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► To cite this version:

Nina Cossin-Sevrin, Antoine Stier, Mikaela Hukkanen, Sandrine Zahn, Vincent Viblanc, et al.. Early-life environmental effects on mitochondrial aerobic metabolism: a brood size manipulation in wild great tits. *Journal of Experimental Biology*, 2023, 226 (21), pp.jeb245932. 10.1242/jeb.245932 . hal-04267175

HAL Id: hal-04267175

<https://hal.science/hal-04267175>

Submitted on 1 Nov 2023

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1 **Early-life environmental effects on mitochondrial aerobic** 2 **metabolism: a brood size manipulation in wild great tits**

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18 **Summary statement**

19 Increasing or reducing brood size affected chick growth patterns but not their cellular
20 metabolism. The actual number of individuals in the nest was associated with different
21 cellular metabolic rates independently of the treatment.

22

23 **Abstract**

24 In avian species, the number of chicks in the nest and subsequent sibling competition for
25 food are major components of the offspring's early-life environment. A large brood size is
26 known to affect chick's growth, leading in some cases to long-lasting effects for the offspring,
27 such as a decrease in size at fledgling and in survival after fledging. An important pathway
28 underlying different growth patterns could be the variation in offspring mitochondrial
29 metabolism through its central role in converting energy. Here, we performed a brood size
30 manipulation in great tits (*Parus major*) to unravel its impact on offspring's mitochondrial
31 metabolism and reactive oxygen species (ROS) production in red blood cells. We
32 investigated the effects of brood size on chicks' growth and survival, and tested for long-
33 lasting effects on juvenile mitochondrial metabolism and phenotype. As expected, chicks
34 raised in reduced broods had a higher body mass compared to enlarged and control groups.
35 However, mitochondrial metabolism and ROS production were not significantly affected by

36 the treatment either at chick or juvenile stages. Interestingly, chicks raised in very small
37 broods were smaller in size and had higher mitochondrial metabolic rates. The nest of
38 rearing had a significant effect on nestling mitochondrial metabolism. The contribution of the
39 rearing environment in determining offspring mitochondrial metabolism emphasizes the
40 plasticity of mitochondrial metabolism in regards to the nest environment. This study opens
41 new avenues regarding the implication of postnatal environmental conditions in shaping the
42 offspring's early-life mitochondrial metabolism.

43 **Key words:** Animal performance, brood size, cellular metabolism, oxidative stress, *Parus*
44 *major*

45 **Introduction**

46 Parents may have the capacity to shape offspring phenotypes by influencing the
47 offspring's environment during development. This phenomenon, referred to as parental
48 effects, is an important influence on offspring phenotype (Badyaev & Uller, 2009; Mousseau
49 & Fox, 1998; Wolf & Wade, 2009). From an evolutionary perspective, parental effects, in
50 general, are thought to improve offspring survival, growth and / or quality, hence improving
51 parental fitness (Bonduriansky & Crean, 2018; Mousseau & Fox, 1998; Yin et al., 2019).
52 However, it is unclear whether parental effects are always adaptive (Bonduriansky & Crean,
53 2018; Burgess & Marshall, 2014; Marshall & Uller, 2007; Sánchez-Tójar et al., 2020; Uller,
54 2008; Uller et al., 2013; Yin et al., 2019).

55 Parental care (e.g. postnatal provisioning) is an important early-life influence affecting
56 offspring phenotype (Uller, 2008). For dependent offspring relying on parents to survive, it is
57 now well established that a deficit in parental care can lead to detrimental long-term
58 consequences (e.g. Developmental Origins of Health and Disease hypothesis), but the
59 mechanism underlying long-lasting effects of early-life environmental conditions on offspring
60 phenotype are not well understood (Gluckman et al., 2007; Hoogland & Ploeger, 2022;
61 Meunier et al., 2022; Rogers & Bales, 2019).

62 In avian species, variation in early-life nutritional conditions and sibling competition
63 have been widely tested by manipulating brood size (enlarging or reducing brood size) with
64 the aim to simulate increased or reduced parental effort, thereby modulating postnatal
65 parental care and assessing the consequences on offspring phenotype and survival. In great
66 tits (*Parus major*), offspring from enlarged broods exhibit decreased body mass and size
67 (wing or tarsus length) at fledging, and decreased recapture probability over the long-term,
68 i.e. a few months after fledging (in zebra finches: De Kogel, 1997; in great tits: Hōrak, 2003;
69 Rytönen & Orell, 2001; Smith et al., 1989). Studies on zebra finches (*Taeniopygia guttata*)
70 reported long-lasting effects of early-life nutritional deficits on fitness related traits, including

71 laying initiation and breaks, hatching success, plasma antioxidant levels and flight
72 performances (Blount et al., 2003, 2006; Criscuolo et al., 2011). Yet, the mechanisms driving
73 the effects of early-life environmental variation (including postnatal provisioning) on the
74 offspring phenotype and survival remain poorly understood.

75 Variation in metabolic rate represents one important candidate pathway underlying
76 variation in growth patterns as it could be involved in energy allocation processes and is
77 thought to be associated with individual fitness (Brown et al., 2018; Burger et al., 2019,
78 2021). Beside nestling body mass and size, several studies examined the impacts of brood
79 size on offspring metabolic rate. In tree swallows (*Tachycineta bicolor*), nestlings from
80 enlarged broods had 15% lower resting metabolic rate compared to individuals from reduced
81 broods (Burness et al., 2000). On the contrary, zebra finches raised in large broods had a
82 9% higher standard metabolic rate at 1-year old compared to birds reared in small broods
83 (Verhulst et al., 2006). While the association between whole-organism metabolic rate has
84 been extensively studied to test the association between a physiological trait and fitness (or
85 proximate traits when fitness cannot be assessed directly, see precautions here: Arnold et
86 al., 2021; Pettersen et al., 2018), only more recently studies have focused on mitochondrial
87 aerobic metabolism (Ballard & Pichaud, 2014; Heine & Hood, 2020; Koch et al., 2021).
88 Studying mitochondrial respiration could reveal the cellular metabolic consequences of
89 brood size manipulation (and thus, how variation of nutritional conditions and sibling
90 competition influence offspring). Increased competition might have a significant effect on
91 mitochondrial respiration since organisms relying on aerobic metabolism use nutrients for
92 producing ATP via a set of metabolic reactions, part of them occurring within mitochondria.
93 ATP production in mitochondria is also associated with constitutive release of damaging sub-
94 products (e.g. reactive oxygen species, ROS), which may lead to oxidative damage that
95 impair protein and lipid structures and promote DNA mutations (Lane, 2011; Mazat et al.,
96 2020; Monaghan et al., 2009; Sastre et al., 2003). Thus, measuring both oxidative
97 phosphorylation (leading to ATP synthesis) and mitochondrial ROS production (byproducts
98 of cellular respiration) allows us to evaluate metabolic constraints and trade-offs at the
99 cellular level (Koch et al., 2021). The efficiency by which mitochondria are able to convert
100 ATP from a fixed amount of substrates and the determinants of this efficiency are
101 challenging to understand as the efficiency varies between species, but also within
102 individuals of the same species, according to age, condition and tissue (Cossin-Sevrin et al.,
103 2022; Koch et al., 2021; Salmón et al., 2022; Stier et al., 2019, 2022).

104 Recent studies have found that early-life environmental stressors might impair
105 mitochondrial function (Gyllenhammer et al., 2020; Zitkovsky et al., 2021). For example food
106 restriction was shown to decrease basal metabolic rate in adult chinese bulbul (*Pycnonotus*
107 *sinensis*) and silky starlings (*Sturnus sericeus*), and to decrease levels of mitochondrial state

108 4 respiration in the liver for both species (Mao et al., 2019; Zhang et al., 2018). Yet, the
109 impact of early-life conditions on mitochondrial function and the long-lasting effects remain
110 poorly understood.

111 Here, we experimentally manipulated brood size in wild great tits (*Parus major*) to
112 test how rearing conditions (altered sibling competition for food and potential change in food
113 availability/quality) affect nestling red blood cell mitochondrial metabolic phenotype: a
114 promising proxy of individual performance. We aimed to test i) if brood size was important in
115 determining nestling mitochondrial metabolism traits and associated ROS production, ii)
116 differences in nestling growth trajectories, and if these were associated with differences in
117 mitochondrial metabolic rates; iii) if differences in mitochondrial metabolic rates affected
118 offspring future survival. We further iv) tested if early-life determination of mitochondrial
119 aerobic metabolism could affect adult phenotype with potential medium-term costs (e.g.
120 consequences on juvenile mitochondrial metabolic rates and ROS production). Finally, our
121 experimental design allowed assessing v) the relative contributions of the foster rearing
122 environment (from 2 to 14 days post-hatching) vs. the combination of genetic background,
123 prenatal effects and early-stage rearing conditions (until 2 days post-hatching) on offspring
124 mitochondrial metabolism. To test the impact of brood size manipulation treatment on
125 postnatal parental care, we recorded parental feeding rates on a subsample of nests. We
126 predicted nestlings raised in enlarged broods to have a lower body mass and size compared
127 to control and reduced brood sizes. According to prior literature, the offspring mitochondrial
128 function is sensitive to postnatal environmental conditions. In rodent models, chronic stress
129 exposure and separation from mother during lactation led in most of the cases to a decrease
130 in mitochondrial complexes activities and increase of ROS production (Picard & McEwen,
131 2018; Zitkovsky et al., 2021). We may therefore expect an enlargement of the brood size
132 and its associated consequences, such as a decrease in parental feeding rates, to create a
133 stressful environment leading to a general decrease of the offspring mitochondrial
134 metabolism and increase of ROS production. Nevertheless, most of the work assessing how
135 stressful early-life environment may impair mitochondrial function have been so far realized
136 on mammals and the consequences in avian species and long-term effects remain elusive.
137 Here we test the importance of brood size as a proxy to early-life environmental rearing
138 conditions in shaping nestling mitochondrial metabolic rates, associated ROS production and
139 later growth and survival patterns.

140 **Material and Methods**

141 a) Field site and population monitoring

142 This study was conducted on Ruissalo Island, Finland (60°26.055' N, 22°10.391' E), in a
143 Great tit population (*Parus major* Linnaeus 1758) breeding in artificial nest boxes (n = 588
144 nest boxes). In Great tit, the average clutch size varies from 7 to 12 eggs (Perrins and
145 McCleery, 1989) and the nestling period lasts from 16 to 22 days. Data for our experiment
146 were collected during the 2020 breeding season (April to July) and during the autumn of
147 2020 (October to November). We monitored the breeding season progress by checking the
148 occupation of nest boxes by great tits once a week. Clutch size, hatching date (\pm 24h) and
149 fledging success were recorded.

150 b) Experimental manipulation of brood size

151 To investigate the effects of brood size on nestling mitochondrial function, growth pattern
152 and subsequent survival, we performed a brood size manipulation experiment, including
153 cross-fostering (Fig.1). We selected two nests (nest-pairs) having the same hatching date (\pm
154 24h) and conducted the brood size manipulation and cross-fostering 2 days after hatching.
155 The initial brood size (i.e. before the manipulation) of each nest was recorded, with an
156 average (\pm SEM) of 7.98 ± 0.07 nestlings per nest (ranging from 4 to 11 nestlings, n = 70
157 nests). Approximately half of the brood was cross-fostered between nest-pairs in order to
158 assess the influence of the nest of origin (representing the contribution of genetic
159 background, prenatal and early postnatal parental effects) vs. the nest of experimental
160 cross-fostering (i.e. nest of rearing). The nest of rearing here reflects postnatal
161 environmental conditions and parental effects from 2 days after hatching until fledging. The
162 experimental design consisted of 3 treatment groups: i) a control group (C) where half of the
163 brood was cross-fostered between nest-pairs without modifying brood size (n = 20 nests), ii)
164 a reduced group (R) where half of the brood was cross-fostered between nest-pairs and 2
165 nestlings were removed from the brood (n = 25 nests), and iii) an enlarged group (E) where
166 half of the brood was cross-fostered between nest-pairs and 2 nestlings were added to the
167 brood (n = 25 nests) (Fig.1).

168 In total, this study included 70 great tit nests resulting in 540 nestlings monitored (n_C
169 = 150, n_E = 236, n_R = 154), of which 227 individuals were cross-fostered and 399 fledged (n_C
170 = 98, n_E = 188, n_R = 113) (see sample sizes for different measurements in Table 1).

171 Before the brood size manipulation, nestlings from nest-pairs were weighed on an
172 electronic scale (body mass \pm 0.1g) and individually marked (nail-clipping). To measure

173 nestling mitochondrial density before the treatment started, we performed blood sampling 2
174 days after hatching, before the brood size manipulation, on a subsample of nestlings (1 -
175 10 μ L from the tarsus vein using heparinized capillaries, 2-4 nestlings/nest, see Table 1).
176 When performing the brood size manipulation and cross-fostering we avoided moving the
177 smallest or biggest nestlings to minimize disturbing sibling competition hierarchies that could
178 have significantly decreased nestlings' survival chances after the manipulation. Body mass
179 of nestlings swapped between nests was as similar as possible and cross-fostered
180 individuals were kept in a warm box during the transfer (using heating pads). To assess if
181 parental feeding rates differed according to the brood size manipulation treatment groups,
182 we video-recorded a subsample of nest boxes 8 days after hatching (see more details in
183 supplementary materials). We found higher rates for E group compared to R group, while
184 parental feeding rate between E and C groups was not significantly different (Fig.S1).

185 Nestlings were ringed 7 days after hatching, weighed and measured with a metal
186 ruler (wing length \pm 1mm) at days 7 and 14 (Table 1). Nestlings were blood sampled at day
187 14 (~30-75 μ L from the brachial vein using heparinized capillaries). Blood samples were used
188 to (1) evaluate mitochondrial aerobic metabolism (fresh samples kept on ice collected on 14-
189 day-old as nestlings and juveniles, Table 1), to (2) measure mitochondrial DNA copy number
190 (i.e. mtDNA $_{cn}$), a proxy of mitochondrial density (measured on frozen blood samples on 2
191 and 14-day-old nestlings and as juveniles when samples were available), and to (3) measure
192 mitochondrial reactive oxygen species (ROS) measured in 14-day-old nestlings and
193 juveniles from the same samples as the mitochondrial aerobic metabolism assay (see below
194 for detailed protocol).

195 Previous data on this population (Ruuskanen, *unpublished data*) showed that
196 dispersion of great tits after fledging is almost entirely limited in this study area as none of
197 the birds ringed as nestlings were recaptured outside of the study area. Thus, we were able
198 to use the recapture probability of nestlings the following autumn (as juveniles, between 9 to
199 20 weeks after fledging) as a proxy of medium-term apparent survival. We conducted mist-
200 nesting with playback at 6 feeding stations inside the study area (3 sessions of ca 2-4h /
201 feeding station over October/November summing up to a total of 14 days and 69 hours of
202 mist-nesting). Juveniles were visually sexed. In total, we recaptured 67 individuals from 34
203 nests: (juveniles/nests) $n_C = 22/9$; $n_E = 31/15$; $n_R = 14/10$, Table 1).

204 c) Mitochondrial DNA copy number

205 We randomly selected a minimum of 2 nestlings per nest (one original and one
206 cross-fostered nestling). Genomic DNA was extracted from 1 to 5 μ L of frozen blood samples

207 (stored at -80°C) using a salt extraction procedure adapted from Aljanabi and Martinez
208 (1997). Due to small volumes, some of the blood samples collected on day 2 could not be
209 analyzed. When data were available (see Table 1), we measured mtDNA $_{cn}$ on the same
210 individuals at day 2, day 14 and as juvenile (i.e. recaptured in autumn 2020). DNA quantity
211 and purity were estimated using a *NanoDrop ND-1000* spectrophotometer. Samples were
212 re-extracted if needed ($[\text{DNA}] < 50\text{ng}/\mu\text{L}$, $260/280$ ratio < 1.80 or $260/230 < 2$). Samples
213 were then diluted to $1.2\text{ng}/\mu\text{L}$ in sterile H_2O and stored at -80°C until qPCR assays. We
214 quantified mtDNA $_{cn}$ using real-time quantitative PCR assays (qPCR) from a protocol
215 described in Cossin-Sevrin et al. (2022). We made some adjustments to the original
216 protocol: samples were automatically pipetted (epMotion® 5070, Eppendorf, Hamburg,
217 Germany) in duplicates in 384-well qPCR plates ($n = 5$ plates) and qPCR were performed
218 with a Biorad instrument (CFX-384, Biorad, Hercules, USA). We used Recombination
219 Activating Gene 1 (*RAG1*) as a single control gene and cytochrome oxidase subunit 2
220 (*COI2*) as specific mitochondrial gene (sequences and procedure of verification are
221 described in Cossin-Sevrin et al., 2022). qPCR reactions were conducted in a total volume of
222 $12\mu\text{L}$, including 6ng of DNA samples, primers at a final concentration of 300nM and $6\mu\text{L}$ of
223 GoTaq® qPCR Mix (Promega, Madison, USA). qPCR conditions were the following : 3min at
224 95°C (polymerase activation), followed by 40 cycles of 10s at 95°C , 15s at 58°C , 10s at
225 72°C . Melting curve program was 5s at 65°C , and $0.5^{\circ}\text{C}/\text{s}$ increased until 95°C . A pooled
226 DNA sample from 14 adult individuals was used as a reference sample (i.e. ratio = 1.0 for
227 mtDNA $_{cn}$) and was included in duplicate on every plate. qPCR efficiencies of *RAG1* and
228 *COI2* genes were respectively (mean \pm SEM): $99.14 \pm 1.17\%$ and $95.74 \pm 0.11\%$.
229 Repeatability of mtDNA $_{cn}$ between sample-duplicates was $R = 0.90$ (CI 95% = [0.88, 0.92]).
230 The samples were distributed randomly on different plates and in order to control for
231 interplate variability, qPCR plate identity was included as a random intercept in our statistical
232 analysis (see details below). DNA integrity of 46 randomly selected samples was evaluated
233 and deemed satisfactory using gel electrophoresis (100ng of DNA, 0.8% agarose gel at
234 100mV for 1 hour).

235 d) Mitochondrial aerobic metabolism

236 In order to test the impact of brood size on nestling mitochondrial respiration, we
237 measured mitochondrial aerobic metabolism in a subsample (1 to 3 nestlings per nest), 14
238 days after hatching (individuals/nest: $n_C = 26/14$, $n_E = 41/21$, $n_R = 35/19$) and in the same
239 individuals as juveniles (recaptured in autumn 2020), when samples were available ($N = 14$
240 individuals). We additionally measured mitochondrial aerobic metabolism from the majority
241 of juveniles recaptured that participated in the manipulation (as nestlings) (in total,

242 juvenile/nest: $n_C = 16/9$, $n_E = 26/15$, $n_R = 12/8$). Blood sample volumes collected on 2-day-old
243 nestlings were unfortunately not large enough for measuring mitochondrial aerobic
244 metabolism at this stage (i.e. 1-10 μ L of blood). Mitochondrial respiration was analyzed using
245 high-resolution respirometry (3 *Oroboros* Instruments, Innsbruck, Austria) at 40°C adapted
246 from a protocol described in Stier et al., (2019): digitonin (20 μ g/mL), pyruvate (5mM), malate
247 (2mM), ADP (1.25mM), succinate (10mM), oligomycin (2.5 μ M), antimycin A (2.5 μ M). We
248 used 20 μ L (nestlings) to 30 μ L (juveniles) of fresh blood when available, suspended in Mir05
249 buffer. Five distinct respiration rates were analyzed: 1) the endogenous cellular respiration
250 rate before permeabilization (*ROUTINE*), 2) the maximum respiration rate fueled with
251 exogenous substrates of complex I, as well as ADP (*C*), 3) the maximum respiration rate
252 fueled with exogenous substrates of complexes I and II, as well as ADP (*CI+II*), 4) the
253 respiration rate contributing to the proton leak (*LEAK*), 5) the respiration rate supporting ATP
254 synthesis through oxidative phosphorylation (*OXPHOS*). We also calculated three
255 mitochondrial flux ratios (FCR): 1) *OXPHOS* coupling efficiency ($OxCE = (CI+CIi-LEAK)/CI+II$),
256 2) the proportion of maximal respiration capacity being used under
257 endogenous cellular condition (i.e. $FCR_{ROUTINE/CI+II}$) and 3) the ratio between the maximal
258 respiration rate of complex I and the maximal respiration capacity (i.e. $FCR_{CI/CI+II}$). *OXPHOS*
259 coupling efficiency FCR provides an index of mitochondrial efficiency in producing ATP,
260 whereas $FCR_{ROUTINE/CI+II}$ reflects the cellular control of mitochondrial respiration by
261 endogenous ADP/ATP turnover and substrate availability. Respiration rates were
262 standardized by the number of cells in each sample, measured by *BIO-RAD* TC20
263 automated cell counter. The technical repeatability of mitochondrial aerobic metabolism
264 measurements was high: *ROUTINE*: $R = 0.985$ (CI 95% = [0.936, 0.997]); *CI+II*: $R = 0.98$ (CI
265 95% = [0.912,0.995]); *LEAK*: $R = 0.979$ (CI 95% = [0.916, 0.995]); *OXPHOS*: $R = 0.977$ (CI
266 95% = [0.898,0.995]) based on 9 duplicates.

267 e) Reactive oxygen species measurements

268 Reactive oxygen species (ROS) were measured in 14-day-old nestlings and juveniles
269 from the same samples as the mitochondrial aerobic metabolism assay (i.e. red blood cells
270 suspended in Mir05 buffer) (see Table 1 for sample-sizes). The relative amount of ROS was
271 estimated by fluorescence, using MitoSOX™ Red kit (MitoSOX™ red mitochondrial
272 superoxide indicator, Thermo Fisher) that specifically measures mitochondrial superoxide
273 (i.e. the primary mitochondrial ROS) in live cells. Samples were supplemented with 4 μ L of
274 MitoSOX™ (final concentration 4 μ M) and incubated for 30 min at 40°C protected from light.
275 After being cooled down (5 min on ice) and centrifuged (2 min, 1000g at 4°C), samples were
276 re-suspended in 250 μ L Mir05 buffer added with 5mM pyruvate, 2.5mM malate, 10mM

277 succinate and 1.25mM ADP. 100 μ L of samples were loaded on a white 96-well plate (n =
278 43) with a transparent bottom. Kinetics of fluorescence were read for 30 min (emission 510
279 nm/ excitation 580 nm) in EnSpire® 2300 Multilabel Reader (PerkinElmer) set at 40°C.
280 Samples were analyzed in duplicates. The slope of relative fluorescence (RFU/min) was
281 then extracted and normalized by the internal control present on each plate (dry
282 *Saccharomyces cerevisiae* diluted at 10mg/mL in Mir05). As a positive control (for
283 mitochondrial ROS production) diluted *Saccharomyces cerevisiae* supplemented with
284 antimycin A was included in each plate. Relative mitochondrial ROS results were
285 standardized by the number of cells present in each well, taking into account dilution factor
286 (cell count estimated with the *BIO-RAD* TC20 automated cell counter). Repeatability of the
287 ROS production measurements between sample-duplicates was $R = 0.924$ (CI 95% = [0.9,
288 0.941]).

289 f) Statistical analysis

290 Statistical analyses were conducted using R v.4.0.2 (R core team, 2020) and performed
291 using linear mixed models (LMMs) or generalized linear mixed models (GLMMs). Results for
292 preliminary tests (see below) were obtained using linear mixed models with the cross-
293 fostering status (yes or no) added as fixed factor and the nest box included as random
294 intercept.

295 *Preliminary tests*

296 Pre-treatment clutch sizes (raw data mean \pm SEM: R = 9.24 ± 0.26 , C = 8.65 ± 0.28 ,
297 E = 8.48 ± 0.17 eggs; ANOVA: $F = 2.97$, $P = 0.06$) and hatching date (C = 58.70 ± 1.21 , E &
298 R = 60.16 ± 1.06 days; ANOVA: $F = 0.54$, $P = 0.59$) were relatively balanced between
299 treatment groups. Initial brood sizes on day 2 post-hatching per treatment groups were the
300 following: (raw data mean \pm SEM [range]) R = 8.00 ± 0.32 [5;11], C = 7.50 ± 0.44 [4;10] and
301 E = 7.68 ± 0.28 [4;9] chicks and were not statistically different between treatment groups
302 before the manipulation (ANOVA: $F = 0.55$, $P = 0.57$). Nestling body mass (raw data mean \pm
303 SEM: R = 2.93 ± 0.07 , C = 2.94 ± 0.05 , E = 2.98 ± 0.05 g; $F = 0.51$, $P = 0.60$) and relative
304 mtDNA cn (raw data mean \pm SEM: R = 7.62 ± 0.91 , C = 8.10 ± 0.42 , E = 8.29 ± 0.88 ; $F =$
305 0.32 , $P = 0.73$) measured before the experimental manipulation (2 days post-hatching) were
306 not statistically different between groups before the assessment of the treatment. We did not
307 find any significant differences between the chicks cross-fostered and non cross-fostered for
308 the responses variables tested throughout this study (i.e. growth metrics, mtDNA cn ,
309 mitochondrial metabolic rates, ROS production, survival metrics, all $F < 2.72$, all $P > 0.1$).

310 *Experimental approach*

311 To investigate the experimental effect of brood size manipulation on response
312 variables, we always included in our models the treatment as a 3-level fixed factor (R,C,E),
313 the hatching date (continuous variable) and the initial brood size (continuous variable) to
314 account for initial differences in brood size across nests (see Table 2A). These analyses are
315 referred to “*experimental approach*” in the text. To test for potential different effects of the
316 treatment according to the initial number of nestlings in the nest, we always tested the
317 interaction between the treatment and initial brood size in our models. Non-significant
318 interactions (treatment* initial brood size) were dropped from the model in order to properly
319 interpret the main effects. Nest box of rearing ID and original nest box ID were included as
320 random intercepts in the models. In case of convergence issues, original nest box ID (and
321 potentially hatching date if needed) were removed from the model (Table 2A). For models
322 that included repeated measures across time (i.e. body mass and mtDNA cn), we initially
323 included the age and treatment, as well as their interaction that was removed from the final
324 model when non-significant. For mtDNA cn and postnatal body mass analysis, the bird ID
325 was included as a random intercept in the model to take into account the non-independence
326 of measures from the same individual.

327 *Correlative approach*

328 To explore the associations between number of nestlings and the measured traits
329 (focusing on the ecological aspect of the brood size rather than experimental), we used
330 another set of models including the actual number of nestlings (on the day of data collection)
331 as a continuous variable (see Table 2B). These analyses are referred to “*correlative*
332 *approach*” in the text. As the number of nestlings per nest nests varied substantially across
333 and within treatment groups (e.g. at day 14 brood size ranged from 2 to 11 nestlings), this
334 analysis reflects the associations between a given brood size and trait of interest. However,
335 given that the dataset using brood size as a continuous variable includes both
336 experimentally manipulated (E, R) and non-manipulated nests (C) we also analyzed the
337 associations between the number of nestlings and target variables using only the non-
338 manipulated nests (C) group to check if patterns might have been confounded by including
339 experimental nests (Table S3). As results were similar, we report results of the full dataset in
340 the main text. In these analyses, we also included hatching date as a continuous variable
341 and the IDs of both original and rearing nest boxes as random intercepts. qPCR plate ID
342 could not be included in the model only including the control group because of convergence
343 issues (Table 2B).

344 The nature of mtDNA cn data did not fulfill the criteria of normality according to a Cullen and
345 Frey plot (*fitdistrplus* package; Delignette-Muller and Dutang, 2015); therefore, we analyzed
346 the effects of the treatment and the number of nestlings across the age of the individual
347 (included as 2-levels fixed factor: day 14 and juveniles) using a GLMM (gamma error
348 distribution, log link).

349 We analyzed mitochondrial respiration rates (recorded on 14-day-old nestlings and juveniles,
350 including *ROUTINE*, *CI*, *CI+II*, *LEAK*, *OXPHOS*) at the mitochondrial level (i.e. respiration
351 measurements controlled for mitochondrial density by inclusion of mtDNA cn as a covariate),
352 which indicates the respiration rate per unit of mitochondria.

353 For mitochondrial respiration rates measured at day 14, we further quantified the variance
354 explained by the random intercepts (i.e. both original nest box ID and nest box of rearing ID
355 included as random intercepts, while treatment, initial brood size, hatching date and
356 mtDNA cn were included as fixed factors), using *RptR* package (gaussian distribution, N
357 bootstraps = 1000) (Nakagawa & Schielzeth, 2010; Stoffel et al, 2017).

358 To investigate the contribution of mitochondrial respiration rates at day 14 on juvenile
359 apparent survival (i.e. recapture probability), we performed GLM on survival (logistic binary
360 distribution of dependent variables: 0 = dead, 1 = alive) and included mitochondrial
361 respiration rates or FCR(s) and hatching date as explanatory factors. As the number of
362 individuals recaptured was less than 2 individuals for several nests, we could not include the
363 nest of rearing ID as a random intercept in our models (convergence issues). Results from
364 these analyses are presented in Table S5.

365 All models were performed using *lme4* package (Bates et al., 2015). Normality and
366 homoscedasticity of the residuals were visually inspected (Q–Q plots) and no clear violation
367 was observed. Results from type III ANOVA tables with *F* values and *P* values (i.e. testing
368 the main effect of each factor and interaction) were calculated based on Satterthwaite's
369 method and are presented in the text. Results from GLMMs (logistic binary distribution) were
370 calculated based on Wald Chisquare tests (type II ANOVA). Model estimates (with
371 associated 95% CI and *P* values) are reported in tables. *emmeans* package was used to
372 conduct multiple *post hoc* comparisons (adjusted with Tukey honest significant differences
373 correction). Effect-sizes (Cohen's *D*) were estimated using *effsize* package (Ben-Shachar et
374 al., 2020). Values were considered as statistically significant for $P < 0.05$.

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Results

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1. Brood size manipulation

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Our treatment led to significant differences in brood size between treatment groups (R, C, E) after the manipulation on day 2: average (\pm SEM, on raw data) brood sizes were R = 6.00 ± 0.32 (initial 8.00 ± 0.32), C = 7.50 ± 0.44 (initial 7.50 ± 0.44), E = 9.68 ± 0.28 (initial 7.68 ± 0.28) nestlings per nest on day 2 (Tukey HSD *post hoc*: all comparisons $P < 0.009$). Brood size remained significantly higher for the E group than C or R during the whole growth period (from day 2 to day 14) (all Cohen's D > 1.50) (Tukey HSD *post hoc*: C vs. E and E vs. R comparisons, all $P < 0.02$), while the differences in brood sizes between C and R groups were not significant at 7 days (Cohen's D with 95% CI = $0.43 [-0.25, 1.11]$) and 14 days after hatching (Cohen's D with 95% CI = $0.37 [-0.31, 1.05]$) (Tukey HSD *post hoc*: C vs. R comparison, all $P > 0.90$). Averages (\pm SEM, on raw data) for R, C and E groups were respectively: R = 4.84 ± 0.54 , C = 5.25 ± 0.72 , E = 7.88 ± 0.76 nestlings at day 7 and R = 4.60 ± 0.54 , C = 4.95 ± 0.68 , E = 7.56 ± 0.75 nestlings at day 14. [To confirm our results presented below, we used the *bootMer* function from *lme4* package \(type settled as parametric and n bootstrap = 1000\). Confidence interval \(95%\) of predicted estimates using a parametric bootstrapping method remained different from zero for factors having a statistically significant effect with generalized linear mixed models.](#)

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2. Nestling growth trajectories

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Postnatal body mass dynamic (from day 7 to 14) was differentially affected by the treatment depending on offspring age (Table 2). Specifically, nestlings from the R group had a higher body mass 14 days after hatching than nestlings E groups (+4.81%), while body mass at day 14 remained similar between R and C groups (Table 2, Fig.2). Body mass at day 14 from nestlings raised in C and E groups were not statistically different (Table 2, Fig.2). We did not find any significant difference in body mass 7 days after hatching (Tukey HSD *post hoc* comparisons: all $t < 1.18$, all $P > 0.36$). Body mass significantly increased with hatching date (Table 2). The treatment did not significantly impact nestling wing length during the growth period (day 7 and day 14) (all $F < 0.68$, all $P > 0.51$). Wing length at day 7 and 14 were significantly and positively associated with the hatching date (all $F > 6.57$, all $P < 0.01$). We found a significant positive correlation of wing length at day 14 and initial brood size (estimate \pm SE = 0.42 ± 0.18 , $F_{1,41.5} = 5.66$, $P = 0.02$). Juvenile body mass and size were not significantly impacted by the treatment (all $F < 0.63$, all $P > 0.54$).

407 3. Mitochondrial DNA copy number

408 While mtDNA cn was not significantly impacted by the treatment ($\chi^2 = 0.49$, $P = 0.78$),
409 mtDNA cn significantly decreased with the age ($\chi^2 = 447.6$, $P < 0.001$) (raw data Cohen's D
410 with 95% CI: day 14 vs. juveniles = 1.35 [1.01, 1.68]).

411 4. Mitochondrial aerobic metabolism

412 We did not find any significant effect of the brood size manipulation treatment or of the initial
413 brood size on the different mitochondrial respiration rates and FCR(s) measured at day 14
414 (Tables 4, Fig.3). Juvenile mitochondrial respiration rates and FCR(s) were not significantly
415 impacted either by the treatment (all $F < 0.75$, all $P > 0.48$) or the initial brood size (all $F <$
416 2.46 , all $P > 0.13$). All mitochondrial respiration rates increased with mtDNA cn at day 14
417 (Tables 4) and in juveniles (all $F > 5.39$, all $P < 0.02$), except for *LEAK* (juveniles: $F_{1, 49} =$
418 3.07 , $P = 0.09$).

419 For all mitochondrial respiration rates measured at day 14, the nest of rearing significantly
420 contributed to explain the variance in our models (all repeatabilities > 0.51 , all $P < 0.001$,
421 Fig.4). Except for *ROUTINE* (repeatability = 0.08, $P = 0.20$), the variance explained by the
422 nest of origin was significantly higher than 0 (all repeatabilities > 0.13 , all $P < 0.02$) but the
423 contribution of the nest of rearing was higher than the nest of origin (Fig.4).

424 5. ROS production

425 In 14-days-old nestlings, mitochondrial ROS production was not significantly affected by the
426 treatment ($F_{2, 45.7} = 0.62$, $P = 0.54$) or the initial brood size ($F_{1, 49.7} = 0.05$, $P = 0.82$). These
427 results remained consistent in juveniles (treatment: $F_{2, 48} = 1.58$, $P = 0.22$; initial brood size:
428 $F_{1, 48} = 0.74$, $P = 0.39$). While mitochondrial ROS production was not significantly associated
429 with mtDNA cn in nestlings ($F_{1, 83} = 0.48$, $P = 0.49$), juvenile mitochondrial ROS production
430 significantly increased with mtDNA cn measured in autumn (estimate \pm SE = 0.003 ± 0.001
431 , $F_{1, 48} = 4.60$, $P = 0.04$).

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433 6. Survival metrics

434 Fledgling success was not significantly affected by the treatment ($\chi^2 = 2.44$, $P = 0.29$, raw
435 data: R = 75.33%, C = 65.79%, E = 77.78%), neither by the initial brood size ($\chi^2 = 0.05$, $P =$
436 0.83) or the hatching date ($\chi^2 = 2.18$, $P = 0.14$). Juvenile recapture probability was not
437 significantly affected by the treatment ($\chi^2 = 2.18$, $P = 0.34$, raw data: R = 12.17%, C =
438 22.22% , E = 18.52%) or the initial brood size ($\chi^2 = 0.03$, $P = 0.87$), but was negatively

439 associated with the hatching date ($\chi^2 = 13.6$, $P < 0.001$). Finally, we did not find any
440 significant associations between juvenile recapture probability, mitochondrial respiration
441 rates and FCR(s) measured at day 14 (all $P > 0.2$, Table S5).

442 7. Correlative approach

443 When analyzing each age separately, in order to account for the number of nestlings in the
444 nest at a given age, nestling body mass at day 7 was negatively associated with the number
445 of nestlings in the nest (Table S1), while we did not find an association for the wing length
446 ($F_{1, 31.10} = 0.38$, $P = 0.54$). On day 14, nestling body mass was not significantly associated
447 with the number of nestlings (Table S1), we found similar results for juvenile body mass ($F_{1, 34.1} = 0.18$, $P = 0.66$). Nestling wing length at day 14 tended to increase with the number of
448 nestlings (Table S1). While mtDNA $_{cn}$ at day 14 was not associated with the number of
449 nestlings in the nest ($P = 0.11$), larger brood sizes a few days before fledging (i.e. day 14)
450 predicted higher mtDNA $_{cn}$ for juveniles (estimate \pm SE = 0.07 ± 0.03 , $P = 0.04$). We found a
451 negative association between the number of nestlings at day 14 and mitochondrial
452 respiration rates measured at day 14 (Table S2, Fig.5). *OXPHOS* coupling efficiency and
453 both FCR $_{ROUTINE/C+II}$ and FCR $_{C/C+II}$ were not significantly associated with the number of
454 nestlings at day 14 (all $F < 1.38$ and all $P > 0.25$). We found similar results when only
455 including individuals raised in the C group (Table S3). As we suspected nestlings from small
456 brood sizes (less than 5 chicks at day 14) with high mitochondrial respiration rates to drive
457 the associations between the number of nestlings and mitochondrial metabolic rates (Fig.5),
458 we performed the same statistical analysis excluding nestlings raised in small broods ($n = 12$
459 nestlings from 8 nests removed from the analysis). In this case, we could not detect any
460 significant associations between the number of nestlings (day 14) on the different
461 mitochondrial respiration rates measured (all $F < 2.23$, all $P > 0.14$, Table S4, Fig.5).
462 Juvenile mitochondrial respiration rates (all $F < 0.21$, all $P > 0.65$) or FCRs (all $F < 0.72$, all P
463 > 0.49), were not associated with the number of nestlings at day 14, except for FCR $_{C/C+II}$ for
464 which we found a negative association (estimate \pm SE = -0.005 ± 0.003 , $F_{1, 62} = 4.36$, $P =$
465 0.04). We did not find significant associations between the number of nestlings at day 14
466 and nestling mitochondrial ROS production (day 14: $F_{1, 53.49} = 0.42$, $P = 0.52$) or in juveniles
467 ($F_{1, 50} = 1.08$, $P = 0.30$). Fledgling success was strongly positively associated with the
468 number of nestlings in the nest at day 14 ($\chi^2 = 61.47$, $P < 0.001$). Juvenile recapture
469 probability was not significantly associated with the number of nestlings day 14 ($\chi^2 = 0.23$, P
470 $= 0.63$).

472 Discussion 473

474 Overall, the experimental brood size manipulation did not significantly affect nestling
475 mitochondrial density, metabolism or ROS production. Despite a mild impact of the treatment
476 on nestling growth trajectories, body mass differences cannot be associated here with
477 variation in mitochondrial metabolism. Furthermore, we did not detect any significant long-
478 lasting effect of the brood size manipulation treatment on juveniles (neither on recapture
479 probability, body mass and size, nor mitochondrial density, metabolism and subsequent
480 ROS production). However, our results emphasized the importance of the actual number of
481 nestlings in the nest regardless of experimental manipulation for nestling mitochondrial
482 respiration. Nestling mitochondrial metabolic rates were negatively associated with the
483 number of nestlings in the nest (but see precautions in interpretations below). Our results
484 also provide evidence that environmental conditions during the growth period (nest of
485 rearing) contribute more to explaining variance in red blood cells mitochondrial metabolism
486 than genetic inheritance pre- and early postnatal parental effects (nest of origin) in great tits.
487 Taken together, our results suggest that (even though modified by the treatment) the actual
488 number of nestlings in the nest (rather than the modification of the initial brood size) is
489 associated with nestling growth pattern and mitochondrial metabolism. Indeed, the number
490 of siblings in a nest may have an influence on many environmental factors, such as food
491 availability and competition between chicks, as well as early-life conditions critical to nestling
492 growth, such as nest temperature (Andreasson et al., 2016; Hope et al., 2021; Nord &
493 Nilsson, 2011).

494 *Experimental approach*

495 Nestling growth trajectories (postnatal body mass) differed according to nestling age and our
496 treatment. As expected, individuals raised in the reduced group had a higher body mass a
497 few days before fledging compared to the enlarged group but not the control group (see also
498 Hōrak, 2003). While we expected nestlings raised in enlarged group to have lower body
499 mass (Hōrak, 2003; Rytönen & Orell, 2001; Smith et al., 1989), nestlings raised in enlarged
500 and control groups had similar body masses over the entire growth period. Moreover,
501 nestling wing length did not differ between treatment groups. It is possible that parents
502 managed to compensate for the brood size augmentation by increasing parental effort, as
503 suggested by results on parental feeding rates (measured on a subsample of nests, Fig.S1).
504 The number of visits was significantly higher in the enlarged group compared to the reduced
505 group and tended to be higher compared to controls (although non-significant). These
506 results would be supported by prior studies suggesting that parents can rear more nestlings
507 than the number of eggs laid (Casti, 2018; Monaghan & Nager, 1997; Vander Werf, 1992).

508 It is worth noting that in our experiment the difference in nestling number between
509 control and reduced groups did not remain significant (small effect-sizes between groups) at
510 the end of the growth period (from day 7 to 14). This likely contributes to explain why our

511 experiment failed to demonstrate large differences between treatment groups. It is
512 interesting that even without differences in the number of chicks at the end of the experiment
513 between control and reduced groups, the reduced group tended to have larger chicks (see
514 hypothesis below).

515 It has been shown that a brood size enlargement can affect nestling metabolism, as
516 brood size decreases whole animal resting rate of oxygen consumption in the short-term
517 (tree swallow), and increases standard metabolic rate in the a long-term (zebra finches)
518 (Burness et al., 2000; Verhulst et al., 2006). In our case, the brood size manipulation
519 treatment did not have an effect on nestling red blood cell mitochondrial metabolism during
520 the growth period or in a longer-term in juveniles. This lack of effects may be explained by
521 the two reasons mentioned above (i.e. increase of parental feeding rates and no differences
522 in chick number between control and reduced groups). Nestling (and juveniles) ROS
523 production were not impacted by the treatment either. This outcome is in accordance with
524 our findings that mitochondrial aerobic metabolism did not differ between treatment groups.
525 Despite the mild effect of brood size manipulation on nestling body mass, nestling fledgling
526 success and apparent medium-term survival (i.e. recapture probability as juvenile) were not
527 significantly impacted by the treatment, likely explained by the increase in parental feeding
528 rates.

529 *Correlative approach*

530 For the reasons mentioned above, our experiment failed to create large differences between
531 treatment groups, and the variation in brood size within treatment groups was large. Thus,
532 we performed another set of statistical analysis beside the experimental, using the actual
533 number of nestlings as explanatory variable. Our results suggest that the actual number of
534 offspring in the nest is associated with nestling postnatal body mass and structural size.
535 Nestling body mass was negatively associated with the number of nestlings in the nest in the
536 middle of the growth period (day 7), but tended to be positively associated with the number
537 of individuals in the nest at the end of the growth period (day 14). This insight was surprising
538 as the opposite results (i.e. negative association between the wing length and the number of
539 chicks in the nest) have been reported in the literature (Hörak, 2003; Rytönen & Orell,
540 2001; Smith et al., 1989). Yet, these results from previous studies have been found in the
541 framework of a brood size manipulation and did not strictly focus on the actual number of
542 chicks in the nest.

543 We found a negative association between mitochondrial metabolism (*ROUTINE*, *CI*,
544 *CI+II*, *LEAK* and *OXPHOS*) and number of nestlings. As both *LEAK* and *OXPHOS* were
545 negatively correlated with the number of nestlings, we did not find an association between
546 *OXPHOS* coupling efficiency and nestling number. The higher mitochondrial metabolic rates
547 observed for nestlings raised in small broods could reflect a higher energetic demand,

548 potentially linked to a higher need for thermogenesis (Andreasson et al., 2016, Bicudo et al.,
549 2001).

550 While these results are in accordance with our predictions (decrease in mitochondrial
551 metabolic rates in larger broods) it is important to note that these negative associations with
552 the number of nestlings did not remain significant when nestlings from very small broods
553 (less than 5 nestlings at day 14, which is quite exceptional for the study species) were
554 excluded from the analysis, meaning that those specific broods drove the patterns.
555 Therefore, we cannot conclude that a relatively large brood size (e.g. via effects of stress) is
556 associated with lower mitochondrial respiration. Interestingly, broods with less than 5
557 nestlings at day 14 had really low survival chances during the growth period (from day 2 to
558 14) compared to the larger broods (> 4 nestlings) (average on raw data: 63.4% vs. 92.4% of
559 survival at day 14, excluding nests without chicks at day 14: n = 12 nests) and most of the
560 nestlings did not reach day 7 (average at day 7: 1.13 nestlings lost in small broods vs. 0.34
561 in larger broods). We therefore suspect nestling growth and mitochondrial metabolic patterns
562 to rather reflect unusual rearing conditions than being general patterns. Our main hypothesis
563 is that these individuals might be at a less-advanced developmental stage, given their
564 smaller structural size, knowing that mitochondrial quantity and/or respiration decreases
565 during postnatal development (Stier et al. 2020; Stier et al. 2022; Cossin-Sevrin et al. 2022,
566 Hsu et al. 2023; but see: Dawson & Salmón, 2020) and potentially more stressed (some
567 environmental stressors may lead to higher metabolic rate, i.e. in interaction with
568 glucocorticoid levels in zebra finches; Jimeno et al., 2017). Alternatively, these small broods
569 with a high unusual mortality during early-growth may be subject to selective disappearance
570 and nestlings surviving until 14 days after hatching represent a non-random pool of
571 individuals that managed to survive and cope with detrimental conditions during early-
572 growth. Despite the negative association between nestling mitochondrial metabolic rates and
573 the number of nestlings, we did not find any association between nestling ROS production
574 and the number of nestlings, and fledging success was positively associated with the
575 number of nestlings. Yet the sample size for small broods was limited, and therefore the
576 results need to be interpreted with caution.

577 Furthermore, our study demonstrates that both genetic inheritance (but also
578 complementary mechanisms, such as parental effects before the cross-fostering) and the
579 rearing environment contribute to variation in offspring mitochondrial traits, but with a larger
580 contribution from the rearing environment. Similar results about lower contribution of familial
581 background have been found for resting metabolic rate in collared flycatcher nestlings
582 (*Ficedula albicollis*) (McFarlane et al., 2021). While the underlying mechanisms of
583 modulation of mitochondria by early-life environmental conditions are unknown, recent
584 research points out that mitochondrial function can respond to environmental cues through

585 changes in gene expression and mitochondrial DNA methylation (Sharma et al., 2019;
586 Wallace, 2016).

587 One objective of this study was to assess if differences in nestling mitochondrial
588 metabolic phenotype could predict different juvenile recapture probabilities. In our case, we
589 did not find any association of nestling mitochondrial metabolic rates on juvenile apparent
590 survival. We may have expected higher mitochondrial metabolism to lead to detrimental
591 consequences through an increase in ROS release (potentially leading to oxidative stress).
592 However, as previously stated, ROS production did not differ between nestlings and both
593 results are concordant. Furthermore, if nestlings that survived until day 14 were subject to
594 selective disappearance, testing for the association between mitochondrial phenotype and
595 survival as juvenile seems challenging.

596 As a limitation in our study, mitochondrial ROS production, substrate preferences and
597 mitochondrial aerobic metabolism are known to vary between tissues (Mailloux, 2020;
598 Salmón et al., 2022). Therefore, one should always be careful when investigating ROS
599 production in a single tissue (Costantini, 2019; Monaghan et al., 2009). However, we
600 focused our study on blood samples to i) estimate nestling survival and potential long-lasting
601 effect of our experiment and ii) since mitochondrial aerobic metabolism measurements in
602 blood samples can be positively associated with other tissues (Koch et al., 2021; Stier et al.,
603 2017). Collecting blood samples allows the use of limited-invasive methods on wild species,
604 and to avoid terminal sampling.

605 Altogether, our results suggest that nestling mitochondrial aerobic metabolism is
606 associated with the actual number of nestlings in the nest, and the contribution of postnatal
607 environmental conditions experienced by the offspring explains a large part of the variation.
608 The effect of rearing conditions on offspring mitochondrial metabolism emphasizes the
609 plasticity of mitochondrial metabolism in changing environments. Further studies would be
610 needed to closely investigate what are the major environmental cues affecting the offspring
611 mitochondrial metabolism during the growth period (e.g. availability of nutrients, ambient
612 temperature) (White & Kearney, 2013), but also to disentangle the role of the brood size in
613 influencing rearing environment (e.g. nest temperature, Andreasson et al., 2016) and its
614 consequences on nestling physiology and fitness-related traits (e.g. body temperature, DNA
615 methylation, ageing) (Andreasson et al., 2018; Koch et al., 2021; Sheldon et al., 2018).

616

617 **Acknowledgements**

618 We are grateful to Toni Laaksonen, Jorma Nurmi, Robin Cristofari, Natacha Garcin, Ida
619 Penttinen, Bin-Yan Hsu and volunteer bird ringers for their help on the field. We thank Tuija
620 Koivisto for the video analysis. We thank Marine Pery for her involvement in this project.

621

622 **Competing interests**

623 We declare we have no competing interests.

624 **Funding**

625 N.C-S was supported by EDUFI Fellowship (Opetushallitus), Maupertuis Grant and the
626 Biology, Geography and Geology doctoral program of the University of Turku at the time of
627 writing. A.S was funded by the Turku Collegium for Science and Medicine, who contributed
628 to fund the field study. A.S acknowledges funding from the European Commission Marie
629 Sklodowska-Curie Postdoctoral Fellowship (#894963) at the time of writing. S.R and M.H
630 acknowledge support from Academy of Finland (#286278 granted to S.R).

631 **Ethics**

632 All procedures were approved by the Animal Experiment Committee of the State Provincial
633 Office of Southern Finland (license no. ESAVI/5454/2020) and by the Environmental Center
634 of Southwestern Finland (license no. VARELY/890/2020) granted to S.R.

635 **Authors contribution**

636 S.R, A.S had the original idea and designed the study with N.C-S. N.C-S, S.R, A.S, M.H
637 collected the data. N.C-S and A.S collected mitochondrial respiration rates measurements.
638 N.C-S performed DNA extractions and conducted qPCR analysis in collaboration with S.Z.
639 N.C-S conducted statistical analyses and wrote the first version of this manuscript under the
640 supervision of S.R and K.A. All co-authors revised the manuscript. S.R, A.S, V.A-V funded
641 experimental work and data collection.

642

643 **Data available statement**

644 Data are available on Figshare DOI: 10.6084/m9.figshare.22354432. (Embargo pending
645 upon publication, private link: <https://figshare.com/s/e9f615b7f9e30e5c5d21>).

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846 Tables

847

848 **Table 1. Sample-sizes according to nestling age, treatment group (R: reduced broods,**
849 **C: control broods, E: enlarged broods) and the different traits measured throughout**
850 **this study. The number of nests is indicated in brackets.**

851

Measurements	Day 2	Day 7	Day 14	Juveniles
Body mass/size	$n_R = 154$ (25)	$n_R = 121$ (21)	$n_R = 115$ (21)	$n_R = 14$ (10)
	$n_C = 150$ (20)	$n_C = 105$ (16)	$n_C = 99$ (16)	$n_C = 22$ (9)
	$n_E = 236$ (25)	$n_E = 194$ (21)	$n_E = 189$ (21)	$n_E = 31$ (15)
Mitochondrial DNA copy number (i.e. proxy of mitochondrial density)	$n_R = 17$ (6)		$n_R = 48$ (20)	$n_R = 12$ (8)
	$n_C = 38$ (10)		$n_C = 46$ (16)	$n_C = 16$ (9)
	$n_E = 16$ (5)		$n_E = 55$ (21)	$n_E = 28$ (15)
Mitochondrial aerobic metabolism			$n_R = 35$ (19)	$n_R = 12$ (8)
			$n_C = 26$ (14)	$n_C = 16$ (9)
			$n_E = 41$ (21)	$n_E = 26$ (15)
ROS production measurements			$n_R = 34$ (18)	$n_R = 11$ (8)
			$n_C = 23$ (14)	$n_C = 16$ (9)
			$n_E = 37$ (20)	$n_E = 26$ (15)

852

853 **Table 2. Summary of the statistical analyses performed according to the experimental**
854 **approach (A) and the correlative approach (B).** To analyze this dataset, we used linear
855 mixed models (LMMs), linear models (LMs), but also generalized linear mixed models
856 (GLMMs) and generalized linear models (GLMs). For each response variable, explanatory
857 variables, both categorical variables and continuous variables (the latter in *italic*) included in
858 the model are presented. Random intercept terms are underlined. In case of convergence
859 issue, the original nest box ID and the hatching date (if needed) have been removed from
860 the models. For the correlative approach, the number of nestlings at the day of the
861 measurement is included for the models with nestlings, and in the models with juveniles the
862 number of nestlings refers to the brood size 14 days post-hatching. For FCRs (i.e. *OXPHOS*
863 coupling efficiency, $FCR_{ROUTINE/CI+II}$, $FCR_{CI/CI+II}$), *mtDNAcn* was not included as covariate in
864 the models.

Responses variables	A) Experimental approach
Postnatal body mass from day 7 to day 14	LMM: treatment, age, <i>initial brood size</i> , <i>hatching date</i> , <u>bird ID</u> , <u>nest box of rearing ID</u> , <u>original nest box ID</u>
Nestling and juvenile body size	LMMs: treatment, <i>initial brood size</i> , <i>hatching date</i> , <u>nest box of rearing ID</u> , <u>original nest box ID</u>
Postnatal <i>mtDNAcn</i> from day 14 to juvenile	GLMM, gamma error distribution, log link: treatment, age, <u>nest box of rearing ID</u> , <u>bird ID</u> , <u>qPCR plate ID</u>
Nestling mitochondrial metabolic rates day 14	LMMs: treatment, <i>initial brood size</i> , <i>hatching date</i> , <i>mtDNAcn</i> , <u>nest box of rearing ID</u> , <u>original nest box ID</u>
Juvenile mitochondrial metabolic rates	LMS: treatment, <i>initial brood size</i> , <i>mtDNAcn</i>
Nestling ROS production day 14	LMM: treatment, <i>initial brood size</i> , <i>mtDNAcn</i> , <i>hatching date</i> , <u>nest box of rearing ID</u>
Juvenile ROS production	LM: treatment, <i>initial brood size</i> , <i>mtDNAcn</i>
Fledging success	GLM, logistic binary distribution of dependent variables (0 = dead, 1 = alive): treatment, <i>initial brood size</i> , <i>hatching date</i> , <u>nest box of rearing ID</u>
Recapture success (survival after fledging)	GLM, logistic binary distribution of dependent variables (0 = dead, 1 = alive): treatment, <i>initial brood size</i> , <i>hatching date</i> , <u>nest box of rearing ID</u> , <u>original nest box ID</u>

865

Responses variables	B) Correlative approach
Nestling and juvenile body mass	LMMs: <i>number of nestlings</i> , <i>previous mass measured</i> , <i>hatching date</i> , <u>nest box of rearing ID</u> , <u>original nest box ID</u>
Nestling and juvenile body size	LMMs: <i>number of nestlings</i> , <i>hatching date</i> , <u>nest box of rearing ID</u> , <u>original nest box ID</u>
Nestling <i>mtDNAcn</i>	GLMM, gamma error distribution, log link: <i>number of nestlings</i> , <i>hatching date</i> , <u>nest box of rearing ID</u> , <u>original nest box ID</u> , <u>qPCR plate ID</u>
Juvenile <i>mtDNAcn</i>	GLMM, gamma error distribution, log link: <i>number of nestlings</i> , <u>nest box of rearing ID</u> , <u>qPCR plate ID</u>
Nestling mitochondrial metabolic rates	LMMs: <i>number of nestlings</i> , <i>hatching date</i> , <i>mtDNAcn</i> , <u>nest box of rearing ID</u> , <u>original nest box ID</u>
Juvenile mitochondrial metabolic rates	LMS: <i>number of nestlings</i> , <i>mtDNAcn</i>
Nestling ROS production	LMM: <i>number of nestlings</i> , <i>hatching date</i> , <i>mtDNAcn</i> , <u>nest box of rearing ID</u>
Juvenile ROS production	LM: <i>number of nestlings</i> , <i>mtDNAcn</i>
Fledging success	GLM, logistic binary distribution of dependent variables (0 = dead, 1 = alive): <i>number of nestlings</i> , <i>hatching date</i> , <u>nest box of rearing ID</u>
Recapture success (survival after fledging)	GLM, logistic binary distribution of dependent variables (0 = dead, 1 = alive): <i>number of nestlings</i> , <i>hatching date</i> , <u>nest box of rearing ID</u> , <u>original nest box ID</u>

866 **Table 3. Results of a LMM testing the effect of age and brood size manipulation**
867 **treatment on nestling body mass.** Day 7: n = 420 observations, day 14: n = 403
868 observations, N = 420 individuals in total. Estimates are reported with their 95% CI. *Post-hoc*
869 comparisons results with Tukey HSD correction are presented for the age of 14 days post-
870 hatching. Bird ID, Original nest box ID and nest box of rearing ID were included as random
871 intercepts in models. σ^2 , within-group variance; τ_{00} , between-group variance. Sample size
872 (n) along with marginal (fixed effects only) and conditional (fixed and random effects). Bold
873 indicates significance ($P < 0.05$).
874

Predictors	Estimates	95% CI	P values
(Intercept)	5.87	2.60 – 9.14	0.001
treatment (E)	-0.42	-1.13 – 0.29	0.240
treatment (R)	-0.09	-0.81 – 0.64	0.809
age (day 14)	5.99	5.62 – 6.36	<0.001
initial brood size at day 2	-0.07	-0.21 – 0.08	0.365
hatching date	0.09	0.04 – 0.14	<0.001
treatment (E) : age (day 14)	0.23	-0.23 – 0.69	0.324
treatment (R) : age (day 14)	0.65	0.194 – 1.15	0.012
<i>post-hoc comparisons for day 14:</i>			
treatment (C) vs. treatment (E)	0.19	-0.66 – 1.04	0.856
treatment (C) vs. treatment (R)	-0.56	-1.44 – 0.32	0.290
treatment (E) vs. treatment (R)	-0.75	-1.34 – -0.16	0.009
Random Effects			
σ^2	1.80		
τ_{00} bird	0.13		
τ_{00} nest of origin	0.66		
τ_{00} nest of rearing	0.31		
n nest of origin	58		
n nest of rearing	58		
n observations	823		
Marginal R^2 / Conditional R^2	0.779 / 0.862		

875 **Table 4: Results of linear mixed model testing the effects of the brood size**
876 **manipulation on mitochondrial respiration rates (A & B) and FCRs (B) measured on**
877 **14-day-old nestlings (N = 102 individuals, n = 55 nest boxes).** Mitochondrial respiration
878 rates (except FCRs, see Methods) were corrected for the mitochondrial DNA copy number
879 (i.e., proxy of mitochondrial density). Linear mixed models (LMM) estimates are reported
880 with their 95% CI. Original nest box ID and nest box of rearing ID were included as random
881 intercepts in the models. σ^2 , within group variance; τ_{00} between-group variance. Bold
882 indicates significance ($P < 0.05$).
883

Table 4.A	<i>ROUTINE</i>			<i>CI</i>			<i>CI + II</i>			<i>LEAK</i>		
Predictors	Estimates	CI 95%	P-values	Estimates	CI 95%	P-values	Estimates	CI 95%	P-values	Estimates	CI 95%	P-values
(Intercept)	4.93	2.29 – 7.58	<0.001	21.69	12.92 – 30.46	<0.001	31.14	17.59 – 44.70	<0.001	3.02	1.17 – 4.88	0.002
treatment (E)	-0.24	-0.81 – 0.33	0.397	-0.56	-2.44 – 1.32	0.551	-1.30	-4.21 – 1.60	0.372	-0.22	-0.62 – 0.17	0.265
treatment (R)	-0.19	-0.76 – 0.39	0.518	-0.13	-2.04 – 1.77	0.889	-0.69	-3.64 – 2.26	0.640	-0.13	-0.53 – 0.28	0.529
initial brood size	-0.08	-0.23 – 0.06	0.268	-0.30	-0.78 – 0.17	0.203	-0.40	-1.13 – 0.34	0.282	-0.07	-0.17 – 0.03	0.179
mtDNA _{cn}	0.35	0.27 – 0.44	<0.001	0.94	0.72 – 1.16	<0.001	1.49	1.15 – 1.83	<0.001	0.19	0.14 – 0.23	<0.001
hatching date	-0.03	-0.07 – 0.01	0.153	-0.20	-0.34 – 0.07	0.004	-0.29	-0.49 – 0.08	0.007	-0.02	-0.05 – 0.01	0.205
Random effects												
σ^2	0.32			1.32			3.16			0.06		
τ_{00} nest of origin	0.06			1.16			2.64			0.04		
τ_{00} nest of rearing	0.40			5.22			12.59			0.24		
Observations	102			102			102			102		
Marginal R ² / Conditional R ²	0.417 / 0.764			0.390 / 0.895			0.392 / 0.896			0.339 / 0.885		

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Table 4.B	<i>OXPHOS</i>			<i>OXPHOS</i> coupling efficiency			<i>FCR ROUTINE/CI+II</i>			<i>FCR CI/CI+II</i>		
Predictors	Estimates	CI 95%	P-values	Estimates	CI 95%	P-values	Estimates	CI 95%	P-values	Estimates	CI 95%	P-values
(Intercept)	28.08	16.14 – 40.03	<0.001	0.92	0.87 – 0.96	<0.001	0.12	0.03 – 0.21	0.011	0.72	0.64 – 0.82	<0.001
treatment (E)	-1.08	-3.64 – 1.48	0.401	2.0e-3	-7.5e-3 – 0.01	0.672	4.1e-3	-0.02 – 0.02	0.682	0.02	-3.7e-3 – 0.03	0.112
treatment (R)	-0.56	3.16 – 2.03	0.664	-2.0e-4	-9.5e-3 – 9.9e-3	0.967	8.1e-4	-0.02 – 0.02	0.936	0.02	-4.3e-3 – 0.03	0.126
initial brood size	-0.33	-0.97 – 0.32	0.314	1.4e-3	-8.9e-4 – 3.7e-3	0.223	-5.7e-4	-5.7e-4 – 4.5e-3	0.823	-1.4e-3	-6.1e-3 – -3.3e-3	0.556
mtDNA _{cn}	1.30	1.00 – 1.61	<0.001	-	-	-	-	-	-	-	-	-
hatching date	-0.27	-0.45 – 0.09	0.004	-9.5e-4	-1.6e-3 – 2.6e-4	0.008	2.1e-3	7.9e-4 – 3.5e-3	0.003	-8.9e-4	-2.2e-3 – 4.6e-4	0.189

Random effects												
σ^2	2.50			<0.001			<0.001			<0.001		
τ_{00} nest of origin	2.13			<0.001			-	-	-	<0.001		
τ_{00} nest of rearing	9.68			<0.001			<0.001			<0.001		
Observations	102			102			102			102		
Marginal R^2 / Conditional R^2	0.394 / 0.894			0.133 / 0.593			0.148 / 0.502			0.061 / 0.567		

888 **Figure legends**

889 **Fig.1: Experimental design of the study presenting the brood size manipulation (A)**
890 **and collection of the data (B).** Sample sizes are presented according to treatment groups:
891 control (C), reduced (R), and enlarged broods (E). The timing of different measurements and
892 analyses are indicated below the time-line (see Methods for details).

893 **Fig.2: Predicted body mass of nestlings from 7 to 14 days post-hatching according to**
894 **brood size manipulation treatment groups: reduced (R), control (C), enlarged (E)**
895 **brood sizes.** For day 7 and day 14: predicted values (in grey) and predicted averages (in
896 black) with their 95% CI and results from Tukey HSD *post hoc* tests are reported. Predicted
897 values are corrected for the average hatching date of the season and the average initial
898 brood size. Stars indicate the significance of the *post hoc* test (** $P < 0.01$) for body mass
899 comparison between chicks raised in reduced vs. enlarged broods (other comparisons were
900 non-significant). $R^2 = 0.89$. See Table 1 for sample-sizes. For body masses measured
901 before treatment (day 2), raw data, raw data averages and standard errors of the mean are
902 reported. Body mass at day 2 was not statistically significant according to brood size
903 manipulation treatment group ($F = 0.51$, $P = 0.60$).

904 **Fig.3: Effect of the brood size manipulation on mitochondrial metabolic rates and flux**
905 **control ratios.** Mitochondrial aerobic metabolism was measured at day 14 between
906 individuals raised in reduced, control and enlarged broods (see sample-sizes Table 1).
907 Standardized effect sizes are based on predicted values of the model and reported with their
908 95% CI. In black, effect sizes between individuals raised in enlarged vs. control broods. In
909 grey, effect sizes between individuals raised in reduced vs. control broods.

910 **Fig.4: Variance explained by the nest of origin (in grey) and the nest of rearing (in**
911 **black) in linear mixed models testing mitochondrial respiration rates at day 14**
912 **according to the number of nestlings (at day 14).** Stars indicate significance to be
913 different from 0 (** $P < 0.01$, *** $P < 0.001$). Repeatabilities are presented with their 95% CI.
914 ns: non-significant. See Table 1 for sample-sizes.

915 **Fig.5: Predicted values of mitochondrial respiration rates on 14 days old nestlings**
916 **according to the number of nestlings at day 14.** Blue color refers to the complete dataset
917 ($N = 102$ individuals), red color refers to a subsample ($N = 90$ individuals) excluding small
918 brood sizes (less than 5 chicks at day 14, $N = 12$ individuals from 8 nest boxes). Predicted
919 values are extracted from linear mixed models (LMMs) presented in Tables S2 and S4.
920 Regression lines and results from the models are presented. Predicted values are corrected
921 for the average hatching date of the season. Mitochondrial respiration rates were corrected
922 for mitochondrial DNA copy number (i.e. proxy of the mitochondrial density). Original nest
923 box ID and nest box of rearing ID were included as random intercepts in the models
924 presented in Table S2 (blue color). Only the nest of rearing ID could be included as random
925 intercepts in the models presented in Table S4 (red color, see methods). R^2 of each model
926 are reported in Tables S2 and S4.