Plastic but repeatable: rapid adjustments of mitochondrial function and density during reproduction in a wild bird species

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Running Head: Mitochondrial plasticity & repeatability

Abstract

Most of the energy fluxes supporting animal performance flow through mitochondria. Hence, inter-individual differences in performance might be rooted in inter-individual variations in mitochondrial function and density. Furthermore, because the energy required by an individual often changes across life stages, mitochondrial function and density are also expected to show within-individual variation (*i.e.* plasticity). No study so far has repeatedly measured mitochondrial function and density in the same individuals to simultaneously test for within-individual repeatability and plasticity of mitochondrial traits. Here, we repeatedly measured mitochondrial DNA copy number (a proxy of density) and respiration rates from blood cells of female pied flycatchers (*Ficedula hypoleuca*) at the incubation and chick-rearing stages. Mitochondrial density and respiration rates were all repeatable (R=[0.45; 0.80]), indicating high within-individual consistency in mitochondrial traits across life-history stages. Mitochondrial traits were also plastic, showing a quick (*i.e.* 10 days) down-regulation from incubation to chick-rearing in mitochondrial density, respiratory activity, and cellular regulation by endogenous substrates and/or ATP demand. These downregulations were partially compensated by an increase in mitochondrial efficiency at the chick-rearing stage. Therefore, our study provides clear evidence for both short-term plasticity and high within-individual consistency in mitochondrial function and density during reproduction in a wild bird species.

Keywords: mitochondria, cellular respiration, metabolism, repeatability, plasticity, physiology/life-history nexus

Introduction

Through oxidative phosphorylation, mitochondria produce more than 90% of the energy fuelling cellular and therefore individual activities [1]. Hence, variation in mitochondrial function and density (*i.e.* amount of mitochondria per cell) have been suggested to account for inter-individual variation in performance [2-4], and in turn individual quality, with high quality individuals consistently outperforming low quality ones. Furthermore, because the energy required by an individual often changes across life stages and contexts (*e.g.* reproduction, hypoxia, cold exposure; [5-7]), mitochondrial function and density are also expected to show within-individual variation (*i.e.* plasticity). Although both hypotheses have received independent support, no study so far has repeatedly measured mitochondrial function and density in the same individuals to simultaneously test for within-individual repeatability and plasticity of mitochondrial traits. Indeed, measures of mitochondrial function almost exclusively rely on invasive sampling that usually prevents repeated sampling on the same individuals [8]. Yet, repeated sampling using muscle biopsies has previously been conducted in large animals including humans, showing for instance a mitochondrial plasticity in response to physical exercise [9], but without evaluating the within-individual repeatability in mitochondrial traits.

To fill this knowledge gap, we repeatedly measured mitochondrial function and density from blood cells of free-living female pied flycatchers (*Ficedula hypoleuca*) at the incubation and chick-rearing stages. We previously demonstrated that birds possess functional mitochondria in their red blood cells [10], and thus that mitochondrial function can be repeatedly measured from the same individuals using a minimally invasive repeated blood sampling approach [11]. Hence, our design allowed testing whether mitochondrial traits are repeatable within individuals, with for instance some individuals having

consistently more mitochondria with greater respiration rates or efficiencies than others, which is an underlying assumption of the individual quality hypothesis. Furthermore, the reproductive cycle of female birds is well known to be divided into egg-laying, incubation and chick-rearing stages that can differ in their energy constraints [12]. Hence, our design also allowed testing whether mitochondrial traits can quickly respond to changes in energy constraints, supporting the hypothesis that mitochondrial traits are plastic.

Material & methods

Fieldwork

Pied flycatcher (*Ficedula hypoleuca*) females breeding in artificial nestboxes in Ruissalo island (Turku, Finland, 60°26.055′N, 22°10.391′E) were captured twice during their reproduction in 2018. Females were captured a first time at day 8 of incubation, and then recaptured at day 7 after hatching (hereafter referred as chick-rearing), leaving a time interval of 10.1 ± 0.1 days between the two sampling occasions. Bird weight was recorded (± 0.1g) and a small blood sample (*i.e.* 25 to 50µL) was taken by puncturating the wing vein with a 26*G* sterile needle and collecting blood using a heparinised capillary (see supplementary methods in ESM for details on blood processing).

Mitochondrial respiration of permeabilized blood cells

We analyzed mitochondrial respiration using a high-resolution respirometry system (Oroboros Instruments, Innsbruck, Austria) at 40°C, adapting the protocol we described in [11] by permeabilizing blood cells in order to get better insights on mitochondrial function (see supplementary methods in ESM and Fig S1). We evaluated 6 mitochondrial respiration rates: 1) *ROUTINE* respiration: endogenous cellular respiration before permeabilization; 2) *complex I*

respiration fuelled by exogenous complex I substrates and ADP; 3) *complex I+II* respiration fuelled by exogenous complex I + II substrates and ADP; 4) *complex II* contribution to respiration fuelled by exogenous complex I and II substrates and ADP; 5) *LEAK* respiration due mostly to mitochondrial proton leak (*i.e.* not producing ATP but dissipating heat); and 6) *OXPHOS* respiration that is supporting ATP synthesis through oxidative phosphorylation. We also calculated 3 mitochondrial *flux control ratios* (FCRs), namely $FCR_{LEAK/I+II}$ indicating the proportion of mitochondrial respiration being linked to proton leak (*i.e.* an indicator of mitochondrial inefficiency to produce ATP), $FCR_{ROUTINE/I+II}$ indicating the proportion of maximal capacity being used under endogenous cellular conditions (*i.e.* reflecting the cellular control of mitochondrial respiration by endogenous ADP/ATP turnover and substrate availability), and $FCR_{I/I+II}$ indicating the relative contribution of complex I to total respiration.

Mitochondrial density

As an indicator of mitochondrial density, we estimated relative mitochondrial DNA copy number (hereafter referred as *mtDNAcn*) by measuring the amount of mitochondrial DNA relative to the nuclear DNA using a relative qPCR protocol routinely used in humans (*e.g.* [13]) and recently adapted in wild birds [14]. Detailed methodology is available in the supplementary methods provided in ESM.

Statistical analysis

Statistical tests were conducted using *R* 3.4.2. We had measures of body mass and mitochondrial density for 40 females captured both at incubation and chick-rearing. However, due to the strong logistical constraints of working with fresh blood samples for the analysis of mitochondrial respiration, we only had measures of mitochondrial respiration rates for 33

females at incubation and 13 females at the chick-rearing stage. Mitochondrial respiration rates were correlated with mitochondrial density (Pearson correlations: all $r > 0.26$ and $p < 0.10$). Therefore, we analyzed both cellular respiration rates (O_2 consumption normalized per cell number, *e.g. LEAK*) and respiration rates corrected for mitochondrial density (residuals of the regressions between cellular respirations rates and *mtDNAcn*, *e.g.* $LEAK_{mt}$) since variations in respiration rates at the cellular level can be explained both by how mitochondria enclosed in those cells are respiring and by the density of mitochondria per cell. Within-individual differences between breeding stages were analyzed using paired t-tests and presented as effect size and 95% C.I. following [15]. Since within-individual changes associated with reproductive stage could be confounded by other factors related to time (*e.g.* changes in weather, instrument drift), we also performed an additional between-individual comparison using unpaired t-tests and associated effect size and 95% C.I following [15]. Specifically, we compared the 13 females sampled at chick-rearing to 13 different females sampled simultaneously (average sampling date for both groups = 15th June), but during incubation. Finally, we evaluated adjusted within-individual repeatability (*i.e.* adjusted for the effects of breeding stage as a fixed factor) and the associated 95% C.I. using the *rptR* package [16].

Results

At the within-individual level, body mass, mtDNA copy number, and mitochondrial/cellular respirations rates (*i.e.* after controlling or not for mtDNA copy number) all significantly decreased from the incubation to the chick-rearing stage (Fig 1). Females also had more efficient mitochondria during chick rearing (lower $FCR_{LEAK/I+II}$) and used less of their mitochondrial maximal capacity (lower $FCR_{R/I+II}$), but the relative contribution of complex I to respiration ($FCR_{I/I+II}$) was not significantly affected (Fig 1). The

between-individual analyses revealed the exact same pattern, although some of the differences became non-significant ($LEAK_{mt}$, $FCR_{LEAK/I+II}$, $FCR_{R/I+II}$; Fig S2).

Despite the major short-term changes observed in mitochondrial traits from incubation to chick-rearing, mitochondrial density and respiration rates were moderately to highly repeatable within an individual, even after accounting for variations in mitochondrial density (Fig 2).

Discussion

Using repeated measures of mitochondrial traits from the same individual pied flycatchers sampled during incubation and chick-rearing, our study demonstrates that variation in mitochondrial respiration and density is significantly repeatable, as well as significantly plastic within individuals. Individuals with high mitochondrial density and respirations during incubation also had higher values during chick-rearing, and this even if within each individual, mitochondrial traits in blood cells were quickly down-regulated from incubation to chick-rearing. The finding that mitochondrial traits are repeatable provides an important pre-requisite for the hypothesis that repeatable variation in individual performance and quality may be explained by inter-individual variation in mitochondrial traits. Our results also suggest that quick (*i.e.* 10 days) adjustments of cellular bioenergetics (at least in blood cells) can occur during reproduction, most likely in response to differences in energy constraints between breeding stages [12].

Currently, we still know very little about how consistent over time are mitochondrial traits measured in the same individuals, and whether variation in mitochondrial traits measured in one tissue mirrors what is happening in other tissues. We have already shown elsewhere that red blood cell mitochondrial traits are moderately correlated with mitochondrial traits in other tissues ([11] in king penguin, AS unpublished results in Japanese quails), and thus in this study we focused on the first knowledge gap by testing within-individual repeatability in mitochondrial traits over an interval of 10 days. Our results show that mitochondrial traits in blood cells were moderately to highly repeatable (mean [min-max] R values = 0.63 [0.45-0.80]) within-individuals, to an extent being similar to what we found for female body mass in our study (R = 0.58). Interestingly, the repeatability estimates for mitochondrial traits were also in the range of what has been reported by a meta-analysis

on whole animal metabolic rates (R = 0.57 [17]). Yet, we have to keep in mind that the sampling interval was short (*i.e.* 10 days), and that within-individual repeatability is likely to decrease with increasing duration between sampling points. Significant repeatability could be explained by genetic differences between individuals in genes coding mitochondrial proteins (*e.g.* [18]), but also by potential long-lasting effects of early-life conditions on mitochondrial function [19]. Although within-individual repeatability estimates establish the upper limit for heritability of mitochondrial traits, almost everything remains to be done to understand the relative importance of genetics *vs.* environmental effects in determining mitochondrial traits in wild populations, and to unravel the relationships between mitochondrial and fitness-related traits. This information is essential to shed light on the importance of mitochondria in shaping variation in individual quality and animal life histories.

Our longitudinal study design also allowed testing for plasticity *per se* in mitochondrial traits by measuring the same individuals under two different environmental conditions rather than, as usually done in the field of mitochondrial biology, by measuring different individuals kept in different environmental conditions. Our results show that both mitochondrial density (*i.e.* estimated as mtDNA copy number) and respiration rates decreased within an individual from incubation to chick-rearing, thus suggesting a down-regulation in cellular metabolism, at least in blood cells. This within-individual down-regulation of cellular metabolism was also confirmed at the between-individual level (Fig S2), thereby ruling out alternative explanations linked for instance to consistent changes in environmental conditions between sampling occasions. The decreases in respiration rates were only moderately explained by changes in mitochondrial density since these decreases remained of moderate to large effect size even after controlling for differences in

191 mitochondrial density. It suggests that female pied flycatchers quickly (10 days) decreased
192 the abundance of respiratory complexes per mitochondria, and to a lesser extent (*i.e.*
193 smaller effect size) the number of mitochondria per cell between incubation and chick-
194 rearing stages. Additionally, mitochondria were also working at a slower pace under
195 endogenous cellular conditions relative to the maximal capacity (*i.e.* lower $FCR_{R/I+II}$),
196 suggesting that the control of mitochondrial respiration by endogenous substrates
197 availability and/or ATP demand was also tighter during chick-rearing. Finally, we found a
198 decrease in the relative proton leak ($FCR_{L/I+II}$) between incubation and chick-rearing. It
199 suggests that individuals might have increased their mitochondrial efficiency (at least in
200 blood cells), which could potentially carry an oxidative cost [20]. Females are likely more
201 energy-constrained during the chick-rearing stage, and therefore increasing mitochondrial
202 efficiency could be advantageous to maximize short-term performance despite potential
203 delayed costs linked to oxidative stress. The higher relative respiration linked to proton leak
204 during incubation could also potentially be related to the higher need for heat
205 production/dissipation during this breeding stage (*i.e.* to keep the eggs warm) than during
206 chick-rearing. Altogether, our results suggest that mitochondrial adjustments in blood cells
207 occur at 4 different levels (*i.e.* density, respiration, endogenous regulation and coupling),
208 and thus that studies on mitochondrial function should carefully consider these 4 levels of
209 regulation. Indeed, studies using isolated mitochondria are likely to miss effects related to
210 mitochondrial density and to cellular regulation by endogenous substrates and/or ATP
211 demand. Studies using permeabilized tissues/cells will not be able to tease apart the effects
212 of mitochondrial density and function if not assessing separately mitochondrial density, and
213 could miss effects linked to cellular regulation by endogenous substrates and/or ATP
214 demand if endogenous respiration (*i.e. ROUTINE*) is not assessed. While more research is

needed to further our understanding of the significance of mitochondria in blood cells at the organismal level, our study demonstrate that using blood cells could be a promising approach to study the contribution of mitochondrial traits in shaping individual quality and responses to environmental changes.

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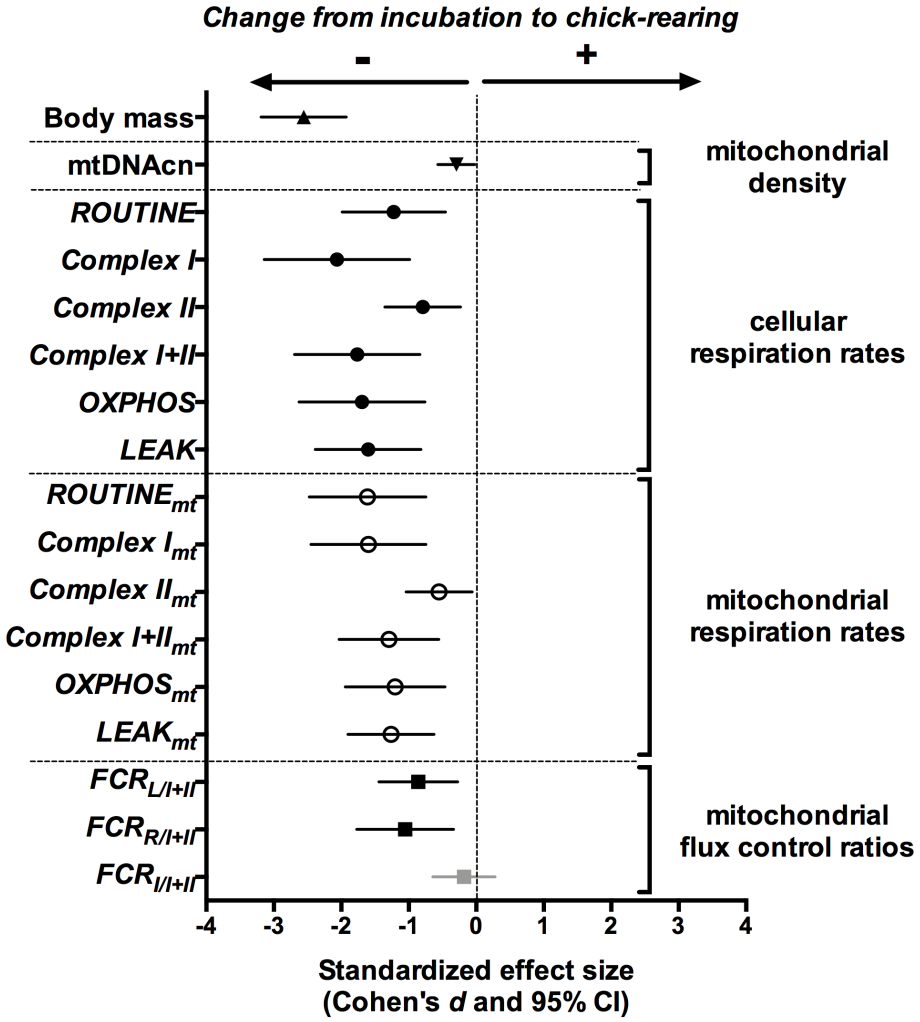
239 **Figures**



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241 **Fig 1**: **Within-individual changes between incubation and chick-rearing stages in body**
242 **mass, mitochondrial copy number, respiration rates and flux control ratios in female pied**
243 **flycatchers.** Standardized effect size *(Cohen's d*) are reported with their 95% confidence
244 interval. Significant differences between breeding stages are presented in black and non-
245 significant ones in grey. For respiration rates, we tested both the effects on cellular
246 mitochondrial respiration (*e.g. LEAK*), therefore including effects linked both to
247 mitochondrial function and density, and the effects after correcting for mitochondrial
248 density (*i.e.* using regression residuals; labelled *e.g.* $LEAK_{mt}$). Detailed information on
249 parameters is given in the method section and ESM.

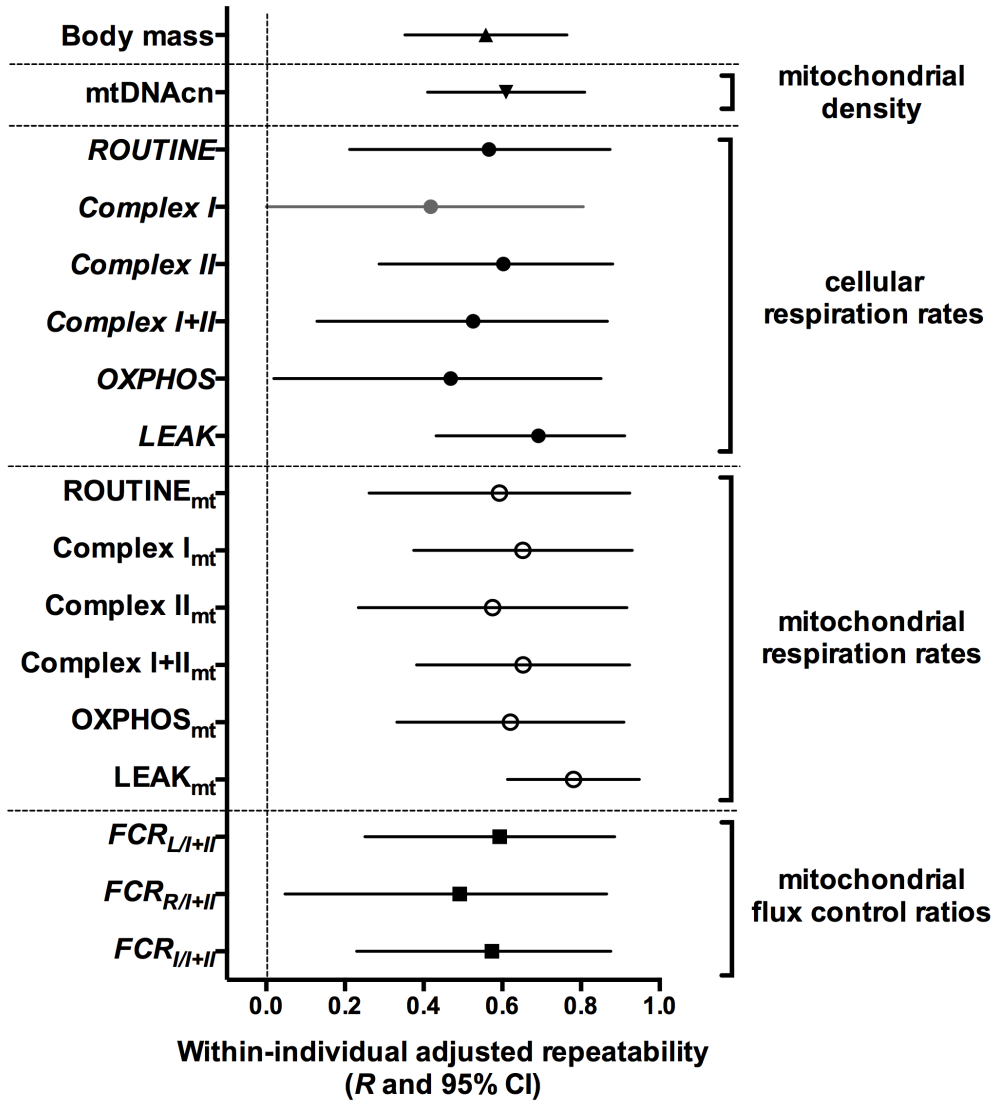

Fig 2: **Within-individual adjusted repeatability (*i.e.* consistency) in body mass, mitochondrial density, respiration rates and flux control ratios between incubation and chick-rearing stages in female pied flycatchers.** Adjusted repeatability estimates *R* (*i.e.* adjusted for breeding stage fixed effect) are reported with their 95% confidence interval. Significant effects are presented in black and non-significant ones in grey. For mitochondrial respiration rates, we tested both the effects on cellular mitochondrial respiration (*e.g. LEAK*), therefore including effects linked both to mitochondrial function and density, and the effects after correcting for mitochondrial density (*i.e.* using regression residuals; labelled *e.g.* $LEAK_{mt}$). Detailed information on parameters is given in the method section and ESM.

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Electronic Supplementary Material (ESM)
ESM1: Supplementary methods

Plastic but repeatable: rapid adjustments of mitochondrial function and density during reproduction in a wild bird species

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Blood processing

Blood samples were kept cold in the field using ice packs before being transferred to the laboratory for mitochondrial analysis (< 2 hours from blood collection). Blood samples were centrifuged 5min at 2000*g* and plasma was removed before re-suspending the blood cells in 1mL of Mir06 buffer pre-equilibrated at 40°C (see below for Mir06 composition). Twenty µL of this solution were diluted in 1mL of PBS in order to count the number of cells per sample using an automatic cell counter (Bio-Rad TC20 cell counter), in order to normalize mitochondrial respiration rates to the number of cells being used in the assay. Avian red blood cells have an approximate lifespan of one month, meaning that most of the red blood cell pool will remain the same between samples collected 10 days apart.

Mitochondrial respiration of permeabilized blood cells.

Mitochondrial respiration of permeabilized blood cells was analyzed using a high-resolution respirometry system (Oroboros Instruments, Innsbruck, Austria) and a protocol adapted from Stier et al. (2017, *Methods Ecol Evol*). Blood cells were diluted in respiration buffer Mir06 (0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM Hepes, 110 mM sucrose, free fatty acid bovine serum albumin (1 g/L), catalase 280U/mL pH 7.1) and added in a closed chamber maintained at 40°C where O_2 consumption was recorded following a standard sequential substrate/inhibitor addition protocol. First endogenous respiration of intact blood cells (*i.e. ROUTINE*) was recorded before adding 5 $ng.mL^{-1}$ of digitonin to permeabilize the cells (to allow substrates and ADP to enter the cells). Substrates of complex I (P: pyruvate 5mM and M: malate 2mM) and a saturating amount of ADP (2mM) were added to stimulate mitochondrial respiration fuelled by complex I (hereafter referred as *complex I*). Substrate of complex II (S: succinate 10mM) was then added to stimulate mitochondrial respiration fuelled by both complexes I and II (hereafter referred as *complex I+II*). The difference between these two rates was then calculated to estimate the contribution of complex II to overall respiration (hereafter referred as *complex II*). ATP synthesis was then inhibited with 2.5 µM of oligomycin to estimate mitochondrial inefficiency being mostly linked to proton leak (hereafter referred as *LEAK*). The difference between *complex I+II* and *LEAK* was then calculated to estimate the contribution of oxidative phosphorylation (*i.e.* ATP synthesis) to maximal respiration, hereafter referred as *OXPHOS.* A sequential titration with 0.5µM of the uncoupler FCCP never stimulated respiration above values of *complex I+II* so was removed from the final protocol. Finally, we inhibited mitochondrial respiration using antimycin A (2.5µM), and this residual non-mitochondrial O_2 consumption was subtracted from the mitochondrial parameters described above. Respiration rates were expressed as pmol $O_2.s^{-1}.10^6$ $cells^{-1}$. Oxygen levels

within the chamber were maintained between 140 and 200µM of O_2 using 0.5µL injections of 200mM H_2O_2 between titration steps. Mitochondrial responses to this chemical titration are presented below in Fig S1. We also calculated 3 mitochondrial *flux control ratios* (FCRs), namely $FCR_{L/I+II}$ indicating the proportion of mitochondrial respiration being linked to proton leak (*i.e.* an indicator of mitochondrial inefficiency to produce ATP), $FCR_{R/I+II}$ indicating the proportion of maximal capacity being used under endogenous cellular conditions (*i.e.* reflecting the cellular control of mitochondrial respiration by endogenous ADP/ATP turnover and substrate availability), and $FCR_{I/I+II}$ indicating the relative contribution of complex I to total respiration. The low amount of blood being available with such small birds prevented us to estimate the technical repeatability in this study, but our previous results in king penguins revealed a high repeatability of the method (Stier et al. 2017, *Methods Ecol Evol*), and the high within-individual repeatability estimates reported here in the main text (Fig 2) confirms that our technical repeatability was high although not directly evaluated.

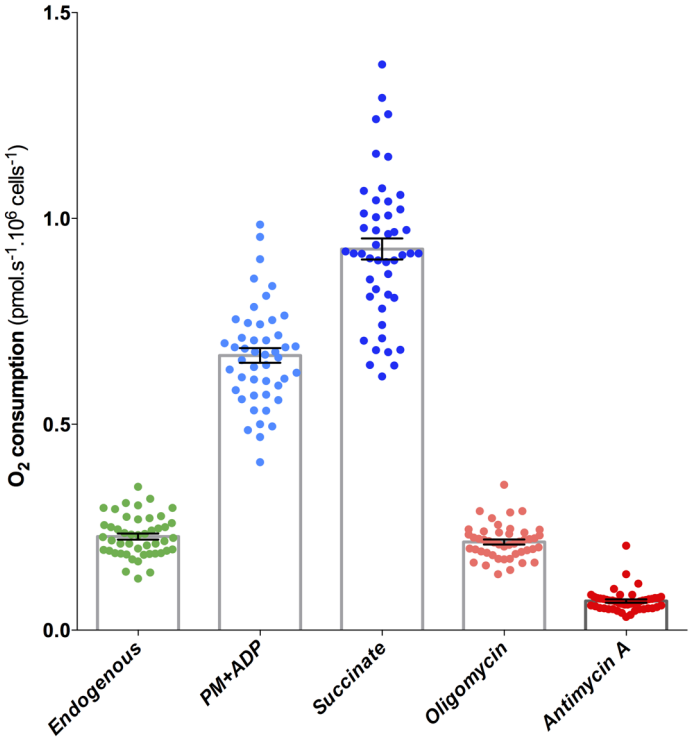

Fig S1: *In-vitro* cellular O_2 consumption of pied flycatcher red blood cells in response to a standard sequential substrate/inhibitor addition protocol. Cells have been permeabilized with 5ng.mL^{-1} of digitonin after recording the endogenous respiration of intact cells. Details about chemical additions are given in the supplementary methods described above. Data are presented as individual data points and mean ± SE.

Mitochondrial density

As an indicator of mitochondrial density, we estimated relative mtDNA copy number by measuring the amount of mitochondrial DNA relative to the nuclear DNA (hereafter referred as *mtDNAcn*) by qPCR on a 384-QuantStudio™ 12K Flex Real-Time PCR System (Thermo Fisher). DNA was extracted (using Macherey-Nagel Blood QuickPure spin columns) from blood samples and diluted to 1.2ng.μL^{-1}. We used RAG1 as a nuclear single-copy control gene (verified using a BLAST analysis on the collared flycatcher *Ficedula albicollis* genome; F: GCAGATGAACTGGAGGCTATAA, R: CAGCTGAGAAACGTGTTGATTC). 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 collared flycatcher genome (F: GGAGACGACCAAGTCTACAATG; R: TTTCCGAACCCTCCGATTATG). Melt curve and electrophoresis analyses confirmed that a single amplicon of the expected length was generated by PCR with the designed primers. For the qPCR assay, the reactions were performed in a total volume of 12µL including 6ng of DNA, primers at a final concentration of 200nM and 6µL of Absolute Blue qPCR Mix SYBR Green low ROX (Thermo Scientific). RAG1 and COI2 reactions were performed in triplicates on the same plates (4 plates in total); the qPCR conditions were: 15 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 60°C and 30s at 72°C. A DNA sample being a pool of DNA from 10 individuals was used as a reference sample and was included in triplicate on every plate. The efficiency of each amplicon was estimated from a standard curve of the reference sample ranging from 1.5 to 24ng. The mean reaction efficiencies were 106.8 ± 1.4% for RAG1 and 91.1 ± 0.7% for COI2. The relative mtDNA copy number (*mtDNAcn*) of each sample was calculated as $(1+Ef_{COI2})^{\Delta CqCOI2}/(1+Ef_{RAG1})^{\Delta CqRAG1}$; *Ef* being the amplicon efficiency, and ΔCq the difference in Cq-values between the reference sample and the focal sample. Intra-plate technical repeatability of *mtDNAcn* based on triplicates was 0.81 (95% C.I. [0.76-0.85]), and the inter-plate technical repeatability based on two repeated plates was 0.92 (95% C.I. [0.88-0.95]).

Electronic Supplementary Material (ESM)
ESM2: Supplementary results

Plastic but repeatable: rapid adjustments of mitochondrial function and density during reproduction in a wild bird species

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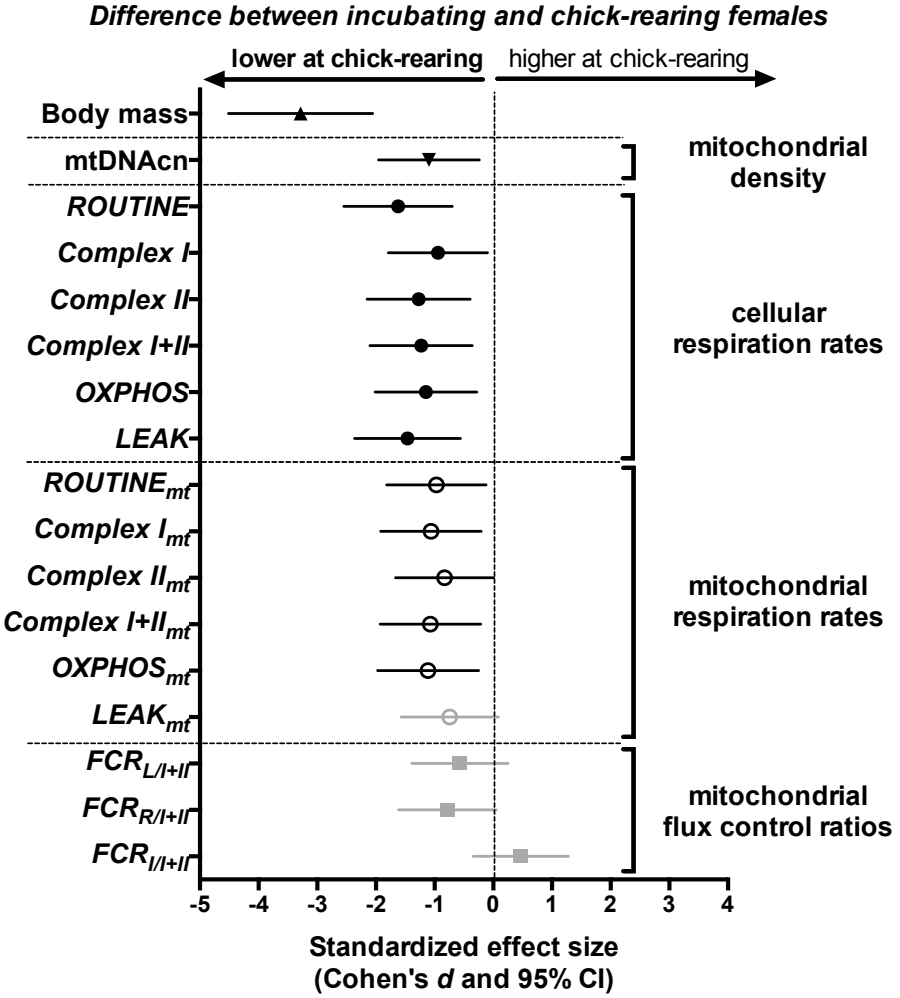

Fig S2: **Between-individual differences between incubation and chick-rearing stages in body mass, mitochondrial copy number, respiration rates and flux control ratios in female pied flycatchers matched for sampling date (15th of June in average for both N = 13 incubating and N = 13 chick-rearing females).** Standardized effect size *(Cohen's d*) are reported with their 95% confidence interval. Significant differences between breeding stages are presented in black and non-significant ones in grey. For respiration rates, we tested both the effects on cellular mitochondrial respiration (*e.g. LEAK*), therefore including effects linked both to mitochondrial function and density, and the effects after correcting for mitochondrial density (*i.e.* using regression residuals; labelled *e.g.* $LEAK_{mt}$). Detailed information on parameters is given in the method section and supplementary methods.

Electronic Supplementary Material (ESM)
ESM1: Supplementary methods

Plastic but repeatable: rapid adjustments of mitochondrial function and density during reproduction in a wild bird species

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Blood processing

Blood samples were kept cold in the field using ice packs before being transferred to the laboratory for mitochondrial analysis (< 2 hours from blood collection). Blood samples were centrifuged 5min at 2000*g* and plasma was removed before re-suspending the blood cells in 1mL of Mir06 buffer pre-equilibrated at 40°C (see below for Mir06 composition). Twenty µL of this solution were diluted in 1mL of PBS in order to count the number of cells per sample using an automatic cell counter (Bio-Rad TC20 cell counter), in order to normalize mitochondrial respiration rates to the number of cells being used in the assay. Avian red blood cells have an approximate lifespan of one month, meaning that most of the red blood cell pool will remain the same between samples collected 10 days apart.

Mitochondrial respiration of permeabilized blood cells.

Mitochondrial respiration of permeabilized blood cells was analyzed using a high-resolution respirometry system (Oroboros Instruments, Innsbruck, Austria) and a protocol adapted from Stier et al. (2017, *Methods Ecol Evol*). Blood cells were diluted in respiration buffer Mir06 (0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM Hepes, 110 mM sucrose, free fatty acid bovine serum albumin (1 g/L), catalase 280U/mL pH 7.1) and added in a closed chamber maintained at 40°C where O_2 consumption was recorded following a standard sequential substrate/inhibitor addition protocol. First endogenous respiration of intact blood cells (*i.e. ROUTINE*) was recorded before adding 5 $ng.mL^{-1}$ of digitonin to permeabilize the cells (to allow substrates and ADP to enter the cells). Substrates of complex I (P: pyruvate 5mM and M: malate 2mM) and a saturating amount of ADP (2mM) were added to stimulate mitochondrial respiration fuelled by complex I (hereafter referred as *complex I*). Substrate of complex II (S: succinate 10mM) was then added to stimulate mitochondrial respiration fuelled by both complexes I and II (hereafter referred as *complex I+II*). The difference between these two rates was then calculated to estimate the contribution of complex II to overall respiration (hereafter referred as *complex II*). ATP synthesis was then inhibited with 2.5 µM of oligomycin to estimate mitochondrial inefficiency being mostly linked to proton leak (hereafter referred as *LEAK*). The difference between *complex I+II* and *LEAK* was then calculated to estimate the contribution of oxidative phosphorylation (*i.e.* ATP synthesis) to maximal respiration, hereafter referred as *OXPHOS.* A sequential titration with 0.5µM of the uncoupler FCCP never stimulated respiration above values of *complex I+II* so was removed from the final protocol. Finally, we inhibited mitochondrial respiration using antimycin A (2.5µM), and this residual non-mitochondrial O_2 consumption was subtracted from the mitochondrial parameters described above. Respiration rates were expressed as pmol $O_2.s^{-1}.10^6$ $cells^{-1}$. Oxygen levels

within the chamber were maintained between 140 and 200µM of O_2 using 0.5µL injections of 200mM H_2O_2 between titration steps. Mitochondrial responses to this chemical titration are presented below in Fig S1. We also calculated 3 mitochondrial *flux control ratios* (FCRs), namely $FCR_{L/I+II}$ indicating the proportion of mitochondrial respiration being linked to proton leak (*i.e.* an indicator of mitochondrial inefficiency to produce ATP), $FCR_{R/I+II}$ indicating the proportion of maximal capacity being used under endogenous cellular conditions (*i.e.* reflecting the cellular control of mitochondrial respiration by endogenous ADP/ATP turnover and substrate availability), and $FCR_{I/I+II}$ indicating the relative contribution of complex I to total respiration. The low amount of blood being available with such small birds prevented us to estimate the technical repeatability in this study, but our previous results in king penguins revealed a high repeatability of the method (Stier et al. 2017, *Methods Ecol Evol*), and the high within-individual repeatability estimates reported here in the main text (Fig 2) confirms that our technical repeatability was high although not directly evaluated.

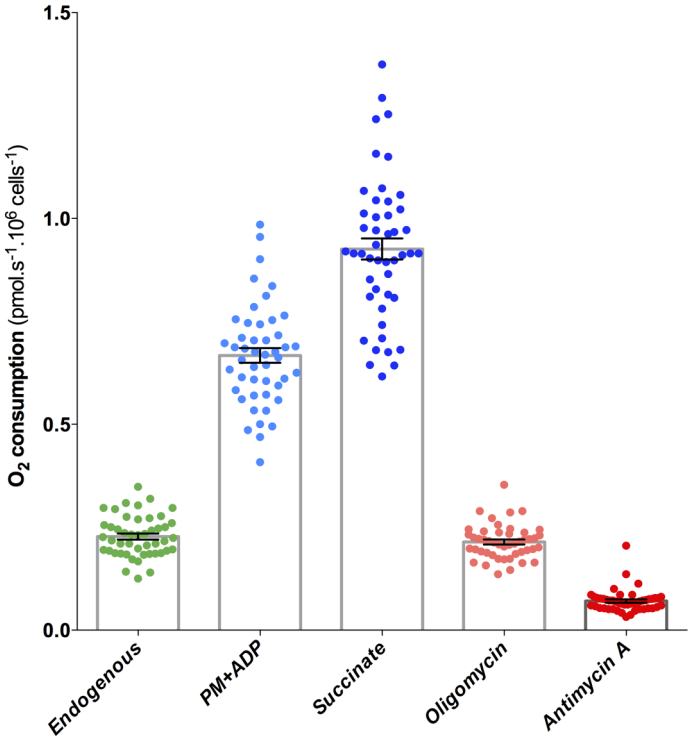

Fig S1: *In-vitro* cellular O_2 consumption of pied flycatcher red blood cells in response to a standard sequential substrate/inhibitor addition protocol. Cells have been permeabilized with 5ng.mL^{-1} of digitonin after recording the endogenous respiration of intact cells. Details about chemical additions are given in the supplementary methods described above. Data are presented as individual data points and mean ± SE.

Mitochondrial density

As an indicator of mitochondrial density, we estimated relative mtDNA copy number by measuring the amount of mitochondrial DNA relative to the nuclear DNA (hereafter referred as *mtDNAcn*) by qPCR on a 384-QuantStudio™ 12K Flex Real-Time PCR System (Thermo Fisher). DNA was extracted (using Macherey-Nagel Blood QuickPure spin columns) from blood samples and diluted to 1.2ng.μL^{-1}. We used RAG1 as a nuclear single-copy control gene (verified using a BLAST analysis on the collared flycatcher *Ficedula albicollis* genome; F: GCAGATGAACTGGAGGCTATAA, R: CAGCTGAGAAACGTGTTGATTC). 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 collared flycatcher genome (F: GGAGACGACCAAGTCTACAATG; R: TTTCCGAACCCTCCGATTATG). Melt curve and electrophoresis analyses confirmed that a single amplicon of the expected length was generated by PCR with the designed primers. For the qPCR assay, the reactions were performed in a total volume of 12µL including 6ng of DNA, primers at a final concentration of 200nM and 6µL of Absolute Blue qPCR Mix SYBR Green low ROX (Thermo Scientific). RAG1 and COI2 reactions were performed in triplicates on the same plates (4 plates in total); the qPCR conditions were: 15 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 60°C and 30s at 72°C. A DNA sample being a pool of DNA from 10 individuals was used as a reference sample and was included in triplicate on every plate. The efficiency of each amplicon was estimated from a standard curve of the reference sample ranging from 1.5 to 24ng. The mean reaction efficiencies were 106.8 ± 1.4% for RAG1 and 91.1 ± 0.7% for COI2. The relative mtDNA copy number (*mtDNAcn*) of each sample was calculated as $(1+Ef_{COI2})^{\Delta CqCOI2}/(1+Ef_{RAG1})^{\Delta CqRAG1}$; *Ef* being the amplicon efficiency, and ΔCq the difference in Cq-values between the reference sample and the focal sample. Intra-plate technical repeatability of *mtDNAcn* based on triplicates was 0.81 (95% C.I. [0.76-0.85]), and the inter-plate technical repeatability based on two repeated plates was 0.92 (95% C.I. [0.88-0.95]).

Electronic Supplementary Material (ESM)
ESM2: Supplementary results

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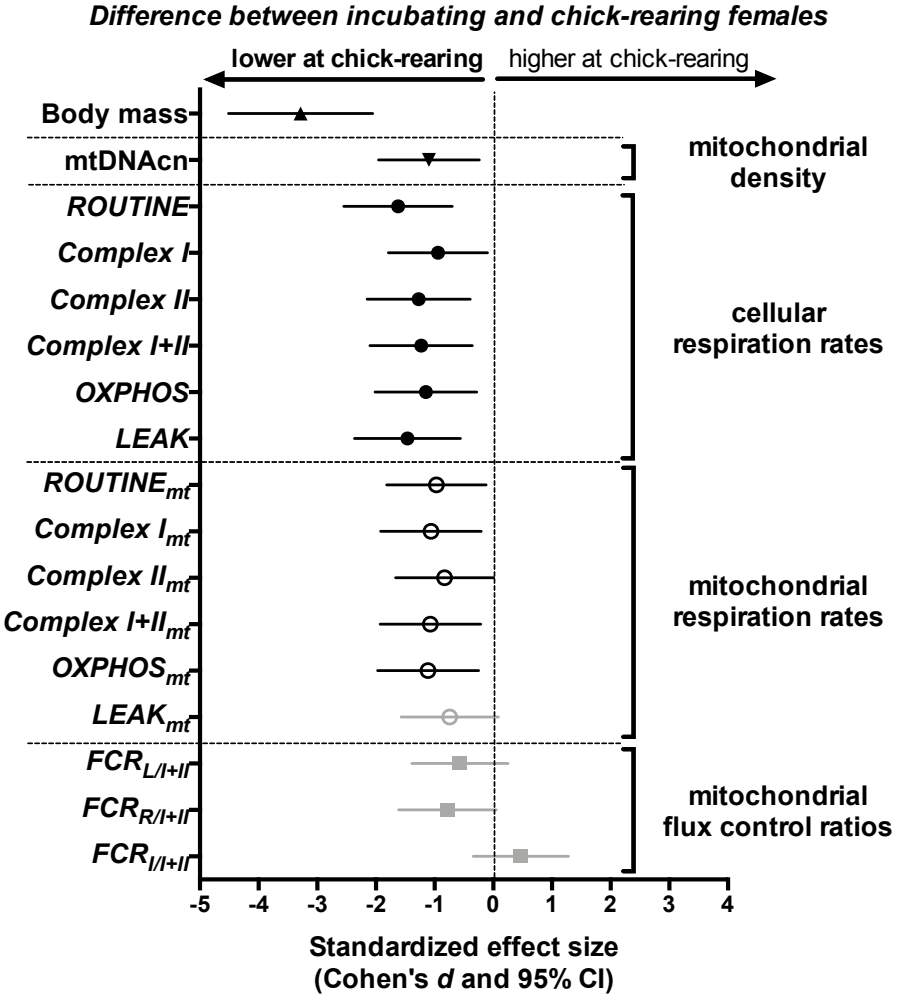

Fig S2: **Between-individual differences between incubation and chick-rearing stages in body mass, mitochondrial copy number, respiration rates and flux control ratios in female pied flycatchers matched for sampling date (15th of June in average for both N = 13 incubating and N = 13 chick-rearing females).** Standardized effect size *(Cohen's d*) are reported with their 95% confidence interval. Significant differences between breeding stages are presented in black and non-significant ones in grey. For respiration rates, we tested both the effects on cellular mitochondrial respiration (*e.g. LEAK*), therefore including effects linked both to mitochondrial function and density, and the effects after correcting for mitochondrial density (*i.e.* using regression residuals; labelled *e.g.* $LEAK_{mt}$). Detailed information on parameters is given in the method section and supplementary methods.