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RESEARCH ARTICLE

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

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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 growth, leading in some cases to long-lasting effects for the offspring, such as a decrease in size at fledgling 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 mitochondrial metabolism and reactive oxygen species (ROS) production in red blood cells. We investigated the effects of brood size on chick 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 with enlarged and control groups. However, mitochondrial metabolism and ROS production were not significantly affected by the treatment at either 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 relation to the nest environment. This study opens new avenues regarding the effect of postnatal environmental conditions in shaping offspring early-life mitochondrial metabolism.

KEY WORDS: 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 and Uller, 2009; Mousseau and Fox, 1998; Wolf and 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 and Crean, 2018; Mousseau and Fox, 1998; Yin et al., 2019). However, it is unclear whether parental effects are always adaptive (Bonduriansky and Crean, 2018; Burgess and Marshall, 2014; Marshall and 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 mechanisms underlying long-lasting effects of early-life environmental conditions on offspring phenotype are not well understood (Gluckman et al., 2007; Hoogland and Ploeger, 2022; Rogers and 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 of simulating 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: Hõrak, 2003; Rytkönen and 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 performance (Blount et al., 2003, 2006; Criscuolo et al., 2011). Yet, the mechanisms driving the effects of early-life environmental variation (including postnatal provisioning) on 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). Besides 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 with individuals from reduced broods (Burness et al., 2000). In contrast, zebra finches raised in large broods had a 9% higher standard metabolic rate at 1 year old compared with birds reared in small broods (Verhulst et al., 2006). While the link with 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:

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Arnold et al., 2021; Pettersen et al., 2018), only more recently have studies focused on mitochondrial aerobic metabolism (Ballard and Pichaud, 2014; Heine and 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 as organisms relying on aerobic metabolism use nutrients for producing ATP via a set of metabolic reactions, some of which occur 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 impairs protein and lipid structures and promotes 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 with 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., 2023; 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) whether brood size is important in determining nestling mitochondrial metabolism traits and associated ROS production; (ii) differences in nestling growth trajectories, and whether these are associated with differences in mitochondrial metabolic rates; and (iii) whether differences in mitochondrial metabolic rate affect offspring future survival. We further tested (iv) whether early-life determination of mitochondrial aerobic metabolism could affect adult phenotype with potential medium-term costs (e.g. consequences for juvenile mitochondrial metabolic rates and ROS production). Finally, our experimental design allowed us to (v) assess the relative contributions of the foster rearing environment (from 2 to 14 days post-hatching) versus 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 would have a lower body mass and size compared with control and reduced brood sizes. According to prior literature, offspring mitochondrial function is sensitive to postnatal environmental conditions. In rodent models, chronic stress exposure and separation from the mother during lactation led in most of the cases to a decrease in mitochondrial complex activity and an increase of ROS production (Picard and 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 offspring mitochondrial metabolism and an increase of ROS production. Nevertheless, most of the work assessing how a stressful early-life environment may impair mitochondrial function has been so far realized on mammals and the consequences in avian species and long-term effects remain elusive. Here, we tested the importance of brood size as a proxy for early-life environmental rearing conditions in shaping nestling mitochondrial metabolic rates, associated ROS production and later growth and survival patterns.

MATERIALS 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 tits, 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.

Ethics

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

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 a mean (±s.e.m.) 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) versus 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 three 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 (C n=150, E n=236, R n=154), of which 227 individuals were cross-fostered and 399 fledged (C n=98, E n=188, R n=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 ±0.1 g) and

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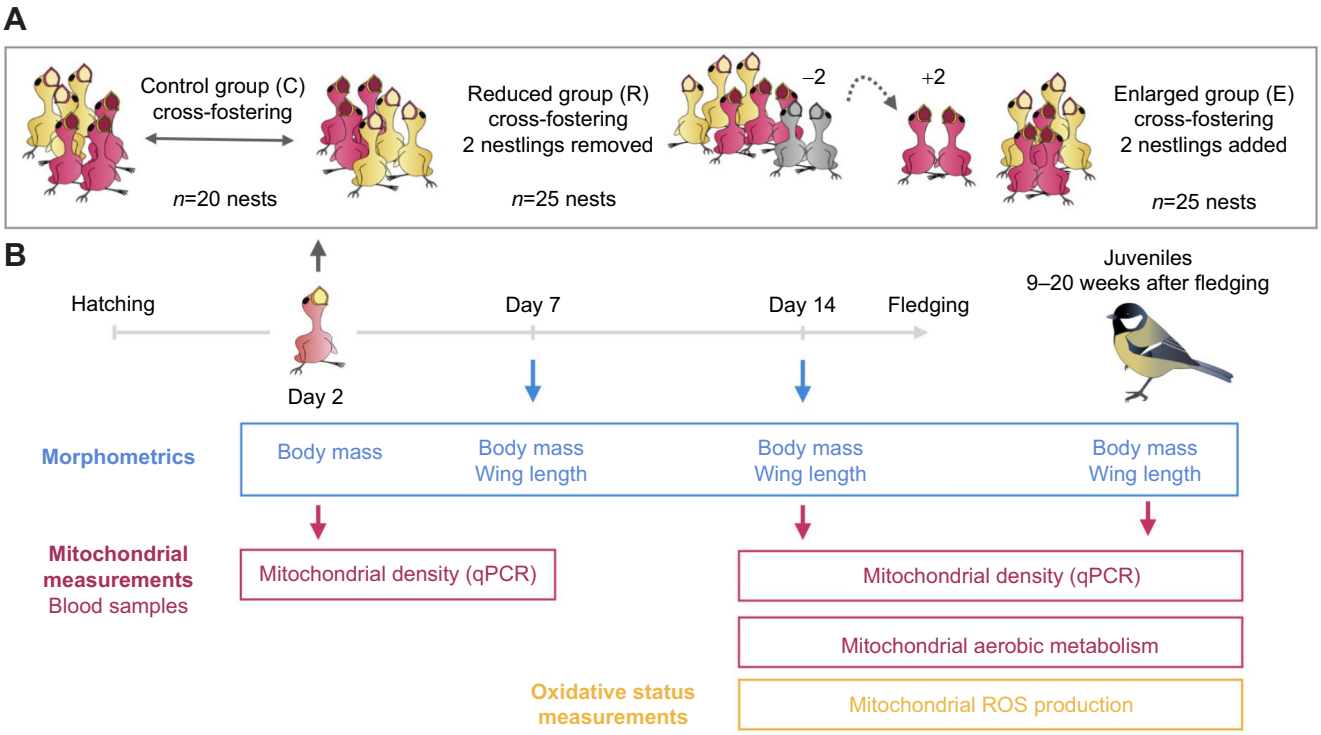

Fig. 1. Experimental design of the study. (A) Brood size manipulation. (B) Data collection. Sample sizes are presented according to treatment groups: control (C), reduced (R) and enlarged (E) broods. The timing of different measurements and analyses is indicated below the time line (see Materials and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

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 µl from the tarsus vein using heparinized capillaries, 2–4 nestlings per 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 whether parental feeding rates differed according to the brood size manipulation treatment groups, we video-recorded a subsample of nest boxes 8 days after hatching (for more details, see Supplementary Materials and Methods). We found higher rates for the E group compared with the R group, while parental feeding rates between the E and C groups were not significantly different (Fig. S1).

Nestlings were ringed 7 days after hatching, weighed and measured with a metal ruler (wing length ±1 mm) at days 7 and 14 (Table 1). Nestlings were blood sampled on day 14 (~30–75 µl from the brachial vein using heparinized capillaries). Blood samples were used to (1) evaluate mitochondrial aerobic metabolism (fresh samples kept on ice collected from 14 day old nestlings and juveniles; Table 1), (2) measure mitochondrial DNA copy number (i.e. mtDNAcn), a proxy for mitochondrial density (measured in frozen blood samples from 2 and 14 day old nestlings and juveniles when samples were available), and (3) measure mitochondrial ROS

Table 1. Sample sizes according to nestling age, treatment group and the different traits measured throughout this study

	Nestlings			
Measurements	Day 2	Day 7	Day 14	Juveniles
Body mass/size	R *n*=154 (25)	R *n*=121 (21)	R *n*=115 (21)	R *n*=14 (10)
	C *n*=150 (20)	C *n*=105 (16)	C *n*=99 (16)	C *n*=22 (9)
	E *n*=236 (25)	E *n*=194 (21)	E *n*=189 (21)	E *n*=31 (15)
mtDNAcn	R *n*=17 (6)		R *n*=48 (20)	R *n*=12 (8)
	C *n*=38 (10)		C *n*=46 (16)	C *n*=16 (9)
	E *n*=16 (5)		E *n*=55 (21)	E *n*=28 (15)
Mitochondrial aerobic metabolism			R *n*=35 (19)	R *n*=12 (8)
			C *n*=26 (14)	C *n*=16 (9)
			E *n*=41 (21)	E *n*=26 (15)
ROS production			R *n*=34 (18)	R *n*=11 (8)
			C *n*=23 (14)	C *n*=16 (9)
			E *n*=37 (20)	E *n*=26 (15)

R, reduced broods; C, control broods; E, enlarged broods; mtDNAcn, mitochondrial DNA copy number (a proxy of mitochondrial density); ROS, reactive oxygen species. The number of nests is indicated in parentheses.

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

mtDNAcn

We randomly selected a minimum of 2 nestlings per nest (one original and one cross-fostered nestling). Genomic DNA was extracted from 1–5 µl of frozen blood samples (stored at −80°C) using a salt extraction procedure adapted from Aljanabi and Martinez (1997). Because of their small volumes, some of the blood samples collected on day 2 could not be analysed. When data were available (see Table 1), we measured mtDNAcn on the same individuals on days 2 and 14 and as juveniles (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]<50 ng µl^{-1}, $A_{260/280}$<1.80 or $A_{260/230}$<2). Samples were then diluted to 1.2 ng µl^{-1} in sterile H_2O and stored at −80°C until real-time quantitative PCR assays (qPCR) assays. We quantified mtDNAcn using 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 duplicate in 384-well qPCR plates (n=5 plates) and qPCR was performed with a Bio-Rad instrument (CFX-384, Bio-Rad, Hercules, CA, USA). We used recombination activating gene 1 (*RAG1*) as a single control gene and cytochrome oxidase subunit 2 (*COI2*) as a 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 µl, including 6 ng of DNA samples, primers at a final concentration of 300 nmol l^{-1} and 6 µl of GoTaq® qPCR Mix (Promega, Madison, WI, USA). qPCR conditions were as follows: 3 min at 95°C (polymerase activation), followed by 40 cycles of 10 s at 95°C, 15 s at 58°C, 10 s at 72°C. The melting curve program was 5 s at 65°C, and increases of 0.5°C s^{-1} to 95°C. A pooled DNA sample from 14 adult individuals was used as a reference sample (i.e. ratio=1.0 for mtDNAcn) and was included in duplicate on every plate. qPCR efficiencies of *RAG1* and *COI2* genes were, respectively (mean±s.e.m.): 99.14±1.17% and 95.74±0.11%. Repeatability of mtDNAcn between sample duplicates was R=0.90 (95% confidence interval CI [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). The DNA integrity of 46 randomly selected samples was evaluated and deemed satisfactory using gel electrophoresis (100 ng of DNA, 0.8% agarose gel at 100 mV for 1 h).

Table 2. Summary of the statistical analyses performed according to the experimental approach and the correlative approach

Responses variables	Analyses
(A) Experimental approach	
Postnatal body mass from day 7 to day 14	**LMM:** treatment, age, *initial brood size*, *hatching date*, bird ID, nest box of rearing ID, original nest box ID
Nestling and juvenile body size	**LMMs:** treatment, *initial brood size*, *hatching date*, nest box of rearing ID, original nest box ID
Postnatal mtDNAcn 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 rate day 14	**LMMs:** treatment, *initial brood size*, *hatching date*, *mtDNAcn*, nest box of rearing ID, original nest box ID
Juvenile mitochondrial metabolic rate	**LMs:** treatment, *initial brood size*, *mtDNAcn*
Nestling ROS production day 14	**LMM:** treatment, *initial brood size*, *mtDNAcn*, *hatching date*, nest box of rearing ID
Juvenile ROS production	**LM:** treatment, *initial brood size*, *mtDNAcn*
Fledging success	**GLM, logistic binary distribution of dependent variables (0=dead, 1=alive):** treatment, *initial brood size*, *hatching date*, nest box of rearing ID
Recapture success (survival after fledging)	**GLM, logistic binary distribution of dependent variables (0=dead, 1=alive):** treatment, *initial brood size*, *hatching date*, nest box of rearing ID, original nest box ID
(B) Correlative approach	
Nestling and juvenile body mass	**LMMs:** *number of nestlings*, *previous mass measured*, *hatching date*, nest box of rearing ID, original nest box ID
Nestling and juvenile body size	**LMMs:** *number of nestlings*, *hatching date*, nest box of rearing ID, original nest box ID
Nestling mtDNAcn	**GLMM, gamma error distribution, log link:** *number of nestlings*, *hatching date*, nest box of rearing ID, original nest box ID, qPCR plate ID
Juvenile mtDNAcn	**GLMM, gamma error distribution, log link:** *number of nestlings*, nest box of rearing ID, qPCR plate ID
Nestling mitochondrial metabolic rate	**LMMs:** *number of nestlings*, *hatching date*, *mtDNAcn*, nest box of rearing ID, original nest box ID
Juvenile mitochondrial metabolic rate	**LMs:** *number of nestlings*, *mtDNAcn*
Nestling ROS production	**LMM:** *number of nestlings*, *hatching date*, *mtDNAcn*, nest box of rearing ID
Juvenile ROS production	**LM:** *number of nestlings*, *mtDNAcn*
Fledging success	**GLM, logistic binary distribution of dependent variables (0=dead, 1=alive):** *number of nestlings*, *hatching date*, nest box of rearing ID
Recapture success (survival after fledging)	**GLM, logistic binary distribution of dependent variables (0=dead, 1=alive):** *number of nestlings*, *hatching date*, nest box of rearing ID, original nest box ID

To analyse 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 issues, 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 on the day of 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 flux control ratios (FCRs; i.e. OXPHOS coupling efficiency, $FCR_{ROUTINE/CI+II}$, $FCR_{CI/CI+II}$), mitochondrial DNA copy number (mtDNAcn) was not included as covariate in the models. ROS, reactive oxygen species.

Mitochondrial aerobic metabolism

In order to test the impact of brood size on nestling mitochondrial respiration, we measured mitochondrial aerobic metabolism in a subsample (1–3 nestlings per nest), 14 days after hatching (individuals per nest: C n=26/14, E n=41/21, R n=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, juveniles per nest: C n=16/9, E n=26/15, R n=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 µl of blood). Mitochondrial respiration was analysed using high-resolution respirometry (3 Oroboros Instruments, Innsbruck, Austria) at 40°C adapted from a protocol described in Stier et al. (2019) with: digitonin (20 µg ml^{-1}), pyruvate (5 mmol l^{-1}), malate (2 mmol l^{-1}), ADP (1.25 mmol l^{-1}), succinate (10 mmol l^{-1}), oligomycin (2.5 µmol l^{-1}), antimycin A (2.5 µmol l^{-1}). We used 20 µl (nestlings) to 30 µl (juveniles) of fresh blood when available, suspended in Mir05 buffer. Five distinct respiration rates were analysed: (1) the endogenous cellular respiration rate before permeabilization (ROUTINE), (2) the maximum respiration rate fuelled with exogenous substrates of complex I, as well as ADP (CI), (3) the maximum respiration rate fuelled with exogenous substrates of complexes I and II, as well as ADP (CI+CII), (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 (flux control ratio, FCR): (1) OXPHOS coupling efficiency (OxCE=(CI+CII−LEAK)/CI+II), (2) the proportion of maximal respiration capacity being used under the endogenous cellular condition (i.e. $FCR_{ROUTINE/CI+II}$) and (3) the ratio between the maximal respiration rate of complex I and the maximal respiration capacity (i.e. $FCR_{CI/CI+II}$). OxCE FCR provides an index of mitochondrial efficiency in producing ATP, whereas $FCR_{ROUTINE/CI+II}$ 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 a Bio-Rad TC20 automated cell counter. The technical repeatability of mitochondrial aerobic metabolism measurements was high: ROUTINE: R=0.985 (95% CI [0.936–0.997]); CI+II: R=0.98 (95% CI [0.912–0.995]); LEAK: R=0.979 (95% CI [0.916–0.995]); OXPHOS: R=0.977 (95% CI [0.898–0.995]) based on 9 duplicates.

ROS measurements

ROS were measured in 14 day old nestlings and juveniles from the same samples as for 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 a 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 4 µl of MitoSOX™ (final concentration 4 µmol l^{-1}) and incubated for 30 min at 40°C, protected from light. After being cooled down (5 min on ice) and centrifuged (2 min, 1000 ***g*** at 4°C), samples were re-suspended in 250 µl Mir05 buffer added with 5 mmol l^{-1} pyruvate, 2.5 mmol l^{-1} malate, 10 mmol l^{-1} succinate and 1.25 mmol l^{-1} ADP. Samples (100 µl) 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) with an EnSpire® 2300 Multilabel Reader (PerkinElmer) set at 40°C. Samples were analysed in duplicates. The slope of relative fluorescence (RFU min^{-1}) was then extracted and normalized by the internal control present on each plate (dry *Saccharomyces cerevisiae* diluted at 10 mg ml^{-1} in Mir05). As a positive control (for mitochondrial ROS production) diluted *S. 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 (95% CI [0.9–0.941]).

Statistical analysis

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

Preliminary tests

Pre-treatment clutch sizes (raw data means±s.e.m.: 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 days, E and 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 group were as follows: (raw data means±s.e.m. [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 means±s.e.m.: R 2.93±0.07 g, C 2.94±0.05 g, E 2.98±0.05 g; F=0.51, P=0.60) and relative mtDNAcn (raw data means±s.e.m.: 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 that were and were not cross-fostered for the response variables tested throughout this study (i.e. growth metrics, mtDNAcn, 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 as 'experimental approach' in the text. To test for potential differences in 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 the 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 mtDNAcn), we initially included age and treatment, as well as their interaction, which was removed from the final model when non-significant. For mtDNAcn and postnatal body mass analysis,

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 the 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 as '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 analysed the associations between the number of nestlings and target variables using only the non-manipulated nests (C) to check whether patterns might have been confounded by including experimental nests (Table S3). As the 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 the original and the 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 mtDNAcn data did not fulfil the criteria of normality according to a Cullen and Frey plot (*fitdistrplus* package; https://CRAN.R-project.org/package=fitdistrplus); therefore, we analysed the effects of 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 analysed mitochondrial respiration rates (recorded in 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 mtDNAcn as a covariate), which indicates the respiration rate per unit of mitochondria.

For mitochondrial respiration rates measured on 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 mtDNAcn were included as fixed factors), using the *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 a general linear model (GLM) on survival (logistic binary distribution of dependent variables: 0=dead, 1=alive) and included mitochondrial respiration rates or FCRs and hatching date as explanatory factors. As the number of individuals recaptured was <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 (https://CRAN.R-project.org/package=lme4). 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 Results. Results from GLMMs (logistic binary distribution) were calculated based on Wald Chisquare tests (type II ANOVA). Model estimates (with associated 95% CI and *P*-values) are reported in tables. *emmeans* package (https://CRAN.R-project.org/package=emmeans) was used to conduct multiple *post hoc* comparisons [adjusted with Tukey honest significant differences (HSD) correction]. Effect sizes (Cohen's *d*) were estimated using *effsize* package (https://CRAN.R-project.org/package=effsize). 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: mean (±s.e.m., 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 for the C or R group during the whole growth period (from day 2 to day 14) (all Cohen's $d>1.50$) (Tukey HSD *post hoc:* C versus E and E versus 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* [95% CI]=0.43 [−0.25–1.11]) and 14 days after hatching (Cohen's *d* [95% CI]=0.37 [−0.31–1.05]) (Tukey HSD *post hoc:* C versus R comparison, all $P>0.90$). Means (±s.e.m., 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). The 95% CI of predicted estimates using a parametric bootstrapping method remained different from zero for factors having a statistically significant effect with GLMMs.

Nestling growth trajectories

Postnatal body mass dynamic (from day 7 to 14) was differentially affected by treatment depending on offspring age (Table 3). Specifically, nestlings from the R group had a higher body mass 14 days after hatching than nestlings from the E group (+4.81%), while body mass at day 14 remained similar between R and C groups (Table 3, Fig. 2). Body mass at day 14 of nestlings raised in the C and E groups was not statistically different (Table 3, 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 3). 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 was significantly and positively associated with 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±s.e.=0.42±0.18, $F_{1,41.5}=5.66$, $P=0.02$). Juvenile body mass and size were not significantly impacted by treatment (all $F<0.63$, all $P>0.54$).

mtDNAcn

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

Mitochondrial aerobic metabolism

We did not find any significant effect of the brood size manipulation treatment or the initial brood size on the different mitochondrial respiration rates and FCRs measured on day 14 (Tables 4 and 5,

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Table 3. Results of a LMM testing the effect of age and brood size manipulation treatment on nestling body mass

Predictors	Estimate	95% CI	*P*
(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**
Post hoc comparisons for day 14:			
Treatment (C) versus treatment (E)	0.19	−0.66–1.04	0.856
Treatment (C) versus treatment (R)	−0.56	−1.44–0.32	0.290
Treatment (E) versus treatment (R)	−0.75	−1.34– −0.16	**0.009**
Random effects			
σ2	1.80		
τ00 bird	0.13		
τ00 nest of origin	0.66		
τ00 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		

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 birds 14 days post-hatching. Bird ID, original nest box ID and nest box of rearing ID were included as random intercepts in models. σ2, within-group variance; τ00, between-group variance. Sample size (*n*) along with marginal (fixed effects only) and conditional (fixed and random effects). Bold indicates significance (P<0.05).

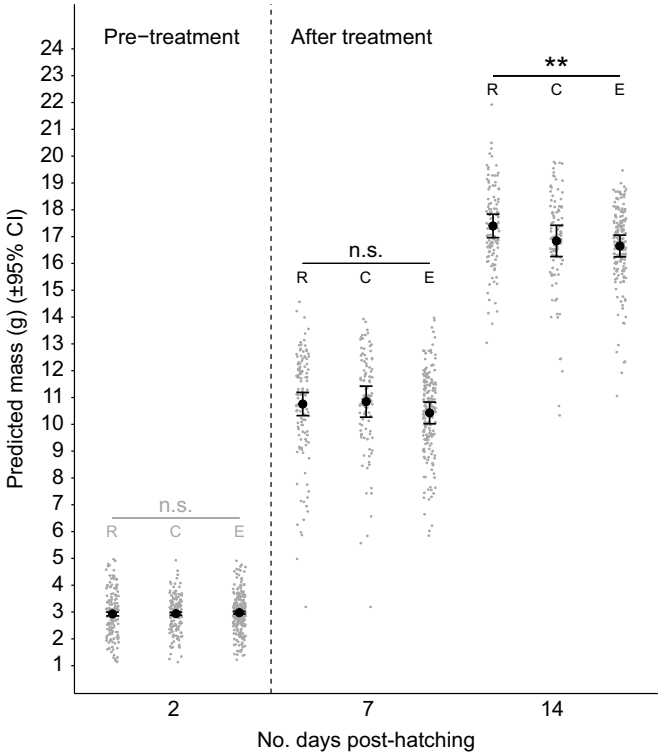

Fig. 2. Predicted body mass of nestlings from 7 to 14 days post-hatching according to brood size of the treatment group (R, C, E). For day 7 and day 14: predicted values (in grey) and predicted averages (in black) with their 95% confidence interval (CI) and results from Tukey HSD *post hoc* tests are reported. Predicted values were corrected for the average hatching date of the season and the average initial brood size. Asterisks indicate the significance of the *post hoc* test (**P<0.01) for body mass comparison between chicks raised in reduced versus enlarged broods (other comparisons were non-significant); R^2=0.89. See Table 1 for sample sizes. For body mass measured before treatment (day 2), raw data, and raw data means±s.e.m. 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). Juvenile mitochondrial respiration rates and FCRs were not significantly impacted by either 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 mtDNAcn at day 14 (Table 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 on 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 that of the nest of origin (Fig. 4).

ROS production

In 14 day old nestlings, mitochondrial ROS production was not significantly affected by 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 mtDNAcn in nestlings ($F_{1,83}$=0.48, P=0.49), juvenile mitochondrial ROS production significantly increased with mtDNAcn measured in autumn (estimate±s.e.=0.003±0.001, $F_{1,48}$=4.60, P=0.04).

Survival metrics

Fledgling success was not significantly affected by treatment (χ^2=2.44, P=0.29, raw data: R 75.33%, C 65,79%, E 77.78%), or by the initial brood size (χ^2=0.05, P=0.83) or the hatching date (χ^2=2.18, P=0.14). Juvenile recapture probability was not significantly affected by treatment (χ^2=2.18, P=0.34, raw data: R 12.17%, C 22.22%, E 18.52%) or the initial brood size (χ^2=0.03, P=0.87), but was negatively associated with hatching date (χ^2=13.6, P<0.001). Finally, we did not find any significant associations between juvenile recapture probability, mitochondrial respiration rates and FCRs measured on day 14 (all P>0.2; Table S5).

Correlative approach

When analysing each age separately, in order to account for the number of nestlings in the nest at a given age, nestling body mass on day 7 was negatively associated with the number of nestlings in the nest (Table S1), whereas we did not find an association for 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 on day 14 tended to increase with the number of nestlings (Table S1). While mtDNAcn on 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 mtDNAcn for juveniles (estimate±s.e.=0.07±0.03, P=0.04). We found a negative association between the number of nestlings on day 14 and mitochondrial respiration rates measured on day 14

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Table 4. Results of LMM testing the effects of the brood size manipulation on mitochondrial respiration rates measured on 14-day-old nestlings (*N*=102 individuals, *n*=55 nest boxes)

	ROUTINE			CI			CI+II			LEAK			OXPHOS			OXPHOS coupling efficiency		
Predictor	Estimate	95% CI	*P*	Estimate	95% CI	*P*	Estimate	95% CI	*P*	Estimate	95% CI	*P*	Estimate	95% CI	*P*	Estimate	95% CI	*P*
(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**	28.08	16.14–40.03	**<0.001**	0.92	0.87–0.96	**<0.001**
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	−1.08	−3.64–1.48	0.401	$2.0e^{-3}$	$-7.5e^{-3}$–0.01	0.672
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	−0.56	3.16–2.03	0.664	$-2.0e^{-4}$	$-9.5e^{-3}$_ $9.9e^{-3}$	0.967
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	−0.33	−0.97–0.32	0.314	$1.4e^{-3}$	$-8.9e^{-4}$_ $3.7e^{-3}$	0.223
mtDNAcn	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**	1.30	1.00–1.61	**<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	−0.27	−0.45–−0.09	**0.004**	$-9.5e^{-4}$	$-1.6e^{-3}$_ $-2.6e^{-4}$	**0.008**
Random effects																		
σ2	0.32			1.32			3.16			0.06			2.50			<0.001		
τ00 nest of origin	0.06			1.16			2.64			0.04			2.13			<0.001		
τ00 nest of rearing	0.40			5.22			12.59			0.24			9.68			<0.001		
Observations	102			102			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			0.394/0.894			0.133/0.593		

Mitochondrial respiration rates (see Materials and Methods) were corrected for the mtDNAcn (i.e. proxy of mitochondrial density). 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. σ2, within group variance; τ00, between-group variance. Bold indicates significance (P<0.05).

(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 on 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 (<5 chicks at day 14) with high mitochondrial respiration rates were driving the association 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) and 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 on day 14, except for $FCR_{CI/CI+II}$, for which we found a negative association (estimate±s.e.=−0.005±0.003, $F_{1,62}$=4.36, P=0.04). We did not find significant associations between the number of nestlings on 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 on day 14 (χ^2=61.47, P<0.001). Juvenile recapture probability was not significantly associated with the number of nestlings on day 14 (χ^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 (either on recapture probability, body mass and size, or on 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 the 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 cell mitochondrial metabolism than genetic inheritance prenatal 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 and Nilsson, 2011).

Experimental approach

Nestling growth trajectories (postnatal body mass) differed according to nestling age and treatment. As expected, individuals raised in the reduced group had a higher body mass a few days before fledging compared with that of those in the enlarged group but not the control group (see also Hõrak, 2003). While we expected nestlings raised in the enlarged group to have lower body mass (Hõrak, 2003; Rytkönen and Orell, 2001; Smith et al., 1989),

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Table 5. Results of LMM testing the effects of the brood size manipulation on FCRs

	$FCR_{ROUTINE/CI+CII}$			$FCR_{CI/CI+II}$		
Predictors	Estimate	95% CI	*P*	Estimate	95% CI	*P*
(Intercept)	0.12	0.03–0.21	**0.011**	0.72	0.64–0.82	**<0.001**
Treatment (E)	$4.1e^{-3}$	−0.02–0.02	0.682	0.02	$-3.7e^{-3}$–0.03	0.112
Treatment (R)	$8.1e^{-4}$	−0.02–0.02	0.936	0.02	$-4.3e^{-3}$–0.03	0.126
Initial brood size	$-5.7e^{-4}$	$-5.7e^{-4}$–$4.5e^{-3}$	0.823	$-1.4e^{-3}$	$-6.1e^{-3}$–$-3.3e^{-3}$	0.556
mtDNAcn	–	–	–	–	–	–
Hatching date	$2.1e^{-3}$	$7.9e^{-4}$–$3.5e^{-3}$	**0.003**	$-8.9e^{-4}$	$-2.2e^{-3}$–$4.6e^{-4}$	0.189
Random effects						
σ2	<0.001			<0.001		
τ00 nest of origin	–	–	–	<0.001		
τ00 nest of rearing	<0.001			<0.001		
Observations	102			102		
Marginal R^2/conditional R^2	0.148/0.502			0.061/0.567		

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. σ2, within group variance; τ00, between-group variance. Bold indicates significance (P<0.05).

nestlings raised in the 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 the results on parental feeding rates (measured on a subsample of nests; Fig. S1). The number of visits was significantly higher in the enlarged group than in the reduced group and tended to be higher compared with controls (although non-significant). These results are supported by prior studies suggesting that parents can rear more nestlings than the number of eggs laid (Casti, 2018; Monaghan and 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 probably contributes to explain why our experiment failed to demonstrate large differences

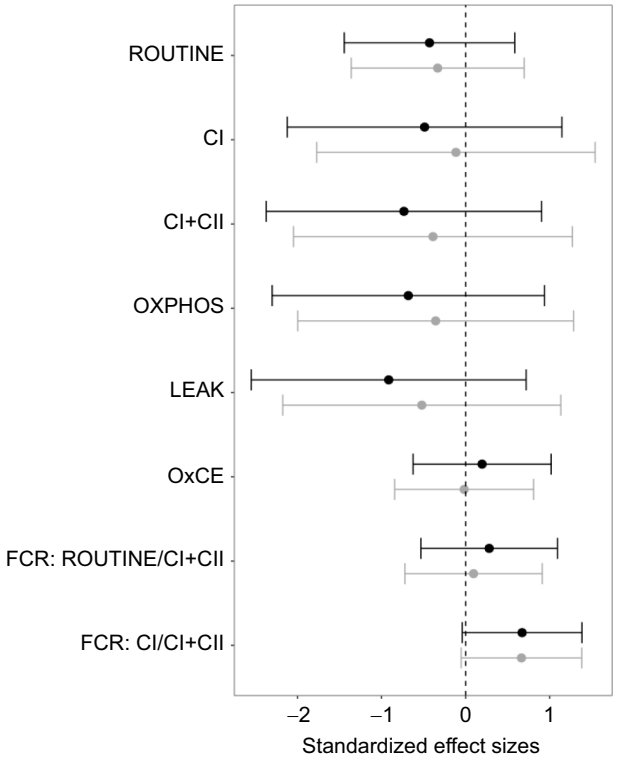

Fig. 3. Effect of brood size manipulation on mitochondrial metabolic rates and flux control ratios. Mitochondrial aerobic metabolism (ROUTINE, endogenous cellular respiration rate; CI, complex I respiration rate; CI+CII, complex I and II respiration rate; OXPHOS, respiration rate supporting ATP synthesis through oxidative phosphorylation; LEAK, respiration leak contributing to proton leak; OxCE, OXPHOS coupling efficiency; FCR, flux control ratio; see Materials and Methods) was measured on day 14 between individuals raised in the R, C and E broods (for sample sizes, see Table 1). Standardized effect sizes are based on predicted values of the model and reported with their 95% CI. Effect sizes between individuals raised in E versus C broods are shown in black; those between individuals raised in R versus C broods are shown in grey.

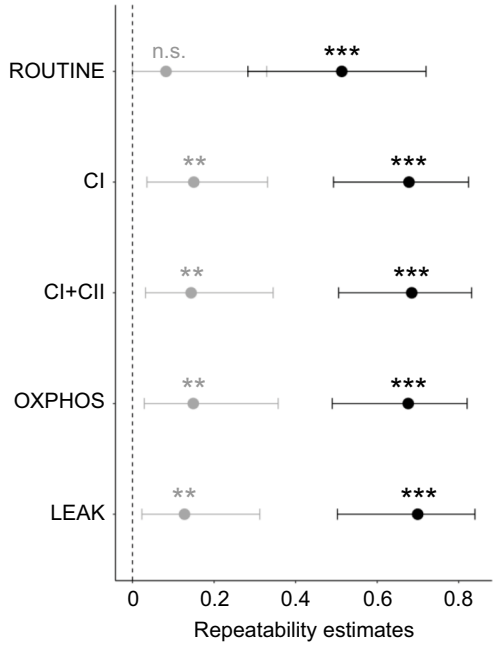

Fig. 4. Variance explained by the nest of origin and the nest of rearing in linear mixed models testing mitochondrial respiration rates on day 14 according to the number of nestlings on day 14. Repeatability estimates are presented with their 95% CI for nest of origin (grey) and nest of rearing (black). Asterisks indicate a significant difference from 0 (***P<0.001, **P<0.01). See Table 1 for sample sizes.

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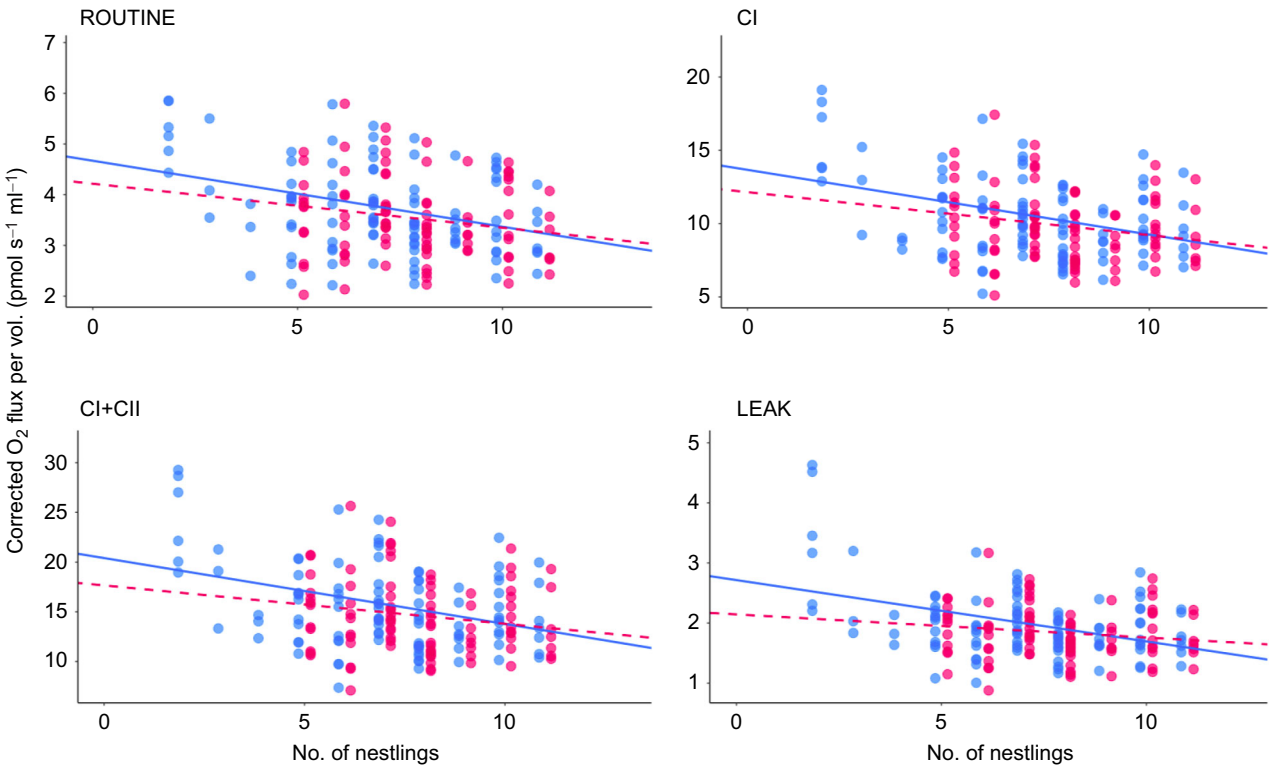

Fig. 5. Predicted values of mitochondrial respiration rates on 14 day old nestlings according to the number of nestlings on day 14. Values are shown for the complete dataset (blue, *N*=102 individuals) and a subsample excluding small brood sizes (red, *N*=90 individuals) (<5 chicks at day 14, *N*=12 individuals from 8 nest boxes). Predicted values were extracted from linear mixed models (LMMs) presented in Tables S2 and S4. Regression lines and results from the models are presented. Predicted values were corrected for the average hatching date of the season. Mitochondrial respiration rates were corrected for mitochondrial DNA copy number (mtDNAcn, 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). Only the nest of rearing ID could be included as a random intercept in the models presented in Table S4 (red, see Materials and Methods). R^2 of each model is reported in Tables S2 and S4.

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 the longer term in juveniles. This lack of effect 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 juvenile) 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 a juvenile) were not significantly impacted by the treatment, which is probably explained by the increase in parental feeding rates.

Correlative approach

For the reasons mentioned above, our experimental manipulation 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 analyses beside the experimental set, using the actual number of nestlings as the 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 finding was surprising as the opposite results (i.e. a negative association between wing length and the number of chicks in the nest) have been reported in the literature (Hõrak, 2003; Rytkönen and Orell, 2001; Smith et al., 1989). However, these results from previous studies were 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+CII, LEAK and OXPHOS) and the 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 (<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 <5 nestlings at day 14 had really low survival chances during the growth period (from day 2 to 14) compared with those from the larger broods (>4 nestlings) (average on raw data: 63.4% versus 92.4% 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 versus 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, 2022; Cossin-Sevrin et al., 2022; Hsu et al., 2023; but see: Dawson and 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 an usually high 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. However, 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 a 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 whether 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 juveniles 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., 2023). 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 (i) to estimate nestling survival and potential long-lasting effects of our experiment and (ii) because mitochondrial aerobic metabolism measurements in blood samples can be positively associated with those in other tissues (Koch et al., 2021; Stier et al., 2017). Collecting blood samples allows the use of limited invasive methods on wild species, and avoids 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 the major environmental cues affecting offspring mitochondrial metabolism during the growth period are (e.g. availability of nutrients, ambient temperature) (White and 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 for 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
The authors declare no competing or financial interests.

Author contributions
Conceptualization: N.C.-S., A.S., S.R.; Methodology: N.C.-S., A.S., S.Z., K.A., S.R.; Formal analysis: N.C.-S., K.A., S.R.; Investigation: N.C.-S., K.A., S.R.; Resources: N.C.-S., A.S., M.H., S.Z., S.R.; Data curation: N.C.-S., S.Z.; Writing - original draft: N.C.-S.; Writing - review & editing: N.C.-S., A.S., M.H., S.Z., V.A.V., K.A., S.R.; Supervision: K.A., S.R.; Project administration: S.R.; Funding acquisition: A.S., V.A.V., S.R.

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Data availability
Data are availablefrom figshare: doi:10.6084/m9.figshare.22354432.v1

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