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

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Abstract

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

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

Introduction

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

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

Whereas the effects of maternal androgens (*e.g.*, testosterone, 5α-dihydrotestosterone, andostenedione) on offspring development have been well studied (Groothuis et al., 2005; Podmokła et al., 2018), less is known on the effects of thyroid hormones (THs). Yet, THs are central growth regulators, and coordinate maturation and differentiation as transcription factors (Darras, 2019; Ruuskanen and Hsu, 2018). Thus, variation in THs during critical periods may have marked effects on offspring development (*e.g.*, neurotrophic signals, cerebellar-mediated motor function, retinal layer) (Darras, 2019; Ruuskanen and Hsu, 2018), and are also known to affect offspring behavior via early-life imprinting (Bett et al., 2016; Yamaguchi et al., 2012). THs modulate metabolism associated with (i) medium to long-term changes in the basal energy expenditure of the organism (Harper and Seifert, 2008; Kim, 2008) and (ii) modulation of the activity of downstream regulatory hormones and growth factors such as insulin, glucagon and catecholamines ((Grøntved et al., 2015; Pucci et al., 2000; Sinha et al., 2018).

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

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

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

As THs are known to stimulate mitochondrial aerobic metabolism and biogenesis while potentially decreasing the efficiency at which nutrients are converted to ATP (Cioffi et al., 2013), we expected nestlings hatched from eggs supplemented with THs to exhibit a higher mitochondrial density and higher mitochondrial respiration rates, but a potentially higher proton leak leading to less efficient mitochondria (Fig. 2). We predicted that such a higher metabolic capacity could boost embryo development and early post-hatching growth and survival, while the lower mitochondrial efficiency might impair body condition and performance later during postnatal development (Salin et al., 2019) leading to a decrease in survival prospects especially after fledging (but see (Hsu et al., 2019; Hsu et al., 2020; Hsu et al., 2021; Ruuskanen et al., 2016; Sarraude et al., 2020), for the contrasted effects of prenatal THs on growth in avian species). Since physiological amounts of GC have been suggested to enhance mitochondrial density and aerobic metabolism (including ATP production, (Manoli et al., 2007), we expected nestlings hatched from eggs supplemented with GC to exhibit a higher mitochondrial density and higher mitochondrial respiration rate, as well as a higher efficiency to produce ATP (Fig. 2, but see (Casagrande et al., 2020)for somewhat opposite effects of high GC levels at the postnatal stage). Thus, we expected these individuals to have a faster growth (both pre- and postnatal) leading to an increase in survival prospects on the short-term (*i.e.* fledging and/or first autumn) but potential long-term costs (Haussmann et al., 2012; Metcalfe and Monaghan, 2001). Finally, we tested if GC and TH hormones had interactions, such as synergistic effects, affecting offspring mitochondrial function, growth and survival (Brown et al., 2014). For instance, it has been shown that postnatal supplementation with THs and GC has synergistic effects on growth (Khangembam et al., 2017). Yet, directional predictions about the effects of prenatal hormones are very difficult to make considering 1. the likely environmental-dependence of their cost-benefit balance, 2. the existence of non-linear dose-responses and 3. the fact that embryos are not passive receivers of maternal hormones but can manipulate such signals (Groothuis et al., 2019).

Material and Methods

Field site and population monitoring

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

Experimental manipulation of glucocorticoids and thyroid hormones

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

Hormone solutions were prepared using crystal T4 (L-thyroxine 98% HPCL, CAS number 51-48-9, Sigma-Aldrich), T3 (3,3',5-triiodo-L-thyronine, >95% HPCL, CAS number 6893-02-3, Sigma-Aldrich) and CORT (Corticosterone VETRANAL®, HPCL, CAS number 50-22-6, Sigma-Aldrich) dissolved in 0.1M NaOH (TH) or 99% EtOH (CORT), and diluted in 0.9% NaCl to the targeted concentrations. We followed the injection procedure as described in (Hsu et al., 2019; Sarraude et al., 2020). We prepared the corresponding hormone solutions for each experimental group (CO, TH, CORT or CORT + TH), so that each egg was injected only once with 2 µl of the corresponding hormone solution and all eggs in one nest received the same hormonal mix. Egg injections started on the day the 5th egg was laid, and every day later on until the last egg was laid. This protocol ensured injections were done before the incubation onset, meanwhile minimizing nest-disturbance (i.e. we avoided visiting the nest every day) and allowing to closely monitor the onset of incubation, given that great tits can start incubation well before clutch completion. When no new eggs were observed for two consecutive days, the clutch was considered complete. Hatching was monitored daily starting 2 days prior to the estimated hatch date. Hatching was considered as "day 0".

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

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

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

Mitochondrial DNA copy number

We randomly selected 2 nestlings per nest (n = 104 individuals) and estimated *mtDNAcn* on the same individuals at day 7, day 14 and as juveniles (autumn 2019) when samples were available (respectively sample-sizes at day 7/ day 14 / juveniles: n_{CO} = 26/27/9 , n_{CORT} = 23/21/10 , n_{TH} = 29/24/7, $n_{CORT + TH}$ = 25/23/11, resulting in 235 samples in total). Genomic DNA was extracted from 5µL of frozen blood samples using a salt extraction procedure adapted from (Aljanabi and Martinez, 1997). DNA quantity and purity were estimated using a *NanoDrop* spectrophotometer*.* Samples were re-extracted if needed ([DNA] < 50ng/µL, 260/280 ratio < 1.80 or 260/230 < 2). DNA integrity of 48 randomly selected samples were evaluated and deemed satisfactory using gel electrophoresis (100 ng of DNA, Midori Green staining, 0.8 % agarose gel at 100 mV for 60 min). Samples meeting our quality checks were then diluted at 1.2 ng/µL in sterile H_2O and stored at -80°C until qPCR assays. *mtDNAcn* was quantified using real-time quantitative PCR (qPCR) assays as previously described for other passerine species (Stier et al., 2019; Stier et al., 2020) and great tits (Hsu et al., 2021; Stier et al., 2021). This technique estimates the relative mtDNAcn by determining the ratio of mtDNA repeat copy number to a nuclear singly copy gene (SCG). qPCR reactions were performed in a total volume of 12µL including 6ng of DNA sample, primers at a final concentration of 300nM and 6µL of SensiFAST^{TM} SYBR® Lo-ROX Kit (Bioline). We used Recombination Activating Gene 1 (RAG1) as a single-copy control gene (SCG) verified using a BLAST analysis on the great tit genome. The gene RAG1 was amplified using the primers RAG1 forward (5'-TCG GCT AAA CAG AGG TGT AAA G-3') and RAG1 reverse (5'-CAG CTT GGT GCT GAG ATG TAT-3'). For *mtDNAcn*, we used cytochrome oxidase subunit 2 (COI2) as a specific mitochondrial gene after verifying that it was not duplicated as a pseudo-gene in the nuclear genome using a BLAST analysis on the great tit genome. We used the primers sequences COI2 forward (5' – CAAAGATATCGGCACCCTCTAC-3') and COI2 reverse (3'-GCCTAGTTCTGCACGGATAAG-5'). Samples were run in triplicates. qPCR conditions were 3 min at 95°C (polymerase activation), followed by 40 cycles of 10s at 95°C, 15s at 58°C, 10s at 72°C (DNA denaturation, primers annealing, DNA extension and fluorescence reading). The melting curve program was 15s at 95°C, 1min at 58°C, 0.1°C/s increase to 95°C, and then hold 15s at 95°C*.* A DNA sample being a pool of DNA from 10 adult individuals was used as a reference sample (*i.e.* ratio = 1.0 for *mtDNAcn*) and was included in triplicates in every plate. qPCR efficiencies of control and mitochondrial genes were 91.4 ± 0.003% and 104.5 ± 0.005%, respectively. Repeatability of mtDNAcn measurements estimated with samples-triplicates was high R = 0.921 ($CI_{95\%}$ = [0.907; 0.934], n = 1287). We also calculated the inter-plate repeatability of *mtDNAcn* measurements using samples being measured on different plates: R = 0.867 ($CI_{95\%}$ = [0.822, 0.916], n = 211). All the qPCR assays (n = 10 plates) were performed on a 384-QuantStudioTM 12K Flex Real-Time PCR System (Thermo Fisher).

Molecular sexing

Nestlings were molecularly sexed using a qPCR approach adapted from (Chang et al., 2008; Ellegren and Fridolfsson, 1997), using blood samples when available (2 nestlings per brood). Forward and reverse sexing primers were 5′- CACTACAGGGAAAACTGTAC-3′ (2987F) and 5′- CCCCTTCAGGTTCTTTAAAA -3′ (3112R), respectively. qPCR reactions were performed in a total volume of 12µL including 6ng of DNA, primers at a final concentration of 800nM and 6µL of SensiFAST^{TM} SYBR® Lo-ROX Kit (Bioline). qPCR conditions were: 3 min at 95°C, followed by 40 cycles of 45 s at 95°C, 60 s at 52°C and 60s at 72°C, then followed by a melting curve analysis (95°C 60s, 45°C 50s, increase to 95°C at 0.1°C/s, 95°C 30s). Samples were run in duplicates in a single plate and 6 adults of known sex were included as positive controls.

Mitochondrial respiration

Mitochondrial respiration was analyzed using high-resolution respirometry (Oroboros Instruments, Innsbruck, Austria) at 40°C, adapted from the protocol described in (Stier et al., 2019) (protocol modifications: mitochondrial respiration rates were estimated using 30µL of fresh blood when available, suspended in Mir05 buffer). We analyzed 4 mitochondrial respiration rates: 1) the endogenous cellular respiration rate before permeabilization (*ROUTINE*), 2) the maximum respiration rate fueled with exogenous substrates of complex I and II, as well as ADP (*CI + II*), 3) the respiration rate contributing to proton leak (*LEAK, i.e*., not producing ATP but dissipating heat), 4) the respiration rate supporting ATP synthesis through oxidative phosphorylation (*OXPHOS*). We also calculated 2 mitochondrial flux ratios (FCRs): 1) *OXPHOS* coupling efficiency: *OxCE* = (1-*LEAK*) / *CI+II*), and 2) the proportion of maximal respiration capacity being used under endogenous cellular condition (*i.e.*, *FCR ROUTINE / CI+II*). The former provides an index of mitochondrial efficiency in producing ATP, whereas the latter reflects the cellular control of mitochondrial respiration by endogenous ADP/ATP turnover and substrate availability. Due to the logistical constraints of respirometry measurements (*i.e., the* need to work on freshly collected samples, > 2 h of processing per 2 samples), the analysis of mitochondrial respiration was limited to 1 nestling per nest (repeated measurements from same individuals at day 7 and day 14), summing up to 89 samples from 48 individuals (respectively sample-sizes at day 7/day 14: n_{CO} = 11/11,

n_{CORT} = 11/10, n_{TH} = 14/12, $n_{CORT + TH}$ = 10/10). Mitochondrial respiration rates were not analyzed from juveniles due to logistical constraints. The technical repeatability of mitochondrial respiration measurements was high: *ROUTINE* : R = 0.989 ($CI_{95\%}$ = [0.957, 0.997]); *CI* + *CII*: R = 0.992 ($CI_{95\%}$ = [0.968, 0.998]); *LEAK*: R = 0.982 ($CI_{95\%}$ = [0.929, 0.995]) ; *OXPHOS:* R = 0.992 ($CI_{95\%}$ = [0.968, 0.998]) based on n = 9 duplicates.

Statistical analyses

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

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

mtDNAcn data distribution did not fulfill the criteria of normality according to a Cullen and Frey plot (‘fitdistrplus’ package, (Delignette-Muller and Dutang, 2015), therefore we evaluated the effects of CORT and TH treatments on *mtDNAcn* using a GLMM (gamma error distribution, log link). We included nest box ID as a random intercept and bird ID as a repeated factor to account for the non-independency of measures from a same individual. All mitochondria respiration rates (recorded at day 7 and day 14; including *ROUTINE, LEAK, OXPHOS, CI+II*) were tested with LMMs. We analyzed mitochondrial respiration rates at both the cellular level (*i.e.*, respiration measurements expressed relative to cell number) that indicates respiration properties per unit of cells, and 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 models including repeated measures across time (body mass, *mtDNAcn*, mitochondrial respiration measurements), we initially included CORT, TH, age and all interactions as fixed factors and removed non-significant interactions following a backward-stepwise procedure to obtain the lowest AIC value.

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

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

Results

Prenatal hormonal effects on hatching, fledging success and developmental time

Hatching success (CO = 55.6%, CORT = 53.4%, TH = 62.7%, CORT+TH = 58.6%) and fledging success (CO = 90%, CORT = 89.8%, TH = 75.7%, CORT+TH = 74.4%) were not significantly affected by the prenatal hormonal manipulation (GLMMs, all $\chi^2 < 2.5$, all $p >$ 0.11). Developmental time was significantly increased (+ 7%) by a prenatal CORT supplementation (LM, CORT *vs.* non-CORT: lsmean ± SE: 12.8 ± 0.2 *vs.*12.0 ± 0.2 days,

$F_{1,49}$ = 6.27, p = 0.015), but significantly decreased (- 5%) by a prenatal TH supplementation (TH *vs.* non-TH: lsmean ± SE: 12.1 ± 0.2 *vs.* 12.7 ± 0.2 days; $F_{1,49}$ = 4.26, *p* = 0.044). However, there was no significant CORT x TH interaction ($F_{1,49}$ = 2.24, p = 0.14).

Prenatal hormonal effects on mitochondrial density

We found a significant effect of the prenatal CORT supplementation in interaction with age on mitochondrial density (overall test for Age x CORT: χ^2 = 8.65, *p* = 0.013, Fig. 3a). Mitochondrial density was significantly influenced by age (χ^2 = 451.7, *p* < 0.001), decreasing from day 7 to day 14 (Tukey HSD post-hoc: *p* < 0.001) and from day 14 to the juvenile stage (Tukey HSD post-hoc: p < 0.001; see Table 1 for estimates of final model). While prenatal CORT did not significantly affect mitochondrial density at day 7 (Tukey HSD post-hoc: *p* = 0.29) or in juveniles (Tukey HSD post-hoc: *p* = 0.92), it significantly decreased mitochondrial density by 27 % at day 14 (Tukey HSD post-hoc: *p* = 0.006, Fig. 3a). We found no significant evidence for an effect of prenatal TH supplementation on mitochondrial density (χ^2 = 0.003, *p* = 0.96, Fig. 3b), nor for an interaction between prenatal TH and CORT (χ^2 = 0.006, *p* = 0.81). Brood size was negatively related to mitochondrial density (χ^2 = 4.31, *p* = 0.036), while hatching date was not significantly related to mitochondrial density (χ^2 = 1.50, *p* = 0.22, Table 1).

Prenatal hormonal effects on mitochondrial aerobic metabolism

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

Contrary to prenatal CORT, there was no significant effect of the prenatal TH supplementation on mitochondrial aerobic metabolism (LMMs, all *F* < 2.26, all *p* > 0.14, Tables 2 & 3). All mitochondrial respiration rates significantly decreased between nestling day 7 and day 14 (LMMs, *ROUTINE: -15.3 %*, *OXPHOS: -12.4 %*, *CI+II: -11.5 %*; all $F > 4.8$, $p < 0.032$, Table 2), except *LEAK* (LMM, $F = 1.70$, $p = 0.20$, Table 2). While $\mathrm{FCR}_{ROUTINE/CI+II}$ was not significantly impacted by age ($F = 1.89$, $p = 0.18$, Table 2), younger chicks had more efficient mitochondria (*i.e.* 2.9% higher *OxCE*, $F = 8.33$, $p = 0.006$, Table 3). Males showed a significantly higher *LEAK* (lsmean: +16.5%, $F = 4.23$, $p = 0.047$) than females when controlling for mitochondrial density (Table 2), but we did not find other significant sex differences in mitochondrial aerobic metabolism (LMMs, all $F < 1.65$, all $p > 0.20$, Table 2). Brood size was not significantly associated with mitochondrial aerobic metabolism traits (LMMs, all $F < 1.69$, all $p > 0.20$, Tables 2 and 3). All mitochondrial aerobic metabolism traits except *ROUTINE* ($F = 0.22$, $p = 0.64$) and *LEAK* ($F = 0.02$, $p = 0.88$) were significantly positively associated with the hatching date (LMMs, all $F > 8.10$, all $p < 0.008$, Tables 2 and 3).

Prenatal hormonal effects on growth

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

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

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

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

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

Discussion

We tested for potential developmental plasticity related to two prenatal hormones in a wild great tit population. By experimentally increasing yolk hormone content to simulate higher maternal deposition of these hormones in the eggs, we investigated the effects of GC, TH, and their interaction on offspring mitochondrial aerobic metabolism, development and survival. Developmental time was significantly increased by prenatal CORT supplementation, but significantly decreased by prenatal TH supplementation. Elevated prenatal CORT exposure significantly reduced mitochondrial density and respiration rates, without significantly affecting mitochondrial coupling efficiency (*OxCE*). Interestingly, such down-regulations of mitochondrial aerobic metabolism might have been partially compensated by a higher usage of maximal mitochondrial capacity (*i.e.* higher $FCR_{ROUTINE/CI+II}$). We did not find very clear effects of prenatal hormonal treatments on growth patterns and recapture probability. Yet, nestlings hatched from CORT-injected eggs were lighter at day 2 and had a tendency to grow faster in early life (*i.e.* day 2 to day 7), although these differences were not statistically significant in our experiment, so that effects of prenatal CORT on nestling's body mass, size and condition should be considered with caution. Recaptured females from CORT group were lighter and in worse condition than juvenile females from non-CORT group, while we did not find a significant difference in males. Despite not being statistically significant, recapture probability was *ca.* 14% higher for juveniles from the CORT group. We expected prenatal TH treatment to promote mitochondrial biogenesis, leading to an increase of mitochondrial density and mitochondrial aerobic metabolism but found no support for such hypothesis. Others studies have also reported a lack of significant effect of prenatal TH supplementation on nestling mitochondrial density in other avian species (Hsu et al., 2020; Hsu et al., 2021; Stier et al., 2020). Several hypotheses may explain the contrasting results in studies focusing on maternal hormonal effects, such as specific dose-dependent or context dependent response of maternal hormones, variation in initial hormones transferred/deposited by the mother or pleiotropic effects of maternal hormones (Groothuis et al., 2019). One limitation in the present study is the estimation of mitochondrial density and mitochondrial aerobic metabolism using blood cells. While it has been previously shown that mitochondrial function in blood cells is to some extent correlated to mitochondrial function in other tissues (Stier et al., 2017; Stier et al., 2022), TH may have tissue-specific effects that we were not able to detect in the present study.

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

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

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

One illustration of potential direct prenatal impact of CORT and TH is the result we obtained regarding developmental time (*i.e.* incubation duration). We found that a prenatal increase of CORT levels increased developmental time *in ovo*, while an increase in prenatal TH levels decreased developmental time. It has been previously shown that an augmentation of TH *in ovo* may accelerate hatching (Hsu et al., 2017). Measuring mitochondrial aerobic metabolism during embryo development will be necessary to understand if such effects on embryo growth might be mediated by mitochondrial metabolism. Yet, as we monitored the nest only once a day to determine hatching date, overall incubation duration is estimated with a potential error of ± 1 day, meaning that this result should be interpreted with caution, but warrants further investigation. Understanding how effects on developmental time may carry-over and affect post-hatching phenotypes also requires further investigation.

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

Conclusion

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

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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/5718/2019) and by the Environmental Center of Southwestern Finland (license no. VARELY/924/2019) granted to S.R.

Competing interests

We declare we have no competing interests.

Author’s contribution

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

Data availability statement

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

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Figures and Tables

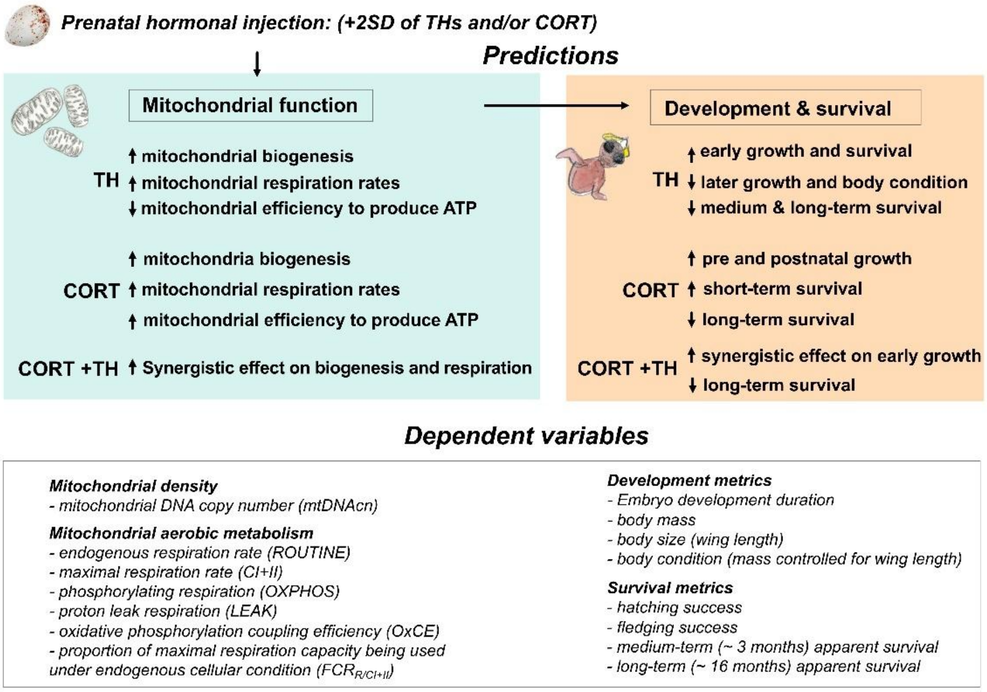

Fig. 1: Experimental timeline of the study, with sample sizes for different response variables. Great tit nestlings fledge around 18 - 20 days after hatching.

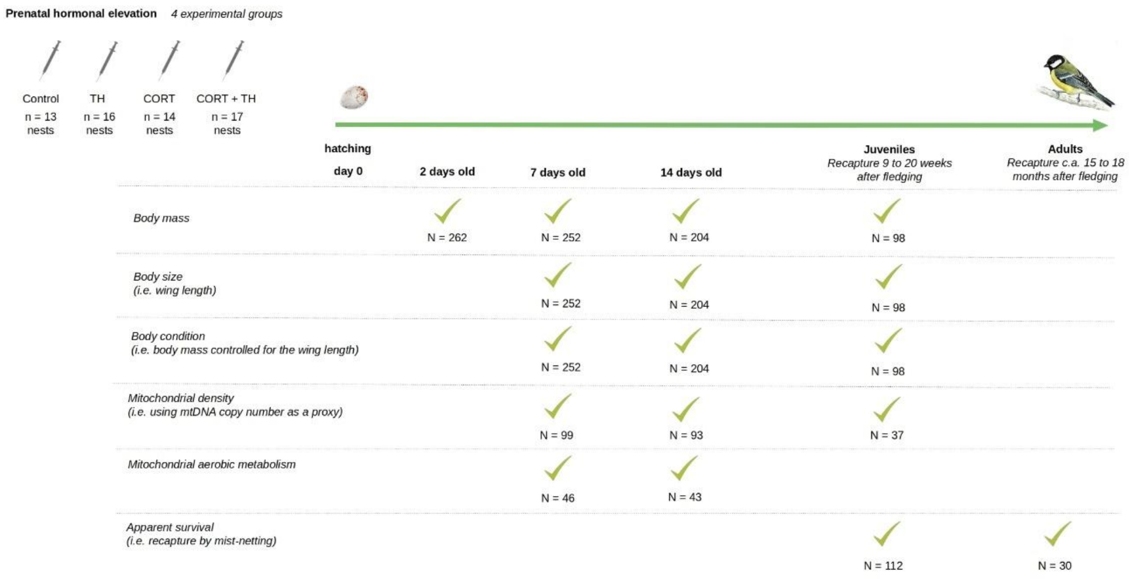

Fig. 2: Predictions related to the experimental manipulation of prenatal thyroid and glucocorticoid hormones.

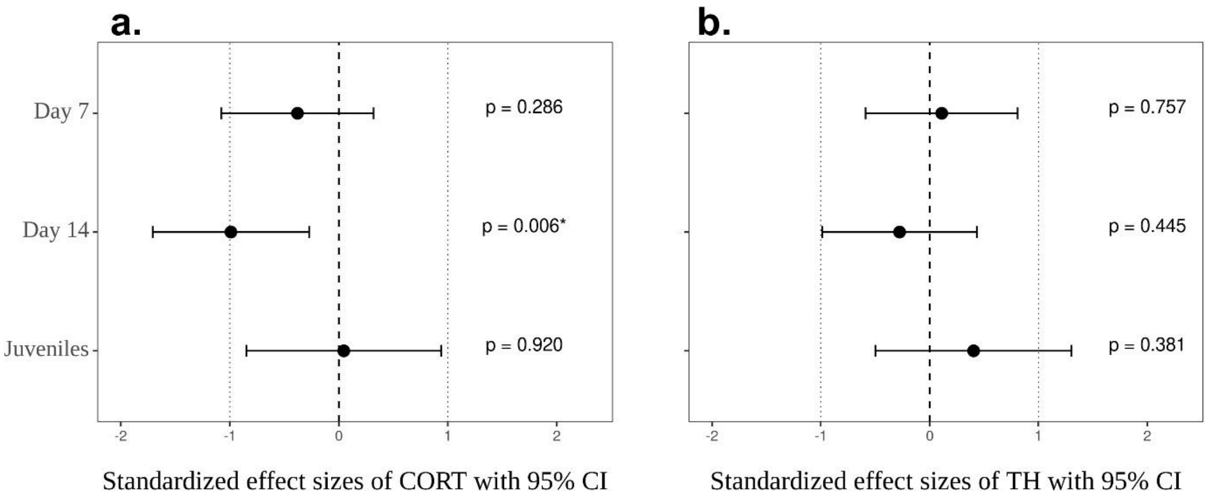

Fig. 3: Effects of prenatal CORT (a) and TH (b) treatments on mitochondrial density at day 7 (n = 99), day 14 (n = 93) and juvenile age (n = 37) (N = 100 individuals). Standardized effect sizes based on predicted values of the model are reported with their 95% confidence intervals. Age x CORT interaction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

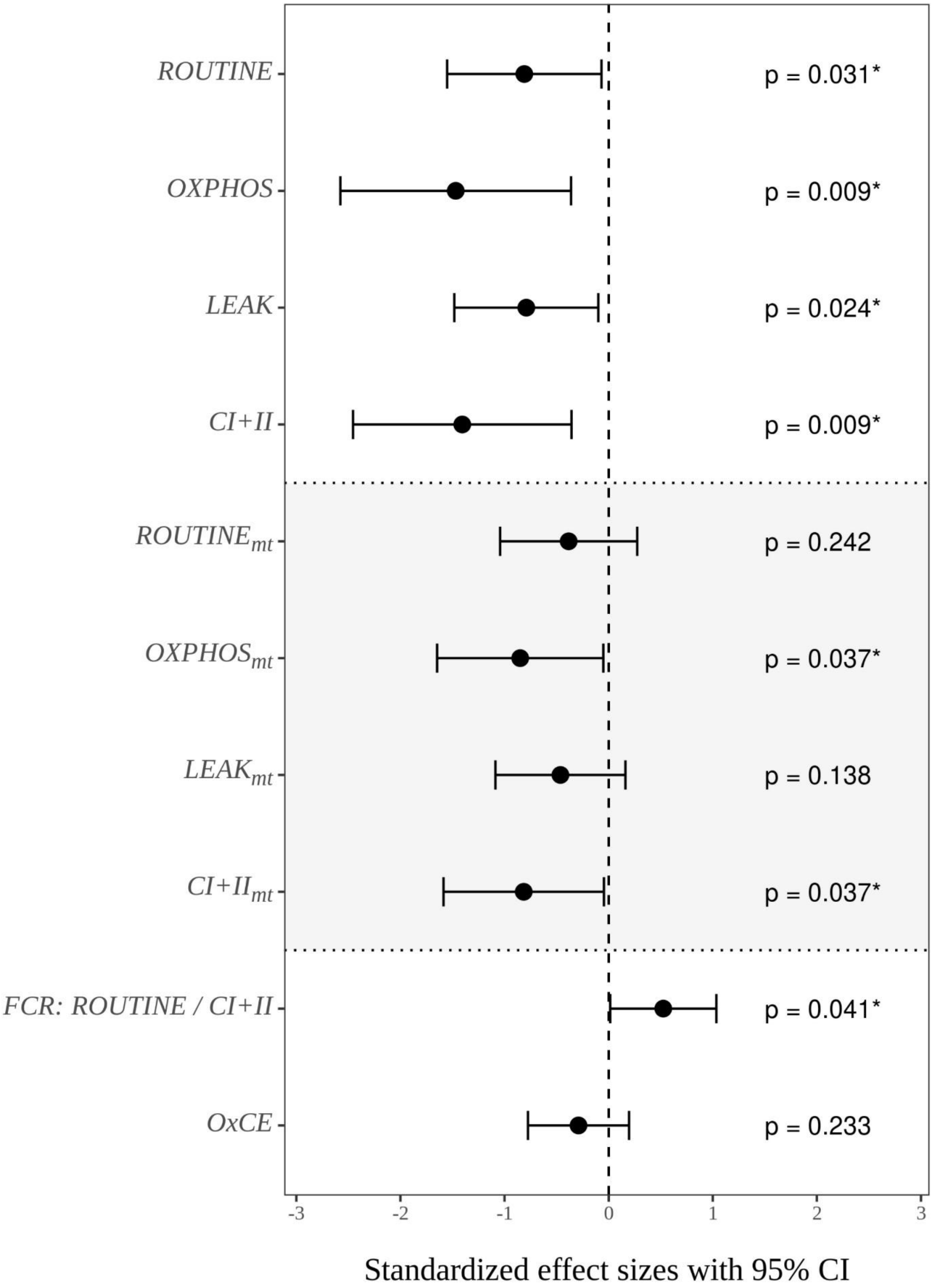
ROUTINE
OXPHOS
LEAK
CI+II
ROUTINE$_{mt}$
OXPHOS$_{mt}$
LEAK$_{mt}$
CI+II$_{mt}$
FCR: ROUTINE / CI+II
OxCE
p = 0.031*
p = 0.009*
p = 0.024*
p = 0.009*
p = 0.242
p = 0.037*
p = 0.138
p = 0.037*
p = 0.041*
p = 0.233
-3
-2
-1
0
1
2
3
Standardized effect sizes with 95% CI

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

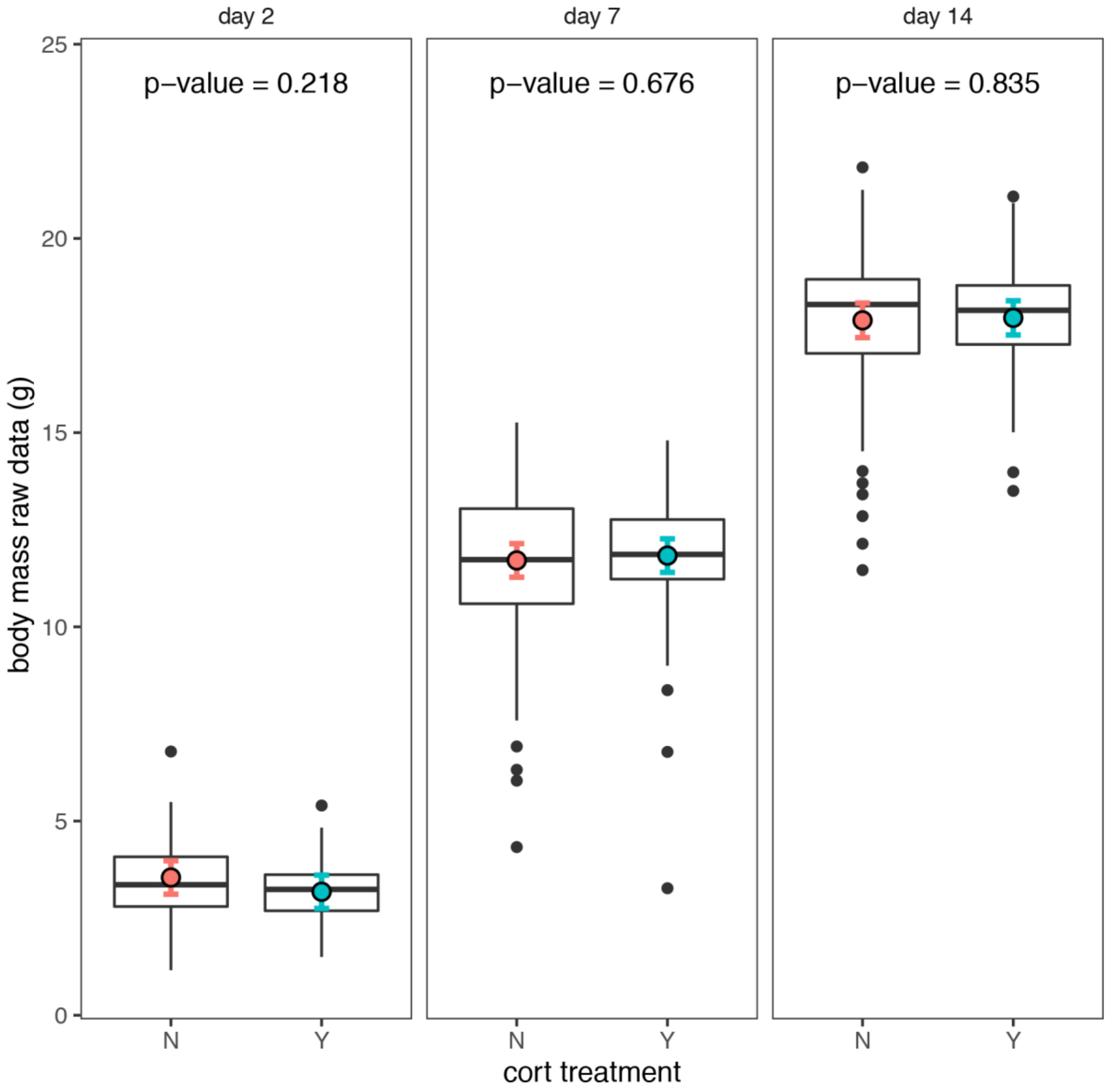

Fig.5: Effects of prenatal CORT treatment on postnatal body mass growth. Raw data distribution is plotted (d2: $n_{CORT/non\text{-}CORT}$ = 129/ 133; d7: $n_{CORT/non\text{-}CORT}$ = 123 / 128; d14: $n_{CORT/non\text{-}CORT}$ = 105/100 individuals) and least square means of statistical model presented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

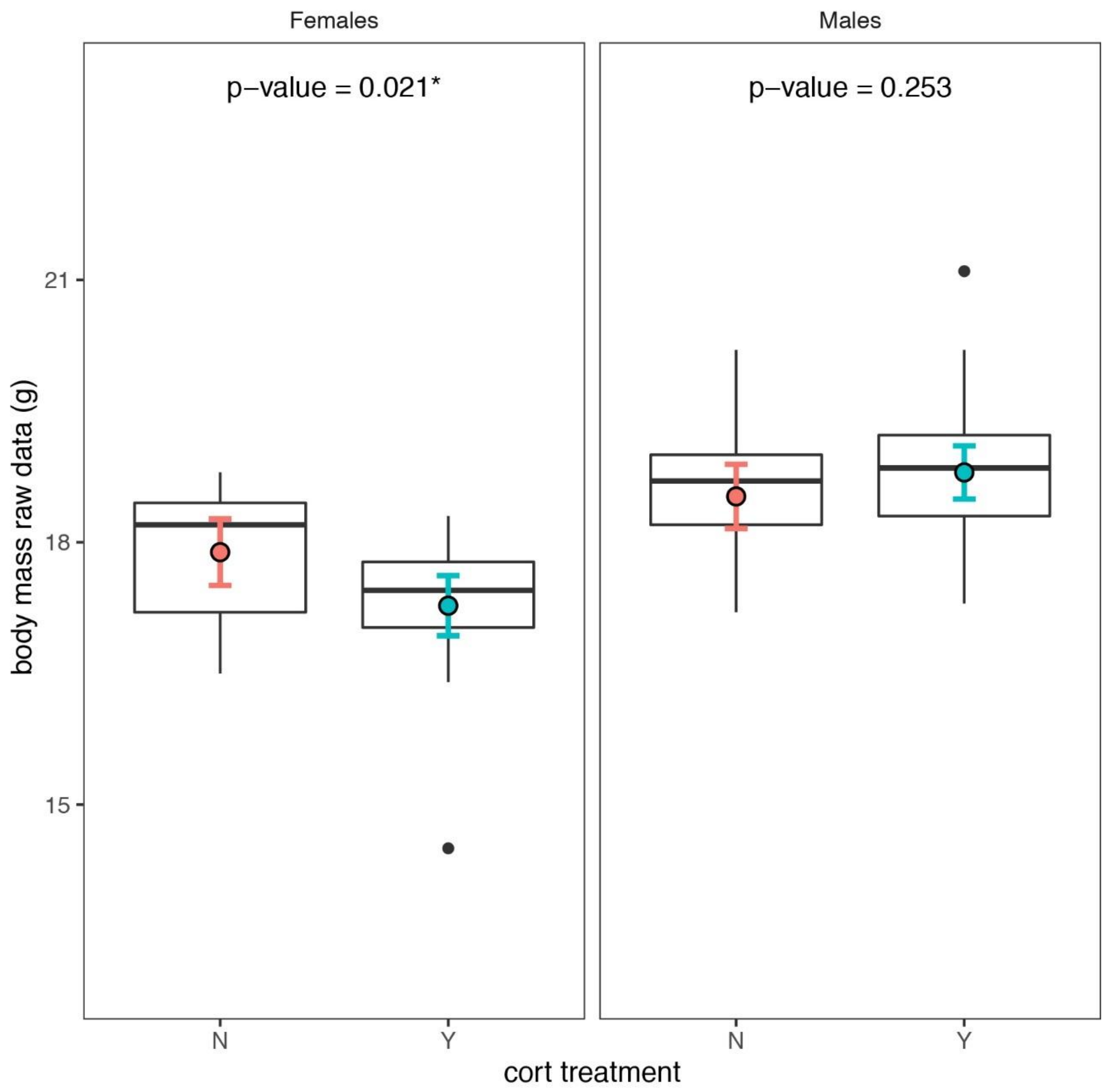

Fig.6: Effects of prenatal CORT treatment and sex on juvenile body mass. Raw data distribution is plotted (Females: $n_{CORT/non\text{-}CORT}$ = 26/19; Males: $n_{CORT/non\text{-}CORT}$ = 32/21 individuals) and least square means of statistical model presented as colored dots, with their 95% confidence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

Table 1: Results of generalized linear mixed model (gamma distribution with log-link) testing the effect of age and prenatal hormonal treatments on mitochondrial density (i.e. mtDNAcn; d7: n = 99 observations, d14: n = 93 observations, Juv: n = 37 observations; N = 100 individuals). Model estimates are reported with their 95% confidence intervals. Chick ID (ring) and nest box of origin (nestbox) were included as random effects in the model. σ2 = within-group variance; τ00 = between-group variance. Sample size along with marginal (fixed effects only) and conditional (fixed and random effects) R^2 are presented.

		mtDNAcn	
Predictors	***Estimates***	***CI***	***p***
(Intercept)	5.80	4.66 – 7.22	**<0.001**
age [day14]	0.54	0.48 – 0.61	**<0.001**
age [juvenile]	0.15	0.12 – 0.17	**<0.001**
CORT [Y]	0.89	0.71 – 1.11	0.286
TH [Y]	0.99	0.81 – 1.23	0.956
sex [M]	1.03	0.88 – 1.20	0.740
hatching date	1.07	0.96 – 1.19	0.221
brood size day 2	0.88	0.78 – 0.99	**0.036**
age [day14] * CORT [Y]	0.82	0.69 – 0.98	**0.028**
age [juvenile] * CORT [Y]	1.15	0.90 – 1.46	0.273
Random Effects			
σ2	0.10		
τ00 ring	0.02		
τ00 nestbox	0.03		
N ring	100		
N nestbox	48		
Observations	229		
Marginal R2 / Conditional R2	0.762 / 0.836		

Table 2: Results of linear mixed model testing the effect of age and prenatal hormonal treatments on mitochondrial respiration rates (corrected for mitochondrial density; d7: n = 46 observations, d14: n = 43 observations, N = 48 individuals)*. Chick ID (ring) was included as random effect in the model. σ2 = within-group variance; τ00 = between-group variance. Sample size along with marginal (fixed effects only) and conditional (fixed and random effects)* R^2 *are presented.*

	ROUTINE			***LEAK***			***OXPHOS***			***CI + CII***		
Predictors	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*
(Intercept)	0.32	0.12 – 0.52	**0.002**	0.32	0.10 – 0.53	**0.004**	0.14	-0.73 – 1.01	0.753	0.45	-0.57 – 1.46	0.387
CORT [Y]	-0.04	-0.10 – 0.02	0.236	-0.05	-0.12 – 0.01	0.131	-0.30	-0.56 – -0.03	**0.029**	-0.35	-0.66 – -0.03	**0.030**
TH [Y]	0.02	-0.04 – 0.08	0.448	0.02	-0.04 – 0.09	0.501	-0.05	-0.32 – 0.22	0.723	-0.03	-0.34 – 0.29	0.869
sex [M]	0.03	-0.04 – 0.09	0.419	0.07	0.003 – 0.144	**0.040**	0.09	-0.20 – 0.38	0.541	0.16	-0.17 – 0.50	0.341
age [day7]	0.09	0.04 – 0.15	**0.001**	0.04	-0.02 – 0.10	0.193	0.25	0.04 – 0.47	**0.021**	0.29	0.03 – 0.55	**0.028**
mtDNAcn	0.05	0.03 – 0.06	**<0.001**	0.04	0.02 – 0.05	**<0.001**	0.18	0.12 – 0.24	**<0.001**	0.22	0.15 – 0.29	**<0.001**
hatching date	0.0005	-0.002 – 0.003	0.641	-0.0002	-0.003 – 0.002	0.882	0.02	0.01 – 0.03	**0.001**	0.02	0.01 – 0.03	**0.004**
brood size day 2	-0.01	-0.03 – 0.01	0.194	-0.01	-0.03 – 0.01	0.467	-0.02	-0.10 – 0.05	-0.03	-0.03	-0.11 – 0.06	0.541
Random effects												
σ2	0.01			0.01			0.12			0.18		
τ00 ring	0.0005			0.01			0.13			0.17		
N ring	48			48			48			48		
Observations	89			89			89			89		
Marginal R2 / Conditional R2	0.639/0.766			0.467/0.627			0.651/0.829			0.647/0.816		

Table 3: Results of linear mixed model testing the effect of age and prenatal hormonal treatments on mitochondrial maximum capacity usage (i.e. $FCR_{ROUTINE/CI+II}$) and OXPHOS coupling efficiency (i.e. OxCE; d7: n = 46 observations, d14: n = 43 observations, N = 48 individuals). Chick ID (ring) was included as a random effect in the model. σ2 = within-group variance; τ00 = between-group variance. Sample size along with marginal (fixed effects only) and conditional (fixed and random effects) R^2 are presented.

	$FCR_{ROUTINE/CI+II}$			***OxCE***		
Predictors	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*
(Intercept)	0.305	0.256 – 0.354	**<0.001**	0.715	0.659 – 0.771	**<0.001**
CORT [Y]	0.017	0.002 – 0.032	**0.029**	-0.010	-0.028 – 0.007	0.251
TH [Y]	0.012	-0.004 – 0.028	0.133	-0.012	-0.030 – 0.006	0.187
sex [M]	-0.007	-0.023 – 0.010	0.441	-0.013	-0.032 – 0.007	0.199
age [day7]	0.009	-0.004 – 0.022	0.169	0.023	0.007 – 0.039	**0.004**
hatching date	-0.001	-0.002 – -0.001	**<0.001**	0.001	0.001 – 0.002	**<0.001**
brood size day 2	-0.0002	-0.004 – 0.004	0.930	-0.001	-0.006 – 0.004	0.676
Random Effects						
σ2	0.001			0.0014		
τ00 ring	0.0002			0.0001		
N ring	48			48		
Observations	89			89		
Marginal R2/Conditional R2	0.299/0.398			0.292/0.349		

Table 4: Results of linear mixed model testing the effect of age and prenatal hormonal treatments on body mass during the rearing period. Chick (ring) and nest box (nestbox) identities were included as random effects in the model. σ2 = within-group variance; τ00 = between-group variance. Sample size along with marginal (fixed effects only) and conditional (fixed and random effects) R^2 are presented; day 2 (n = 262 observations), day 7 (n = 251 observations) and day 14 after hatching (n = 205 observations).

	Body mass		
Predictors	*Estimates*	*CI*	*p*
(Intercept)	6.05	4.37 – 7.73	**<0.001**
age [day7]	8.18	7.94 – 8.42	**<0.001**
age [day14]	14.36	14.09 – 14.62	**<0.001**
CORT [Y]	-0.39	-0.97 – 0.19	0.183
TH [Y]	-0.27	-0.80 – 0.27	0.330
hatching date	-0.04	-0.06 – -0.02	**<0.001**
brood size day 2	-0.01	-0.15 – 0.13	0.852
age [day7] * CORT [Y]	0.49	0.14 – 0.83	0.006
age [day14] * CORT [Y]	0.43	0.05 – 0.80	**0.025**
Random Effects			
σ2	0.98		
τ00 ring	0.25		
τ00 nestbox	0.84		
N ring	265		
N nestbox	52		
Observations	717		
Marginal R2 / Conditional R2	0.945 / 0.974		

Table S1. Results of linear mixed model testing the effect of prenatal hormonal treatments on body mass at day 2 post-hatching. Nest box identity (nestbox) was included as random effect in the model. σ2 = within-group variance; τ00 = between-group variance. Sample size along with marginal (fixed effects only) and conditional (fixed and random effects) R^2 are presented.

		Body mass day 2	
Predictors	*Estimates*	*CI*	*p*
(Intercept)	4.81	3.52 – 6.09	**<0.001**
CORT [Y]	-0.30	-0.73 – 0.12	0.165
TH [Y]	0.18	-0.25 – 0.62	0.403
brood size day 2	-0.02	-0.12 – 0.09	0.756
hatching date	-0.02	-0.04 – -0.01	**0.004**
Random effects			
σ2	0.27		
τ00 nestbox	0.53		
N nestbox	52		
Observations	262		
Marginal R2 / Conditional R2	0.119 / 0.705		

Table S2. Results of linear mixed models testing the effect of prenatal hormonal treatments on day 7: a. body mass gain (i.e. body mass at day 7 controlled for body mass at day 2); b. wing length (i.e. body size); and c. body condition (i.e. body mass corrected for body size). Nest box identity (nestbox) was included as random effect in the model. σ2 = within-group variance; τ00 = between-group variance. Sample size along with marginal (fixed effects only) and conditional (fixed and random effects) R^2 are presented.

a.	**Body mass day 7**		
Predictors	*Estimates*	*CI*	*p*
(Intercept)	6.32	4.38 – 8.27	**<0.001**
CORT [Y]	0.58	-0.02 – 1.18	0.056
TH [Y]	-0.27	-0.88 – 0.35	0.391
mass day 2	1.64	1.50 – 1.78	**<0.001**
brood size day 2	0.07	-0.08 – 0.22	0.373
hatching date	-0.01	-0.03 – 0.01	0.400
Random effects			
σ2	0.30		
τ00 nestbox	1.01		
N nestbox	49		
Observations	248		
Marginal R2 / Conditional R2	0.623 / 0.914		

b.	**Wing length day 7**		
Predictors	*Estimates*	*CI*	*p*
(Intercept)	24.08	19.75 – 28.42	**<0.001**
CORT [Y]	-0.60	-1.98 – 0.78	0.393
TH [Y]	-0.73	-2.10 – 0.65	0.300
brood size day 2	0.17	-0.20 – 0.53	0.377
hatching date	-0.08	-0.13 – -0.03	**0.004**
Random effects			
σ2	4.83		
τ00 nestbox	4.73		
N nestbox	49		
Observations	251		
Marginal R2 / Conditional R2	0.118 / 0.555		

c.	**Body mass day 7**		
Predictors	*Estimates*	*CI*	*p*
(Intercept)	3.84	2.28 – 5.40	**<0.001**
CORT [Y]	0.30	-0.10 – 0.70	0.140
TH [Y]	-0.04	-0.44 – 0.36	0.839
wing length day 7	0.44	0.40 – 0.48	**<0.001**
brood size day 2	-0.06	-0.17 – 0.04	0.233
hatching date	-0.01	-0.02 – 0.01	0.255
Random effects			
σ2	0.48		
τ00 nestbox	0.37		
N nestbox	49		
Observations	251		
Marginal R2 / Conditional R2	0.708 / 0.835		

Table S3. Results of linear mixed models testing the effect of prenatal hormonal treatments on day 14: a. body mass gain (i.e. body mass at day 14 controlled for body mass at day 7); b. wing length (i.e. body size); and c. body condition (i.e. body mass corrected for body size). Nest box identity (nestbox) was included as random effect in the model. σ2 = within-group variance; τ00 = between-group variance. Sample size along with marginal (fixed effects only) and conditional (fixed and random effects) R^2 are presented.

a.	**Body mass day 14**		
Predictors	*Estimates*	*CI*	*p*
(Intercept)	15.08	12.26 – 17.90	**<0.001**
CORT [Y]	0.24	-0.54 – 1.02	0.552
TH [Y]	0.15	-0.56 – 0.87	0.674
mass day 7	0.52	0.42 – 0.61	**<0.001**
brood size day 2	-0.15	-0.36 – 0.07	0.177
hatching date	-0.05	-0.08 – -0.01	**0.004**
Random effects			
σ2	0.61		
τ00 nestbox	1.37		
N nestbox	41		
Observations	204		
Marginal R2 / Conditional R2	0.385 / 0.811		

b.	**Wing length day 14**		
Predictors	*Estimates*	*CI*	*p*
(Intercept)	58.18	52.30 – 64.06	**<0.001**
CORT [Y]	-0.17	-2.01 – 1.66	0.852
TH [Y]	-1.13	-2.87 – 0.61	0.204
brood size day 2	0.20	-0.30 – 0.70	0.430
hatching date	-0.14	-0.21 – -0.07	**<0.001**
Random effects			
σ2	5.99		
τ00 nestbox	6.99		
N nestbox	41		
Observations	204		
Marginal R2 / Conditional R2	0.224 / 0.642		

c.	**Body mass 14**		
Predictors	*Estimates*	*CI*	*p*
(Intercept)	10.11	6.34 – 13.88	**<0.001**
CORT [Y]	0.32	-0.41 – 1.06	0.390
TH [Y]	0.06	-0.64 – 0.75	0.876
wing length day 14	0.21	0.16 – 0.26	**<0.001**
brood size day 2	-0.19	-0.39 – 0.01	0.063
hatching date	-0.03	-0.06 – -0.001	**0.042**
Random effects			
σ2	0.76		
τ00 nestbox	1.18		
N nestbox	41		
Observations	204		
Marginal R2 / Conditional R2	0.339 / 0.740		

Table S4. Results of linear mixed models testing the effect of prenatal hormonal treatments on juvenile: a. body mass; b. wing length (i.e. body size); and c. body condition (i.e. body mass corrected for body size). Nest box identity (nestbox) was included as random effect in the model. σ2 = within-group variance; τ00 = between-group variance. Sample size along with marginal (fixed effects only) and conditional (fixed and random effects) R^2 are presented.

a.	**Body mass juvenile**		
Predictors	*Estimates*	*CI*	*p*
(Intercept)	18.32	17.03 – 19.61	**<0.001**
sex [M]	0.64	0.17 – 1.10	**0.009**
CORT [Y]	-0.61	-1.11 – -0.12	**0.019**
TH [Y]	-0.11	-0.48 – 0.26	0.569
hatching date	-0.01	-0.03 – 0.02	0.548
sex [M] * CORT [Y]	0.89	0.29 – 1.49	**0.005**
Random effects			
σ2	0.49		
τ00 nestbox	0.11		
N nestbox	36		
Observations	98		
Marginal R2 / Conditional R2	0.398 / 0.509		

b.	**Wing length juvenile**		
Predictors	*Estimates*	*CI*	*p*
(Intercept)	76.59	74.15 – 79.02	**<0.001**
CORT [Y]	0.13	-0.63 – 0.90	0.731
TH [Y]	-0.05	-0.82 – 0.73	0.904
sex [M]	2.70	2.20 – 3.21	**<0.001**
hatching date	-0.01	-0.06 – 0.03	0.508
Random effects			
σ2	1.33		
τ00 nestbox	0.74		
N nestbox	36		
Observations	98		
Marginal R2 / Conditional R2	0.471 / 0.660		

c.	**Body mass juvenile**		
Predictors	*Estimates*	*CI*	*p*
(Intercept)	12.40	3.60 – 21.20	**0.007**
sex [M]	0.41	-0.16 – 0.98	0.161
CORT [Y]	-0.64	-1.13 – -0.14	**0.015**
TH [Y]	-0.11	-0.48 – 0.27	0.573
wing length juvenile	0.08	-0.04 – 0.19	0.186
hatching date	-0.01	-0.03 – 0.02	0.620
sex [M] * CORT [Y]	0.91	0.31 – 1.51	**0.004**
Random effects			
σ2	0.48		
τ00 nestbox	0.11		
N nestbox	36		
Observations	98		
Marginal R2 / Conditional R2	0.407 / 0.520		