supplementation on mitochondrial aerobic metabolism (LMMs, all $F < 2.26$, all $p > 0.14$, Tables
398 2 & 3). All mitochondrial respiration rates significantly decreased between nestling day 7 and
399 day 14 (LMMs, *ROUTINE*: -15.3 %, *OXPHOS*: -12.4 %, *CI+II*: -11.5 %; all $F > 4.8$, $p < 0.032$,
400 Table 2), except *LEAK* (LMM, $F = 1.70$, $p = 0.20$, Table 2). While $FCR_{ROUTINE/CI+II}$ was not
401 significantly impacted by age ($F = 1.89$, $p = 0.18$, Table 2), younger chicks had more efficient
402 mitochondria (*i.e.* 2.9% higher *OxCE*, $F = 8.33$, $p = 0.006$, Table 3). Males showed a
403 significantly higher *LEAK* (lsmean: +16.5%, $F = 4.23$, $p = 0.047$) than females when controlling
404 for mitochondrial density (Table 2), but we did not find other significant sex differences in
405 mitochondrial aerobic metabolism (LMMs, all $F < 1.65$, all $p > 0.20$, Table 2). Brood size was
406 not significantly associated with mitochondrial aerobic metabolism traits (LMMs, all $F < 1.69$,

407 all $p > 0.20$, Tables 2 and 3). All mitochondrial aerobic metabolism traits except *ROUTINE* (F
408 = 0.22, $p = 0.64$) and *LEAK* ($F = 0.02$, $p = 0.88$) were significantly positively associated with
409 the hatching date (LMMs, all $F > 8.10$, all $p < 0.008$, Tables 2 and 3).

410

411 *Prenatal hormonal effects on growth*

412 When analyzing body mass dynamics during postnatal growth (from day 2 to day 14),
413 there was a significant interaction between age (d2 vs. d7 vs. d14) and CORT treatment
414 factors ($F_{2,460} = 4.40$, $p = 0.013$, Table 4, Fig. 5), but no significant effect of the prenatal TH
415 supplementation ($F_{1,50} = 0.95$, $p = 0.33$, Table 4). Specifically, nestlings from CORT-
416 supplemented eggs were slightly lighter (-11.3%) at day 2 than offspring from non-CORT-
417 supplemented eggs (lsmean \pm SE: $3.54 \pm 0.22\text{g}$ vs. $3.14 \pm 0.21\text{g}$), but reached the body mass
418 of chicks from the non-CORT-supplemented group at day 7 and 14 (Fig. 5), although these
419 differences were not statistically significant in post-hoc analyses (Tukey HSD post-hoc: all p
420 > 0.18).

421 Analyzing the different postnatal stages separately (day 2, day 7 and day 14) for body
422 mass gain (*i.e.* body mass at time t analyzed with body mass at time $t-1$ as covariate), body
423 size and body condition did not reveal any significant effect of prenatal hormonal treatments
424 (*i.e.*, CORT and TH), either as main factors (all $F < 3.65$, $p > 0.06$, Tables S1-S3) or in
425 interaction (CORT x TH: all $F < 3.75$, all $p > 0.05$). Yet, there was a non-significant trend for
426 CORT chicks to gain more body mass between day 2 and day 7 ($F_{1,43.7} = 3.65$, $p = 0.063$,
427 Table S2), and for an interaction between CORT and TH in explaining body size at day 7 ($F_{1,47}$
428 = 3.74, $p = 0.059$) with chicks that received both hormones having smaller wings than others
429 (lsmmeans \pm SE: CORT+TH: 18.5 ± 0.7 ; no-CORT/no-TH: 19.9 ± 0.7 ; CORT/no-TH: 20.7 ± 0.7 ;
430 TH/no-CORT: 20.4 ± 0.7).

431 For juveniles (*i.e.* subsample of individuals recaptured in autumn and morphologically
432 sexed), we found a significant interaction between CORT treatment and sex on body mass (F
433 = 8.36, $p = 0.005$) and condition ($F = 8.91$, $p = 0.004$) but not on body size ($F = 0.42$, $p = 0.52$;
434 Table S4). Body mass was 3.4% lower for females that received a prenatal CORT treatment
435 than females from the non-CORT group ($p = 0.021$), while there was no significant effect of
436 the prenatal CORT treatment on male body mass ($p = 0.25$, Fig. 6). We found similar results
437 for female body condition (CORT: -3.3%, $p = 0.016$) and no significant differences between
438 males ($p = 0.25$). Prenatal TH supplementation did not significantly affect body mass, condition
439 or size in juveniles (all $F < 0.33$, all $p > 0.56$; Table S4), neither in interaction with CORT
440 treatment (CORT x TH: all $F < 4.06$, all $p > 0.05$).

441

442 *Prenatal hormonal effect on recapture probability (i.e. proxy of apparent survival)*

443 Recapture probabilities were not significantly affected by prenatal hormonal treatments
444 either on the short-term (juveniles in 2019: 56.03% and 42.34% for CORT vs. non-CORT, χ^2
445 = 2.35, $p = 0.12$; and 50.00% and 48.62% for TH vs. non-TH, $\chi^2 = 0.01$, $p = 0.93$) or long-term
446 (adults in 2020: 15.52% and 10.81% for CORT vs. non-CORT, $\chi^2 = 0.68$, $p = 0.41$; and 15.25%
447 and 11.01% for TH vs. non-TH, $\chi^2 = 0.59$, $p = 0.44$). There was no significant interaction
448 between prenatal CORT and TH treatments on the aforementioned parameters (all $\chi^2 < 0.56$
449 and all $p > 0.45$).

450

451 **Discussion**

452 We tested for potential developmental plasticity related to two prenatal hormones in a
453 wild great tit population. By experimentally increasing yolk hormone content to simulate higher
454 maternal deposition of these hormones in the eggs, we investigated the effects of GC, TH,
455 and their interaction on offspring mitochondrial aerobic metabolism, development and survival.
456 Developmental time was significantly increased by prenatal CORT supplementation, but
457 significantly decreased by prenatal TH supplementation. Elevated prenatal CORT exposure
458 significantly reduced mitochondrial density and respiration rates, without significantly affecting
459 mitochondrial coupling efficiency (*OxCE*). Interestingly, such down-regulations of
460 mitochondrial aerobic metabolism might have been partially compensated by a higher usage
461 of maximal mitochondrial capacity (*i.e.* higher $FCR_{ROUTINE/CI+II}$). We did not find very clear
462 effects of prenatal hormonal treatments on growth patterns and recapture probability. Yet,
463 nestlings hatched from CORT-injected eggs were lighter at day 2 and had a tendency to grow
464 faster in early life (*i.e.* day 2 to day 7), although these differences were not statistically
465 significant in our experiment, so that effects of prenatal CORT on nestling's body mass, size
466 and condition should be considered with caution. Recaptured females from CORT group were
467 lighter and in worse condition than juvenile females from non-CORT group, while we did not
468 find a significant difference in males. Despite not being statistically significant, recapture
469 probability was *ca.* 14% higher for juveniles from the CORT group. We expected prenatal TH
470 treatment to promote mitochondrial biogenesis, leading to an increase of mitochondrial density
471 and mitochondrial aerobic metabolism but found no support for such hypothesis. Others
472 studies have also reported a lack of significant effect of prenatal TH supplementation on
473 nestling mitochondrial density in other avian species (Hsu et al., 2020; Hsu et al., 2021; Stier
474 et al., 2020). Several hypotheses may explain the contrasting results in studies focusing on
475 maternal hormonal effects, such as specific dose-dependent or context dependent response
476 of maternal hormones, variation in initial hormones transferred/deposited by the mother or
477 pleiotropic effects of maternal hormones (Groothuis et al., 2019). One limitation in the present
478 study is the estimation of mitochondrial density and mitochondrial aerobic metabolism using

479 blood cells. While it has been previously shown that mitochondrial function in blood cells is to
480 some extent correlated to mitochondrial function in other tissues (Stier et al., 2017; Stier et al.,
481 2022), TH may have tissue-specific effects that we were not able to detect in the present study.

482 Mitochondrial density was significantly reduced by a prenatal CORT increase, but in
483 an age-specific manner since a significant effect was only observed at day 14 (a few days
484 before fledging), suggesting that prenatal CORT had a delayed and transient effect (*i.e.* no
485 evidence of developmental plasticity). This mitochondrial density reduction contributed to an
486 apparent decrease of all respiration rates at the cellular level, including oxidative
487 phosphorylation (as measured through *OXPPOS*). At the mitochondrial level (*i.e.*
488 independently from mitochondrial density), CORT significantly decreased respiration related
489 to both oxidative phosphorylation (*OXPPOS*) and maximal respiration capacity (*CI+II*). Since
490 the effect of prenatal CORT was consistent across time (*i.e.* at day 7 and 14, no significant
491 Age x CORT interactions), it is possible that prenatal CORT induced proper developmental
492 plasticity, although effects later in life will have to be assessed to verify this hypothesis.
493 Because of a decrease in the maximum capacity of mitochondria in the CORT group,
494 mitochondria in that group were functioning, on average, significantly closer to their metabolic
495 maximum (as measured through a significant increase in $FCR_{ROUTINE(CI+II)}$), yet without any clear
496 change in coupling efficiency (no significant effect on *OxCE*). Therefore, the down-regulation
497 of mitochondrial density and aerobic metabolism might have been partially compensated by a
498 higher endogenous usage of maximal mitochondrial capacity, but not by an increase in
499 coupling efficiency. This effect of prenatal CORT on blood cell aerobic metabolism is in sharp
500 contrast with results from a recent study on the same species that experimentally increased
501 CORT levels after hatching (Casagrande et al., 2020): postnatal CORT supplementation led
502 to an increase in respiration rate being linked to proton leak and a concomitant decrease in
503 coupling efficiency (Casagrande et al., 2020). This suggests that the same hormone can have
504 contrasting effects on mitochondrial aerobic metabolism depending on the timing of exposure.
505 Alternatively to a direct effect of prenatal CORT on mitochondrial density, it is possible that
506 the effect we observed could be related to an effect on prenatal CORT on blood cell
507 maturation. To the best of our knowledge, there is no information on blood cell maturation
508 related to prenatal CORT increase in avian species. Yet, it is known that prenatal GC
509 contribute to the maturation of erythropoiesis in mammals (Tang et al., 2011). According to
510 our results and other related studies (Hsu et al., 2021; Stier et al., 2020), mitochondrial density
511 in avian blood cells decreases sharply along postnatal development. Thus, if the effect of
512 CORT we observed (*i.e.* decreased mitochondrial density at day 14) was related to an effect
513 of prenatal CORT on blood cells maturation, it would likely mean that an increase in prenatal
514 CORT can accelerate the maturation of blood cells.

515 Despite reduced mitochondrial density and lower mitochondrial aerobic metabolism,
516 CORT-supplemented nestlings reached, on average, a fledging body mass, body size and
517 body condition similar to non-CORT individuals. The CORT-treatment may have led to lower
518 energy requirements enabling individuals to reach similar mass/size despite lower
519 mitochondrial density and aerobic metabolism. An alternative hypothesis could be that CORT-
520 nestlings obtained more food from their parents, which would be in line with the known effect
521 of CORT on nestling begging rate (e.g. (Rubolini et al., 2005)). An interesting aspect of our
522 results is that we found a medium-term sex-specific effect of the CORT treatment on juveniles
523 the following autumn (i.e., 9 to 20 weeks after fledging). Prenatal CORT supplementation
524 significantly decreased body mass and condition of juvenile females, suggesting that the
525 treatment may lead to some delayed deleterious effects. The mechanisms underlying the
526 delayed effect of CORT on body mass and condition at the juvenile stage remain however
527 unclear. Sex-specific effects of prenatal GC on adult metabolism have been recently
528 documented in laboratory conditions on mammalian models (Kroon et al., 2020; Ruiz et al.,
529 2020). Thus, it could be possible that the sex-specific effect observed here on body mass
530 could be related to metabolic alterations at the juvenile stage. Further studies are needed to
531 test this hypothesis, for instance by measuring the effect of prenatal CORT on both whole-
532 body and mitochondrial aerobic metabolism at the juvenile stage.

533 Contrary to our expectations and what has been found in a previous study on the same
534 population (Hsu et al., 2021), the prenatal increase of TH in our study did not affect nestling
535 growth patterns. Several hypotheses may explain these contrasting results. The impact of
536 prenatal TH supplementation may depend on the original amount of TH deposited in eggs,
537 which in itself varies between individuals and environmental conditions, such as ambient
538 temperature or food availability (Ruuskanen and Hsu, 2018). Also, the effect may depend on
539 postnatal environmental conditions, as maternal effects are context-dependent (Groothuis et
540 al., 2020). It is also possible that TH impacted traits that we did not measure in this study (e.g.,
541 specific target tissues, behavioral strategies). In addition, all traits were measured post-
542 hatching and prenatal TH effects may be not visible anymore after hatching. These
543 hypotheses may also explain why we were not able to detect significant interactions (e.g.
544 permissive, synergistic or antagonistic effects) between CORT and TH treatments, although
545 there was a non-significant trend towards a negative effect of the interaction between prenatal
546 CORT and TH on body size at day 7.

547 One illustration of potential direct prenatal impact of CORT and TH is the result we
548 obtained regarding developmental time (i.e. incubation duration). We found that a prenatal
549 increase of CORT levels increased developmental time *in ovo*, while an increase in prenatal
550 TH levels decreased developmental time. It has been previously shown that an augmentation

551 of TH *in ovo* may accelerate hatching (Hsu et al., 2017). Measuring mitochondrial aerobic
552 metabolism during embryo development will be necessary to understand if such effects on
553 embryo growth might be mediated by mitochondrial metabolism. Yet, as we monitored the
554 nest only once a day to determine hatching date, overall incubation duration is estimated with
555 a potential error of ± 1 day, meaning that this result should be interpreted with caution, but
556 warrants further investigation. Understanding how effects on developmental time may carry-
557 over and affect post-hatching phenotypes also requires further investigation.

558 One objective of this study was to investigate the effects of both prenatal TH and CORT
559 on offspring short and long-term survival. Prenatal hormonal treatments did not significantly
560 affect recapture probabilities (a proxy of apparent survival) in the following autumns (juveniles
561 catching in 2019 and adults catching in 2020) even if we found a significant negative impact
562 of CORT on the body mass and body condition of juvenile females. Yet, recapture probability
563 seemed to be higher for juveniles from the CORT group, calling for further studies on the
564 mechanisms by which prenatal hormones may induce differences in medium-term survival. It
565 is worth noting that our results are based on a moderate sample size ($N \approx 200$ per age group
566 for phenotypic data, and $N \approx 45$ per age group for high-resolution respirometry) and that further
567 exploration with larger samples may be necessary to strengthen our conclusions.

568 **Conclusion**

569 Our experimental approach mimicking an increase in maternal hormonal deposition
570 in eggs showed that an increase in CORT exposure *in ovo* decreases postnatal mitochondrial
571 density and metabolism in blood cells, without markedly affecting mitochondrial coupling
572 efficiency or nestling growth patterns. As mitochondrial function is expected to be central in
573 the nexus between development, growth and metabolism, exploring how variation in
574 mitochondrial function modulates offspring phenotype and fitness-related traits would help
575 better understanding the pathways through which maternal effects (including maternal
576 hormones) operate. Exploring the impacts of prenatal maternal hormones on offspring
577 mitochondrial function offers a novel perspective in explaining variation in offspring growth
578 trajectories. Since prenatal effects may have long term-consequences up into adulthood
579 (Groothuis et al., 2019; Groothuis et al., 2020), and as we indeed found decreased body mass
580 and condition of CORT-treated juvenile females in our study, further investigations should
581 focus on the long-term effects of prenatal hormones on mitochondrial aerobic metabolism later
582 in life (in juvenile and adult birds).

583

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588

589 **Ethics**

590 All procedures were approved by the Animal Experiment Committee of the State Provincial
591 Office of Southern Finland (license no. ESAVI/5718/2019) and by the Environmental Center
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593

594 **Competing interests**

595 We declare we have no competing interests.

596

597 **Author's contribution**

598 S.R, A.S, and B-Y.H designed the study. A.S, B-Y.H, C.M, S.R and N.C.S conducted the
599 fieldwork and collected the samples. A.S and C.M conducted the mitochondrial respirometry
600 measurements. N.C.S performed DNA extractions and qPCR measurements. N.C.S analyzed
601 the data with the support of S.R, V-A.V and A.S. N.C.S, S.R, V-A.V and A.S co-wrote the
602 manuscript. B-Y.H and C.M. commented on the manuscript. S.R and A.S shared the senior
603 authorship of this article and contributed equally to this work.

604

605 **Data availability statement**

606 Data are available on Figshare DOI: 10.6084/m9.figshare.15141138,
607 <https://figshare.com/s/3c05173c4cc5ebd0c3f4>

608

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616

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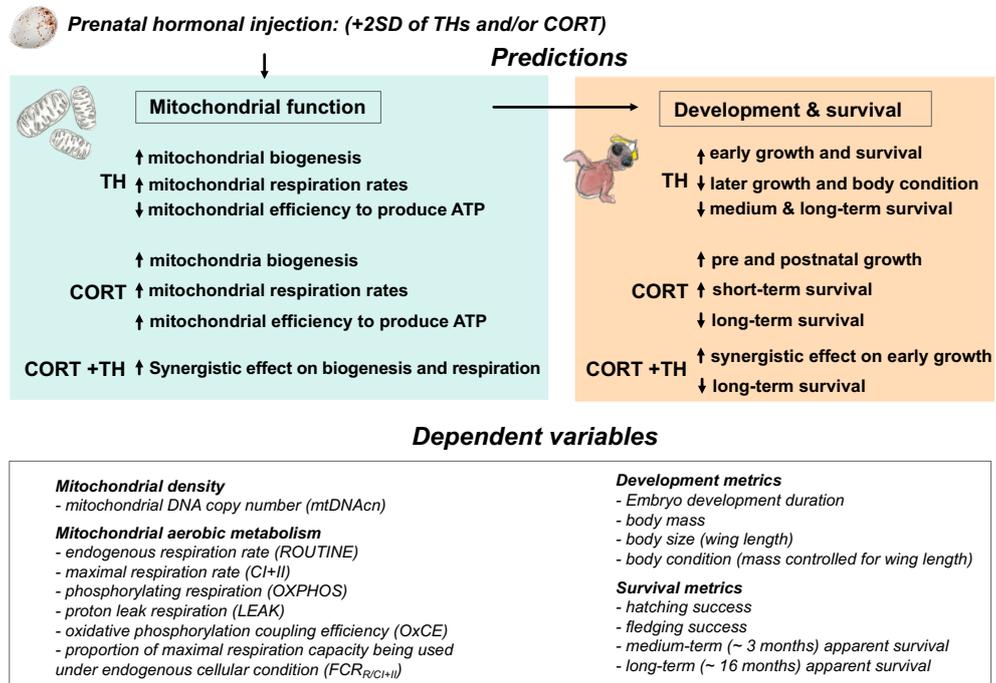
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839 **Figures**

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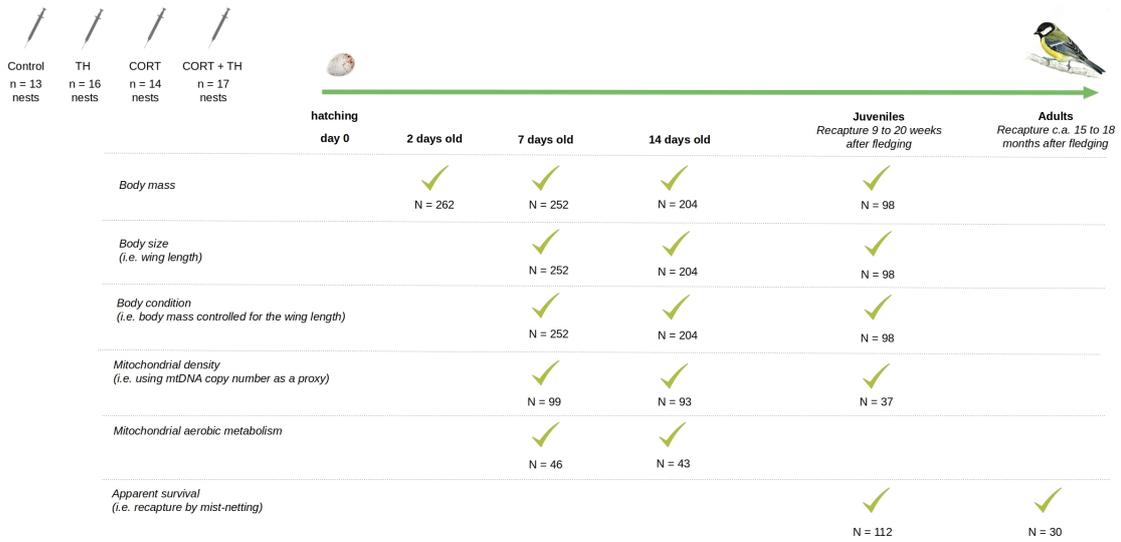
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843 **Fig. 1: Experimental timeline of the study, with sample sizes for different response**

844 **variables.** Great tit nestlings fledge around 18 - 20 days after hatching.

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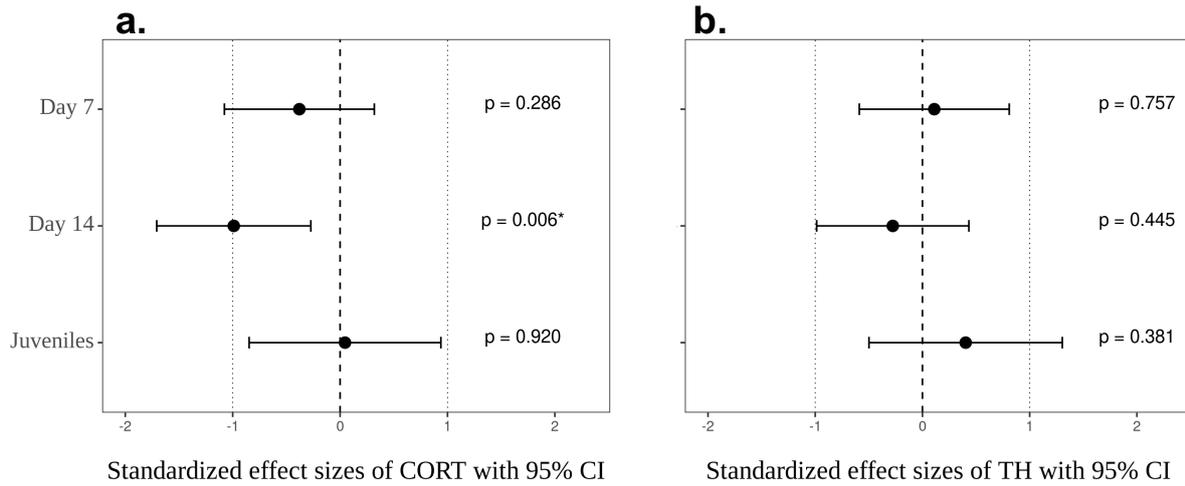
Prenatal hormonal elevation 4 experimental groups



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847 **Fig. 2: Predictions related to the experimental manipulation of prenatal thyroid and**
 848 **glucocorticoid hormones.**

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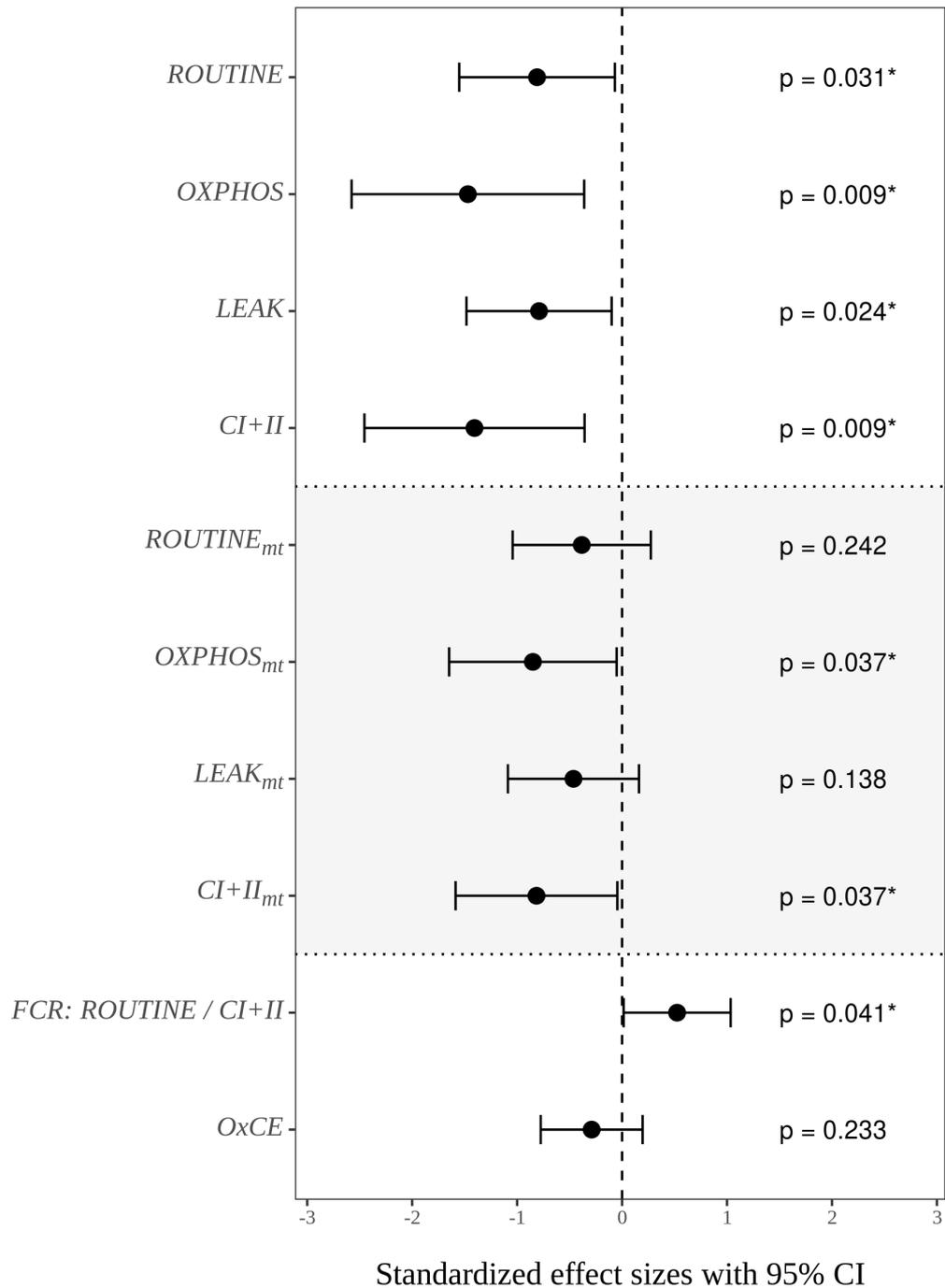
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852 **Fig. 3: Effects of prenatal CORT (a) and TH (b) treatments on mitochondrial density at**
 853 **day 7 (n = 99), day 14 (n = 93) and juvenile age (n = 37) (N = 100 individuals).** Standardized
 854 effect sizes based on predicted values of the model are reported with their 95% confidence
 855 intervals. Age x CORT interaction was significant ($\chi^2 = 8.65$, $p = 0.013$), and post-hoc tests
 856 revealed a significant effect of CORT at day 14 only ($p = 0.006$).

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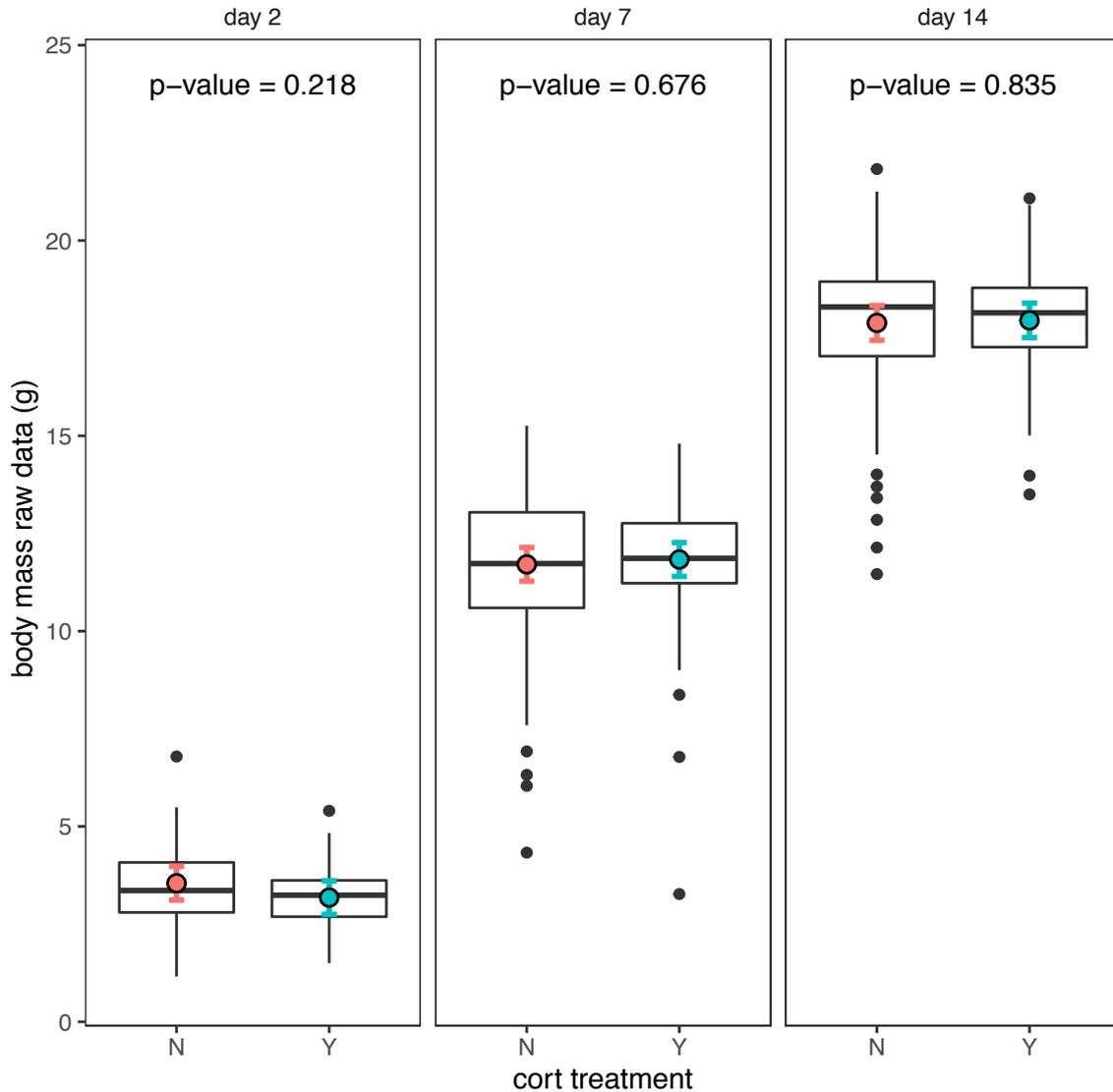
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860 **Fig.4: Effect of a prenatal CORT treatment on mitochondrial aerobic metabolism (d7:**
 861 **n_{CORT/non-CORT} = 21/25; d14: n_{CORT/non-CORT} = 20/23 individuals).** Standardized effect sizes
 862 based on predicted values of the model are reported with their 95% confidence intervals. Age
 863 x CORT interactions were not statistically significant. Response variables indicated as _{mt} are
 864 corrected for the mitochondrial density (*mtDNAcn* included as a covariate in models).

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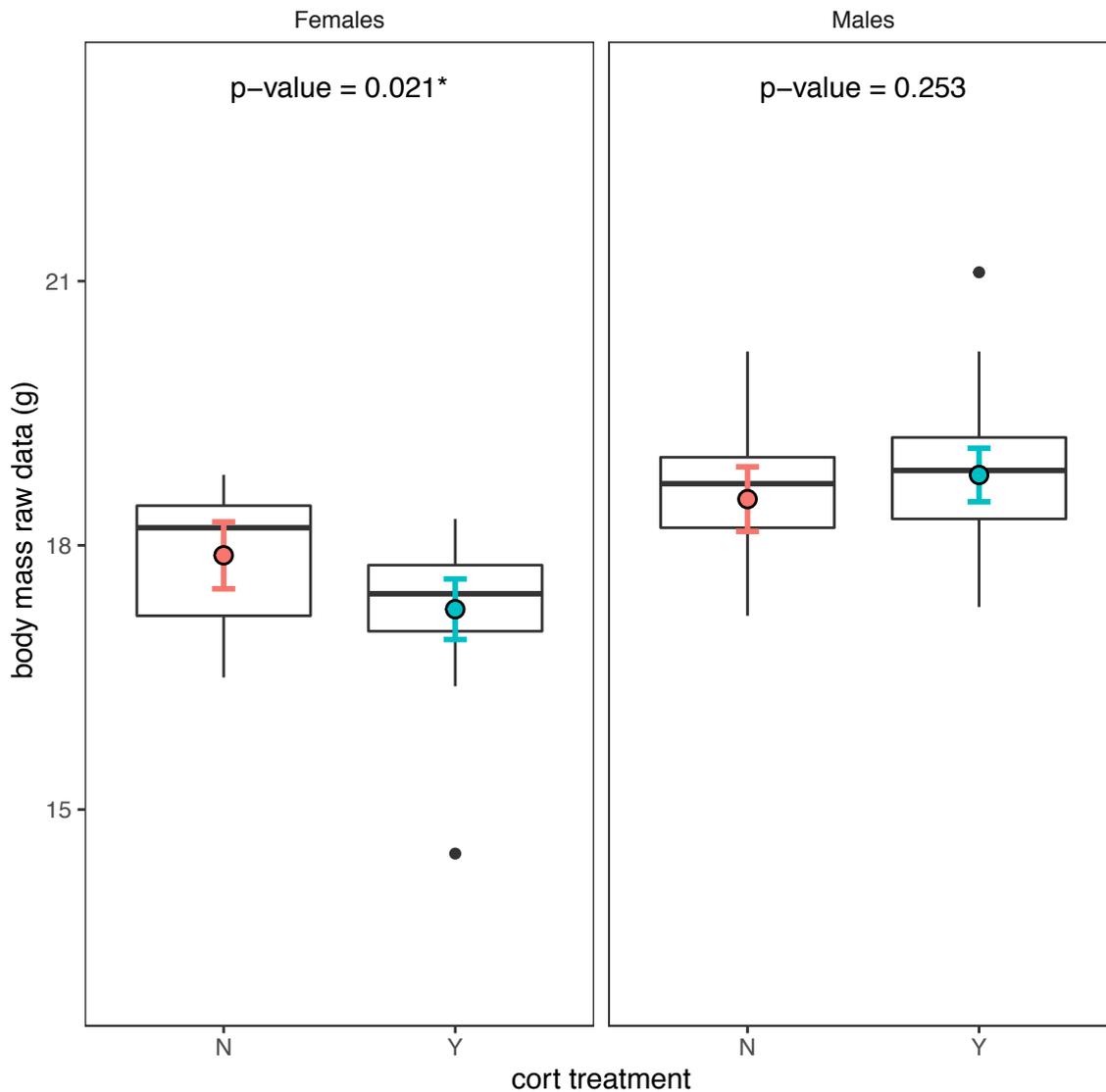


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867 **Fig.5: Effects of prenatal CORT treatment on postnatal body mass growth.** Raw data
 868 distribution is plotted (d2: n_{CORT/non-CORT} = 129/ 133; d7: n_{CORT/non-CORT} = 123 / 128; d14: n_{CORT/non-}
 869 _{CORT} = 105/100 individuals) and least square means of statistical model presented as colored
 870 dots, with their 95% confidence interval. The interaction age x CORT was statistically
 871 significant (overall test for the interaction $F_{2,460} = 4.40$, $p = 0.013$), but none of the post-hoc
 872 tests performed were significant (all $p > 0.18$).

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877 **Fig.6: Effects of prenatal CORT treatment and sex on juvenile body mass.** Raw data
 878 distribution is plotted (Females: $n_{\text{CORT/non-CORT}} = 26/19$; Males: $n_{\text{CORT/non-CORT}} = 32/21$ individuals)
 879 and least square means of statistical model presented as colored dots, with their 95%
 880 confidence interval. The interaction CORT*sex was statistically significant ($F = 8.36$, $p =$
 881 0.005). p -values of Tukey HSD post-hoc tests are reported for each sex.

882 **Table 1: Results of generalized linear mixed model (gamma distribution with log-link) testing**
883 **the effect of age and prenatal hormonal treatments on mitochondrial density (i.e. mtDNAcn;**
884 **d7: n = 99 observations, d14: n = 93 observations, Juv: n = 37 observations; N = 100**
885 **individuals). Model estimates are reported with their 95% confidence intervals. Chick ID (ring) and**
886 **nest box of origin (nestbox) were included as random effects in the model. σ^2 = within-group**
887 **variance; τ_{00} = between-group variance. Sample size along with marginal (fixed effects only) and**
888 **conditional (fixed and random effects) R^2 are presented.**
889

mtDNAcn			
Predictors	Estimates	CI	p
(Intercept)	5.80	4.66 – 7.22	<0.001
age [day14]	0.54	0.48 – 0.61	<0.001
age [juvenile]	0.15	0.12 – 0.17	<0.001
CORT [Y]	0.89	0.71 – 1.11	0.286
TH [Y]	0.99	0.81 – 1.23	0.956
sex [M]	1.03	0.88 – 1.20	0.740
hatching date	1.07	0.96 – 1.19	0.221
brood size day 2	0.88	0.78 – 0.99	0.036
age [day14] * CORT [Y]	0.82	0.69 – 0.98	0.028
age [juvenile] * CORT [Y]	1.15	0.90 – 1.46	0.273
Random Effects			
σ^2	0.10		
τ_{00} ring	0.02		
τ_{00} nestbox	0.03		
N ring	100		
N nestbox	48		
Observations	229		
Marginal R2 / Conditional R2	0.762 / 0.836		

890

891 **Table 2: Results of linear mixed model testing the effect of age and prenatal hormonal treatments on mitochondrial respiration rates**
892 **(corrected for mitochondrial density; d7: n = 46 observations, d14: n = 43 observations, N = 48 individuals).** Chick ID (ring) was included
893 as random effect in the model. σ^2 = within-group variance; τ_{00} = between-group variance. Sample size along with marginal (fixed effects only)
894 and conditional (fixed and random effects) R^2 are presented.

895

Predictors	ROUTINE			LEAK			OXPHOS			CI + CII		
	Estimates	CI	p	Estimates	CI	p	Estimates	CI	p	Estimates	CI	p
(Intercept)	0.32	0.12 – 0.52	0.002	0.32	0.10 – 0.53	0.004	0.14	-0.73 – 1.01	0.753	0.45	-0.57 – 1.46	0.387
CORT [Y]	-0.04	-0.10 – 0.02	0.236	-0.05	-0.12 – 0.01	0.131	-0.30	-0.56 – -0.03	0.029	-0.35	-0.66 – -0.03	0.030
TH [Y]	0.02	-0.04 – 0.08	0.448	0.02	-0.04 – 0.09	0.501	-0.05	-0.32 – 0.22	0.723	-0.03	-0.34 – 0.29	0.869
sex [M]	0.03	-0.04 – 0.09	0.419	0.07	0.003 – 0.144	0.040	0.09	-0.20 – 0.38	0.541	0.16	-0.17 – 0.50	0.341
age [day7]	0.09	0.04 – 0.15	0.001	0.04	-0.02 – 0.10	0.193	0.25	0.04 – 0.47	0.021	0.29	0.03 – 0.55	0.028
mtDNAcn	0.05	0.03 – 0.06	<0.001	0.04	0.02 – 0.05	<0.001	0.18	0.12 – 0.24	<0.001	0.22	0.15 – 0.29	<0.001
hatching date	0.0005	-0.002 – 0.003	0.641	-0.0002	-0.003 – 0.002	0.882	0.02	0.01 – 0.03	0.001	0.02	0.01 – 0.03	0.004
brood size day 2	-0.01	-0.03 – 0.01	0.194	-0.01	-0.03 – 0.01	0.467	-0.02	-0.10 – 0.05	-0.03	-0.03	-0.11 – 0.06	0.541
Random effects												
σ^2	0.01			0.01			0.12			0.18		
τ_{00} ring	0.0005			0.01			0.13			0.17		
N ring	48			48			48			48		
Observations	89			89			89			89		
Marginal R2 / Conditional R2	0.639/0.766			0.467/0.627			0.651/0.829			0.647/0.816		

896

897 **Table 3: Results of linear mixed model testing the effect of age and prenatal hormonal**
898 **treatments on mitochondrial maximum capacity usage (i.e. $FCR_{ROUTINE/CI+II}$) and OXPPOS**
899 **coupling efficiency (i.e. OxCE; d7: n = 46 observations, d14: n = 43 observations, N =**
900 **48 individuals).** Chick ID (ring) was included as a random effect in the model. σ^2 = within-
901 group variance; τ_{00} = between-group variance. Sample size along with marginal (fixed effects
902 only) and conditional (fixed and random effects) R^2 are presented.

903
904

Predictors	$FCR_{ROUTINE/CI+II}$			OxCE		
	Estimates	CI	p	Estimates	CI	p
(Intercept)	0.305	0.256 – 0.354	<0.001	0.715	0.659 – 0.771	<0.001
CORT [Y]	0.017	0.002 – 0.032	0.029	-0.010	-0.028 – 0.007	0.251
TH [Y]	0.012	-0.004 – 0.028	0.133	-0.012	-0.030 – 0.006	0.187
sex [M]	-0.007	-0.023 – 0.010	0.441	-0.013	-0.032 – 0.007	0.199
age [day7]	0.009	-0.004 – 0.022	0.169	0.023	0.007 – 0.039	0.004
hatching date	-0.001	-0.002 – -0.001	<0.001	0.001	0.001 – 0.002	<0.001
brood size day 2	-0.0002	-0.004 – 0.004	0.930	-0.001	-0.006 – 0.004	0.676
Random Effects						
σ^2	0.001			0.0014		
τ_{00} ring	0.0002			0.0001		
N ring	48			48		
Observations	89			89		
Marginal R2/Conditional R2	0.299/0.398			0.292/0.349		

905
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907

908 **Table 4: Results of linear mixed model testing the effect of age and prenatal hormonal**
 909 **treatments on body mass during the rearing period.** Chick (ring) and nest box (nestbox)
 910 identities were included as random effects in the model. σ^2 = within-group variance; τ_{00} =
 911 between-group variance. Sample size along with marginal (fixed effects only) and conditional
 912 (fixed and random effects) R^2 are presented; day 2 ($n = 262$ observations), day 7 ($n = 251$
 913 observations) and day 14 after hatching ($n = 205$ observations).

914
 915

Body mass			
<i>Predictors</i>	<i>Estimates</i>	<i>CI</i>	<i>p</i>
(Intercept)	6.05	4.37 – 7.73	<0.001
age [day7]	8.18	7.94 – 8.42	<0.001
age [day14]	14.36	14.09 – 14.62	<0.001
CORT [Y]	-0.39	-0.97 – 0.19	0.183
TH [Y]	-0.27	-0.80 – 0.27	0.330
hatching date	-0.04	-0.06 – -0.02	<0.001
brood size day 2	-0.01	-0.15 – 0.13	0.852
age [day7] * CORT [Y]	0.49	0.14 – 0.83	0.006
age [day14] * CORT [Y]	0.43	0.05 – 0.80	0.025
Random Effects			
σ^2	0.98		
τ_{00} ring	0.25		
τ_{00} nestbox	0.84		
N ring	265		
N nestbox	52		
Observations	717		
Marginal R2 / Conditional R2 0.945 / 0.974			

916

Electronic Supplementary Material of:

Developmental plasticity of mitochondrial aerobic metabolism, growth and survival by prenatal glucocorticoids and thyroid hormones: an experimental test in wild great tits

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Table S1: Results of linear mixed model testing the effect of prenatal hormonal treatments on body mass at day 2 post-hatching. Nest box identity (*nestbox*) was included as random effect in the model. σ^2 = within-group variance; τ_{00} = between-group variance. Sample size along with marginal (fixed effects only) and conditional (fixed and random effects) R^2 are presented.

Body mass day 2			
Predictors	Estimates	CI	p
(Intercept)	4.81	3.52 – 6.09	<0.001
CORT [Y]	-0.30	-0.73 – 0.12	0.165
TH [Y]	0.18	-0.25 – 0.62	0.403
brood size day 2	-0.02	-0.12 – 0.09	0.756
hatching date	-0.02	-0.04 – -0.01	0.004
Random effects			
σ^2	0.27		
τ_{00} nestbox	0.53		
N nestbox	52		
Observations	262		
Marginal R2 / Conditional R2	0.119 / 0.705		

Table S2: Results of linear mixed models testing the effect of prenatal hormonal treatments on day 7: a. body mass gain (i.e. body mass at day 7 controlled for body mass at day 2); b. wing length (i.e. body size); and c. body condition (i.e. body mass corrected for body size). Nest box identity (nestbox) was included as random effect in the model. σ^2 = within-group variance; τ_{00} = between-group variance. Sample size along with marginal (fixed effects only) and conditional (fixed and random effects) R^2 are presented.

a. Body mass day 7			
<i>Predictors</i>	<i>Estimates</i>	<i>CI</i>	<i>p</i>
(Intercept)	6.32	4.38 – 8.27	<0.001
CORT [Y]	0.58	-0.02 – 1.18	0.056
TH [Y]	-0.27	-0.88 – 0.35	0.391
mass day 2	1.64	1.50 – 1.78	<0.001
brood size day 2	0.07	-0.08 – 0.22	0.373
hatching date	-0.01	-0.03 – 0.01	0.400
Random effects			
σ^2	0.30		
τ_{00} nestbox	1.01		
N nestbox	49		
Observations	248		
Marginal R2 / Conditional R2	0.623 / 0.914		

b. Wing length day 7			
<i>Predictors</i>	<i>Estimates</i>	<i>CI</i>	<i>p</i>
(Intercept)	24.08	19.75 – 28.42	<0.001
CORT [Y]	-0.60	-1.98 – 0.78	0.393
TH [Y]	-0.73	-2.10 – 0.65	0.300
brood size day 2	0.17	-0.20 – 0.53	0.377
hatching date	-0.08	-0.13 – -0.03	0.004
Random effects			
σ^2	4.83		
τ_{00} nestbox	4.73		
N nestbox	49		
Observations	251		
Marginal R2 / Conditional R2	0.118 / 0.555		

c.			
Body mass day 7			
<i>Predictors</i>	<i>Estimates</i>	<i>CI</i>	<i>p</i>
(Intercept)	3.84	2.28 – 5.40	<0.001
CORT [Y]	0.30	-0.10 – 0.70	0.140
TH [Y]	-0.04	-0.44 – 0.36	0.839
wing length day 7	0.44	0.40 – 0.48	<0.001
brood size day 2	-0.06	-0.17 – 0.04	0.233
hatching date	-0.01	-0.02 – 0.01	0.255
Random effects			
σ^2	0.48		
τ_{00} nestbox	0.37		
N nestbox	49		
Observations	251		
Marginal R2 / Conditional R2	0.708 / 0.835		

Table S3: Results of linear mixed models testing the effect of prenatal hormonal treatments on day 14: a. body mass gain (i.e. body mass at day 14 controlled for body mass at day 7); b. wing length (i.e. body size); and c. body condition (i.e. body mass corrected for body size). Nest box identity (nestbox) was included as random effect in the model. σ^2 = within-group variance; τ_{00} = between-group variance. Sample size along with marginal (fixed effects only) and conditional (fixed and random effects) R^2 are presented.

a.			
Body mass day 14			
<i>Predictors</i>	<i>Estimates</i>	<i>CI</i>	<i>p</i>
(Intercept)	15.08	12.26 – 17.90	<0.001
CORT [Y]	0.24	-0.54 – 1.02	0.552
TH [Y]	0.15	-0.56 – 0.87	0.674
mass day 7	0.52	0.42 – 0.61	<0.001
brood size day 2	-0.15	-0.36 – 0.07	0.177
hatching date	-0.05	-0.08 – -0.01	0.004
Random effects			
σ^2	0.61		
τ_{00} nestbox	1.37		
N nestbox	41		
Observations	204		
Marginal R2 / Conditional R2	0.385 / 0.811		

b.			
Wing length day 14			
<i>Predictors</i>	<i>Estimates</i>	<i>CI</i>	<i>p</i>
(Intercept)	58.18	52.30 – 64.06	<0.001
CORT [Y]	-0.17	-2.01 – 1.66	0.852
TH [Y]	-1.13	-2.87 – 0.61	0.204
brood size day 2	0.20	-0.30 – 0.70	0.430
hatching date	-0.14	-0.21 – -0.07	<0.001
Random effects			
σ^2	5.99		
τ_{00} nestbox	6.99		
N nestbox	41		
Observations	204		
Marginal R2 / Conditional R2	0.224 / 0.642		

c.			
Body mass 14			
<i>Predictors</i>	<i>Estimates</i>	<i>CI</i>	<i>p</i>
(Intercept)	10.11	6.34 – 13.88	<0.001
CORT [Y]	0.32	-0.41 – 1.06	0.390
TH [Y]	0.06	-0.64 – 0.75	0.876
wing length day 14	0.21	0.16 – 0.26	<0.001
brood size day 2	-0.19	-0.39 – 0.01	0.063
hatching date	-0.03	-0.06 – -0.001	0.042
Random effects			
σ^2	0.76		
τ_{00} nestbox	1.18		
N nestbox	41		
Observations	204		
Marginal R2 / Conditional R2	0.339 / 0.740		

Table S4: Results of linear mixed models testing the effect of prenatal hormonal treatments on juvenile: a. body mass; b. wing length (i.e. body size); and c. body condition (i.e. body mass corrected for body size). Nest box identity (nestbox) was included as random effect in the model. σ^2 = within-group variance; τ_{00} = between-group variance. Sample size along with marginal (fixed effects only) and conditional (fixed and random effects) R^2 are presented.

a.			
Body mass juvenile			
<i>Predictors</i>	<i>Estimates</i>	<i>CI</i>	<i>p</i>
(Intercept)	18.32	17.03 – 19.61	<0.001
sex [M]	0.64	0.17 – 1.10	0.009
CORT [Y]	-0.61	-1.11 – -0.12	0.019
TH [Y]	-0.11	-0.48 – 0.26	0.569
hatching date	-0.01	-0.03 – 0.02	0.548
sex [M] * CORT [Y]	0.89	0.29 – 1.49	0.005
Random effects			
σ^2	0.49		
τ_{00} nestbox	0.11		
N nestbox	36		
Observations	98		
Marginal R2 / Conditional R2	0.398 / 0.509		

b.			
Wing length juvenile			
<i>Predictors</i>	<i>Estimates</i>	<i>CI</i>	<i>p</i>
(Intercept)	76.59	74.15 – 79.02	<0.001
CORT [Y]	0.13	-0.63 – 0.90	0.731
TH [Y]	-0.05	-0.82 – 0.73	0.904
sex [M]	2.70	2.20 – 3.21	<0.001
hatching date	-0.01	-0.06 – 0.03	0.508
Random effects			
σ^2	1.33		
τ_{00} nestbox	0.74		
N nestbox	36		
Observations	98		
Marginal R2 / Conditional R2	0.471 / 0.660		

c.			
Body mass juvenile			
<i>Predictors</i>	<i>Estimates</i>	<i>CI</i>	<i>p</i>
(Intercept)	12.40	3.60 – 21.20	0.007
sex [M]	0.41	-0.16 – 0.98	0.161
CORT [Y]	-0.64	-1.13 – -0.14	0.015
TH [Y]	-0.11	-0.48 – 0.27	0.573
wing length juvenile	0.08	-0.04 – 0.19	0.186
hatching date	-0.01	-0.03 – 0.02	0.620
sex [M] * CORT [Y]	0.91	0.31 – 1.51	0.004
Random effects			
σ^2	0.48		
τ_{00} nestbox	0.11		
N nestbox	36		
Observations	98		
Marginal R2 / Conditional R2	0.407 / 0.520		