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

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Summary statement

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

Abstract

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

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

Keywords: Animal performance, brood size, cellular metabolism, oxidative stress, *Parus major*

Introduction

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

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

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

reported long-lasting effects of early-life nutritional deficits on fitness related traits, including laying initiation and breaks, hatching success, plasma antioxidant levels and flight performances (Blount et al., 2003, 2006; Criscuolo et al., 2011). Yet, the mechanisms driving the effects of early-life environmental variation (including postnatal provisioning) on the offspring phenotype and survival remain poorly understood.

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

Recent studies have found that early-life environmental stressors might impair mitochondrial function (Gyllenhammer et al., 2020; Zitkovsky et al., 2021). For example food restriction was shown to decrease basal metabolic rate in adult chinese bulbul (*Pycnonotus*

sinensis) and silky starlings (*Sturnus sericeus*), and to decrease levels of mitochondrial state 4 respiration in the liver for both species (Mao et al., 2019; Zhang et al., 2018). Yet, the impact of early-life conditions on mitochondrial function and the long-lasting effects remain poorly understood.

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

Material and Methods

Field site and population monitoring

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

Experimental manipulation of brood size

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

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

Before the brood size manipulation, nestlings from nest-pairs were weighed on an electronic scale (body mass \pm 0.1g) and individually marked (nail-clipping). To measure nestling mitochondrial density before the treatment started, we performed blood sampling 2 days after hatching, before the brood size manipulation, on a subsample of nestlings (1 - 10 μ L from the tarsus vein using heparinized capillaries, 2-4 nestlings/nest, see Table 1). When performing the brood size manipulation and cross-fostering we avoided moving the smallest or biggest nestlings to minimize disturbing sibling competition hierarchies that could have significantly decreased nestlings' survival chances after the manipulation. Body mass of nestlings swapped between nests was as similar as possible and cross-fostered individuals were kept in a warm box during the transfer (using heating pads). To assess if parental feeding rates differed according to the brood size manipulation treatment groups, we video-recorded a subsample of nest boxes 8 days after hatching (see more details in supplementary materials). We found higher rates for E group compared to R group, while parental feeding rate between E and C groups was not significantly different (Fig.S1).

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

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

Mitochondrial DNA copy number

We randomly selected a minimum of 2 nestlings per nest (one original and one cross-fostered nestling). Genomic DNA was extracted from 1 to 5 μ L of frozen blood samples (stored at -80°C) using a salt extraction procedure adapted from Aljanabi and Martinez (1997). Due to small volumes, some of the blood samples collected on day 2 could not be analyzed. When data were available (see Table 1), we measured mtDNA cn on the same individuals at day 2, day 14 and as juvenile (i.e. recaptured in autumn 2020). DNA quantity and purity were estimated using a *NanoDrop ND-1000* spectrophotometer. Samples were re-extracted if needed ([DNA] < 50ng/ μ L, 260/280 ratio < 1.80 or 260/230 < 2). Samples were then diluted to 1.2ng/ μ L in sterile H₂O and stored at -80°C until qPCR assays. We quantified mtDNA cn using real-time quantitative PCR assays (qPCR) from a protocol described in Cossin-Sevrin et al. (2022). We made some adjustments to the original protocol: samples were automatically pipetted (epMotion® 5070, Eppendorf, Hamburg, Germany) in duplicates in 384-well qPCR plates (n = 5 plates) and qPCR were performed with a Biorad instrument (CFX-384, Biorad, Hercules, USA). We used Recombination Activating Gene 1 (*RAG1*) as a single control gene and cytochrome oxidase subunit 2 (*COI2*) as specific mitochondrial gene (sequences and procedure of verification are described in Cossin-Sevrin et al., 2022). qPCR reactions were conducted in a total volume of 12 μ L, including 6ng of DNA samples, primers at a final concentration of 300nM and 6 μ L of GoTaq® qPCR Mix (Promega, Madison, USA). qPCR conditions were the following : 3min at 95°C (polymerase activation), followed by 40 cycles of 10s at 95°C, 15s at 58°C, 10s at 72°C. Melting curve program was 5s at 65°C, and 0.5°C/s increased until 95°C. A pooled DNA sample from 14 adult individuals was used as a reference sample (i.e. ratio = 1.0 for mtDNA cn) and was included in duplicate on every plate. qPCR efficiencies of *RAG1* and *COI2* genes were respectively (mean \pm SEM): 99.14 \pm 1.17% and 95.74 \pm 0.11%. Repeatability of mtDNA cn between sample-duplicates was R = 0.90 (CI 95% = [0.88, 0.92]). The samples were distributed randomly on different plates and in order to control for interplate variability, qPCR plate identity was included as a random intercept in our statistical analysis (see details below). DNA integrity of 46 randomly selected samples was evaluated and deemed satisfactory using gel electrophoresis (100ng of DNA, 0.8% agarose gel at 100mV for 1 hour).

Mitochondrial aerobic metabolism

In order to test the impact of brood size on nestling mitochondrial respiration, we measured mitochondrial aerobic metabolism in a subsample (1 to 3 nestlings per nest), 14

days after hatching (individuals/nest: $n_C = 26/14$, $n_E = 41/21$, $n_R = 35/19$) and in the same individuals as juveniles (recaptured in autumn 2020), when samples were available (N = 14 individuals). We additionally measured mitochondrial aerobic metabolism from the majority of juveniles recaptured that participated in the manipulation (as nestlings) (in total, juvenile/nest: $n_C = 16/9$, $n_E = 26/15$, $n_R = 12/8$). Blood sample volumes collected on 2-day-old nestlings were unfortunately not large enough for measuring mitochondrial aerobic metabolism at this stage (i.e. 1-10 μ L of blood). Mitochondrial respiration was analyzed using high-resolution respirometry (3 *Oroboros* Instruments, Innsbruck, Austria) at 40°C adapted from a protocol described in Stier et al., (2019): digitonin (20 μ g/mL), pyruvate (5mM), malate (2mM), ADP (1.25mM), succinate (10mM), oligomycin (2.5 μ M), antimycin A (2.5 μ M). We used 20 μ L (nestlings) to 30 μ L (juveniles) of fresh blood when available, suspended in Mir05 buffer. Five distinct respiration rates were analyzed: 1) the endogenous cellular respiration rate before permeabilization (*ROUTINE*), 2) the maximum respiration rate fueled with exogenous substrates of complex I, as well as ADP (*C*), 3) the maximum respiration rate fueled with exogenous substrates of complexes I and II, as well as ADP (*C*+*II*), 4) the respiration rate contributing to the proton leak (*LEAK*), 5) the respiration rate supporting ATP synthesis through oxidative phosphorylation (*OXPHOS*). We also calculated three mitochondrial flux ratios (FCR): 1) *OXPHOS* coupling efficiency ($OxCE = (C+II-LEAK)/C+II$), 2) the proportion of maximal respiration capacity being used under endogenous cellular condition (i.e. $FCR_{ROUTINE/C+II}$) and 3) the ratio between the maximal respiration rate of complex I and the maximal respiration capacity (i.e. $FCR_{C/C+II}$). *OXPHOS* coupling efficiency FCR provides an index of mitochondrial efficiency in producing ATP, whereas $FCR_{ROUTINE/C+II}$ reflects the cellular control of mitochondrial respiration by endogenous ADP/ATP turnover and substrate availability. Respiration rates were standardized by the number of cells in each sample, measured by *BIO-RAD* TC20 automated cell counter. The technical repeatability of mitochondrial aerobic metabolism measurements was high: *ROUTINE*: R = 0.985 (CI 95% = [0.936, 0.997]); *C*+*II*: R = 0.98 (CI 95% = [0.912,0.995]); *LEAK*: R = 0.979 (CI 95% = [0.916, 0.995]); *OXPHOS*: R = 0.977 (CI 95% = [0.898,0.995]) based on 9 duplicates.

Reactive oxygen species measurements

Reactive oxygen species (ROS) were measured in 14-day-old nestlings and juveniles from the same samples as the mitochondrial aerobic metabolism assay (i.e. red blood cells suspended in MiR05 buffer) (see Table 1 for sample-sizes). The relative amount of ROS was

estimated by fluorescence, using MitoSOX™ Red kit (MitoSOX™ red mitochondrial superoxide indicator, Thermo Fisher) that specifically measures mitochondrial superoxide (i.e. the primary mitochondrial ROS) in live cells. Samples were supplemented with 4µL of MitoSOX™ (final concentration 4µM) and incubated for 30 min at 40°C protected from light. After being cooled down (5 min on ice) and centrifuged (2 min, 1000g at 4°C), samples were re-suspended in 250µL Mir05 buffer added with 5mM pyruvate, 2.5mM malate, 10mM succinate and 1.25mM ADP. 100µL of samples were loaded on a white 96-well plate (n = 43) with a transparent bottom. Kinetics of fluorescence were read for 30 min (emission 510 nm/ excitation 580 nm) in EnSpire® 2300 Multilabel Reader (PerkinElmer) set at 40°C. Samples were analyzed in duplicates. The slope of relative fluorescence (RFU/min) was then extracted and normalized by the internal control present on each plate (dry *Saccharomyces cerevisiae* diluted at 10mg/mL in Mir05). As a positive control (for mitochondrial ROS production) diluted *Saccharomyces cerevisiae* supplemented with antimycin A was included in each plate. Relative mitochondrial ROS results were standardized by the number of cells present in each well, taking into account dilution factor (cell count estimated with the *BIO-RAD* TC20 automated cell counter). Repeatability of the ROS production measurements between sample-duplicates was $R = 0.924$ (CI 95% = [0.9, 0.941]).

Statistical analysis

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

Preliminary tests

Pre-treatment clutch sizes (raw data mean \pm SEM: R = 9.24 ± 0.26 , C = 8.65 ± 0.28 , E = 8.48 ± 0.17 eggs; ANOVA: $F = 2.97$, $P = 0.06$) and hatching date (C = 58.70 ± 1.21 , E & R = 60.16 ± 1.06 days; ANOVA: $F = 0.54$, $P = 0.59$) were relatively balanced between treatment groups. Initial brood sizes on day 2 post-hatching per treatment groups were the following: (raw data mean \pm SEM [range]) R = 8.00 ± 0.32 [5;11], C = 7.50 ± 0.44 [4;10] and

E = 7.68 ± 0.28 [4;9] chicks and were not statistically different between treatment groups before the manipulation (ANOVA: $F = 0.55$, $P = 0.57$). Nestling body mass (raw data mean \pm 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 mtDNA $_{cn}$ (raw data mean \pm SEM: R = 7.62 ± 0.91 , C = 8.10 ± 0.42 , E = 8.29 ± 0.88 ; $F = 0.32$, $P = 0.73$) measured before the experimental manipulation (2 days post-hatching) were not statistically different between groups before the assessment of the treatment. We did not find any significant differences between the chicks cross-fostered and non cross-fostered for the responses variables tested throughout this study (i.e. growth metrics, mtDNA $_{cn}$, mitochondrial metabolic rates, ROS production, survival metrics, all $F < 2.72$, all $P > 0.1$).

Experimental approach

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

Correlative approach

To explore the associations between number of nestlings and the measured traits (focusing on the ecological aspect of the brood size rather than experimental), we used another set of models including the actual number of nestlings (on the day of data collection) as a continuous variable (see Table 2B). These analyses are referred to “*correlative*

approach” in the text. As the number of nestlings per nest varied substantially across and within treatment groups (e.g. at day 14 brood size ranged from 2 to 11 nestlings), this analysis reflects the associations between a given brood size and trait of interest. However, given that the dataset using brood size as a continuous variable includes both experimentally manipulated (E, R) and non-manipulated nests (C) we also analyzed the associations between the number of nestlings and target variables using only the non-manipulated nests (C) group to check if patterns might have been confounded by including experimental nests (Table S3). As results were similar, we report results of the full dataset in the main text. In these analyses, we also included hatching date as a continuous variable and the IDs of both original and rearing nest boxes as random intercepts. qPCR plate ID could not be included in the model only including the control group because of convergence issues (Table 2B).

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

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

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

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

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

Results

Brood size manipulation

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.

Nestling growth trajectories

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 $P > 0.54$).

Mitochondrial DNA copy number

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

Mitochondrial aerobic metabolism

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

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

ROS production

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

Survival metrics

Fledgling success was not significantly affected by the treatment ($\chi^2 = 2.44$, $P = 0.29$, raw data: R = 75.33%, C = 65.79%, E = 77.78%), neither by the initial brood size ($\chi^2 = 0.05$, $P = 0.83$) or the hatching date ($\chi^2 = 2.18$, $P = 0.14$). Juvenile recapture probability was not significantly affected by the treatment ($\chi^2 = 2.18$, $P = 0.34$, raw data: R = 12.17%, C = 22.22%, E = 18.52%) or the initial brood size ($\chi^2 = 0.03$, $P = 0.87$), but was negatively associated with the hatching date ($\chi^2 = 13.6$, $P < 0.001$). Finally, we did not find any significant associations between juvenile recapture probability, mitochondrial respiration rates and FCR(s) measured at day 14 (all $P > 0.2$, Table S5).

Correlative approach

When analyzing each age separately, in order to account for the number of nestlings in the nest at a given age, nestling body mass at day 7 was negatively associated with the number of nestlings in the nest (Table S1), while we did not find an association for the wing length ($F_{1, 31.10} = 0.38$, $P = 0.54$). On day 14, nestling body mass was not significantly associated 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 nestlings (Table S1). While mtDNA $_{cn}$ at day 14 was not associated with the number of nestlings in the nest ($P = 0.11$), larger brood sizes a few days before fledging (i.e. day 14) predicted higher mtDNA $_{cn}$ for juveniles (estimate \pm SE = 0.07 ± 0.03 , $P = 0.04$). We found a negative association between the number of nestlings at day 14 and mitochondrial respiration rates measured at day 14 (Table S2, Fig.5). OXPHOS coupling efficiency and both FCR $_{ROUTINE/CI+II}$ and FCR $_{CI/CI+II}$ were not significantly associated with the number of nestlings at day 14 (all $F < 1.38$ and all $P > 0.25$). We found similar results when only including individuals raised in the C group (Table S3). As we suspected nestlings from small

brood sizes (less than 5 chicks at day 14) with high mitochondrial respiration rates to drive the associations between the number of nestlings and mitochondrial metabolic rates (Fig.5), we performed the same statistical analysis excluding nestlings raised in small broods ($n = 12$ nestlings from 8 nests removed from the analysis). In this case, we could not detect any significant associations between the number of nestlings (day 14) on the different mitochondrial respiration rates measured (all $F < 2.23$, all $P > 0.14$, Table S4, Fig.5). Juvenile mitochondrial respiration rates (all $F < 0.21$, all $P > 0.65$) or FCRs (all $F < 0.72$, all $P > 0.49$), were not associated with the number of nestlings at day 14, except for FCR_{CI/CI+II} for which we found a negative association (estimate \pm SE = -0.005 ± 0.003 , $F_{1,62} = 4.36$, $P = 0.04$). We did not find significant associations between the number of nestlings at day 14 and nestling mitochondrial ROS production (day 14: $F_{1,53.49} = 0.42$, $P = 0.52$) or in juveniles ($F_{1,50} = 1.08$, $P = 0.30$). Fledgling success was strongly positively associated with the number of nestlings in the nest at day 14 ($\chi^2 = 61.47$, $P < 0.001$). Juvenile recapture probability was not significantly associated with the number of nestlings day 14 ($\chi^2 = 0.23$, $P = 0.63$).

Discussion

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

growth, such as nest temperature (Andreasson et al., 2016; Hope et al., 2021; Nord & Nilsson, 2011).

Experimental approach

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

It is worth noting that in our experiment the difference in nestling number between control and reduced groups did not remain significant (small effect-sizes between groups) at the end of the growth period (from day 7 to 14). This likely contributes to explain why our experiment failed to demonstrate large differences between treatment groups. It is interesting that even without differences in the number of chicks at the end of the experiment between control and reduced groups, the reduced group tended to have larger chicks (see hypothesis below).

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

significantly impacted by the treatment, likely explained by the increase in parental feeding rates.

Correlative approach

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

We found a negative association between mitochondrial metabolism (*ROUTINE*, *CI*, *CI+II*, *LEAK* and *OXPHOS*) and number of nestlings. As both *LEAK* and *OXPHOS* were negatively correlated with the number of nestlings, we did not find an association between *OXPHOS* coupling efficiency and nestling number. The higher mitochondrial metabolic rates observed for nestlings raised in small broods could reflect a higher energetic demand, potentially linked to a higher need for thermogenesis (Andreasson et al., 2016, Bicudo et al., 2001).

While these results are in accordance with our predictions (decrease in mitochondrial metabolic rates in larger broods) it is important to note that these negative associations with the number of nestlings did not remain significant when nestlings from very small broods (less than 5 nestlings at day 14, which is quite exceptional for the study species) were excluded from the analysis, meaning that those specific broods drove the patterns. Therefore, we cannot conclude that a relatively large brood size (e.g. via effects of stress) is associated with lower mitochondrial respiration. Interestingly, broods with less than 5 nestlings at day 14 had really low survival chances during the growth period (from day 2 to 14) compared to the larger broods (> 4 nestlings) (average on raw data: 63.4% vs. 92.4% of survival at day 14, excluding nests without chicks at day 14: n = 12 nests) and most of the nestlings did not reach day 7 (average at day 7: 1.13 nestlings lost in small broods vs. 0.34 in larger broods). We therefore suspect nestling growth and mitochondrial metabolic patterns to rather reflect unusual rearing conditions than being general patterns. Our main hypothesis

is that these individuals might be at a less-advanced developmental stage, given their smaller structural size, knowing that mitochondrial quantity and/or respiration decreases during postnatal development (Stier et al. 2020; Stier et al. 2022; Cossin-Sevrin et al. 2022, Hsu et al. 2023; but see: Dawson & Salmón, 2020) and potentially more stressed (some environmental stressors may lead to higher metabolic rate, i.e. in interaction with glucocorticoid levels in zebra finches; Jimeno et al., 2017). Alternatively, these small broods with a high unusual mortality during early-growth may be subject to selective disappearance and nestlings surviving until 14 days after hatching represent a non-random pool of individuals that managed to survive and cope with detrimental conditions during early-growth. Despite the negative association between nestling mitochondrial metabolic rates and the number of nestlings, we did not find any association between nestling ROS production and the number of nestlings, and fledging success was positively associated with the number of nestlings. Yet the sample size for small broods was limited, and therefore the results need to be interpreted with caution.

Furthermore, our study demonstrates that both genetic inheritance (but also complementary mechanisms, such as parental effects before the cross-fostering) and the rearing environment contribute to variation in offspring mitochondrial traits, but with a larger contribution from the rearing environment. Similar results about lower contribution of familial background have been found for resting metabolic rate in collared flycatcher nestlings (*Ficedula albicollis*) (McFarlane et al., 2021). While the underlying mechanisms of modulation of mitochondria by early-life environmental conditions are unknown, recent research points out that mitochondrial function can respond to environmental cues through changes in gene expression and mitochondrial DNA methylation (Sharma et al., 2019; Wallace, 2016).

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

As a limitation in our study, mitochondrial ROS production, substrate preferences and mitochondrial aerobic metabolism are known to vary between tissues (Mailloux, 2020; Salmón et al., 2022). Therefore, one should always be careful when investigating ROS production in a single tissue (Costantini, 2019; Monaghan et al., 2009). However, we

focused our study on blood samples to i) estimate nestling survival and potential long-lasting effect of our experiment and ii) since mitochondrial aerobic metabolism measurements in blood samples can be positively associated with other tissues (Koch et al., 2021; Stier et al., 2017). Collecting blood samples allows the use of limited-invasive methods on wild species, and to avoid terminal sampling.

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

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Competing interests

We declare we have no competing interests.

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Ethics

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

Authors contribution

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 collected the data. N.C-S and A.S collected mitochondrial respiration rates measurements. N.C-S performed DNA extractions and conducted qPCR analysis in collaboration with S.Z. N.C-S conducted statistical analyses and wrote the first version of this manuscript under the supervision of S.R and K.A. All co-authors revised the manuscript. S.R, A.S, V.A-V funded experimental work and data collection.

Data available statement

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

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Figures and Tables

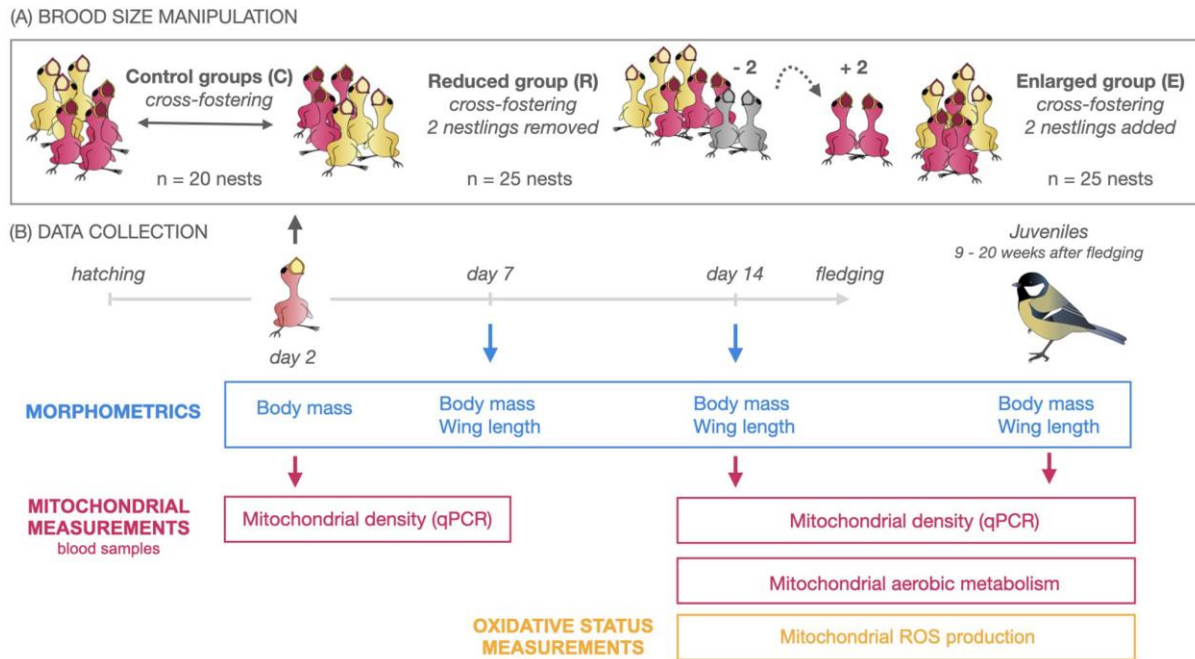


Fig. 1. Experimental design of the study presenting the brood size manipulation (A) and collection of the data (B). Sample sizes are presented according to treatment groups: control (C), reduced (R), and enlarged broods (E). The timing of different measurements and analyses are indicated below the time-line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

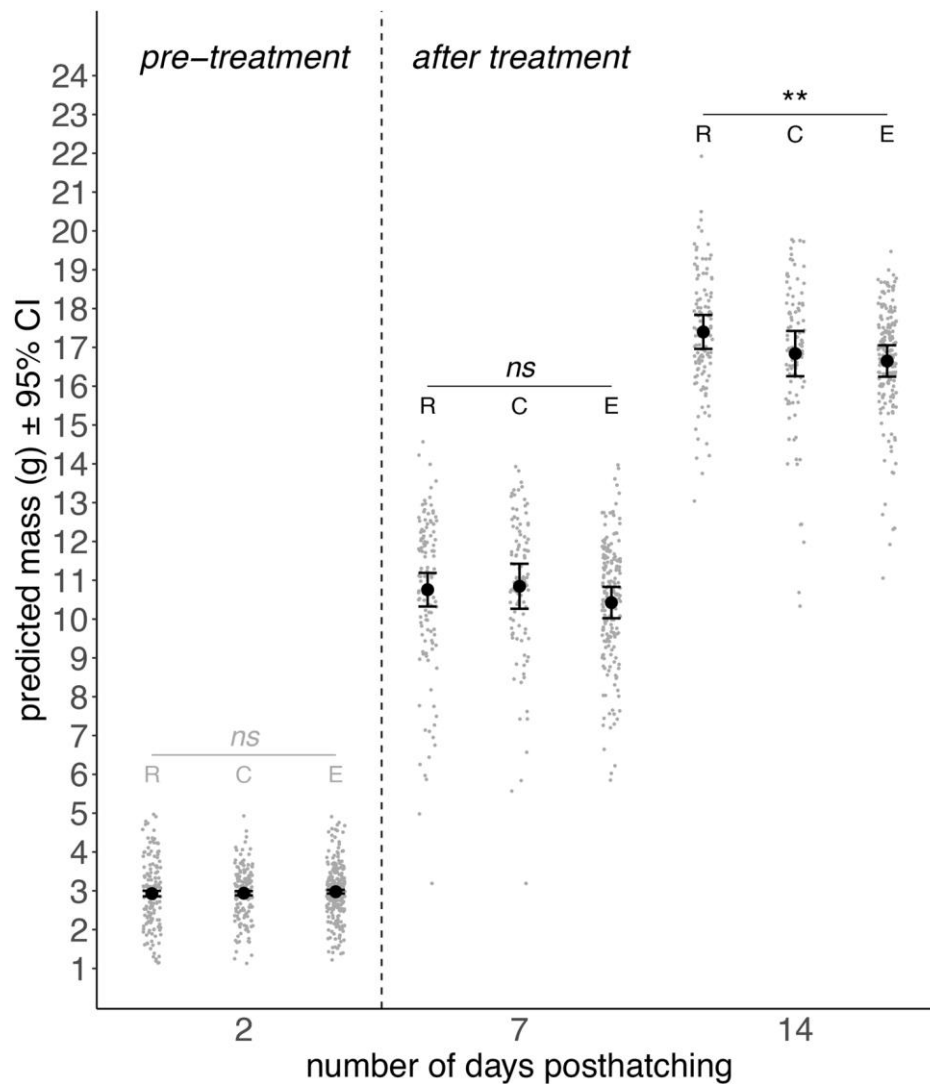


Fig. 2. Predicted body mass of nestlings from 7 to 14 days post-hatching according to brood size manipulation treatment groups: reduced (R), control (C), enlarged (E) brood sizes. For day 7 and day 14: predicted values (in grey) and predicted averages (in black) with their 95% CI and results from Tukey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

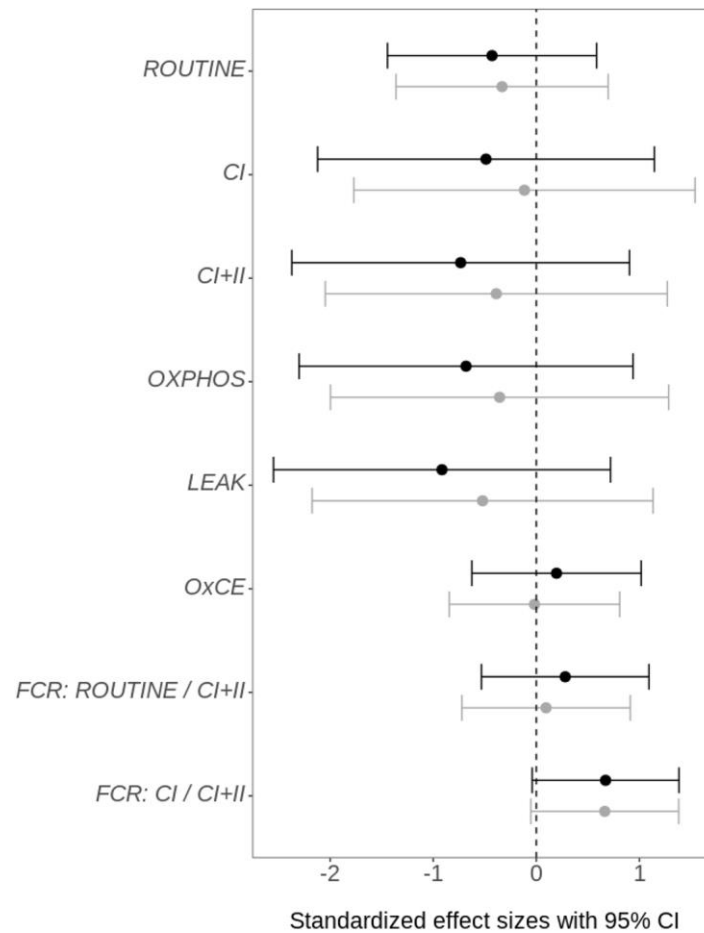


Fig. 3. Effect of the brood size manipulation on mitochondrial metabolic rates and flux control ratios. Mitochondrial aerobic metabolism was measured at day 14 between individuals raised in reduced, control and enlarged broods (see sample-sizes Table 1). Standardized effect sizes are based on predicted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

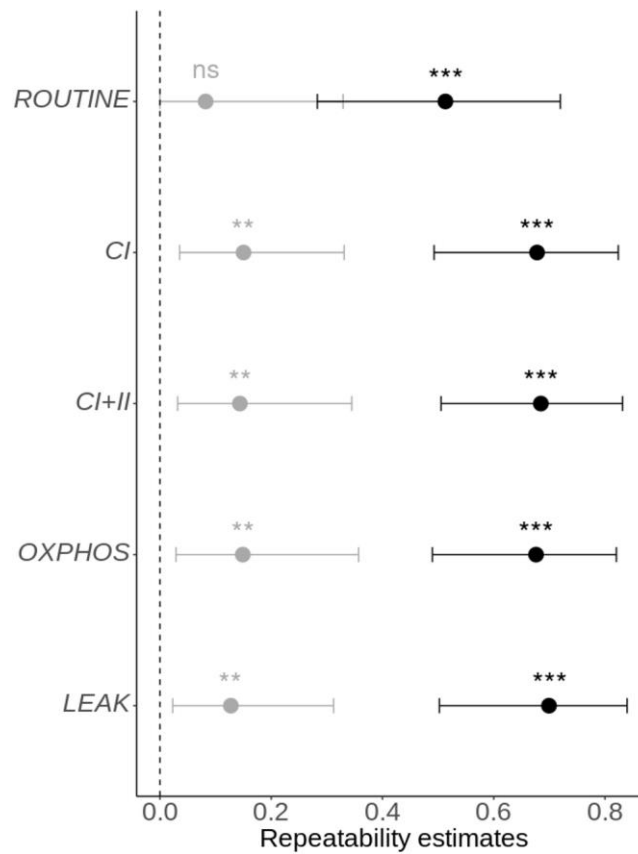


Fig. 4. Variance explained by the nest of origin (in grey) and the nest of rearing (in black) in linear mixed models testing mitochondrial respiration rates at day 14 according to the number of nestlings (at day 14). Stars indicate significance to be different from 0 ($P < 0.01$, *** $P < 0.001$). Repeatabilities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

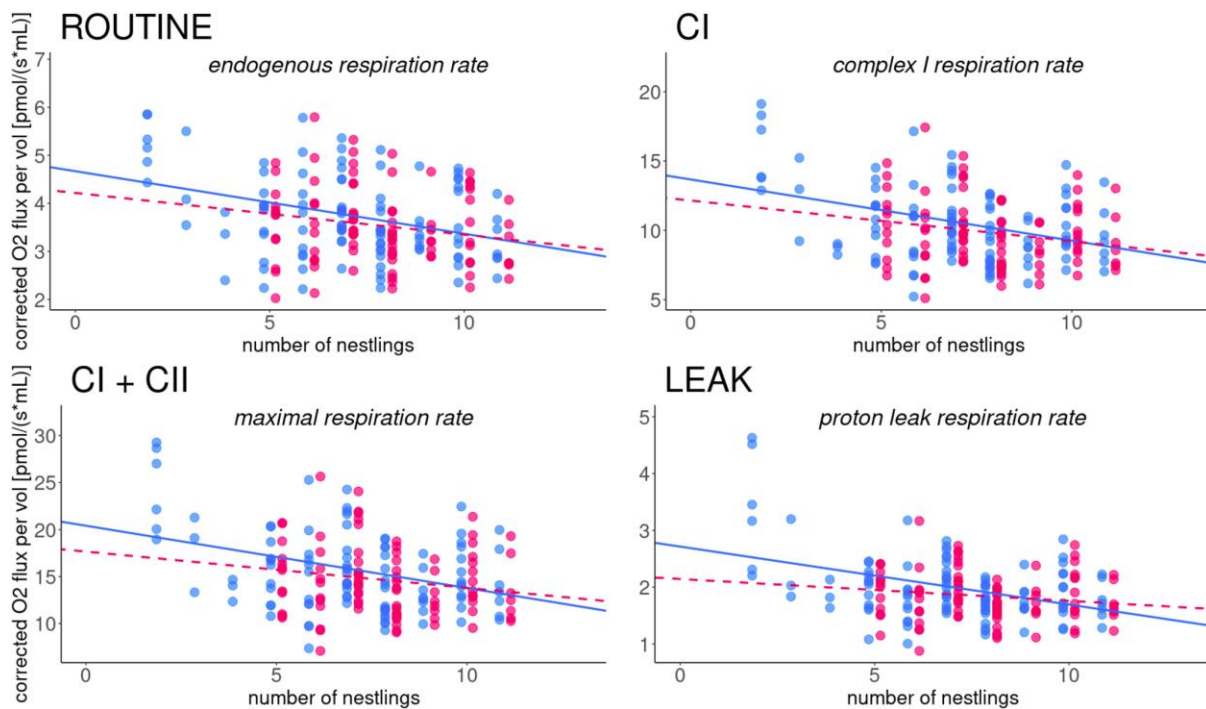


Fig. 5. Predicted values of mitochondrial respiration rates on 14 days old nestlings according to the number of nestlings at day 14. Blue color refers to the complete dataset (N = 102 individuals), red color refers to a subsample (N = 90 individuals) excluding small brood sizes (less than 5 chicks at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

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

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

Table 2. Summary of the statistical analyses performed according to the experimental approach (A) and the correlative approach (B). To analyze this dataset, we used linear mixed models (LMMs), linear models (LMs), but also generalized linear mixed models (GLMMs) and generalized linear models (GLMs). For each response variable, explanatory variables, both categorical variables and continuous variables (the latter in *italic*) included in the model are presented. Random intercept terms are underlined. In case of convergence issue, the original nest box ID and the hatching date (if needed) have been removed from the models. For the correlative approach, the number of nestlings at the day of the measurement is included for the models with nestlings, and in the models with juveniles the number of nestlings refers to the brood size 14 days post-hatching. For FCRs (i.e. *OXPHOS* coupling efficiency, $FCR_{ROUTINE/CI+II}$, $FCR_{CI/CI+II}$), *mtDNAcn* was not included as covariate in 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> , bird ID, nest box of rearing ID, original nest box ID
Nestling and juvenile body size	LMMs: treatment, <i>initial brood size</i> , <i>hatching date</i> , nest box of rearing ID, original nest box ID
Postnatal <i>mtDNAcn</i> from day 14 to juvenile	GLMM, gamma error distribution, log link: treatment, age, nest box of rearing ID, bird ID, qPCR plate ID
Nestling mitochondrial metabolic rates day 14	LMMs: treatment, <i>initial brood size</i> , <i>hatching date</i> , <i>mtDNAcn</i> , nest box of rearing ID, original nest box ID
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> , nest box of rearing ID
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> , nest box of rearing ID
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> , nest box of rearing ID, original nest box ID

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> , nest box of rearing ID, original nest box ID
Nestling and juvenile body size	LMMs: <i>number of nestlings</i> , <i>hatching date</i> , nest box of rearing ID, original nest box ID
Nestling <i>mtDNAcn</i>	GLMM, gamma error distribution, log link: <i>number of nestlings</i> , <i>hatching date</i> , nest box of rearing ID, original nest box ID, qPCR plate ID
Juvenile <i>mtDNAcn</i>	GLMM, gamma error distribution, log link: <i>number of nestlings</i> , nest box of rearing ID, qPCR plate ID
Nestling mitochondrial metabolic rates	LMMs: <i>number of nestlings</i> , <i>hatching date</i> , <i>mtDNAcn</i> , nest box of rearing ID, original nest box ID
Juvenile mitochondrial metabolic rates	LMs: <i>number of nestlings</i> , <i>mtDNAcn</i>

Nestling ROS production	LMM: <i>number of nestlings, hatching date, mtDNAcn, nest box of rearing ID</i>
Juvenile ROS production	LM: <i>number of nestlings, mtDNAcn</i>
Fledging success	GLM, logistic binary distribution of dependent variables (0 = dead, 1 = alive): <i>number of nestlings, hatching date, nest box of rearing ID</i>
Recapture success (survival after fledging)	GLM, logistic binary distribution of dependent variables (0 = dead, 1 = alive): <i>number of nestlings, hatching date, nest box of rearing ID, original nest box ID</i>

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

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		

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

Table 4.A	ROUTINE			CI			CI + II			LEAK		
	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		

Table 4.B	OXPHOS			OXPHOS coupling efficiency			FCR ROUTINE/CI+II			FCR CI/CI+II		
	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		

A. Parental feeding rates

a) Material and Methods

In order to test if parental feeding rates changed following the brood size manipulation, we video-recorded a subsample of nest boxes ($n_C = 8$, $n_E = 15$, $n_R = 14$ nest boxes) 8 days after hatching. The cameras were concealed at ca. 2 m distance from the nest boxes. Videos were recorded for approximately 2h (mean \pm SD = 137.58 \pm 25.19 min) between 7 and 12 am. Standardized parental feeding rate differences (number of nest visits divided by the total length of the video starting from the first visit) was quantified using *BORIS* software (Friard & Gamba, 2016), by a single observer blind to the experimental treatment.

Standardized parental feeding rate differences (i.e. total number of visits per hour in the nest by both parents) were tested according to treatment groups and the initial brood size, but also according to the number of nestlings at day 7, using in both cases a linear model without random effects (LM). We included the starting time of the video recordings as a covariate in models to account for differences in feeding rates during the day.

b) Results

Parental feeding rate (8 days after hatching) was significantly affected by the treatment ($F_{2,32} = 4.64$, $P = 0.02$, see Fig.2A) with higher rates for the E group (raw data mean \pm SE = 41.26 \pm 6.03 visits per hour) compared to R group (raw data mean \pm SE = 25.75 \pm 4.05) (Tukey HSD *post hoc* comparison: $P = 0.04$). Differences in parental feeding rate between E and C groups (C: raw data mean \pm SE = 28.49 \pm 5.22) were close to significance (Tukey HSD *post hoc* comparison: $P = 0.051$). Parental feeding rate significantly increased with initial brood size (estimate \pm SE = 2.76 \pm 1.55, $F_{1,32} = 7.91$, $P = 0.008$) and significantly decreased with time of day (estimate \pm SE = -2.67 \pm 6.13e-10, $F_{1,32} = 19.01$, $P < 0.001$).

Parental feeding rate significantly increased with the number of nestlings recorded 7 days after hatching (estimate \pm SE = 4.28 \pm 1.01, $F_{1,34} = 22.41$, $P < 0.001$).

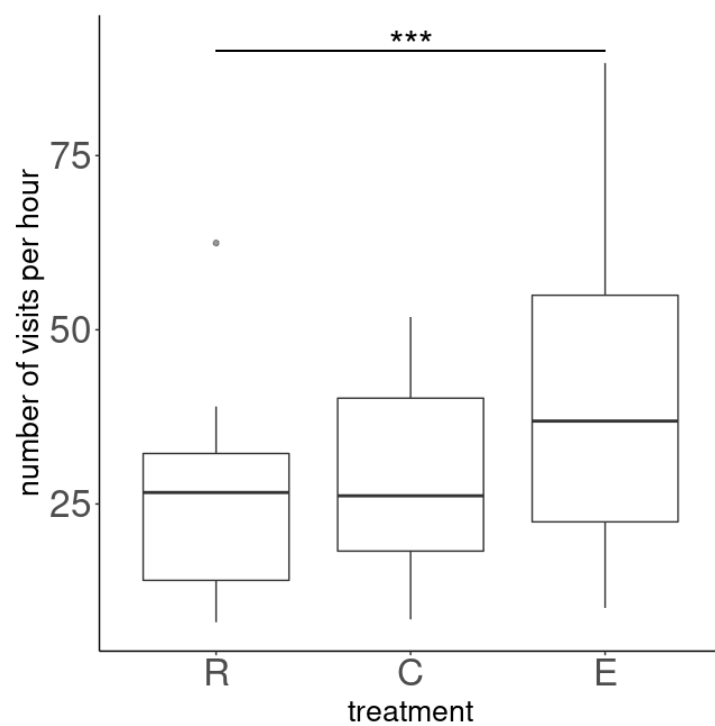


Fig. S1. Parental feeding rate according to the brood size manipulation treatment groups: reduced (R), control (C), enlarged (E) brood sizes. Raw data distribution is presented with boxplots ($n_C = 8$, $n_E = 15$, $n_R = 14$ nest boxes). Stars indicate the significance of Tukey HSD *post hoc* test (** $P < 0.001$). $R^2 = 0.53$.

B) Results for the correlative approach

Table S1. Results of linear mixed model testing the associations between the number of nestlings in the nest and A) nestling body mass at day 7, B) nestling body mass at day 14, C) nestling wing length at day 14. For A), nestling body mass measured at day 2 was included as covariate in the model. For B), nestling body mass measured at day 7 was included as covariate in the model. Linear mixed models (LMM) estimates are reported with their 95% CI. Original nest box ID and nest box of rearing ID were included as random intercepts in the models. σ^2 , within group variance; τ_{00} between-group variance. Bold indicates significance ($P < 0.05$).

Predictors	A) Mass day 7			B) Mass day 14		
	Estimates	CI 95%	P-value	Estimates	CI 95%	P-value
(Intercept)	2.15	-1.34 - 5.65	0.223	4.42	0.32 - 8.51	0.035
previous mass measured (day 2 or day 7)	1.91	1.75 - 2.06	<0.001	0.55	0.49 - 0.62	<0.001
number of nestlings	-0.16	-0.29 - -0.03	0.017	0.03	-0.13 - 0.18	0.726
hatching date	0.07	0.01 - 0.12	0.027	0.11	0.04 - 0.18	0.003
Random effects						
σ^2	0.60			0.60		
τ_{00} nest of origin	0.27			0.14		
τ_{00} nest of rearing	1.07			1.75		
N observations	419			403		
Marginal R^2 / Conditional R^2	0.525/ 0.852			0.348/ 0.844		

Predictors	C) Wing length day 14		
	Estimates	CI 95%	P-value
(Intercept)	20.79	13.77 - 27.81	<0.001
number of nestlings	0.23	-0.003 - 0.46	0.053
hatching date	0.42	0.30 - 0.54	<0.001
Random effects			
σ^2	5.60		
τ_{00} nest of origin	2.68		
τ_{00} nest of rearing	2.30		
N observations	403		
Marginal R^2 / Conditional R^2	0.345/ 0.653		

Table S2. Results of linear mixed models testing the associations between the number of nestlings in the nest (14 days after hatching) and mitochondrial respiration rates measured on 14-day-old nestlings (N = 102 individuals, n = 55 nest boxes). Mitochondrial respiration rates were corrected for the mitochondrial DNA copy number (i.e., proxy of mitochondrial density). Linear mixed models (LMM) estimates are reported with their 95% CI. Original nest box ID and nest box of rearing ID were included as random intercepts in the models. σ^2 , within group variance; τ_{00} between-group variance. Bold indicates significance ($P < 0.05$).

Predictors	ROUTINE			CI			CI + II			LEAK		
	Estimates	CI 95%	P-value	Estimates	CI 95%	P-value	Estimates	CI 95%	P-value	Estimates	CI 95%	P-value
(Intercept)	4.55	2.37 – 6.72	<0.001	20.12	12.93 - 27.31	< 0.001	29.39	18.21 – 40.57	<0.001	2.70	1.20 – 4.20	<0.001
number of nestlings	-0.13	-0.22 – -0.04	0.005	-0.44	-0.72 – -0.17	0.002	-0.66	-1.09 – -0.23	0.003	-0.10	-0.16 – -0.04	<0.001
mtDNA $_{cn}$	0.34	0.25 – 0.42	<0.001	0.91	0.69 – 1.12	<0.001	1.44	1.10 – 1.77	<0.001	0.18	0.14 – 0.23	<0.001
hatching date	-0.02	-0.06 – 0.02	0.305	-0.17	-0.29 – -0.04	0.009	-0.24	-0.43 – -0.05	0.013	-0.01	-0.04 – 0.01	0.384
Random effects												
σ^2	0.32			1.31			3.13			0.06		
τ_{00} nest of origin	0.05			1.10			2.52			0.04		
τ_{00} nest of rearing	0.33			4.21			10.35			0.19		
Observations	102			102			102			102		
Marginal R^2 / Conditional R^2	0.488 / 0.767			0.487 / 0.898			0.483 / 0.899			0.454 / 0.889		

C. Complementary analyses, results for the control group only

Table S3. Results of linear mixed models testing the associations between the number of nestlings in the nest (14 days after hatching) and mitochondrial respiration rates measured on 14-day-old nestlings (N = 26 individuals from the control group only). We found similar results as in statistical analyses conducted on the whole data set (same direction for significant effects). Mitochondrial respiration rates were corrected for the mitochondrial DNA copy number (i.e., proxy of mitochondrial density). Linear mixed models (LMM) estimates are reported with their 95% CI. The original nest box ID and the nest box of rearing ID were both included as random intercepts in the models. σ^2 , within group variance; τ_{00} between-group variance. Bold indicates significance ($P < 0.05$).

	ROUTINE			CI			CI + II			LEAK		
Predictors	Estimates	CI 95%	P-value	Estimates	CI 95%	P-value	Estimates	CI 95%	P-value	Estimates	CI 95%	P-value
(Intercept)	6.85	1.13 – 12.57	0.026	27.36	12.74 – 41.98	0.002	42.52	18.77 – 66.28	0.002	4.84	2.21 – 7.48	0.002
number of nestlings	-0.26	-0.65 – 0.12	0.162	-1.18	-2.14 – -0.22	0.020	-1.82	-3.38 – -0.25	0.026	-0.25	-0.42 – -0.09	0.007
mtDNAcn	0.29	-0.03 – 0.60	0.070	0.54	-0.26 – 1.34	0.175	0.88	-0.39 – 2.16	0.163	0.14	0.01 – 0.26	0.037
hatching date	-0.04	-0.15 – 0.07	0.433	-0.18	-0.46 – 0.10	0.179	-0.30	-0.75 – 0.16	0.181	-0.03	-0.08 – 0.02	0.252
Random effects												
σ^2	0.53			2.95			6.58			0.05		
τ_{00} nest of origin	0.01			1.47			3.28			0.08		
τ_{00} nest of rearing	0.45			2.56			8.35			0.09		
Observations	26			26			26			26		
Marginal R ² / Conditional R ²	0.483 / 0.724			0.585 / 0.824			0.571 / 0.845			0.684 / 0.932		

D. Complementary analyses, results without including the small brood sizes

Table S4. Results of linear mixed models testing the associations between the number of nestlings in the nest (14 days after hatching) and mitochondrial respiration rates measured on 14-day-old nestlings (N = 90 individuals from 46 nests, broods having less than 5 nestlings at day 14 are not included in the analyses). Mitochondrial respiration rates were corrected for the mitochondrial DNA copy number (i.e. proxy of mitochondrial density). Linear mixed models (LMM) estimates are reported with their 95% CI. The nest box of rearing ID was included as random intercept in the models. Nest box of origin could not be included as random intercept because of convergence issues. σ^2 , within group variance; τ_{00} between-group variance. Bold indicates significance ($P < 0.05$).

Predictors	ROUTINE			CI			CI + II			LEAK		
	Estimates	CI 95%	P-value	Estimates	CI 95%	P-value	Estimates	CI 95%	P-value	Estimates	CI 95%	P-value
(Intercept)	3.84	1.53 – 6.16	0.002	16.69	9.32 – 24.07	<0.001	24.27	12.82 – 35.72	<0.001	2.37	1.06 – 3.69	0.001
number of nestlings	-0.09	-0.20 – 0.03	0.143	-0.29	-0.66 – 0.08	0.117	-0.39	-0.96 – 0.19	0.182	-0.04	-0.10 – 0.03	0.247
mtDNAcn	0.30	0.20 – 0.40	<0.001	0.74	0.48 – 1.00	<0.001	1.18	0.78 – 1.59	<0.001	0.15	0.10 – 0.20	<0.001
hatching date	-0.01	-0.05 – 0.03	0.563	-0.12	-0.24 – -0.002	0.055	-0.18	-0.36 – 0.01	0.062	-0.01	-0.03 – 0.01	0.246
Random effects												
σ^2	0.33			2.09			4.91			0.08		
τ_{00} nest of rearing	0.32			3.91			9.51			0.12		
Observations	90			90			90			90		
Marginal R^2 / Conditional R^2	0.293 / 0.643			0.250 / 0.739			0.248 / 0.744			0.248 / 0.682		

E. Results for the association between mitochondrial respiration rates in 14-days-old nestlings and survival as juvenile

Table S5. Results of generalized linear mixed models (GLMM ,with logistic binary distributions of the dependent variables, survival: 0=dead, 1=alive) testing whether mitochondrial respiration rates measured at day 14 predict juvenile recapture probability (i.e. proxy of medium-term apparent survival). Models only include individuals for which mitochondrial metabolic rates have been measured at day 14 (N = 102 individuals). 67 individuals (from 34 nests) have been recaptured as juveniles. Random intercepts could not be included in the models because of convergence issues. Odds ratios are reported with their 95% CI. R^2 values are estimated from the *coefficient of determination D* (Tjur's approach). Bold indicates significance ($P < 0.05$).

Predictors	ROUTINE			CI			CI+II			LEAK		
	Odds Ratios	CI 95%	P-value	Odds Ratios	CI 95%	P-value	Odds Ratios	CI 95%	P-value	Odds Ratios	CI 95%	P-value
(Intercept)	82.24	0.43 – 1.7e4	0.10	60.96	0.26 – 1.4e4	0.14	75.12	0.35 – 1.7e4	0.11	114.31	0.67 – 2.2e4	0.07
Mitochondrial respiration rate d14	1.30	0.87 – 1.95	0.20	1.08	0.95 – 1.23	0.24	1.04	0.96 – 1.14	0.31	1.33	0.66 – 2.47	0.38
hatching date	0.89	0.80 – 0.97	0.01	0.89	0.81 – 0.98	0.01	0.89	0.80 – 0.97	0.01	0.89	0.80 – 0.98	0.01
Observations	102			102			102			102		
R^2	0.09			0.09			0.08			0.08		

References

Friard, O., & Gamba, M. (2016). BORIS: a free, versatile open- source event- logging software for video/audio coding and live observations. *Methods in ecology and evolution*, 7(11), 1325-1330.