

Mitochondria as the powerhouses of sexual selection: Testing mechanistic links between development, cellular respiration, and bird song

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ABSTRACT

The developmental environment can affect the expression of sexually selected traits in adulthood. The physiological mechanisms that modulate such effects remain a matter of intense debate. Here, we test the role of the developmental environment in shaping adult mitochondrial function and link mitochondrial function to expression of a sexually selected trait in males (bird song). We exposed male zebra finches (*Taeniopygia guttata*) to corticosterone (CORT) treatment during development. After males reached adulthood, we quantified mitochondrial function from whole red blood cells and measured baseline CORT and testosterone levels, body condition/composition, and song structure. CORT-treated males had mitochondria that were less efficient ($FCR_{L/R}$) and used a lower proportion of maximum capacity ($FCR_{R/ETS}$) than control males. Additionally, CORT-treated males had higher baseline levels of CORT as adults compared to control males. Using structural equation modelling, we found that the effects of CORT treatment during development on adult mitochondrial function were indirect and modulated by baseline CORT levels, which are programmed by CORT treatment during development. Developmental treatment also had an indirect effect on song peak frequency. Males treated with CORT during development sang songs with higher peak frequency than control males, but this effect was modulated through increased CORT levels and by a decrease in $FCR_{R/ETS}$. CORT-treated males had smaller tarsi compared to control males; however, there were no associations between body size and measures of song frequency. Here, we provide the first evidence supporting links between the developmental environment, mitochondrial function, and the expression of a sexually selected trait (bird song).

1. Introduction

Animals from a diversity of taxa use behavioral displays or elaborate ornaments to attract potential mates (Andersson and Iwasa, 1996; Collins et al., 1994). Uncovering the proximate mechanisms and environmental factors that influence the expression of displays and ornaments has been foundational in understanding the signal content of such sexually selected traits (Qvarnstrom and Price, 2001; Wilkinson et al., 2015; Zera and Harshman, 2001). Since the publication of Mousseau and Fox (1998), there has been substantial interest in the effect of the developmental environment on the expression of sexually selected traits

in adulthood (e.g., Buchanan et al., 2003; Naguib and Nemitz, 2007; Walker et al., 2013). Sexually selected traits that are influenced by the developmental environment may provide information about the maternal environment a potential mate experienced and/or how well they coped with perturbations during development (Miller and Moore, 2007; Nowicki et al., 2002; Spencer and MacDougall-Shackleton, 2011). A body of research has linked elevated levels of the glucocorticoid (GC) hormones during development to decreased expression of sexually selected traits in adulthood (Spencer and MacDougall-Shackleton, 2011; Crino and Breuner, 2015; Leary and Baugh, 2020). However, the proximate cellular mechanisms that link elevated levels of GCs during

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development to changes in trait expression in adulthood remain unknown.

In recent years, it has been proposed that mitochondrial function plays an integral role in modulating the expression of sexually selected traits (Hill, 2018; Hill and Johnson, 2013; Koch and Hill, 2018; Koch et al., 2017) and could potentially be a cellular mechanism that links developmental perturbations to the expression of sexually selected traits in adults. Mitochondria are referred to as the ‘powerhouses’ of life because they produce 90% of the adenosine triphosphate (ATP) animals need to survive (Nicholls and Ferguson, 2002). As part of oxidative phosphorylation, mitochondria establish an electrochemical gradient by pumping protons from the matrix into the intermembrane space. ATP is synthesised from adenosine diphosphate (ADP), as protons pass down this gradient through ATP synthase (Nicholls and Ferguson, 2002). Increased mitochondrial membrane ‘leakiness’ can alter the flow of electrons, resulting in reduced efficiency of ATP production as electrons pass across the inner membrane without activating ATP synthase (Brand, 2005). Mitochondria vary, both between individuals and across environmental conditions, in their efficiency of ATP production and associated production of reactive oxygen species (ROS; Salin et al., 2015; Salin et al., 2016b). ROS are necessary by-products of oxidative phosphorylation (OXPHOS; Nicholls and Ferguson, 2002) and serve as key signalling molecules in the regulation of mitochondrial function, but are also implicated in oxidative stress, cell damage, and increased senescence (Apel and Hirt, 2004; Chen et al., 2007). Thus, variation in mitochondrial efficiency can have consequences for both energy production and somatic damage.

Condition-dependent sexually selected traits may have evolved as indicators of efficient mitochondrial function (i.e., energy production) and/or an individual’s ability to counteract the harmful effects of oxidative stress resulting from elevated levels of ROS (Hill, 2014, 2015a; Koch and Hill, 2018; Koch et al., 2017). Recent evidence in house finches (*Haemorhous mexicanus*) shows positive associations between mitochondrial function and the redness of feathers and provides the first evidence linking mitochondrial function to a sexually selected ornament (Hill et al., 2019). Other recent studies have established that the conditions experienced during development can have sustained effects on mitochondrial function (Casagrande et al., 2020; Stier et al., 2022; Udino et al., 2021). Further studies that test associations between mitochondrial function and other sexually selected ornaments and displays will show if mitochondrial function is a widespread driver of individual- and population-level variation in the expression of sexually selected traits.

Bird song is a widely studied, condition-dependent sexually selected trait (e.g., Buchanan et al., 2013; Catchpole, 1987; Searcy and Andersson, 1986). Passerines learn a species-specific song during development (Marler, 1981). Exposure to disturbances, such as food restriction or elevated levels of corticosterone (CORT; the dominant avian GC), during development decreases the volume of the HVC, a neural structure associated with the production of complex song (e.g., Bell et al., 2018; Buchanan et al., 2004; MacDonald et al., 2006; Nowicki et al., 2002). Although there is a rich literature examining the effects of the developmental environment on song learning/production, the mechanisms that link elevated levels of CORT, food restriction, or other compromised developmental conditions to reduced song quality have not yet been identified. Mitochondrial function is a likely, but untested, mechanism. Efficient mitochondrial function is critical for neurogenesis (Cheng et al., 2010; Mandal et al., 2011) and compromised mitochondrial function early in life could have lasting damage on neural structures through energy limitation and increased ROS production (Kamsler and Segal, 2003; Park et al., 2003). Additionally, developmentally induced changes in mitochondrial function that affect body size and condition could affect song in adult birds by changing the ability to produce energy needed to perform song displays and/or by changing motor performance (Koch and Hill, 2018). For example, exposure to elevated CORT during development decreases growth and can have lasting effects

on adult body size (Crino et al., 2014a; Kraft et al., 2019). Song frequency can be affected by muscle performance, body size, and morphology, and is likely under sexual selection, given its robust relationship with sexual size dimorphism (Francis and Wilkins, 2021; Friis et al., 2021; Mikula et al., 2021; Podos, 2001). Taken together, this evidence suggests that developmental conditions may affect components of bird song via changes in mitochondrial function, with consequences for reproductive success.

Here, we examined the mechanistic pathway linking the early developmental environment to sustained effects on mitochondrial function and the expression of a sexually selected trait (song) in male zebra finches (*Taeniopygia guttata*). Zebra finches are widely used in studies of song structure/production, sexual selection, and endocrinology (Griffith and Buchanan, 2010) and thus provide an ideal model system to test our experimental questions. We exposed males to elevated levels of CORT during the nestling period and examined the effects of this developmental treatment on mitochondrial function and song in adulthood. Exposure to elevated CORT levels during development can have programmatic effects on the neuroendocrine pathway that regulates the release of GCs (the hypothalamic pituitary adrenal (HPA) axis), such that animals exposed to elevated CORT during development release higher levels of CORT as adults (e.g., Spencer et al., 2009). GCs can decrease the expression of condition-mediated sexually selected traits (reviewed in Leary and Baugh, 2020) and act on mitochondrial physiology and biogenesis (Du et al., 2009; Hunter et al., 2016; Picard et al., 2018; Weber et al., 2002). For this reason, we examined associations between mitochondrial function, baseline CORT levels, body condition, and song. We also examined associations between testosterone levels and song structure because of the well-established effects of testosterone on song in passerines (Hau et al., 2000; Ritschard et al., 2011).

We predicted that CORT exposure during development would reduce mitochondrial respiration, either through a reduction in basal cellular metabolic rates or by reducing coupling efficiency (similar to findings by Casagrande et al., 2020). We predicted that CORT exposure during development would alter aspects of song structure, including the frequency (pitch), length, and consistency of songs (Spencer et al., 2005; Zann and Cash, 2008). Additionally, we predicted that a reduction in song length, complexity, and consistency would be associated with decreased mitochondrial efficiency (based on Hill, 2014, 2015a, 2015b; Koch et al., 2021; Koch and Hill, 2018). We predicted that CORT treatment during development would program HPA axis function, resulting in elevated levels of baseline CORT in adulthood similar to previous studies (Spencer et al., 2009). Consequently, we predicted that elevated levels of baseline CORT stemming from CORT treatment during development would be associated with decreased testosterone production in adults, due to the suppressive effects of GCs on the hypothalamic-pituitary-gonadal axis (Wingfield and Sapolsky, 2003). Similarly, we predicted that adults would have reduced body condition due to elevated levels of CORT (like past results, e.g., Kraft et al., 2019). We provide the first empirical study to test mechanistic links between the developmental environment, mitochondrial function, and bird song.

2. Methods

2.1. Experimental timeline

Males were dosed with CORT treatments from 5 to 18 days post-hatching (see below). After reaching adulthood (mean age = 579.5 days, range 397–778), males were sampled for body composition and condition, baseline CORT levels, testosterone levels, mitochondrial function, and song structure (see below for treatments). Males used in this study varied in age because they were sourced opportunistically from a separate experiment. There was no difference in the mean age of the males allocated to the CORT treatment compared to the control treatment (ANOVA $F_{1,27} = 1.14$, $\eta^2 = 0.04$, $p = 0.30$; CORT-treated = 599.5 days, control = 558.0 days).

2.2. Housing and husbandry

We housed 32 pairs of adult zebra finches across four indoor aviaries measuring $3.0 \times 1.0 \times 2.0$ m at Deakin University, Wauran Ponds, Australia. We housed birds on a 14:10 light/dark cycle at 20°C ($\pm 1^\circ\text{C}$) with $\approx 50\%$ humidity. We provided breeding birds with an *ad libitum* commercial seed diet, water, cuttlefish, grit, and a daily provision of supplemental hardboiled egg, spinach, and cucumber. We provided birds with wooden nest boxes, wicker nesting baskets, and shredded burlap and commercially available grass for nesting. Birds paired and bred freely over sequential breeding attempts. We inspected nests daily to determine breeding activity and the hatching date of nestlings. The male offspring used in this study remained in aviaries with their parents until they developed sex-specific plumage (<45 days post-hatching), after which they were housed in single-sex aviaries prior to use in this experiment.

2.3. Developmental CORT treatment

Nestlings were treated with an oral CORT or control treatment twice daily (4–6 h between doses) from 5 to 18 days post-hatching. The first hatched nestling of a clutch was randomly assigned a treatment by flipping a coin; each subsequent nestling was assigned the opposite treatment to the previously hatched nestling. CORT-treated nestlings twice received 0.25 mg/ml of CORT dissolved in 25 μl of peanut oil, for a total daily dose of 6.2 μg of CORT. This treatment has previously been shown to have sustained effects on physiology, behaviour, and reproductive success in zebra finches (Crino et al., 2014a; Crino et al., 2014b; Kraft et al., 2021; Kraft et al., 2019; Spencer et al., 2009). Control nestlings received 25 μl of peanut oil twice a day. We used only one bird per nest for this study to avoid pseudoreplication due to nest effects.

2.4. Cellular respiration

We transported birds to a Monash University field station (Newstead, Victoria) to quantify mitochondrial respiration. After transport, we allowed 24 h for birds to acclimate to their novel housing environment before collecting data. We housed birds in three cages ($100.0 \times 40.0 \times 33.0$ cm) with five birds per cage in an isolated room. We provided birds with an *ad libitum* commercial seed diet and water. We measured mitochondrial function in two sampling periods, which allowed us to minimise disturbances to the birds prior to data collection. CORT levels were not affected by the sampling order of birds ($F_{1,27} = 0.78$, $n^2 = 0.03$, $p = 0.68$).

We measured mitochondrial respiration from whole red blood cells using an Oxygraph-2 k high resolution respirometer (Oroboros Instruments, Innsbruck, Austria), following methods by Stier et al. (2017). To collect blood from birds, we used a 27-gauge needle to puncture the brachial vein and collected $\approx 250 \mu\text{l}$ of blood into heparinised microcapillary tubes. We collected blood samples within 5 min of disturbing birds ($\bar{x}=153.66$ s, range = 68–300 s). CORT levels increase with handling and are often elevated above baseline levels 3 min or less after disturbance (Romero and Reed, 2005). For this reason, we accounted for handling time in statistical analyses (see below). We centrifuged half of the blood at 10,000 rpm for 5 min and isolated the plasma for use in hormone assays. The plasma was stored at -20°C immediately following collection and then stored at -80°C until assayed. We kept the remaining blood on crushed ice (<10 min) before centrifuging it at 3000 g for 10 min to separate whole red blood cells from plasma. We removed the plasma and pipetted 40 μl of red blood cells into 1 ml of ice-cold phosphate buffer solution (PBS), homogenized the sample, and then washed the sample by centrifuging the tube at 600 g for 5 min. We then resuspended the red blood cells in 1 ml of respiratory buffer MiR06 [0.5 mM EGTA, 3 mM MgCl₂, 60 mM K-lactobionate, 20 mM taurine, 210 mM KH₂PO₄, 20 mM Hepes, 100 mM sucrose, free fatty acid bovine serum albumin (1 g L⁻¹, pH 7.1)]. We then added 1 ml of the red blood

cell suspension to 1 ml of MiR06 buffer that was equilibrated at 40°C in the respirometer chamber.

To measure mitochondrial function in the resuspended red blood cells, we serially added mitochondrial agonists and antagonists and sequentially measured four mitochondrial parameters: basal respiration (*Routine*), oxygen consumption used for ATP synthesis (*OXPHOS*), oxygen consumption resulting from proton leak (*Leak*), and maximum capacity of the electron transport chain (*ETS*) (Stier et al., 2017; Table 1). From these parameters, we calculated three flux control ratios (FCRs), which quantify the efficiency of mitochondrial function: coupling efficiency of mitochondria between O₂ consumption and ATP output under endogenous conditions (*FCR_{L/R}*), the coupling efficiency of mitochondria between O₂ consumption and ATP output under stimulated conditions (*FCR_{L/ETS}*), and the proportion of ETS maximum capacity used by the mitochondria under endogenous conditions (*FCR_{R/ETS}*) (Stier et al., 2017; Table 1). All mitochondrial parameters were normalized by the total amount of protein in the aliquot of red blood cells using Bradford assays (Thermo Fisher Scientific, 23236).

2.5. Hemoglobin concentrations

For a subset of birds, we measured whole blood hemoglobin concentration (g/dL) using a Hemocue photometer (Hemocue, Ängelholm, Sweden) at the time of blood collection ($n = 7$ control, 10 CORT-treated). We chose to measure hemoglobin concentration because measuring mitochondrial function from whole red blood cells is a relatively new method (Stier et al., 2017). Although it is proving to be an increasingly common method to measure mitochondrial function, there are many outstanding questions about current methodology and the interpretation of measurements. We had no *a priori* predictions about the potential of hemoglobin to interact with mitochondrial measurements from whole red blood cells. However, we measured it with the intent of contributing foundational knowledge to a new area of research.

2.6. Body condition and composition

Immediately prior to transporting birds from Deakin University, we used a quantitative magnetic resonance (QMR) body composition analyzer (EchoMRI-B, Echo Medical Systems, Houston, USA) to quantify total fat and lean mass (non-structural tissues such as muscle). Prior to use, we calibrated the instrument with a 34.9 g canola oil standard. To measure body composition, we placed birds in a ventilated falcon tube within a plastic holding tube and inserted them into the QMR instrument. We adjusted the body composition values using calibration equations for zebra finches (fat (g) $\times 0.94$ and lean mass (g) $\times 1.021$;

Table 1

Description of mitochondrial parameters measured from whole red blood cells. We calculated the flux control ratios (*FCR_{L/R}*, *FCR_{L/ETS}*, and *FCR_{R/ETS}*) from the measured mitochondrial parameters (methods from Stier et al., 2017).

Variable	Description
<i>Routine</i>	Baseline mitochondrial O ₂ consumption under endogenous cellular conditions
<i>OXPHOS</i>	O ₂ consumption used to produce ATP via oxidative phosphorylation (OXPHOS)
<i>Leak</i>	O ₂ consumption due to proton leak of the mitochondria
<i>ETS</i>	Maximum O ₂ consumption due to the electron transport system (ETS)
<i>FCR_{L/R}</i>	Fraction of <i>Routine</i> respiration attributed to proton LEAK: indicative of the efficiency of mitochondria to couple ATP production to oxygen consumption under endogenous conditions
<i>FCR_{L/ETS}</i>	Fraction of maximum <i>ETS</i> respiration attributed to proton LEAK: indicative of the efficiency of mitochondria under stimulated conditions
<i>FCR_{R/ETS}</i>	Proportion of <i>ETS</i> maximum respiration under endogenous conditions

Guglielmo et al., 2011). We calculated two body composition parameters from QMR: relative fat (fat (g)/tarsus (mm)) and relative lean tissue (lean tissue (g)/tarsus (mm)). In addition to body composition, we calculated body condition as the scaled mass index derived from tarsus length and body mass (Peig and Green, 2009).

2.7. Song structure – recordings and analysis

In zebra finches, a typical song bout consists of repeated introductory syllables followed by a stereotyped syllable sequence (known as a song, song-phrase, or motif) that is repeated for the bout's duration (Zann, 1996). We recorded male songs in individual cages in an isolated, sound-attenuated room at Deakin University. After a 2- to 3-minute acclimation period, we introduced one of ten companion females to the male's cage. We recorded songs using a Sennheiser MKE 2P condenser tie-clip microphone (Sennheiser electronic GmbH, Germany) attached to a Zoom H6 handy recorder (Zoom Corporation, Japan). We recorded male songs during five-minute recording sessions, with a minimum of three sessions to account for intra-individual variation in songs. Two of the males did not sing within ten recording sessions. From every other male, we randomly selected five songs for bioacoustic analysis, excluding introductory syllables.

Using the software Sound Analysis Pro 2011 (Tchernichovski et al., 2000), we measured six acoustic parameters across each of the five songs individually: song duration (ms), mean frequency (Hz), peak frequency (Hz), goodness of pitch, number of syllables, and song consistency (Table 2). The first four variables were measured using the 'Explore & Score' function with default settings. Mean frequency is the centre of distribution of power across frequencies, while peak frequency is the frequency with the highest power. Goodness of pitch is an estimate of the periodicity of harmonic pitch, with higher values indicating more harmonic syllable structures. The prevalence of harmonic syllables may indicate song quality in zebra finches, since males that underwent a tracheosyringeal nerve transection produced fewer harmonic syllables and more noisy syllables (Tomaszycki and Adkins-Regan, 2005). The number of syllables per song was determined based on their separation in time or structure from other elements of the song, via visual inspection of sonograms by a single observer (A.C.K.) who was blind to treatments. We quantified the consistency of song structure, a potential measure of song quality (e.g., Botero et al., 2009; de Kort et al., 2009), using the 'Similarity Batch' function, which measures the similarity of songs based on their pitch, amplitude modulation, frequency modulation, Weiner entropy and goodness of pitch (Tchernichovski et al., 2000). Using default settings, we compared each song with four other songs from the same individual to calculate %Similarity (the percentage of sounds from the first song included in the second), which we interpreted as a measure of song consistency. For each individual, we calculated mean values for each of the six song parameters measured.

2.8. Hormone assays

We measured CORT levels with Enzyme Immunoassay (EIA) kits (Cat No. 901-097, Enzo Life Sciences). We used a raw plasma dilution of 1:25

Table 2
Description of song parameters evaluated in this study.

Variable	Description
Duration (ms)	Mean song duration
Goodness of pitch	Periodicity of harmonic pitch, with higher values indicating more harmonic syllable structures
Mean frequency (Hz)	Mean frequency across song
Peak frequency (Hz)	Frequency at which the song is the loudest
Syllables	Mean number of syllables in a song
Similarity (%)	Broad-scale song similarity, a measure of song consistency

with a 1.5% solution of steroid displacement buffer to determine CORT levels (optimized in Wada et al., 2008). We ran our samples in triplicate against a six-point standard curve ranging from 6.4 to 20,000 pg/ml. An external standard of 500 pg/ml was run on every plate and used to calculate inter-plate variation. Plates were read on a VarioskanLUX microplate reader at 405 nm corrected at 580 nm. Intra- and inter-plate variation were 5.47% and 21.69%, respectively.

Testosterone was quantified using EIA kits (Cat No. 900-097, Enzo Life Sciences). We assayed samples in triplicate using a raw plasma dilution of 1:20. An external standard of 500 pg/ml was run on every plate and used to calculate inter-plate variation. Plates were read on a Varioskan LUX microplate reader at 405 nm corrected at 580 nm. Levels of testosterone were determined from a five-point standard curve ranging from 7.81 to 2000 pg/ml. Intra- and inter-plate variation were 5.71% and 11.23%, respectively.

2.9. Statistics

All data were analysed in R version 4.1.1 using the lme4, MuMin, and piecewiseSEM packages (Barton, 2009; Bates et al., 2015; Lefcheck, 2016a). Baseline CORT levels were positively correlated with the time to collect blood samples ($F_{1,27} = 9.14$, $p = 0.005$, $r^2 = 0.25$). For this reason, we used the residuals of CORT level regressed against blood collection time in all analyses involving CORT as an explanatory factor (hereafter: CORT levels). Testosterone levels were not affected by the time to collect blood samples ($F_{1,22} = 1.08$, $p = 0.31$) and raw values were used in all analyses. For all models, we checked model residuals for normality using Shapiro-Wilk tests. When residuals were non-normal, generalized linear effects models (GLMs) with gamma distributions and log-link functions were used to account for right-skewed data. We evaluated collinearity of model terms using variance inflation factors. Means are provided with one standard error. We calculated effect sizes using Cohen's D for pair-wise comparisons and eta squared for ANOVAs (Lakens, 2013).

Sample sizes varied between models because (1) we were unable to record songs from all males, (2) there was not enough plasma to measure CORT and T levels for all birds, and (3) some red blood cells were not viable for collecting all mitochondrial respiration parameters. The sample sizes for CORT assays were $n = 14$ control and $n = 15$ for CORT-treated. Analyses that examine body condition or body composition include CORT levels as a covariate; therefore, the sample sizes for these models are also $n = 14$ control and $n = 15$ CORT-treated. The sample sizes for testosterone assays were $n = 13$ control and $n = 11$ CORT-treated. All sample sizes for other analyses are listed in the associated tables.

General linear analyses – We examined the effects of nestling developmental treatment on adult baseline CORT levels using a generalized linear mixed effects model with a gamma distribution and CORT levels as the dependent variable and time to collect blood sample, scaled body mass, and clutch size as explanatory variables. We tested the effect of developmental treatment on testosterone levels using GLMs. We included clutch size as a covariate because it can affect nestling CORT levels in some species (e.g., Saino et al., 2003). To examine the effect of developmental treatment on adult testosterone levels, we used a GLM with developmental treatment as a fixed effect and body condition and CORT levels as covariates. We included CORT levels because of the known suppressive effects of CORT on testosterone (Deviche et al., 2014; McGuire et al., 2013) and body condition because low body condition can be associated with elevated CORT levels (Crino et al., 2017; Crino et al., 2018; Perez-Rodriguez et al., 2006).

We used GLMs to examine the effects of developmental treatment on body composition (lean tissue only) and condition (scaled mass index) with developmental treatment, clutch size, and CORT levels as covariates. We included clutch size as a covariate because of the sustained effects of clutch size on body size/condition in small passerines (Crino et al., 2014a; Kraft et al., 2019). Relative lean tissue measures were log-

transformed prior to analysis. We tested differences in body size between treatment groups using GLMs with tarsus length or body mass as the dependent variables and developmental treatment and clutch size as covariates. Many males had measures of fat mass equal to zero grams as determined by QMR ($n = 6$ control and $n = 8$ CORT-treated). For this reason, we used a chi-square analysis to examine if males exposed to the CORT treatment were more likely to have zero fat reserves than control males.

We used GLMs to examine how mitochondrial respiration was affected by developmental treatment. For these models, we used mitochondrial respiration parameters as the dependent variables with developmental treatment and bird age as explanatory variables. We included bird age as an explanatory factor because of the known effects of senescence on mitochondrial function (Dawson and Salmon, 2020; Kokoszka et al., 2001).

We used GLMs to test associations between mitochondrial efficiency and song structure. We used song parameters (Table 2) as dependent variables and developmental treatment, mitochondrial parameters ($FCR_{L/R}$ or $FCR_{R/ETS}$), and CORT levels as explanatory factors. We chose to use $FCR_{R/ETS}$ and $FCR_{L/R}$ in analyses because flux ratios are internally normalized and provide the most useful measures of mitochondrial function (Stier et al., 2017). We included body mass as a covariate in analyses with song frequency measures as the dependent variable because body mass can explain variation in fundamental song frequency across bird species (but see Cardoso et al., 2008; Mikula et al., 2021). We found no associations between measures of song frequency and body mass (mean frequency $F_{1,24} = 0.03$, $\eta^2 = 0.001$, $p = 0.86$; peak frequency $F_{1,24} = 0.01$, $\eta^2 < 0.001$, $p = 0.93$) or tarsus length (mean frequency $F_{1,24} = 0.01$, $p = 0.92$, $\eta^2 < 0.001$; peak frequency $F_{1,24} = 0.001$, $\eta^2 < 0.001$, $p = 0.97$). However, statistical results for models with and without body mass as a covariate were statistically equivalent and we report results for models that include body mass.

Structural equation modelling: teasing apart direct and indirect treatment effects – Treatment with CORT during development has well-established programmatic effects on HPA axis function, such that animals exposed to elevated CORT during development secrete greater amounts of CORT at later life history stages (Crino et al., 2014a; Schmidt et al., 2014; Spencer et al., 2009). In this way, treatment with CORT during development could affect mitochondrial function and song structure through indirect programmatic effects on the HPA axis (i.e., an intermediate outcome; Nakagawa et al., 2017). As in past studies, we found that adults treated with CORT during development had elevated levels of baseline CORT (see Results). To tease apart the direct and indirect effects of the CORT treatment on mitochondrial function and song structure, we conducted post hoc piecewise structural equation modelling (SEM). SEM uses multiple predictors and explanatory variables in a network to infer causality based on known information from observations or experimentation (Lefcheck, 2016b). In this way, SEM can reveal cascading effects between variables that may otherwise be hidden when relationships are evaluated in separate models (Grace, 2006). SEM is increasingly being used as a tool to evaluate variables that are influenced by multiple physiological metrics (e.g., Frauendorf et al., 2021; Sudnick et al., 2021).

We tested two hypothetical pathways by which the developmental treatment could affect mitochondrial function via effects on CORT production. In these pathways, song structure can be affected directly by the developmental treatment or indirectly via changes in mitochondrial function and CORT production. We evaluated these pathways with two sets of models that used song mean frequency as the response variable and either $FCR_{L/R}$ or $FCR_{R/ETS}$ as the mitochondrial parameters. We chose to use these variables because we found that developmental treatment affected $FCR_{L/R}$ and $FCR_{R/ETS}$ and we found an association between $FCR_{R/ETS}$ and song mean frequency (see Results). We calculated standardized path coefficients and corresponding significance (p -values), overall fit of the model using Fisher's C, and compared models using AIC in the PIECEWISESEM package in R. We did not have *a priori*

predictions for the outcomes of SEM models because these analyses were conducted following initial statistical analyses.

Post-hoc analyses – baseline CORT levels and mitochondrial function and hemoglobin and mitochondrial function – Following the outcome of the structural equation models, we conducted post hoc GLMs to examine the effects of baseline CORT levels on mitochondrial function. For these analyses, we used baseline CORT as the independent variable and $FCR_{L/R}$ and $FCR_{R/ETS}$ as dependent variables in separate models. Additionally, we used GLMs to analyse the association between hemoglobin levels and mitochondrial function ($FCR_{L/R}$ and $FCR_{R/ETS}$). Hemoglobin levels were log-transformed to meet assumptions of normality for GLM. Hemoglobin was measured without *a priori* predictions.

3. Results

3.1. Developmental treatment – effects on cellular respiration

Adult males exposed to CORT during development had less efficient mitochondrial function, as measured by higher respiratory control ratios $FCR_{L/R}$ ($F_{1,18} = 5.60$, $\eta^2 = 0.22$, $p = 0.03$; Fig. 1a), and used a lower proportion of their maximum mitochondrial capacity, as measured by the respiratory control ratio $FCR_{R/ETS}$ ($F_{1,18} = 5.26$, $\eta^2 = 0.21$, $p = 0.03$; Fig. 1b). Developmental treatment had no effect on Routine, OXPHOS, Leak, ETS, or $FCR_{L/R/ETS}$ mitochondrial function ($F < 2.60$, $p > 0.12$ for all; effect sizes in Table 3). There were no associations between any mitochondrial parameters and bird age ($F < 3.61$, $p > 0.08$; effect sizes in Table 3). However, statistical outcomes were equivalent if bird age was

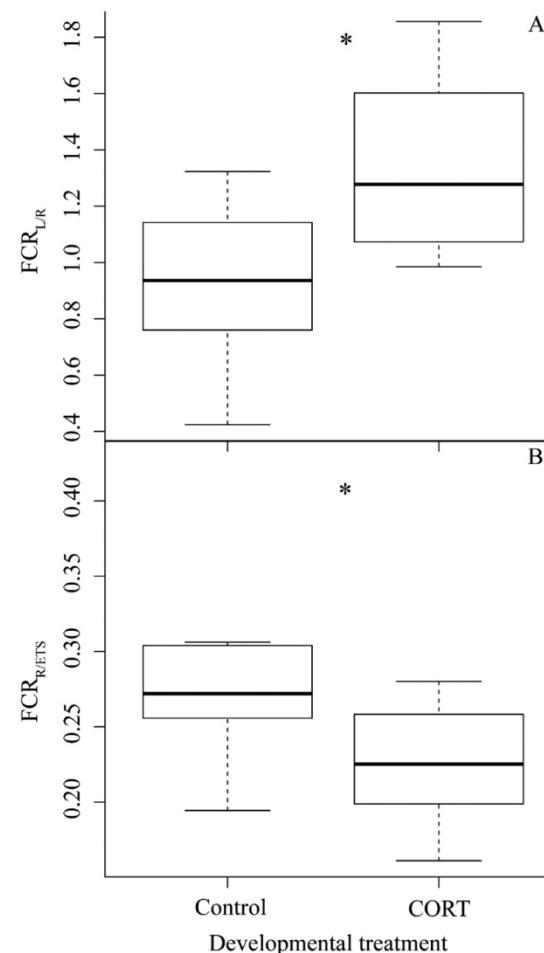


Fig. 1. The effects of developmental treatment on mitochondrial A) coupling efficiency ($FCR_{L/R}$), and B) electron transport chain function in relation to basal metabolic rate ($FCR_{R/ETS}$). * $p < 0.05$.

Table 3

Model effects from GLMs examining the effects of developmental treatment and age associations on mitochondrial parameters.

Dependent variable	Independent variables	N			d.f.	F	p	η^2
			CORT	Control				
<i>Routine</i>	Treatment	12	14	1,23	2.60	0.12	0.10	
	Bird age				0.41	0.53	0.02	
<i>OXPHOS</i>	Treatment	9	7	1,12	0.11	0.75	0.01	
	Bird age				3.61	0.08	0.21	
<i>Leak</i>	Treatment	8	13	1,19	0.004	0.95	<0.01	
	Bird age				3.58	0.07	0.17	
<i>ETS</i>	Treatment	9	13	1,18	1.59	0.22	0.08	
	Bird age				2.48	0.13	0.11	
<i>FCR_{L/R}</i>	Treatment	9	13	1,18	6.79	0.02	0.22	
	Bird age				2.04	0.17	0.10	
<i>FCR_{L/ETS}</i>	Treatment	8	13	1,18	1.47	0.24	0.08	
	Bird age				0.67	0.42	0.04	
<i>FCR_{R/ETS}</i>	Treatment	9	13	1,18	6.95	0.02	0.21	
	Bird age				0.002	0.97	<0.01	

Bold values indicate significant factors ($p < 0.05$).

removed from models; for this reason, age was retained in the final models presented here.

3.2. Song structure, developmental treatment, and mitochondrial function

Song mean and peak frequency were negatively associated with the mitochondrial flux control ratio ($FCR_{R/ETS}$), which represents the

proportion of maximum mitochondrial respiration rate used endogenously ($F_{1,15} = 5.52, 5.27; p = 0.03, 0.04$ respectively; Fig. 2). However, there were no associations between $FCR_{L/R}$ and song mean frequency or peak frequency ($F < 1.11, p > 0.31$ for all; effect sizes in Table 4). Similarly, there were no associations between $FCR_{L/R}$ and $FCR_{R/ETS}$ and song duration, number of syllables, goodness of pitch, or song consistency ($F < 3.15, p > 0.10$ for all; effect sizes in Table 4). Developmental treatment did not affect song mean frequency ($F_{1,15} = 1.63, \eta^2 = 0.11, p = 0.22$) or peak frequency ($F_{1,15} = 1.86, \eta^2 = 0.01, p = 0.19$). Body mass did not affect song mean frequency ($F_{1,15} = 1.79, \eta^2 = 0.12, p = 0.20$) or peak frequency ($F_{1,15} = 1.38, \eta^2 = 0.10, p = 0.26$). There were no effects of the developmental treatment on song duration, number of syllables, goodness of pitch, or song consistency ($F < 1.83, p > 0.20$ for all; effect sizes in Table 4).

3.3. Developmental treatment – effects on corticosterone, testosterone, and body condition

Males treated with CORT during development had higher baseline CORT levels compared to control males ($\bar{x}_{\text{control}} = 4.92 \pm 0.93$ (ng/ml), $\bar{x}_{\text{CORT-treated}} = 9.31 \pm 1.32$ (ng/ml); $F_{1,25} = 7.59, \eta^2 = 0.20, p = 0.01$; Fig. 3). Baseline CORT levels were not associated with clutch size ($F_{1,25} = 3.25, \eta^2 = 0.11, p = 0.08$) or adult body condition ($F_{1,25} = 0.27, \eta^2 = 0.01, p = 0.61$). Testosterone levels did not vary in relation to developmental treatment ($F_{1,20} = 0.52, \eta^2 = 0.02, p = 0.48$), body condition ($F_{1,20} = 0.01, \eta^2 < 0.01, p = 0.91$), or CORT levels ($F_{1,20} = 0.13, \eta^2 = 0.01, p = 0.72$).

Body condition, calculated as the scaled mass index, was not affected by the developmental treatment ($F_{1,25} = 0.44, \eta^2 = 0.02, p = 0.51$), clutch size ($F_{1,25} = 0.30, \eta^2 = 0.01, p = 0.59$), or CORT levels ($F_{1,25} = 0.51, \eta^2 = 0.02, p = 0.48$). Lean tissue mass determined from QMR was not affected by developmental treatment ($F_{1,25} = 0.21, \eta^2 = 0.003, p = 0.65$), clutch size ($F_{1,25} = 0.004, \eta^2 < 0.01, p = 0.95$), or CORT levels ($F_{1,25} = 0.91, \eta^2 = 0.03, p = 0.35$). Males exposed to CORT were not more likely than control males to have a fat mass of zero ($\chi^2 = 0.4, d = 0.24, d.f. = 1, p = 0.85$). Males exposed to CORT during development had smaller tarsi compared to control birds ($F_{1,26} = 4.83, \eta^2 = 0.15, p = 0.04$), but did not differ from control males in adult body mass ($F_{1,25} = 1.92, \eta^2 = 0.07, p = 0.18$). There was no association between clutch size and tarsus length ($F_{1,25} = 0.49, \eta^2 = 0.02, p = 0.49$) or clutch size and body mass ($F_{1,25} < 0.001, \eta^2 < 0.01, p = 0.96$). Adult CORT levels were not associated with body mass ($F_{1,25} = 0.17, \eta^2 = 0.01, p = 0.90$).

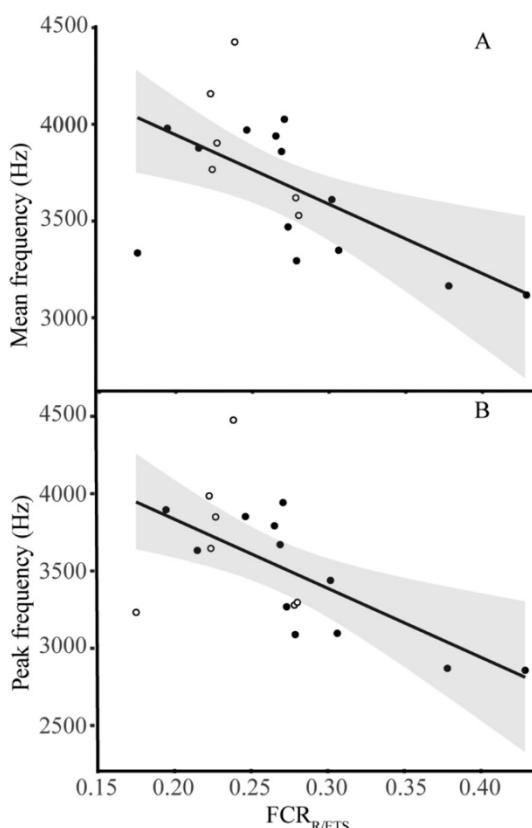


Fig. 2. Associations between the proportion of maximum capacity used by mitochondria ($FCR_{R/ETS}$) and A) song mean frequency ($p = 0.01$, partial $R^2 = 0.31$) and B) song peak frequency ($p = 0.01$, partial $R^2 = 0.27$). Closed circles indicate control group, open circles indicate CORT-treated group, shaded area indicates 95% confidence interval.

Table 4

Model effects from GLMs examining the effects of developmental treatment and mitochondrial function on song parameters.

Dependent variable	Independent variables	N		d.f.	F	p	η^2
		CORT	Control				
Duration (ms)	Treatment	7	14	1,16	0.96	0.34	0.05
	$FCR_{L/R}$				1.79	0.20	0.10
	$FCR_{R/ETS}$				3.15	0.10	0.16
Peak frequency (Hz)	Treatment	6	14	1,15	1.83	0.20	0.01
	$FCR_{L/R}$				0.70	0.42	0.04
	$FCR_{R/ETS}$				8.08	0.01	0.35
Mean frequency (Hz)	Body mass			1,15	1.72	0.21	0.10
	Treatment	6	14		1.94	0.18	0.11
	$FCR_{L/R}$				1.11	0.31	0.07
Syllables	$FCR_{R/ETS}$			1,16	7.61	0.01	0.34
	Body mass				2.01	0.18	0.12
	Treatment	7	14		0.37	0.55	0.02
Goodness of pitch	$FCR_{L/R}$			1,16	0.39	0.54	0.02
	$FCR_{R/ETS}$				0.56	0.47	0.03
	Treatment	7	14		0.09	0.77	0.01
Similarity (%)	$FCR_{L/R}$			1,16	0.18	0.67	0.01
	$FCR_{R/ETS}$				0.58	0.46	0.03
	Treatment	7	14		0.40	0.54	0.02
	$FCR_{L/R}$			1,16	0.18	0.67	0.01
	$FCR_{R/ETS}$				0.41	0.53	0.02

Bold values indicate significant factors ($p < 0.05$).

3.4. Path analysis – testing the links between developmental treatment, baseline CORT, cellular respiration, and song structure

The best supported pathway containing the respiratory control ratio $FCR_{L/R}$ (Fig. 4A) explained 47.4% of the variation in song mean frequency (AI = 24.31, Fisher's C = 2.31, df = 2, $p = 0.32$; where $p > 0.05$ indicates the pathway is suitable for inference; Lefcheck, 2016b). In this pathway, the developmental treatment has an indirect effect on mitochondrial function via effects on baseline CORT levels. Specifically, males exposed to CORT during development secrete higher levels of CORT as adults ($\beta = 0.62$, df = 18, $p = 0.003$), which, in turn, decreases mitochondrial efficiency as measured by an increase in the respiratory

control ratio $FCR_{L/R}$ ($\beta = 0.48$, df = 18, $p = 0.03$). Higher $FCR_{L/R}$ (lower efficiency) positively affected song peak frequency such that males with less efficient mitochondria sang songs with higher peak frequency ($\beta = 0.56$, df = 16, $p = 0.02$; Fig. 5). Song peak frequency was indirectly affected by the developmental treatment via changes in baseline CORT, such that frequency decreased in response to elevated levels of CORT ($\beta = -0.70$, df = 16, $p = 0.01$). In this pathway, there was no direct effect of developmental treatment on song peak frequency ($\beta = 0.45$, df = 18, $p = 0.08$).

The best supported pathway containing the respiratory control ratio $FCR_{R/ETS}$ (Fig. 4B) explained 64.1% of the variation in song peak frequency (AIC = 24.40, Fisher's C = 2.40, df = 2, $p = 0.29$). In this pathway, the developmental treatment has an indirect effect on song via effects on baseline CORT levels. Specifically, males exposed to CORT during development secrete higher levels of baseline CORT as adults ($\beta = 0.61$, df = 17, $p = 0.01$), which, in turn, decreases song peak frequency ($\beta = -0.68$, df = 15, $p = 0.004$). The developmental treatment did not directly affect song peak frequency ($\beta = 0.38$, df = 15, $p = 0.08$). Birds with mitochondria that functioned with a greater proportion of maximum mitochondrial capacity (higher $FCR_{R/ETS}$) produced songs with decreased song peak frequency ($\beta = -0.70$, df = 15, $p = 0.001$). There was no effect of CORT levels on $FCR_{R/ETS}$ ($\beta = -0.37$, df = 17, $p = 0.12$).

3.5. Post-hoc analyses – baseline CORT levels and mitochondrial function and hemoglobin

Mitochondrial inefficiency ($FCR_{L/R}$) was positively associated with baseline CORT levels ($F_{1,19} = 4.59$, $\eta^2 = 0.15$, $p = 0.045$; Fig. 6). $FCR_{R/ETS}$ was not associated with baseline CORT levels ($F_{1,19} = 1.49$, $\eta^2 = 0.05$, $p = 0.24$).

Hemoglobin levels were positively associated with mitochondrial inefficiency ($FCR_{L/R}$; $F_{1,13} = 8.22$, $\eta^2 = 0.36$, $p = 0.01$; Supplemental Fig. 2). There was no association between hemoglobin levels and mitochondrial $FCR_{R/ETS}$ ($F_{1,13} = 0.04$, $\eta^2 = 0.001$, $p = 0.84$).

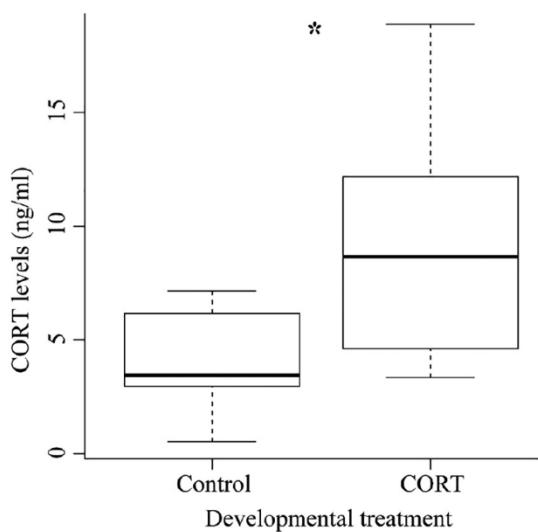


Fig. 3. The effect of development treatment (increased CORT or control) on adult baseline CORT levels. * $p = 0.01$.

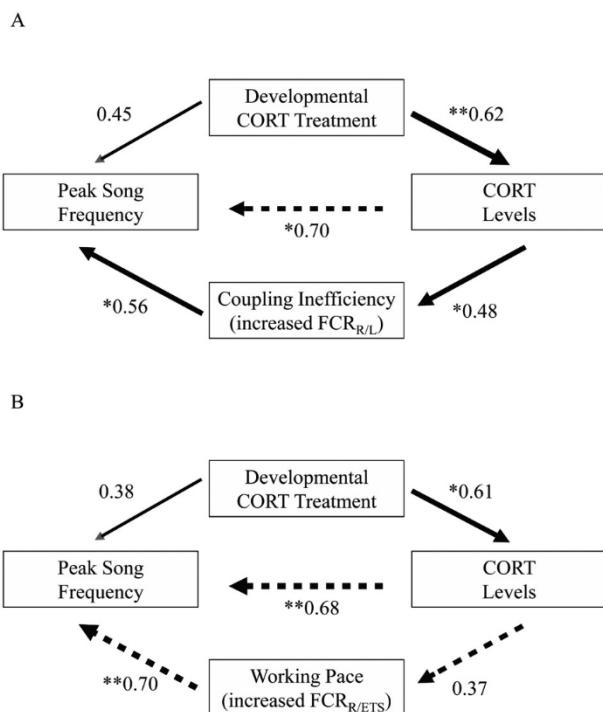


Fig. 4. Effects of developmental treatment on song peak frequency (Hz) via direct and indirect effects. Arrows represent unidirectional relationships between treatment and measured variables. Solid lines indicate positive relationships, dashed lines indicate negative relationships, relative thickness of lines indicates the magnitude of the standardized regression coefficient, $^{**}p < 0.01$, $^*p < 0.05$.

4. Discussion

Here, we provide the first evidence supporting links between the developmental environment, mitochondrial function, and the expression of a sexually selected display (directed song peak frequency). We show that exposure to elevated levels of CORT during development has sustained effects on mitochondrial function in adulthood. However, the effects of exposure to CORT during development on mitochondrial function are indirect and are modulated by adult baseline CORT levels, which are themselves programmed by CORT treatment during development. We found that the developmental treatment indirectly affected song peak frequency. Specifically, males treated with CORT during development sang songs with higher frequency than control males, but this effect was modulated through changes in CORT levels and mitochondrial function. Although males exposed to CORT during development had smaller tarsi compared to control males, there were no associations between body size and measures of song frequency in our study. We did not find treatment effects on testosterone levels, body condition, or lean tissue composition.

Males exposed to CORT during development had mitochondria that used a lower proportion of their maximum capacity (lower $FCR_{R/ETS}$) and had less efficient coupling ability ($FCR_{L/R}$, for which higher levels indicate lower efficiency; Fig. 1). Structural equation models revealed that CORT treatment during development affected mitochondrial function in adulthood through programmatic effects on HPA axis function rather than through direct effects on mitochondrial function. We found a positive association between baseline CORT levels and coupling efficiency, indicating that elevated CORT levels decrease mitochondrial efficiency. Our results parallel Casagrande et al. (2020), who found that nestling great tits (*Parus major*) exposed to elevated CORT levels had red blood cells with greater mitochondrial proton leak and lower efficiency (i.e., higher $FCR_{L/R}$). Together, these results suggest that elevated CORT

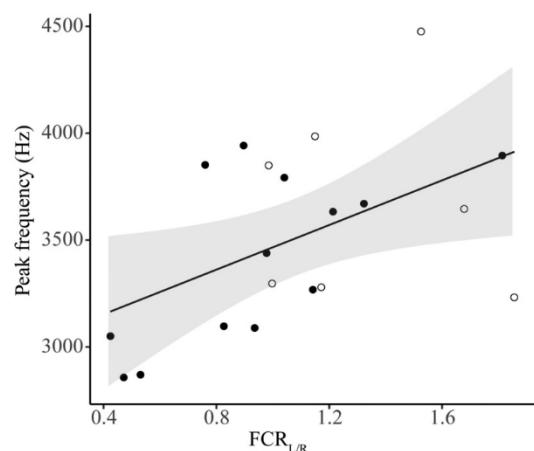


Fig. 5. Association between mitochondrial coupling efficiency ($FCR_{L/R}$) and song peak frequency. Closed circles indicate control group, open circles indicate CORT-treated group, shaded area indicates 95% confidence interval, $p = 0.02$, partial $R^2 = 0.27$.

levels in early life decrease ATP production of red blood cells.

There is growing interest in the use of red blood cells to quantify mitochondrial function (Dawson and Salmon, 2020; Nord et al., 2021; Stier et al., 2019; Ton et al., 2021; Udino et al., 2021). Using red blood cells to quantify mitochondrial function is advantageous because it does not necessitate sacrificing the focal animal and, thus, allows for longitudinal sampling and reduced harm of animals. However, there is currently no consensus about how mitochondrial function in red blood cells correlates with mitochondrial function across tissues (Koch et al., 2021). In king penguins (*Adenodytes patagonica*), mitochondrial function in red blood cells is moderately correlated with mitochondrial function in pectoral muscle (Stier et al., 2017). However, mitochondrial function can vary greatly between tissue types and can be correlated to different aspects of whole-animal metabolic rate (Mowry et al., 2017; Salin et al., 2016a). In great tits, mitochondrial function in red blood cells correlates with whole-animal metabolic rate, but only in birds that had low activity and CORT levels (Malkoc et al., 2021). Our results show that mitochondrial function is affected by elevated CORT levels and support the suggestion by Malkoc et al. (2021) that CORT levels should be accounted for when measuring whole-animal metabolic rate and

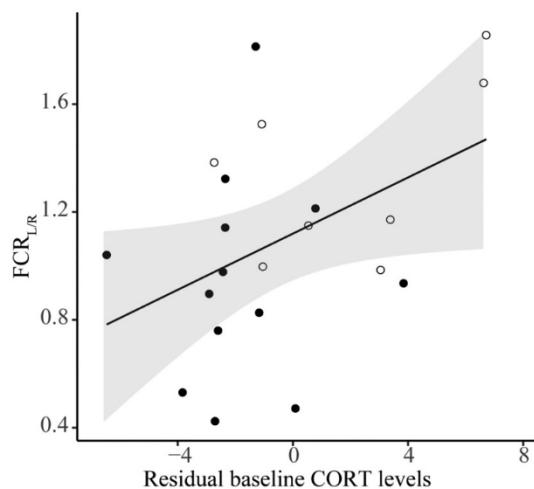


Fig. 6. Association between residual baseline CORT levels and mitochondrial coupling efficiency ($FCR_{L/R}$). Residuals were calculated using linear regression to account for time to collect blood samples. Closed circles indicate control group, open circles indicate CORT-treated group, shaded area indicates 95% confidence interval, $p = 0.045$, adjusted $R^2 = 0.15$.

metabolic rate at the tissue level. Studies that do not have the capacity to measure CORT levels could lessen the effect of CORT on metabolic measurements by minimising disturbance and handling time prior to collecting metabolic measurements or by using handling time as a covariate in analyses.

We found that males exposed to CORT during development sang songs with higher peak frequency compared to control males. Using structural equation modelling, we found statistical support that the effect of development treatment on song frequency in this experiment was indirectly regulated through treatment-induced changes in mitochondrial function rather than resulting directly from CORT treatment. Structural equation modelling is a useful statistical method for teasing apart intercorrelations and indirect effects within a data set. The results of our structural equation models suggest a possible link between mitochondrial efficiency and song frequency, although it is not possible to infer a mechanistic connection between mitochondrial function and song frequency from our experiment. Future studies could directly test the causal link between mitochondrial function and song frequency by manipulating mitochondrial efficiency and testing changes in song frequency. Oxidative phosphorylation can be partially decoupled (resulting in less efficient mitochondria) with drugs such as 2,4-Dinitrophenol (DNP). Administration of DNP has been used to test associations between mitochondrial function and life history traits (Stier et al., 2021; Stier et al., 2014) and could be used to disentangle interrelationships between CORT, mitochondria function, and the expression of sexually selected traits.

We describe the first evidence linking mitochondrial function to song structure. Of the six song variables we used in analyses, only measures of song frequency were associated with mitochondrial function ($FCR_{R/ETS}$ and $FCR_{R/L}$). Recent studies across bird species have shown that song frequency decreases with increased male-biased sexual size dimorphism (Mikula et al., 2021) and increased sexual dichromatism (Francis and Wilkins, 2021), suggesting that song frequency may be under sexual selection in some species. We know of no studies that have linked song frequency to variation in reproductive success in zebra finches. However, studies in zebra finches have linked other components of song structure to female preference or mate choice. For example, female zebra finches prefer males that sing longer songs (Clayton and Prove, 1989; Neubauer, 1999). Other studies have identified motif number, song complexity, song type (directed versus undirected), and song rate as important traits for mate choice (Holbeck and Riebel, 2007; Leadbeater et al., 2005; Spencer et al., 2005; Vyas et al., 2009; Woolley and Doupe, 2008). Cumulatively, these studies show that there is no clear consensus on which song variables are important for mate choice in zebra finches. Similarly, past studies that have examined the effects of elevated CORT or food restriction (often collectively termed 'developmental stress') on adult song production have found variable effects of developmental treatments on song structure/production (reviewed in MacDougall-Shackleton and Spencer, 2012). Results could vary across studies because of differences in developmental treatment, husbandry, lab methodology, stock lines, and study population (Griffith et al., 2017). Future studies could clarify functional links between mitochondrial function, the expression of sexually selected traits, and fitness by pairing song analysis with mate choice trials or measures of reproductive success.

Our results show clear positive effects of CORT exposure during development on adult CORT levels, and link adult CORT levels to reduced mitochondrial efficiency and increased song frequency. However, we are unable to identify the causal mechanism that links changes in mitochondrial function to changes in song frequency. Song frequency is affected by many factors, including the contraction of syringeal muscles (Riede et al., 2010), which can change song frequency by shortening tracheal length and altering the tension of the sound-generating membranes (Daley and Goller, 2004; Doppler et al., 2018; Goller and Suthers, 1996). Potentially, changes in mitochondrial function could affect song frequency through action on syringeal muscles

when performing at extreme rates, since muscular function is known to be crucial for song performance in songbirds, including zebra finches (Mean et al., 2008). However, a reduction in mitochondrial performance and ATP production should arguably be associated with a reduction in fundamental frequency; therefore, it seems more plausible that the change in frequency may be associated with cryptic reductions in muscle or syrinx size, which would generate an increase in song frequency. Our study does not test this potential mechanism, although future studies could do so by measuring mitochondrial function in syringeal muscles.

We found that males exposed to experimentally elevated levels of CORT during development secreted higher levels of CORT as adults compared to control males. Our results are consistent with many other studies showing that developing birds exposed to CORT, food restriction, or adverse perturbations exhibit altered HPA axis function across life-history stages (Crino et al., 2014a; Schmidt et al., 2014; Spencer et al., 2009; Zimmer et al., 2013). Exposure to elevated CORT levels in one generation can even have intergenerational effects, such that birds exposed to elevated CORT levels during development rear offspring that have elevated HPA axis function (Kraft et al., 2021). This growing body of research demonstrates that the developmental environment can have strong, programmatic effects on HPA axis function. The most salient question to emerge from this research is: what are the fitness consequences associated with developmental programming of the HPA axis? One hypothesis is that exposure to CORT during development induces phenotypic changes that match animals to their postnatal environment (Crino and Breuner, 2015; Monaghan, 2008; Monaghan and Haussmann, 2015). However, for developmentally induced phenotypic changes to be adaptive, the natal environment must be highly predictive of the postnatal environment (Burgess and Marshall, 2014; Mateo, 2014; Monaghan, 2008). Most animals live in variable environments, and it is unlikely that a single cue experienced during development could predict similar conditions in the postnatal environment (Potticary and Duckworth, 2020). However, GCs increase in response to a wide variety of environmental conditions and perturbations, and it is possible that exposure to elevated levels of GCs during development provides an integrated signal to animals about the postnatal environment (Potticary and Duckworth, 2020). Our results presented here show that developmental programming of the HPA axis can affect mitochondrial function, with consequences for the expression of a sexually selected trait. Incorporating measures of mitochondrial function into experiments that examine developmental programming of the HPA axis may elucidate the evolutionary significance of this effect.

The information conveyed by sexually selected traits has long been a source of debate in the field of behavioral ecology. It has recently been proposed that mitochondrial function underlies variation in the expression of sexually selected traits (Hill, 2014, 2015a; Koch and Hill, 2018; Koch et al., 2017), but few studies to date have directly tested this hypothesis (but see Hill et al., 2019). Here, we found that exposure to experimentally elevated levels of CORT during development altered mitochondrial function in adult zebra finches. However, the effects of developmental treatment on mitochondrial function were indirect and modulated through changes in HPA axis function. Baseline CORT levels were negatively associated with song frequency, and mitochondrial function was negatively associated with song frequency. Future studies that test relationships between mitochondrial function and acoustic traits in other species will provide further insight into the signal content of bird song. Additionally, studies that examine associations between mitochondrial function, mate choice, and reproductive success will provide insight into how mitochondria impact fitness and life-history strategies.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.yhbeh.2022.105184>.

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