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Blood mitochondrial metabolism predicts flight performance without lasting effects of early-life thyroid hormones

Highlights

- Early-life thyroid hormones do not have lasting effects on adult mitochondrial metabolism
- Mitochondrial metabolism declines from growth to adulthood in zebra finches
- High intensity escape-flight is linked to higher mitochondrial metabolism
- Low intensity exploratory-like behavior is not linked to mitochondrial metabolism

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In brief

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Blood mitochondrial metabolism predicts flight performance without lasting effects of early-life thyroid hormones

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SUMMARY

Early-life conditions can shape adult phenotypes by influencing processes linked to energetic homeostasis. Thyroid hormones (THs) enhance mitochondrial electron transport chain capacity (ETS) in fledglings, but whether this effect is permanent and influences fitness-related traits is unclear. We tested whether ETS remains elevated in adult zebra finches treated with TH during development, predicting that higher ETS would be reflected in energetically demanding traits such as exploratory-like behavior and escape flight. Early-life TH did not alter adult erythrocyte mitochondrial aerobic metabolism. Mitochondrial aerobic metabolism decreased from the energetically demanding growth phase to the less demanding adulthood. Independent of the treatment, mitochondrial cellular metabolic rate and oxidative phosphorylation were positively associated with high-intensity escape flight but not with exploratory-like activity. These results do not support early-life TH as a driver of long-term metabolic or behavioral traits but instead show a link between blood mitochondrial metabolism and energetically costly avian flight performance.

INTRODUCTION

The regulation of metabolic energy production is critical during development. Early life is a period of particularly high energetic demands due to growth, and these demands are further amplified under adverse conditions.[1] This is relevant because challenging early-life environments can leave long-lasting marks on adult physiology and behavior, ultimately affecting survival and reproduction.[2,3] Mitochondria are key components in this context because they produce about 90% of the cellular adenosine tri-phosphate (ATP) needed to live,[4] and exposure to short-term environmental stress during early life can have long-lasting effects on mitochondrial function.[5–7] For instance, early-life poor nutrition in rats leads to lasting changes in mitochondrial gene expression during adulthood.[7] In great tits (*Parus major*), rearing conditions were associated with mitochondrial function in nestlings (mitochondria numbers were higher in juveniles reared in harsher conditions), but had no effects on mitochondria function in later stages.[8] These contrasting findings illustrate that the long-term impact of developmental environments on mitochondrial function is highly context-dependent and remains incompletely understood.

To understand whether early-life conditions affect mitochondria in the long-term, it is vital to examine the mechanisms that determine these connections. Intriguingly, both the production and use of ATP are largely regulated by hormones[4,9] and thyroid hormones (THs) are particularly important during early life. This is because of their anabolic effect in regulating energy metabolism[10] and growth.[11,12] Importantly, TH-secretion is influenced by environmental conditions,[13,14] such as temperature[15,16] and food availability.[17,18] Furthermore, TH are generally known to promote mitochondrial function,[19,20] mainly by increasing mitochondrial respiration rates, mitochondrial biogenesis and mitochondrial gene expression.[21] This makes TH great candidates in mediating environmental conditions to regulate the energy metabolism in the mitochondria. It has previously been shown that TH enhance mitochondrial capacity for energy production—specifically by increasing the electron transport system (ETS) activity—in zebra finch (*Taeniopygia guttata*) chicks raised in enlarged broods.[22] ETS determines the potential of ATP-production capacity.[4,23] Therefore, TH likely supports chicks in coping with unforeseen adverse events that necessitate a high ATP supply. However, whether these TH-induced changes in mitochondrial metabolism are long lasting until adulthood is not yet known. Any enduring effect could arise from organizational changes in mitochondrial structure or function, such as alterations in cristae formation,[24] membrane composition,[25] or gene expression.[7] Such modifications can influence the efficiency of electron transport activity and ATP synthesis, potentially leading to long-term adjustments in cellular energy metabolism.[20]

Because TH regulate genes involved in mitochondrial biogenesis and membrane remodeling,[19] early-life exposure may

establish persistent differences in mitochondrial architecture and performance that endure into adulthood. Early-life conditions mediated by TH can not only shape mitochondrial energy metabolism, but can also influence behavioral and performance-related traits through these metabolic changes. In birds, the most important kind of physical performance for survival is probably flapping flight to escape predators.[26] It has been shown that energetically costly early-life conditions can have negative consequences for adult flight performance in European starlings (*Sturnus vulgaris)*[27] and in zebra finches,[28] but the underlying mechanism are not yet clear. Flight is largely determined by the efficiency of the pectoral muscles to produce ATP, especially for vertical take-off flights exhibited to escape imminent danger.[29] While escape flights can be largely powered by anaerobic metabolism due to their short duration and intensity, underlying mitochondrial aerobic capacity still plays a key role, e.g., by efficiently regenerating phosphocreatine between bursts of activity, as this process depends on mitochondrial ATP production.[30] However, to our knowledge the association between mitochondrial metabolism and escape flight performance has not yet been tested, nor the role of early-life TH in this context.

Fitness and life-history traits depend not only on high-intensity abilities such as escape flight, but also on lower-intensity behaviors like the exploration of unfamiliar environments.[31] Individuals can differ largely in their strategies to cope with the exposure to novel stimuli. These differences are part of life-history strategies, which vary along a fast-slow-life-history continuum, as described by the pace-of-life syndrome.[32] Behavioral strategies are hereby understood to be determined by an integration of a range of physiological traits, including the stress response (mediated by the hypothalamic-pituitary-adrenal axis) and metabolic functions.[32,33] In this context, mitochondrial metabolism is important because continuous locomotion, sensory processing, and information gathering during exploration all incur steady aerobic energy costs, see also Careau and Garland (2012).[34] It is plausible that birds that explore more extensively expend more ATP per unit time than conspecifics that remain relatively stationary, influencing how resources are allocated to growth, maintenance, and reproduction. Therefore, variation in mitochondrial capacity to produce ATP is expected to underlie individual differences in exploratory effort and its associated energetic expenditure, as shown in mammals by measuring whole-organism metabolic rate.[35] However, for other taxonomic groups this link is less clear.[34]

Importantly, whole-organism metabolic rate lacks the fine-scale resolution needed to understand the efficiency with which ATP is produced in the mitochondria (a consideration that also hold true for other whole-organismal methods such as doubly-label ed water[36] or accelerometry[37]). Mitochondrial efficiency is highly context dependent,[38,39] varies among individuals,[40] is related to organismal fitness[4,40,41] and can be altered by hormones.[42,43] While in adults TH have been related to behaviors associated to exploration, in combination with increased metabolism via effects on the frontal cortex,[44] the long-term effects of early-life TH on adult exploratory-like behavior via mitochondrial adjustments have thus far not been tested.

It was previously shown that elevated TH levels throughout the main nestling phase increased the ETS activity at fledging in zebra finch (*Taeniopygia guttata*) hatchlings raised in enlarged broods, compared to untreated chicks raised in the same conditions.[22] This suggests that TH may enhance mitochondrial capacity to support increased energetic demands. Here, we therefore tested whether these early-life, TH-induced mitochondrial phenotypes persist into adulthood and translate into differences in behavior and performance. Specifically, we assessed mitochondrial metabolism in adults and examined whether variation in mitochondrial function relates to exploratory-like activity and vertical escape flight performance. We hereby focused on vertical escape flight (as opposed to horizontal), because this type of flight involves generating lift sufficient enough to overcome the full force of gravity, often without the aid of relative airflows that typically enhance wing carrying capacity. Therefore vertical escape flight in particular represents one of the most energetically demanding physical activities for birds[45] and is thus especially relevant in relation to mitochondrial energetics. We assessed mitochondrial metabolism in the birds' erythrocytes, allowing us to obtain relevant information with a minimally invasive sampling procedure. Blood mitochondrial metabolism is a suitable proxy for our hypothesis, as it is related to whole-organismal energetic expenditure,[46,47] and correlates positively with mitochondrial metabolism in a range of other tissues, including the pectoral muscle,[23,46,48,49] which is important for performance and activity. Recent work in house sparrows (*Passer domesticus*) found that mitochondrial aerobic metabolism in blood cells reflects flight endurance, further supporting the utility of blood mitochondrial metrics.[50] We assessed mitochondrial aerobic metabolism from blood samples taken directly after the behavioral test to capture the energetic properties of the mitochondria during the behavioral test. Recent studies in zebra finches have documented robust, environment-dependent changes in blood mitochondrial physiology, for example following thermal stress[51,52] or corticosterone exposure.[43] Together, these findings make zebra finches a strong model for testing mechanistic hypotheses about how environmental conditions shape mitochondrial function.

We aimed to address the following hypothesis.

1. Early-life THs have lasting effects on mitochondrial metabolism until adulthood: we expected TH-treated birds to retain higher ETS levels[22] in adulthood, compared to birds that did not receive TH in early life. We additionally tested whether the TH-treatment had effects on adult cellular metabolic rate (CMR), oxidative phosphorylation (the rate of ATP production, OxPhos), leak respiration (the portion of mitochondrial respiration unrelated to ATP production) or mitochondrial inefficiency (ratio between Leak and CMR).
2. Exploratory-like behavior and flight performance are linked to mitochondrial metabolism and therefore related to early-life THs:
   2.1 We expected high flight performance and exploratory-like behavior to be associated with high mitochondrial function (i.e., high CMR, OxPhos, ETS, combined with low Leak and mitochondrial inefficiency). Hence, high mitochondrial function should be associated with both high intensity activities such

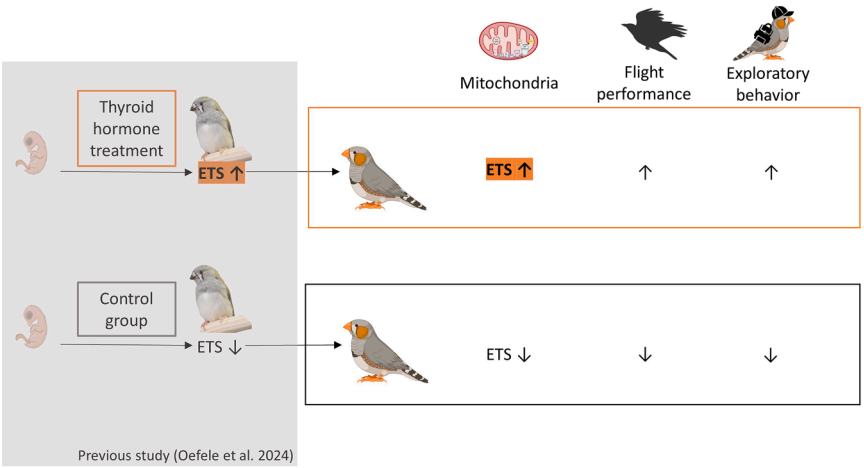

Figure 1. Hypotheses addressed by the study

Hypothesis addressed in this study regarding the long-term effects of early-life thyroid hormones. ETS, maximal electron transport chain capacity; ETS was elevated by early-life thyroid hormone (TH) treatment (shown in a previous study) and here we assessed whether this effect persisted into adulthood, potentially influencing exploratory-like behavior and flight performance (marked in orange). Created with BioRender.com. Photo credit: Marlene Oefele.

as flight performance; as well as with high levels of steady activity during exploration.

2.2 If TH-treated birds retained higher ETS, TH-treated birds exhibit greater flight performance and explore novel environments more proactively, compared to untreated birds. Higher ETS is related to greater energy availability, supporting both exploratory-like behavior and flight performance, which are energy-demanding activities.

Hypothesis addressed in this study regarding the long-term effects of early-life THs are illustrated in Figure 1.

RESULTS

Early-life thyroid hormones do not affect adult mitochondrial metabolism. Mitochondrial metabolism decreases from the energetically demanding growth phase to adulthood

The study longitudinal experimental design, including age in days post hatch and measurements taken at each timepoint, is depicted in Figure 2.

CMR, OxPhos, maximal ETS and Leak declined between the fledgling and first adult timepoints, whereas mitochondrial inefficiency (FCR1) remained unchanged (Table 1 and Figure 3). Full model results are reported in Table S2.

The decline in ETS between fledgling and first adult was stronger in TH-treated birds compared to untreated birds, as indicated a meaningful group*timepoint interaction (estimate, −0.31 [95% CrI, −0.55; −0.07], Table S2). However, groups did not differ in ETS at the adult stage (first adult estimate, 0.08 [95% CrI, −0.11; 0.26]). As previously shown,[22] ETS was initially elevated in the TH-treated group at the timepoint fledgling. Other traits (CMR, OxPhos, Leak, and FCR1) did not differ between the groups at any timepoint (Table S2).

Mitochondrial function predicts escape flight performance

Higher CMR and OxPhos were associated with better (i.e., faster) flight performance (estimates, CMR –1.31 [95% CrI, −2.38; −0.24]; OxPhos −1.05 [95% CrI, −1.98; −0.03], Table S6). Note that negative flight performance scores depict faster flights. ETS was marginally correlated to flight performance (estimate −0.72 [95% CrI, −1.35; 0.01], $p < 0 = 0.983$) indicating strong posterior support for a relationship despite the credible interval overlapping zero. Leak and FCR1 were unrelated to flight performance (estimates, Leak 0.01 [95% CrI, −1.45; 1.29]; FCR1 0.21 [95% CrI, −0.26; 0.66], Table S6). The association between mitochondrial traits and flight performance are depicted in Figure 4. ETS measured at fledging did not predict adult flight performance (Table S12).

Mitochondrial function is not related to exploratory-like behavior

Mitochondrial traits were unrelated to exploratory behavior (estimates, CMR 1.96 [95% CrI, −9.59; 13.23]; OxPhos 5.24 [95% CrI, −4.26; 14.3]; ETS –0.34 [95% CrI, −6.26; 5.62]; Leak −7.18 [95% CrI, −19.03; 5.13]; FCR1 –3.53 [95% CrI, −7.61; 0.59], Table S7 and Figure 5). The associations between mitochondrial traits and exploratory-like behavior are depicted in Figure 4. ETS at fledging was also unrelated to exploratory-like behavior in adulthood (Table S13). None of the mitochondrial traits were associated with changes in body condition during the behavioral experiment, sex (Table S4) or daily sampling order (Table S5). Corticosterone (CORT) showed no main or interactive effects on mitochondrial traits or behavior (Table S11).

Early-life thyroid treatment does not affect flight or exploratory-like behavior

The treatment groups did not differ in flight performance (Table S6) or exploratory-like behavior (Table S7). No meaningful mitochondria*group interactions were detected.

Corticosterone measured after exploration of the new environment did not differ between groups and was not related to mitochondrial traits of behavior

CORT was not different between the groups or sexes (Table S9). CORT was not related to the change in body condition (SMI difference) over the course of the behavioral experiment (Table S8). CORT was not related to any of the mitochondrial traits (CMR, OxPhos, ETS, Leak, and FCR1), see Table S10. Furthermore, exploratory behavior was not related to CORT and there was no meaningful three-way interaction between any of the

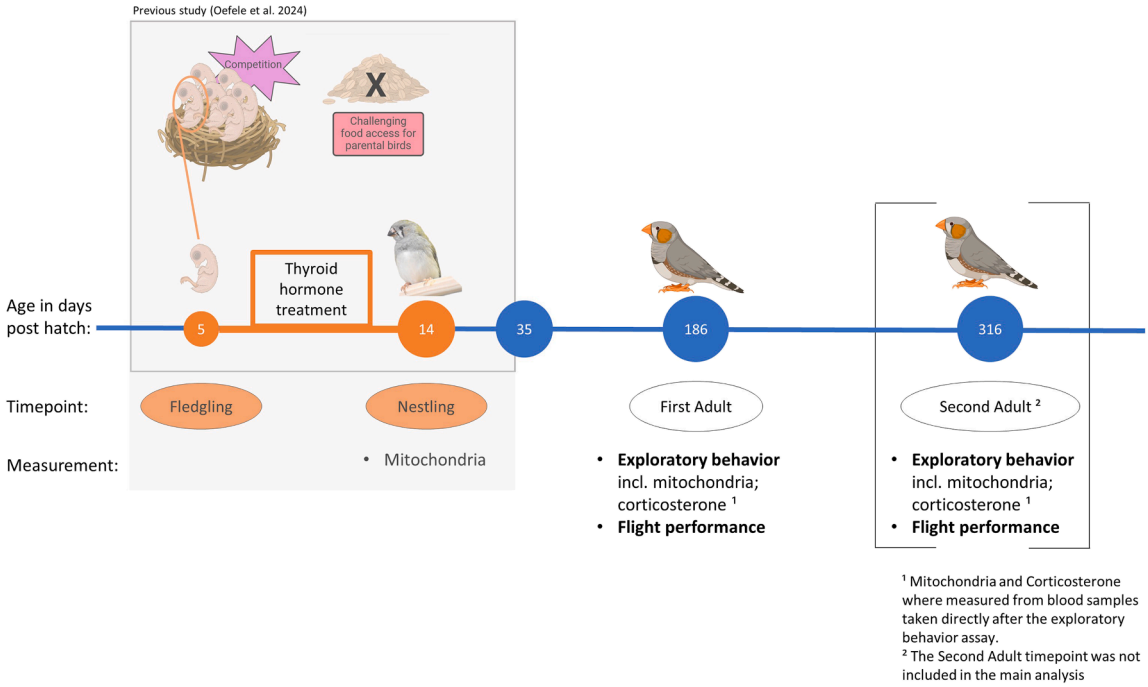

Figure 2. Design of the study

Diagram of longitudinal experimental design, including age in days post hatch and measurements taken at each timepoint. Created with BioRender.com. Photo credit: Marlene Oefele.

mitochondrial traits, group and CORT on exploratory behavior (Table S11). CORT values ranged from 0.34 ng/mL to 63.95 ng/mL (average 13.1 ng/mL, sd 9.7). CORT values had a repeatability score of 0.17. The distribution of the CORT values can be seen in Figure S2. For comparison, baseline CORT values taken at fledging ranged from 0.06 to 6 ng/mL (average 1.1; sd 1.47).

Results are summarized in a diagram in Figure 6.

DISCUSSION

In this study, we aimed to assess the long-term consequences of TH exposure during the main growth phase of zebra finches (*Taeniopygia guttata*), which led to enhanced maximal ETS at the time of fledging.[22] Contrary to what we expected, ETS levels of the TH-treated birds did not remain elevated in adulthood (at 186 days post hatch). Further, adult CMR, OxPhos, Leak, mitochondrial inefficiency (FCR1), exploratory-like behavior or escape flight performance were not affected by the early-life TH treatment. These results indicate that early exposure to elevated TH did not have long-lasting effects on mitochondria or behavior/performance. Regardless of the TH-treatment, mitochondrial aerobic metabolism dropped between the highly energy demanding growth phase and adulthood. Furthermore, mitochondrial metabolism was linked to flight performance, but not to activity levels during exploration.

Contrary to our expectations, the TH-induced increase in ETS at fledging did not last into adulthood, which indicates that TH did not have long-term organizational effects on mitochondrial function in our study species. Generally, TH can modulate mitochondria via genomic and non-genomic effects. Genomic effects refer to the expression of components of ETS,[53] while non-genomic effects likely target the phospholipid composition of the membrane and specific signaling pathways (reviewed by Cioffi et al., 2013[21]). Genomic effects are generally slower than non-genomic ones, and both pathways can enable plastic adjustments (reviewed by Lanni et al., 2016[19]), consistent with the need for mitochondrial function to be flexibly adjusted to

Table 1. Summary of changes in mitochondrial traits between the fledgling and first adult timepoints

Change in mitochondrial traits fledging to adulthood					
	Log (CMR)	log(OxPhos)	log(ETS)	log(Leak)	FCR1
Estimates (95% CrI)	−0.46 (-0.55; −0.38)	−0.50 (-0.63; −0.37)	−0.62 (-0.73; −0.52)	−0.57 (-0.78; −0.37)	0.00 (−0.05; 0.06)
Change in %	−37.04%	−39.35%	−46.21%	−43.45%	–

n = 137.

Values are presented as posterior estimates of log-transformed traits with 95% credible intervals (CrIs), and corresponding percentage decreases are shown for interpretation.

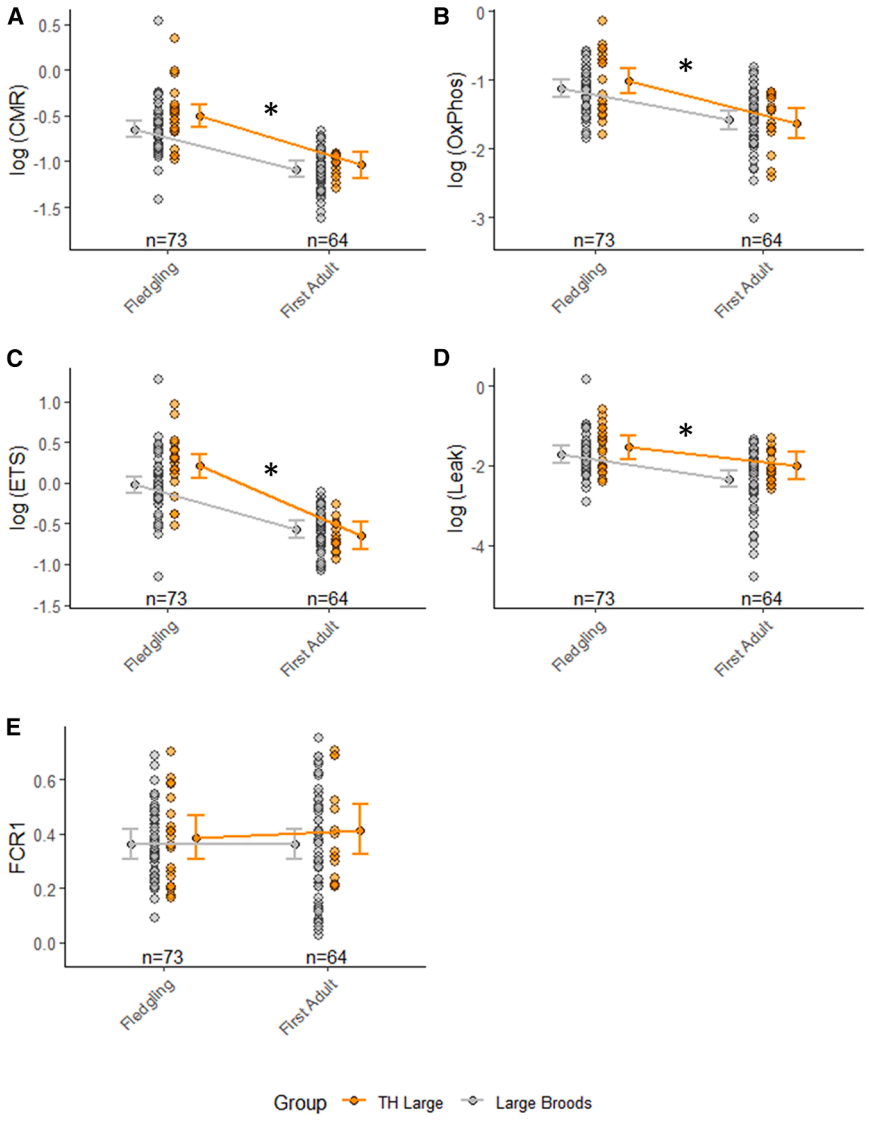

Figure 3. Results about mitochondrial traits

Mitochondrial traits (respiration rates in pmol ml^{-1} on *y* axis) in the two experimental groups: "TH Large" (chicks treated with TH during the main growth phase, reared in enlarged broods, orange circles), "Large Broods" (chicks reared in enlarged broods but not treated, gray circles). Timepoints on *x* axis: fledgling (14 dph); first adult (186 dph). Group estimates (offset circles) with 95% CrIs (error bars), raw data points (circles) and sample sizes (n) are shown for each timepoint.

(A) Log-transformed cellular metabolic rate (CMR).

(B) Log-transformed oxidative phosphorylation (OxPhos).

(C) Log-transformed maximal electron transport chain capacity (ETS).

(D) Log-transformed leak respiration (Leak).

(E) Mitochondria inefficiency leak/CMR (FCR1). Asterisks indicate meaningful treatment-independent changes between the timepoints for CMR (−0.46 [95% CrI, −0.55; −0.38]), OxPhos (−0.50 [95% CrI, −0.63; −0.37]), ETS (−0.62 [95% CrI, −0.73; −0.52]), and Leak (−0.57 [95% CrI, −0.78; –0.37]).

meet the current energetic needs. Such mitochondrial flexibility has previously been shown in other life stages. For example, in female pied flycatchers (*Ficedula hypoleuca*), mitochondrial efficiency and respiration rates matched the energetic demands of reproduction.[54] Our study adds to this evidence by showing a clear decrease in mitochondrial metabolism from the energetically demanding growth phase[55,56] to adulthood, but our results are the first to indicate that ETS can be flexibly adjusted to current energetic needs during early life, without becoming rigidly established. Maintaining flexibility in mitochondrial traits is likely especially important in organisms that show highly variable energetic demands throughout different life stages—as it is the case for small passerines.[57] Indeed, the absence of a clear TH effect in adulthood may reflect the lower maintenance level energy requirements of the adults: once the intense, growth driven phase has passed, any transient TH induced up regulation of mitochondrial capacity could be down scaled to match the reduced demands of daily activity of adults that do not experience the energetic demands of growth.

Overall, mitochondrial function (CMR, OxPhos, ETS, and Leak) decreased around 40% between the end of the growth phase and adulthood, irrespectively of the early-life TH-treatment. These results are expected, because the time of growth is a life-history stage with relatively high energetic demands[58,59] and mitochondrial function is generally known to decline with age.[60,61] A study on zebra finch erythrocytes showed that CMR (also called ROUTINE) and OxPhos declined between 36 (young adults) and 91 (old adults) weeks of age,[62] and in humans energy expenditure is highest during childhood and declines until it stabilizes during adulthood,[63] but see Moe et al. (2007).[64] We do not think that the decline in mitochondrial function observed between fledging and adulthood was due to methodological reasons, although fledging samples were taken directly in the home aviary, with minimal disturbance, while adult samples were taken directly after the behavioral test, thus in a stressful situation and with no access to food. However, mitochondrial traits were not related to the decrease in body condition observed before and after the behavioral test, or to circulating CORT values (indices of stress exposure). Thus, while methodological factors may have influenced some measurements, they do not fully explain the observed patterns.

Regardless of the early-life TH-treatment, we showed here for the first time, that CMR and OxPhos were positively related to a highly energy-demanding physical performance like vertical escape flapping flight. We also observed a similar trend for an

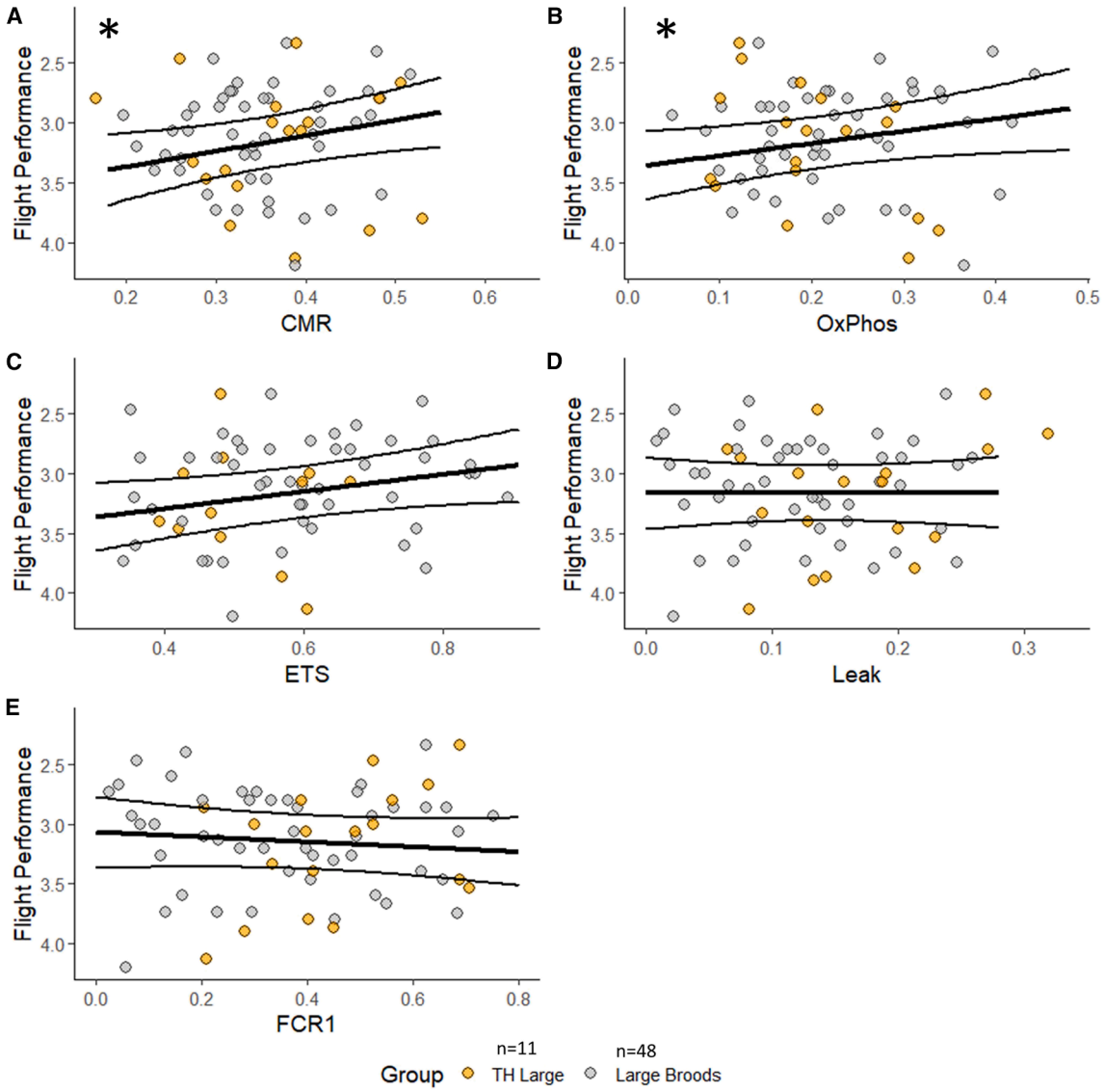

Figure 4. Associations between mitochondrial traits and vertical escape flight

Mitochondrial traits (respiration rates in pmol ml^{-1} on *x* axis) and flight performance in adulthood at timepoint "first adult" (186 dph) in the experimental groups: "TH Large" (chicks treated with TH during the main growth phase, reared in enlarged broods, orange triangles) and "Large Broods" (chicks reared in enlarged broods, gray dots). Black lines are group-independent regression lines with 95% CrIs. Sample sizes per group are shown (n). Flight performance scores are shown reversed on *y* axis, since lower scores indicate faster flights.

(A) Cellular metabolic rate (CMR).

(B) Oxidative Phosphorylation (OxPhos).

(C) Maximal electron transport chain capacity (ETS).

(D) Leak respiration (Leak).

(E) Mitochondrial inefficiency Leak/CMR (FCR1). Regression lines and 95% CrIs are depicted. Asterisks indicate statistically meaningful associations for CMR (−1.31 [95% CrI, −2.38; −0.24]) and OxPhos (−1.05 [95% CrI, −1.98; −0.03]).

association between flight performance and ETS. Although the credible interval did overlap 0, there was a 98.3% probability (determined by calculating the posterior probability) that ETS was related to fast flight performances nevertheless. This is in line with our predictions that for birds to be able to fly faster, they would need to have an enhanced ability to produce ATP, see also Ton et al. 2025.[50] Short bursts of vertical flight are extremely costly for a bird. In zebra finches, short repeated flights lead to an energy expenditure up to 28x their basal metabolic rate, in the most extreme cases.[65] The positive association of mitochondrial function with flight performance furthermore indicates that erythrocyte mitochondrial function can be an informative or even predictive measure of physical quality. Several studies have shown that mitochondria in the blood are directly

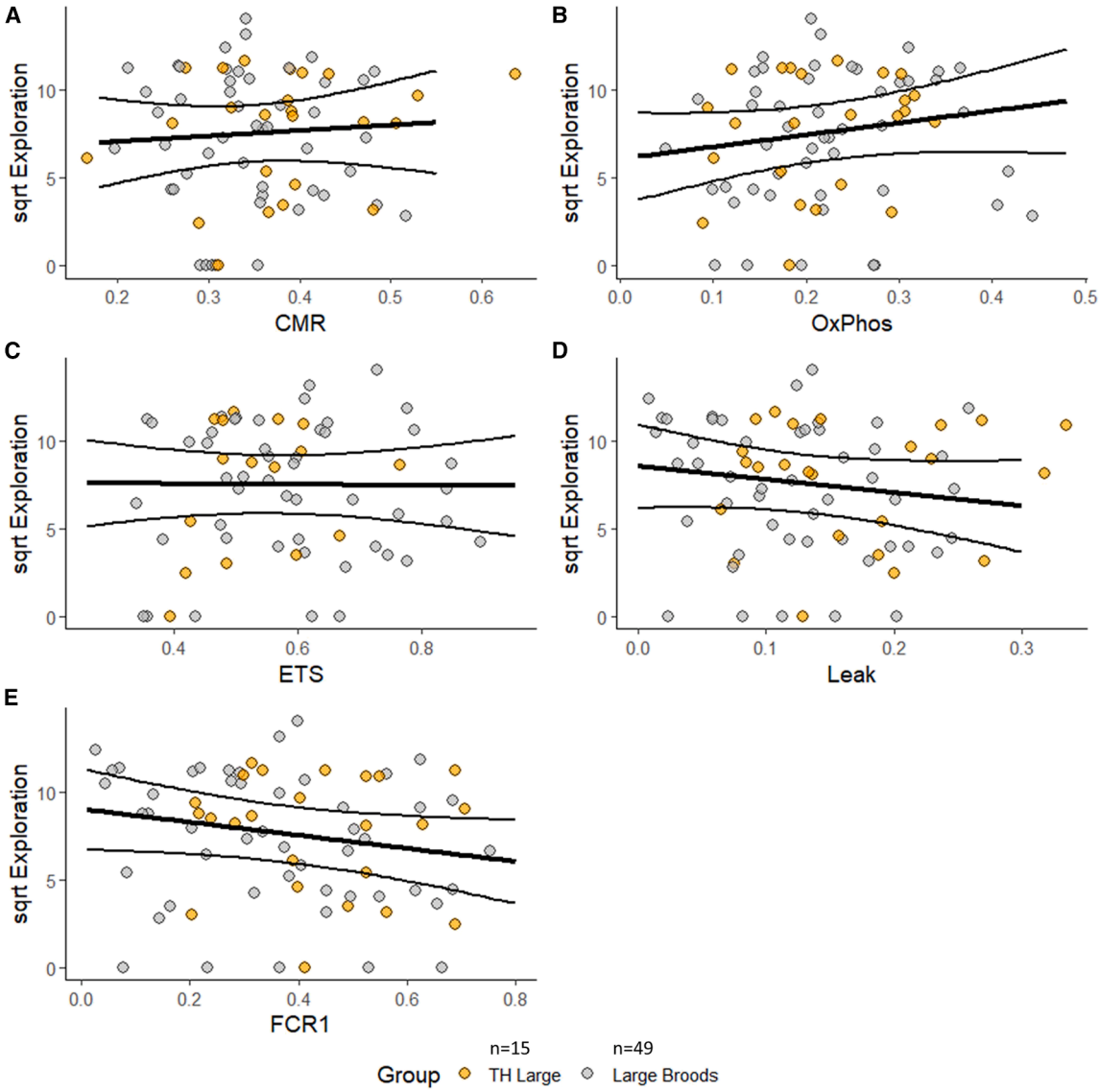

Figure 5. Associations between mitochondrial traits and exploratory-like behavior

Mitochondrial traits (respiration rates in pmol ml^{-1} on *x* axis) and exploration behavior in adulthood at timepoint "first adult" (186 dph) in the experimental groups: "TH Large" (chicks treated with TH during the main growth phase, reared in enlarged broods, orange circles) and "Large Broods" (chicks reared in enlarged broods, gray circles). Black lines are group-independent regression lines with 95% CrIs. Sample sizes per group are shown (n). Exploration scores on *y* axis are square-root transformed.

(A) Cellular metabolic rate (CMR).

(B) Oxidative Phosphorylation (OxPhos).

(C) Maximal electron transport chain capacity (ETS).

(D) Leak respiration (Leak).

(E) Mitochondrial inefficiency Leak/CMR (FCR1).

related to whole organismal energetic needs[46,49,54] and are closely correlated with mitochondrial function in the skeletal muscle,[46,48] where OxPhos and ETS can indeed determine exercise capacity in humans.[66]

While mitochondrial metabolism was related to escape flight performance, contrary to our prediction we did not find an association with exploratory-like behavior. Here, we aimed to test the physiological basis underlying exploration of a novel environment by examining the relationship between exploratory-like behavior and mitochondrial metabolism in a food-limited state. Behaviors related to exploration have consistently been shown to be highly context dependent.[67–69] A meta-analysis found that individual differences in the ability to obtain resources are a stronger determinant of correlations between various behaviors (including those associated with exploration) and fitness than the respective life-history strategy.[70] Since the birds in

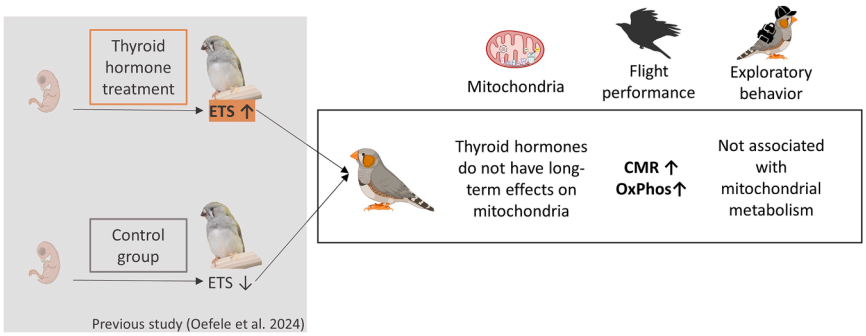

Figure 6. Summary of main results

ETS was elevated by early-life thyroid hormone (TH) treatment (shown in a previous study) and this effect did not persist into adulthood. Regardless of the early-life TH treatment, flight performance was correlated with cellular metabolic rate (CMR) and oxidative phosphorylation (OxPhos). Mitochondrial metabolism was not associated with exploratory-like behavior. Created with BioRender.com. Photo credit: Marlene Oefele.

our study were food-limited, the need to acquire resources may have masked individual differences that would have potentially been evident in a non-fasted setting. Note that exploratory behavior in birds is often times tested without food deprivation before the trial.[71,72] However, we chose to implement mild food-deprivation to be able to standardize the feeding state of the individuals, which we accounted for statistically by controlling for testing order. Therefore, the potential effects of fasting on exploratory-like behavior should be interpreted with caution. In fact, we did find a meaningful effect of the experimental testing order on exploratory-like behavior, with birds that were waiting for their trials longer having higher exploratory scores. Notably, mitochondrial traits were not related to the testing order, suggesting that mitochondrial function was maintained normally, while the exploration activity was adjusted to the given circumstances. It is also possible that mitochondrial function in the liver cells, which are directly involved in nutrient processing, is more acutely related to exploration behavior in a fasted state, compared to mitochondria in the blood. Mitochondria in the blood hereby appear to be less sensitive to short-term food limitation, but rather reflect the general physical condition of the bird. This is evidenced by the positive association between blood mitochondrial metabolism and flight performance observed in the present study, as well as by previous findings in European starlings (*Sturnus vulgaris*), where blood mitochondrial oxygen consumption correlated strongly with that of the pectoral muscle but not with liver metabolism.[46] The latter likely reflects the fact that in that study, birds were sampled while sleeping and in a post-absorptive state, when liver activity is expected to be low.[73] We suggest future studies to conduct such behavioral tests in fasted vs. non-fasted settings to relate these behavioral scores to mitochondrial function, as well as to consider different tissues such as the liver.

Individual differences in exploratory behavior may not only depend on the nutritional state, but also on the exposure to stressors, such as being handled, exposed to novel surroundings and visual isolation (since cages were covered with towels).[74] However, we did not find a relationship between CORT and mitochondrial function or CORT and exploratory behavior or an interaction. Stress-induced CORT concentrations are secreted in response to unpredictable challenges[75] and bold personality types (aka proactive explorers of novel environments) are often related to a lower reactivity of the hypothalamic-pituitary-adrenal axis.[32] While we showed here that the absolute values of CORT that the birds exhibited directly after behavioral test were not related to exploratory behavior, additionally assessing baseline CORT values in future studies could reveal how the individual increase in CORT from baseline to stress-level may relate to such behaviors. Against our expectation, CORT values were also not related to mitochondrial function. It is known that mitochondrial function can be dynamically adjusted to the current energetic needs[54] and on the cellular level, CORT can increase mitochondrial biogenesis and enzyme activity, while chronic CORT exposure is generally associated with oxidative damage and mitochondrial apoptosis (reviewed by Manoli et al., 2007[76]). However, the exact timing of mitochondrial adjustments to acutely elevated CORT levels is not known, as far as we know. The main action of CORT is to re-establish homeostasis by mobilizing energetic resources after the stressful event,[77] while catecholamines can have a faster, "real-time" effect in mobilizing mitochondrial substrates.[76] However, the timescale and the generalizability among tissues of these reactions is not clear and should be assessed by future studies.

Previously, it was demonstrated that TH-administration at physiological levels increases ETS in zebra finch nestlings,[22] suggesting that TH likely mediate environmental adjustments by increasing mitochondrial functions. Here we showed that these TH-dependent mitochondrial changes were not permanent, disappearing in adulthood. It is known that conditions in early life can have lasting effects on fitness-related traits. For example, the so-called silver spoon effect predicts that individuals growing up in less favorable conditions, have fitness disadvantages later in life, and vice-versa.[2] Our results indicate that TH-action on mitochondria may be a mechanistic component of developmental plasticity,[78] whereby mitochondrial function can flexibly adjust to the current energetic needs. Importantly, this plasticity may be particularly relevant in a rapidly changing climate: TH levels are often upregulated when birds face energetic challenges such as cold spells[15,79] or limited food,[80] but the lack of long-term organizational effects observed here suggests that TH-mediated mitochondrial responses serve as short-term compensatory adjustments rather than lasting developmental programming (see also Gyllenhammer et al., 2020[5]). In addition, our within-brood design effectively controlled for between-nest variation in parental care and microclimate; however, because all chicks within a brood experienced the same overall environmental conditions, we cannot entirely exclude the possibility that shared environmental stressors contributed to the

observed patterns. We also provide first evidence that red blood cell mitochondrial metabolism is directly related to a highly energy-demanding performance like escape vertical flight, indicating that blood mitochondrial function can be a predictor of physical quality. Although 1 s take-off flight relies primarily on anaerobic pathways,[50] our results indicate that even short (<5 s) vertical flights involve a measurable aerobic contribution, consistent with the high mitochondrial capacity of avian flight muscles.[45] Further, we did not find evidence that exploratory-like behavior was related to mitochondrial function. The meaning of risk-related behaviors such as exploratory-like behavior and its association with metabolic traits has been debated widely[34,70,81,82] and accumulating evidence suggest that the nature of this link is complex, multifaced and highly context dependent[81]—a pattern also reflected in our findings. Overall, our findings suggest that THs enable short-term metabolic resilience during development, but do not fix mitochondrial phenotypes in adulthood or influence adult performance.

Limitations of the study

While our study provides novel insights, a few considerations should be noted: hatchlings were exposed to early-life thyroid hormones in mildly challenging conditions, which have been terminated after fledging, when fledglings were moved to standard conditions. Future studies should therefore test whether mitochondrial adjustments would consolidate if exposure to such conditions were prolonged beyond the early growth phase, in line with, for example, the "environmental matching hypothesis."[83] Furthermore, mitochondrial metabolism has been assessed in red blood cells, and despite its positive correlation with mitochondrial metabolism in other tissues,[23,46,48,49] tissue-specific adjustments of mitochondria in the liver of the skeletal muscle are likely related to behavior and nutrient processing, and should therefore be assessed by future studies. Exploratory-like behavior was measured under mild food deprivation, which may mask individual differences, as well as potential association with mitochondrial metabolism. Future studies should assess exploratory-like behavior and mitochondria in various contexts, as well as consider assessing baseline corticosterone levels in addition to stress-related levels, see also Malkoc et al. (2025).[47] Finally, future studies should test the association of blood mitochondrial metabolism with flight performance in different avian species. Notably, Ton et al. (2025)[50] found a similar link between flight performance and blood mitochondrial metabolism in house sparrows, suggesting a general association across passerines.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Stefania Casagrande (stefania.casagrande@bi.mpg.de).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Raw data have been deposited at Max Planck Digital Repository EDMOND and are publicly available as of the date of publication at https://doi.org/10.17617/3.UHTWTS.
- All original code has been deposited at Max Planck Digital Repository EDMOND: https://doi.org/10.17617/3.UHTWTS.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

Conceptualization, M.O., M.H., S.R., and S.C.; methodology, M.O. and S.R.; investigation, M.O.; writing – original draft, M.O.; writing – review & editing, M.H., S.R., and S.C.; funding acquisition, M.H.; supervision, M.H. and S.C.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Zebra finch (*Taeniopygia guttata*) blood samples	Taken from study subjects (see experimental model and study participant details)	N/A
Deposited data		
Raw and analyzed data	This paper	https://doi.org/10.17617/3.UHTWTS
Experimental models: Organisms/strains		
Zebra finch (*Taeniopygia guttata*)	Local Seewiesen population, acquired in 2002 from a colony held since 1985 by T. R. Birkhead at Sheffield University, UK. For more information, see population no. 18 in Forstmeier et al. (2007)[84]	N/A
Software and algorithms		
Code	This paper	https://doi.org/10.17617/3.UHTWTS
R, version 4.2.2	R Core Team (2020)[85]	http://www.r-project.org/index.html
R-package "rstanarm", version 2.21.3	Goodrich et al. (2023)[86]	https://mc-stan.org/rstanarm/

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Zebra finches (*Taeniopygia guttata*)

Zebra finches (*Taeniopygia guttata*) originated from a local captive population and were allowed to breed in communal indoor aviaries. The local Seewiesen population was acquired in 2002 from a colony held since 1985 by T. R. Birkhead at Sheffield University, UK. For more information, see population no. 18 in Forstmeier et al. (2007).[84] Birds were breeding in wooden nest boxes under a light:dark photoperiod of 14:10 h with a humidity of 59%.

Study subjects were offspring derived from these birds. Both males and females were included in this study, ages ranged from fledging 14 days post hatching (dph) to 363 dph. Potential sex and age-specific effects have been statistically tested and reported below.

All experimental procedures followed strict animal welfare guidelines of animal experimentation laws of the European Union (Directive 2010/63/EU) as well as the German Animal Welfare Act and were conducted under the approval of the Regierungspräsidium von Oberbayern, license no. ROB-55.1-2532.Vet_02-20-115.

METHOD DETAILS

Study species and housing

To imitate natural resource challenges, food access was hampered by providing seeds and egg food in a 2:1 food to husk ratio,[87] in open containers (60 × 40 × 12 cm) placed on the ground. Newly hatched chicks from a total of 47 nests of birth were cross-fostered 1- or 2-day post hatch (dph), to create either reduced (2–3 chicks) or enlarged broods (6 or 7 chicks). All chicks included in this study were raised in enlarged broods to simulate challenging developmental conditions via social competition in the nest.[88] The average brood size during the main growth phase between 1/2 dph and 14 dph was 5.56, sd: 0.699 (because of asynchronous fledging and naturally occurring loss of nestlings). Broods were enlarged within natural ranges of brood sizes (wild zebra finches typically raise 4 to 6 chicks per nest[74]), and the intensity of the food treatment (2:1) was chosen to induce a mild challenge without causing severe stress (it was shown that a husk ratio of 2:1 does not to affect body mass of hatchlings[89]). The aim was hereby to produce naturally challenging developmental conditions, by promoting normal sibling competition and avoiding the unrealistic energetic abundance typical of *ad libitum* feeding conditions in captivity. Birds have been moved to standard conditions (*ad libitum* food) after fledging. This study includes 67 untreated individuals (control group for TH treatment: "Large Broods") and 20 individuals treated with thyroid hormones (TH) during the main growth phase ("TH Large"). Sample sizes are unbalanced because we used a within-brood design, see "Thyroid hormone treatment". The TH-treatment did not affect growth parameters, see Oefele et al. (2024).[22]

Thyroid hormone treatment

Thyroid hormones (TH) consist of thyroxine (T4) and triiodothyronine T3. We chose to administer T3, since T3 is considered the biologically active form of TH because of its higher cell receptor affinity compared to its precursor T4.[90] The treatment is described in detail in Oefele et al. (2024).[22] In short, T3 was given in a within-brood design, so that each enlarged nest contained both treatment chicks (''TH Large'') and control chicks (''Large Broods''), ensuring a shared early environment. T3 was administered on alternating days (to minimize handling stress) between 5 dph and 14 dph (the main growth period) with a dosage of 5 ng T3 per gram body mass. The control group ''TH Large'' received the vector solution without T3. The amount of TH or vector solution that was administered was adjusted to the body mass of the chicks on each given treatment day. The aim was to subject the hatchlings to regularly elevated peaks of T3 within physiological ranges. The effectiveness of the treatment was validated by assessing T3 plasma levels at the end of the treatment. Within each enlarged nest (~6 nestlings), one chick was randomly assigned to the ''TH Large'' group and treated with TH, the remaining chicks of the nest were assigned to the untreated control group ''Large Broods''.

Exploratory-like behavior

To test behaviors that are related to the exploration of novel environments (i.e., exploratory-like behavior), we employed a novel open field test in line with Verbeek et al. (1994).[91] We used an observation room (4.0 × 2.4 × 2.3 m) with a one-way observation window.[71] The room contained five wooden artificial trees with heights of 1.5 m and four 20 cm cylindrical branches each. Furthermore, we added natural branches to stimulate exploration. The birds had not been exposed to natural branches at any time before the first behavioral test. 12 birds were tested per day. These individuals were randomly caught from their home aviaries on the evening before the test and placed into single holding cages next to the observational room. At capture, we assessed tarsus length (in mm) and body mass (in g) to calculate the scaled mass index (SMI),[92] with the purpose of obtaining a measure of the body condition before the onset of the experiment. Birds had access to *ad libitum* seeds and water over night. At 7 a.m. the light was switched on and birds had the opportunity to feed for 30 min. At 7:30 a.m. the food was removed and light was switched off. The behavioral test began at 9:30 a.m. Food was removed 2 h prior to the test to ensure that all birds were in a similar metabolic state at the time of testing. For the behavioral test, each individual was assessed one by one. The focal bird was taken out of the holding cage by hand and placed inside the dark observational room. All birds were placed on the same marked spot in the observational room. To begin the behavioral test, the light was switched on and the behavior was scored from outside of the observation room. To quantify exploratory-like behavior, we calculated exploratory scores defined as the number of transitions between distinct locations (e.g., flights between wooden 'trees', walls, or the floor). Further, we included hops within each location (within a tree, on the floor, etc.). The behavioral test lasted 10 min, after which the light was switched off, the bird was caught by hand and a blood sample was taken within 3 min of the end of the behavioral test. Mitochondrial function was assessed from these blood samples (from individuals in the same order as the behavioral test), together with glucocorticoid concentrations (corticosterone in birds, CORT) to account for stress-related changes in metabolism and behavior. CORT is also known as a metabolic hormone able to effect both behavior[93] and mitochondrial function.[43] CORT concentrations were hereby expected to be positively related to CMR and mitochondrial inefficiency.[42] Body mass was taken at this time and SMI values calculated to determine the body condition of the birds after the experiment. We used the difference in body condition values (SMI) before and after the behavioral experiment to statistically test for the effect of the body mass loss during the experiments on the focal parameters, see below ''Statistics''. The change in body condition was positively correlated with the daily testing sequence (Pearsońs correlation: 0.35, p-value = <0.001). The birds had an average body mass of 15.12 g (sd ± 0.9) before the experiment and lost on average of 2.16 g (sd ± 0.53), see Figure S3. For more details, see supplemental information.

Blood sampling

A maximum volume of blood that corresponded to 1% of the individualś body mass was taken via venipuncture. Blood samples were kept on ice and processed within 2 h by separating red blood cells from plasma via centrifugation at 2000 x g for 10 min. Mitochondrial analysis on red blood cells was conducted immediately after processing of the blood and remaining red blood cells and plasma were stored at −80°C.

Escape flight performance

The flight performance test was conducted about one week after the behavioral test. We employed a flight tunnel set up similar to the earlier one used by Kullberg et al. (2002),[94] which consisted of a plexiglass vertical tube (160 cm L x 40 cm W) with a holding cage at the top. Black marks placed every 20 cm along the tunnel defined 6 distinct sections of the tube were used to measure the speed of the vertical flight. On the day of the flight performance test, birds were randomly captured from their home aviaries and placed in holding cages with *ad libitum* access to seeds and water. To minimize disturbance, the cages were covered with towels. Birds were allowed to feed freely before the trials to ensure the assessment reflected their maximal flight capacity (with full energy stores). To simulate a vertical escape flight, the bird was placed in the bottom part of the tube in complete darkness and motivated to fly via a hand movement after switching on a light at the top of tunnel. Birds that did not fly up the tunnel after several attempts were excluded from the experiment. Because the reasons for these failures could not be determined, we excluded all 17 such cases from the analyses. A logistic mixed model showed that there was no difference in failed attempts between groups or timepoints, see Table S14. Linear mixed models revealed that mitochondrial traits were not related affected by failed attempts, see Table S15. Each individual was tested three times consecutively. Flights were recorded with a video camera. To determine the flight performance, the flight

speed was assessed by counting the number of video frames (each frame covering 0.02 s) during which the bird was flying through the tube. We then divided the number of video frames by the number of sections that were crossed by the bird in straight flight. For example, when the bird hovered in one section, but still reached the top after hovering, only 5 sections were considered, instead of all 6 sections. The flight performance score was averaged over all three trials (average CV across trials: 0.09). Flight trials were conducted between 9:30 and 14:00. The analysis of the flight performance video recordings was done by an independent investigator who was not aware of the respective treatment groups of the individuals, nor of the predictions and hypothesis of this study.

Mitochondrial bioenergetics

We used intact red blood cells to assess mitochondrial aerobic metabolism in their natural state,[23] including the cellś substrate availability at the time of sampling. Up to 30 μL RBCs were collected from the bottom of the sampling tube with a cut-off pipette tip after centrifugation. RBCs where gently resuspended in cold respiratory Mir05 buffer (0.5 mmol L−1 EGTA, 3 mmol L−1 MgCl2, 60 mmol L−1 potassium lactobionate, 20 mmol L−1 taurine, 10 mmol L−1 KH2PO4, 20 mmol L−1 HEPES, 110 mmol L−1 sucrose, 15 mmol L−1 fatty acid-free bovine serum albumin, pH 7.1). The RBC-buffer solution was centrifuged at 500 g for 5 min and the supernatant was removed. The pellet, including the RBCs, was resuspended in fresh Mir05 buffer at 40 °C, which represent the average body temperature of our birds. After adding the sample to the respirometer chamber, cellular oxygen consumption was assessed according to the following protocol[23]: (1) CMR – the basal respiration of the cell; (2) 1 μg mL−1 oligomycin, an ATPase inhibitor, was added to the chamber to quantify Leak; (3) the mitochondrial uncoupler carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP) was titrated in 1 μmol L−1 steps to quantify ETS; (4) 5 μmol L−1 antimycin was added to determine non-mitochondrial oxygen consumption. Non-mitochondrial oxygen consumption was subtracted from all measure above. Measurements were normalized for the volume of RBCs used for each sample.

Using a Clark electrode high resolution respirometer (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria), we quantified 5 mitochondrial traits: (1) CMR (cellular metabolic rate); (2) Leak (the part of mitochondrial respiration directed toward proton leak); (3) OxPhos (oxidative phosphorylation, proportion of oxygen consumption rate used for ATP-production, calculated by subtracting leak respiration from CMR); (4) ETS (maximal working capacity of electron transport chain, the maximum ATP-production capacity under the given cellular conditions); (5) FCR1 (mitochondrial inefficiency, ratio between Leak and CMR).[23] Due to an unexpected degradation of one of the reagents (CCCP, a mitochondrial uncoupler) during measurements at the Second Adult timepoint, we had to exclude ETS values for this timepoint, which resulted in a low sample size for ETS measurements (TH Large n = 1, Large Broods, n = 13). Other mitochondrial measures were not affected. In a total of 37 cases, we were not able to retrieve enough blood to conduct the mitochondrial analysis (Table S1).

Corticosterone assay

An enzyme immunoassay kit (cat. no. K014-H1, Arbor Assays, Corticosterone ELISA Kit) was used to determine corticosterone (CORT) values in 10 μL of plasma samples.[42] A total of 7 plates was used. A double diethyl ether extraction was used to extract corticosterone (CORT). CORT was then re-dissolved in assay buffer and allowed to reconstitute overnight. The following day, 50 μL of each sample was added in duplicate to wells on an assay plate. Duplicate samples with a coefficient of variation of above 10% were not considered. The average intra-assay coefficient of variation was 2.25% between duplicates. Two stripped chicken plasma controls (with corticosterone added at concentrations of 10 and 5 ng/mL, respectively) and pure assay buffer were used as plate control samples. The inter-assay coefficient of variation was 13.56%.

QUANTIFICATION AND STATISTICAL ANALYSIS

Sample sizes (n) indicate individuals analyzed at each respective timepoint, and are indicated in all figures, in Table S1, and in the full model outputs in the supplemental information (Tables S1–S17). Statistical analysis was conducted in R, version 4.2.2,[85] using the package "rstanarm", version 2.21.3.[86] Generalized linear models in a Bayesian framework were employed to calculate posterior means and 95% credible intervals (CrI), from 4000 simulated values from the normally distributed joint posterior distribution. We used weakly informative priors, with scaling based on the distribution of the data, centering at 0 and standard deviations of each prior were calculated by rstanarm based on the data. The specific prior parameters can be found in the supplemental information (Table S18). Statistically meaningful effects were inferred when the 95% credible intervals (CrIs) of predictors did not include zero. Model fit was assessed by plotting residuals against leverage, normal quantile-quantile plots and Tukey-Ascombe plots. Model convergence was determined by inspecting R-hat values, effective sample sizes, and Bayesian R^2 values with the function "shinystan". The package "ggplot2" was used to generate plots.[95] The package "emmeans", version 1.8.9,[96] was used to conduct post-hoc contrasts and comparisons. Repeatability scores R were calculated with the r-package "rptR"[97] to confirm the robustness of our hormonal and behavioral measurements between all available measurements of the First and Second Adult timepoint. We calculated repeatability scores R as the proportion of the phenotypic variance explained by the individual, in line with Lessells and Boag.[98] Repeatability scores R were calculated for exploratory-like behavior, flight performance and CORT values. Exploratory-like behavior was influenced by the daily testing order (Table S7). Therefore, we adjusted the repeatability score for the daily testing order. Flight performance was not correlated with exploratory-like behavior (Pearsonś correlation: 0.02, p-value = 0.84). Exploratory-like behavior

had a repeatability score R of 0.324 (adjusted for daily order) and flight performance had a repeatability score of 0.277, which can be considered good to moderate for behavioral measures, e.g., Dingemanse et al. (2002).[71]

Sampling timepoints

Each individual was blood sampled five times during its life. For this study, we focused on three of those timepoints (the other two timepoints were used in other studies): 1. ''Fledgling'' (14 dph): At the end of the thyroid hormone (TH) treatment and approximate time of fledging. These measurements were used to assess longitudinal changes in mitochondrial traits between fledging and adulthood; 2. ''First Adult'' (average age 186 dph, range 126–232 dph): The first sampling timepoint in adulthood. This was the focal timepoint for our main questions; 3. ''Second Adult'' (average age 316 dph, four months after ''First Adult''). Exact individual ages at ''First Adult'' and ''Second Adult'' were included in statistical analysis as a covariate, but later removed because they did not have any effect on the dependent variables. We included further analysis showing that individual ages did not affect mitochondrial traits (Table S16), flight performance or exploratory-like behavior (Table S17) at the First Adult timepoint in the supplemental information. Exploratory-like behavior and escape flight performance were measured at both First and Second Adult. Blood samples were collected immediately after the behavior assay, and mitochondrial traits and corticosterone were measured from these samples. Sample sizes at the Second Adult timepoint were reduced for two reasons: (1) some individuals from the Large Broods group were used in another study (Table S1), and (2) a laboratory issue reduced mitochondrial measurements (specifically ETS). Because mitochondrial traits did not differ between the First and Second Adult timepoints (see section *Statistics*/linear mixed models), and sample sizes were lower at the Second Adult stage, we restricted all mitochondrial analyses to the First Adult timepoint. However, we used both adult timepoints to test the robustness of behavior and flight performance. Repeatability scores were calculated between the two timepoints to confirm that our assays captured consistent aspects of individual life history strategies (see section *Statistics*). We did not calculate repeatability for mitochondrial traits due to the smaller sample size at the Second Adult stage. Figure 2 shows a diagram of the longitudinal experimental design. Tarsus length and body mass were measured at each timepoint and the scaled mass index (SMI) was calculated using these two measures.[92]

Linear mixed models

To account for genetic similarities between siblings, we included the nest of birth (''nest of origin'') as a random effect in all models. We also included sex to control for possible sex-dependent differences in all models. We ran separate models for each of the five mitochondrial traits (''CMR'', ''OxPhos'', ''ETS'', ''Leak'' and ''FCR1''), either as dependent or predictor variables depending on the specific question. Whenever mitochondrial traits were used as the dependent variable, we log-transformed ''CMR'', ''OxPhos'', ''ETS'' and ''Leak'' to achieve the best model fit. To assess the effects of the early-life TH-treatment on the dependent variables of interest, we included the two-level predictor ''Group'' (''TH Large'' and ''Large Broods'') in the respective model. Whenever we included interactions, the model was run with and without the interaction for interpretation of the main effects.

To assess longitudinal changes in mitochondria, we ran the following models with each of the five mitochondrial traits as the dependent variable with individual ID as a random factor.

1. Fledgling to First Adult: ''Timepoint'' was included as a two-level predictor (''Fledgling'', ''First Adult'') in interaction with the predictor ''Group''. To determine group differences at First Adult, we used pairwise comparisons using *emmeans.*
2. Between Adult timepoints: We ran separate models with ''Timepoint'' included as a two-level predictor (''First Adult'', ''Second Adult'') in interaction with the predictor ''Group''. Mitochondrial traits did not differ between the two adult timepoints (Table S3 and Figure S1).

The variable ''Daily order'' was included in the respective models below to account for the daily testing sequence in the behavioral and flight performance test. Since birds were fasting during the experiment, we assessed the effect of the change in body condition over the course of the behavioral treatment on mitochondrial traits. Models were run with each of the mitochondrial traits as the dependent variable and the change in SMI (''SMI difference'') as the predictor variable. SMI difference did not have an effect on any of the mitochondrial traits (Table S4). We further tested for effects of the testing sequence on mitochondria, by running separate models with each of the mitochondrial traits as the dependent variable and ''Daily order'' was as the predictor. ''Daily order'' hereby reflects the testing sequence during the behavioral test, as well as the variability in handling time of the blood samples, as mitochondrial analysis in the laboratory was done in the same order as the behavioral test. ''Daily order'' did not have an effect on any of the mitochondrial traits (Table S3).To assess the association between flight performance and mitochondrial traits, flight performance scores were used as the dependent variable and each of the mitochondrial traits was used as the predictor variable in interaction with the predictor ''Group''. To assess the association between exploratory-like behavior and mitochondrial traits, we used the square root transformed exploration score as the dependent variable. We used a square root transformation to achieve the best model fit and to best account for zero values (birds that did not move at all during the behavioral trial). We used exploratory scores as the dependent variable to account for the effect of daily order on behavior (Table S7). Since the fasting during the experiment may affect plasma CORT values (e.g., Woodward et al. (1991)[99]), we tested several relationships involving CORT: (1) The effect of the change in body condition over the course of the behavioral treatment on CORT values. Log-transformed CORT values were used as the dependent variable and ''SMI difference'' as the predictor variable. (2) Whether CORT was different between the two

experimental groups, because it is possible that TH has long-term effects on CORT production, e.g., Rousseau et al. (2021).[100] Log-transformed CORT values as the dependent variable and "Group" as predictor. (3) The association between CORT and each of the mitochondrial traits. CORT values were used as predictors and each of the mitochondrial trait as the dependent variable. (4) To test whether CORT mediates the relationship between mitochondrial traits and exploratory behavior. Exploratory behavior was used as the dependent variable and each of the mitochondrial traits as he predictor in a three-way interaction with the "Group" and CORT values. We additionally tested whether the TH-induced elevation in ETS at fledging predicted flight performance or exploratory behavior in adulthood, as early elevation of ETS may entail delayed costs for behavior and performance (see also "cost of plasticity"[101]). For this purpose, we used the flight performance score, as well as the exploratory score, at First Adult as the dependent variables and ETS at Fledgling as the predictor variable in interaction with the predictor "Group". The posterior correlation of the predictors "ETS at Fledgling" and the "Group" was 0.415, indicating low collinearity. To test whether the early-life TH-treatment affected exploratory behavior and flight performance, we included the predictor "Group" in the respective models above.