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ORIGINAL PAPER

Mitochondrial oxidative phosphorylation response overrides glucocorticoid-induced stress in a reptile

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Abstract

Stress hormones and their impacts on whole organism metabolic rates are usually considered as appropriate proxies for animal energy budget that is the foundation of numerous concepts and models aiming at predicting individual and population responses to environmental stress. However, the dynamics of energy re-allocation under stress make the link between metabolism and corticosterone complex and still unclear. Using ectopic application of corticosterone for 3, 11 and 21 days, we estimated a time effect of stress in a lizard (*Zootoca vivipara*). We then investigated whole organism metabolism, muscle cellular O_2 consumption and liver mitochondrial oxidative phosphorylation processes (O_2 consumption and ATP production) and ROS production. The data showed that while skeletal muscle is not impacted, stress regulates the liver mitochondrial functionality in a time-dependent manner with opposing pictures between the different time expositions to corticosterone. While 3 days exposition is characterized by lower ATP synthesis rate and high H_2O_2 release with no change in the rate of oxygen consumption, the 11 days exposition reduced all three fluxes of about 50%. Oxidative phosphorylation capacities in liver mitochondria of lizard treated with corticosterone for 21 days was similar to the hepatic mitochondrial capacities in lizards that received no corticosterone treatment but with 40% decrease in H_2O_2 production. This new mitochondrial functioning allows a better capacity to respond to the energetic demands imposed by the environment but do not influence whole organism metabolism. In conclusion, global mitochondrial functioning has to be considered to better understand the proximal causes of the energy budget under stressful periods.

Keywords Corticosterone · Lizard · Metabolism · Mitochondrial efficiency · ROS and ATP production · Oxygen consumption

Introduction

Glucocorticoids (GCs) secreted by the hypothalamus–pituitary–adrenal axis vertebrates are crucial hormones in orchestrating the tradeoff between survival and different physiological processes during stressful situations (Angelier and Wingfield 2013). However, physiological consequences are known to be different in accordance to short-term versus long-term elevations in GCs production (i.e., Dickens et al. 2009). While many of the acute effects of GCs consist of mobilizing energy, chronically elevated circulating GCs enhance energy storage (Harris 2015; Sapolsky et al. 2000). Similarly, acute GC elevations can activate the adaptive immune system (even if it could depends on life-history stages (Berger et al. 2005)), whereas chronic GC elevations are linked to suppressed cell-mediated leucocyte trafficking (Sorrells et al. 2009; Dhabhar and McEwen 1997). The

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underlying mechanisms of such biphasic responses in which acute and chronic stress or corticosteroid exposure induce contrasting and sometimes opposite effects have not yet been fully elucidated. While some studies stipulate potential differential actions of different nuclear receptors of GCs (e.g., Joels and de Kloet 1992), others link it to larger magnitude changes in gene expression under acute stress rather than chronic stress (e.g., Wang et al. 2004).

Glucocorticoids regulate the nuclear encoded oxidative phosphorylation gene expression but the detection of glucocorticoid receptor in mitochondria of different cell types also gave large support to a direct regulation of mitochondrial gene expression by glucocorticoids (Du 2009; Lee et al. 2013). Similarly, to physiological mechanisms mentioned before, acute stress shows a pattern of broad, larger-magnitude changes in gene expression, whereas chronic stress shows a less pronounced change, often in a different direction from the response evoked by acute stress or corticosteroid treatment (Hunter et al. 2016). Such contrasted gene expression could explain why in vitro (with cortical neuronal cultures) and in vivo (with brains from male rats) experiments demonstrated that metabolism (e.g., mitochondrial oxidation, membrane potential, mitochondrial complex I activity) and the corresponding genes (i.e., NADH dehydrogenase 1, 3, 6 and ATP synthase 6) are modulated by corticosterone in a time-dependent manner (Du et al. 2009). In parallel, a reduction of the activity of specific mitochondrial electron transport chain complexes together with an increased mitochondrial reactive oxygen species (ROS) production has been shown under chronic glucocorticoid treatment (Manoli et al. 2007), which could impact the oxidative balance (Costantini 2011; Vagasi et al. 2020). Altogether, these physiological studies thus demonstrated (i) a direct linkage between GC secretion in response to stressor and mitochondrial physiology and (ii) a time-dependent regulation of mitochondrial function by GCs.

These observations led to the emergent concept of "mitochondrial allostatic load". This concept can be defined as the "deleterious structural and functional changes that mitochondria undergo" in response to elevated glucocorticoid secretion under a chronic stress situation (Picard et al. 2014, 2018). The mitochondria are the major ATP cell generators (Lehninger et al. 1993) sustaining physiological processes underlying both maintenance and physiological responses to environmental variations. Mitochondrial energy transduction system also continually produces ROS which can be involved in cell signaling and/or physiopathology (Starkov 2008). Unfortunately, at that time, only rare assessments of mitochondrial functionality parameters like ATP and ROS under chronic GCs production were available (Stier et al. 2019). Yet such data are crucial to understand functional consequences of previously described responses to GC secretion, such as alteration of gene expression and/or epigenetic modifications (Hunter 2016), increased oxidized mtDNA and mitochondrial fission (Picard et al. 2014; Eisner et al. 2018).

The objective of this study was to explore the time-dependent metabolic responses at the whole-body and mitochondrial levels in lizards (*Zootoca vivipara*) exposed to 3, 11 and 21 days of corticosterone treatment. This protocol using cross-sectional sampling provides the temporal dynamic of the mitochondrial responses to GC secretion and answers the following question: Are oxygen consumption, ATP and ROS productions and the subsequent ratio (ATP/O, ROS/O and ROS/ATP) equivalently impacted by GCs? In addition, the present data bring information about the link between whole organism and cellular metabolisms that is still an open question (Salin et al. 2016; Gutiérrez et al. 2019).

Materials and methods

Capture and rearing condition

The common lizard (*Zootoca vivipara*) is a small lacertid species (adult's snout-vent length SVL ranging from 50 to 70 mm) widely distributed across Eurasia. In spring 2016, 49 sub-adult males (1-year-old) were captured by hand in outdoor enclosures (10 × 10 m) at CEREEP (Centre de Recherche en Ecologie Expérimentale et Prédictive; Saint-Pierre-lès-Nemours, France, 48° 17′ N, 2° 41′ E) field station from May 16th to May 25th. Animals were measured for body size (SVL, ± 0.5 mm) and body mass (± 1 mg). All animals were maintained in individual terraria (25 × 15 × 16 cm) with a shelter, peat soil as substrate and opportunities for optimal thermoregulation. We used incandescent light bulbs (25 W) for 8 h per day from 09:00 to 17:00 local time to ensure a thermal gradient ranging from 17–23 to 35–38 °C. We provided lizards with water ad libitum and, every other day, with 300 ± 20 mg of food (*Acheta domestica*).

Experimental design and whole organism assays

The study was performed between 12th of June and 12th of July in 2016. At the start of the experiment, animals were distributed in six groups corresponding to two experimental groups (corticosterone-treated [CORT] and control [CONT]) and three treatment exposure: 3 days [3D], 11 days [11D] and 21 days [21D]. Similar sample sizes ($N = 8$) were used in each group except in the CORT-21D group ($N = 9$). The body mass and whole organism metabolic expenditure have been assessed 3 days before the start of the experiments and the day before the cellular and mitochondrial measurements meaning days 2, 10 and 20 for 3D, 11D and 21D groups

respectively (Fig. 1). Before each whole organism assay, the animals were left without food for 3 days to reach the post-absorptive state. After the final assay, the animals were fed and treated with corticosterone according to their treatment group. Then, the animals were euthanized to perform functional analyses of mitochondria (see below and Fig. 1).

Whole organism metabolic expenditure at night was quantified with closed respirometry techniques as previously described (Foucart et al. 2014). We measured oxygen consumption and carbon production overnight (approximately 20:00–08:00 h) in a dark climatic chamber (AQUALYTIC® TC 135S, Dortmund, Germany). Trials were carried out at 25 ± 1 °C, which correspond to body temperature at which functional analyses of mitochondria were performed in vitro (see below). The lizards were placed individually into glass jars (ca. 1000 ml) within the chamber and allowed to acclimatize for 1 h. A baseline air sample (two 140 ml syringes) was collected at the onset of the trial, and the glass vial was then carefully sealed. The trial duration was set to achieve adequate oxygen suppression based on previous studies and preliminary trials (mean = 11.8 h, range = 11.2–12.1 h). A final sample of air was collected with two 140 ml syringes connected to a stopcock. Oxygen and CO_2 concentrations (% of total volume) of the air samples were determined using fuel-cell O_2 and infrared CO_2 sensors (FOXBOX, Sable Systems, Las Vegas, NV, USA) at room temperature with a constant flow (60 ml.min^{-1}) after water absorption through a column of Drierite. Oxygen consumption (VO_2, in ml min^{-1}) and CO_2 production (VCO_2) were calculated as: (final %–initial %) × exact chamber volume (ml)/trial duration (min). Body mass was recorded to the nearest mg before each trial. Respiratory quotient (RQ) was calculated like VCO_2/VO_2 and ranged from 0.60 to 0.82 [mean = 0.72, 95% CI = 0.71–0.73]. We found no treatment effect on RQ (ANOVA, $F_{1,47} = 0.65$, $P = 0.42$), but parallel, temporal changes in RQ for both control and treated lizards ($F_{3,96} = 36.59$, $P < 0.0001$). Since the theoretical RQ value for lipid substrates is 0.71, we therefore assumed that most lizards used stored lipids as the main energy source and used energy equivalents for lipids (19.8 J.mL^{-1}) to convert VO_2 into standard metabolic rates (SMR) values (in J.min^{-1}). These metabolic measurements proved to be extremely reliable and repeatable compared to our earlier studies (Foucart et al. 2014).

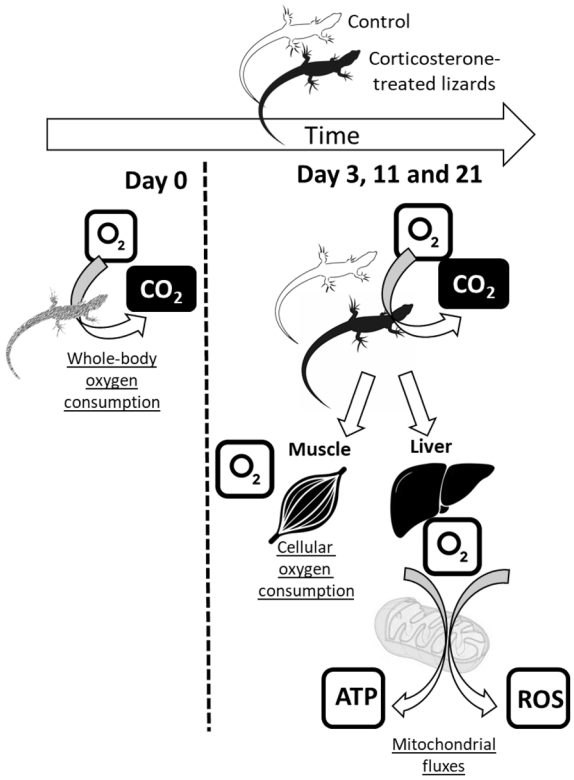

Fig. 1 Experimental design to assess the time effect of corticosterone exposition on whole organism, cellular and mitochondrial metabolism in the common lizard (*Zootoca vivipara*)

Hormonal manipulation and plasma corticosterone assays

Circulating levels of corticosterone were increased using a non-invasive method designed by Meylan et al. (2003). We diluted corticosterone (Sigma-Aldrich, France, C2505-500 mg 92%, $C_{21}H_{30}O_4$) in commercial sesame oil according to 3 µg of corticosterone per 1µL of sesame oil. Each evening between 20:00 h and 21:00 local time (when lizards are mostly inactive), 4.5 µL of corticosterone mixture (CORT) or pure sesame oil (CONT) were topically applied on the backs of the lizards. To check effects on plasma levels of corticosterone, blood was sampled from the infraorbital sinus of the lizards 7 days before the start of the experiment and at the end of the treatment exposure using 2–3 20 µL micro-hematocrit tubes. To standardize the measurements, all samples were collected between 15:00 h and 16:00 local time within 3 min of removal of an animal from its home cage to avoid the handling-induced increase in plasma corticosterone levels (Dauphin-Villemant and Xavier 1987). Plasma was obtained by centrifugation at c.a. 5000 g for 5 min of the blood samples and was stored at − 40 °C for subsequent measurements of plasma levels of corticosterone. Corticosterone levels were measured in duplicates with a competitive enzyme-immunoassay method using corticosterone EIA (IDS Corticosterone EIA kit, ref AC-14F1, IDS EURL Paris, France) after 1:10 dilution of all samples following previously published guidelines (Mugabo 2017). This method using a polyclonal corticosterone antibody is based on a highly repeatable colorimetric assay of absorbance at 450 nm (intra-plate repeatability, 17 plates with 4 repeats per plate: coefficient of intra-class correlation,

$\rho = 0.89$, $F_{3,60} = 103$, $P < 0.001$; inter-plate repeatability: $\rho = 0.93$, $F_{16,47} = 51.3$, $P < 0.001$).

Respiratory capacities of muscular permeabilized fibers

At the end of the experiment, animals were euthanized by decapitation. All skeletal muscles were rapidly and entirely dissected, weighed and then placed in an ice-cold isolation buffer (BIOPS containing 2.77 mM Ca-EGTA, 7.23 mM, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM DTT, 6.56 mM $MgCl_2$, 5.77 mM ATP, 15 mM phosphocreatine, pH 7.2). Skeletal muscle fibers were permeabilized in BIOPS solution supplemented with saponin (50 µg ml^{-1}) according to a standard protocol (Pesta and Gnaiger 2012). Permeabilized fibers were weighed and their respiration were monitored with a high-resolution respirometer (Oxygraph-2 k, Oroboros Instruments; Austria) in a hyper-oxygenated respiratory buffer maintained at 25 °C (110 mM sucrose, 0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 1 g l^{-1} fatty acid-free bovine serum albumin, 20 mM HEPES, pH 7.1). A mixture of respiratory substrates (5 mM pyruvate, 2.5 mM malate and 5 mM succinate) was added to obtain the basal respiration (state 2). Cellular ATP synthesis was initiated by the addition of 1 mM ADP. The maximal fully uncoupled respiration (state 3_{unc}) was initiated by addition of 2 µM carbonyl cyanide p-tri-fluoro-methoxy-phenyl-hydrazone (FCCP), in presence of oligomycin (2 µg ml^{-1}), an inhibitor of ATP synthase.

Extraction of liver and functional analyses of mitochondria

After weighing the liver, the mitochondria were isolated in an ice-cold isolation buffer (250 mM sucrose, 1 mM EGTA, 20 mM Tris–HCl, pH 7.4 at 4 °C) as previously described (Voituron et al. 2017). Briefly, the isolation procedure involved Potter–Elvehjem homogenization (three passages) and differential centrifugations (all steps at 4 °C), with the liver mitochondria being pelleted at 9000 × *g* (10 min). The protein concentration of mitochondrial suspension was spectrophotometrically determined at 540 nm by a Biuret method with bovine serum albumin as a standard. Because lizard mitochondrial preparations contained a dark pigment that absorbed at 540 nm, the absorbance of the same volume of mitochondria in a solution containing 0.06% Na deoxycholate, 0.6% K–Na–tartrate and 3% NaOH was subtracted.

Rates of oxygen consumption and ATP synthesis were measured in a closed glass cell fitted with a Clark oxygen electrode (Rank Brothers Ltd, UK) at 25 °C in a respiratory buffer containing 120 mM KCl, 5 mM KH_2PO_4, 1 mM EGTA, 2 mM $MgCl_2$, 0.3% fatty acid-free bovine serum albumin, 1.6 U/ml hexokinase, 20 mM glucose, 3 mM HEPES (pH 7.4). Liver mitochondria (0.5–1.5 mg/mL) were energized with a mixture of substrates (5 mM pyruvate, 2.5 mM malate and 5 mM succinate). Mitochondrial ATP synthesis was initiated by the addition of 500 µM ADP. After recording the phosphorylating respiration rate for 3 min, four 100 µL samples were withdrawn from the suspension every 1 min and were quenched in 100 µL ice-cold perchloric acid solution consisting of 10% $HClO_4$ and 25 mM EDTA. After centrifugation (15,000 × *g*, 5 min) and neutralization of the resulting supernatant with a KOH solution (2 M KOH, 0.3 M MOPS), ATP production was determined from the slope of the linear accumulation of glucose-6-phosphate content of samples (Voituron et al. 2017). To make sure that the rates, we measured were specific of the mitochondrial ATP synthase activity and not associated with contaminated ATPase activity such as adenylate kinase, we determined oxygen consumption and ATP synthesis rates in the presence of oligomycin (2 µg/mL). These values were taken into account to calculate the rate of mitochondrial ATP synthesis that is associated with mitochondrial ATP synthase activity and oxygen consumption (ATP/O) defining as the mitochondrial efficiency.

Mitochondrial reactive oxygen species production

The rate of H_2O_2 released by isolated liver mitochondria was measured at 25 °C in a respiratory buffer supplemented with 5 U/mL horseradish peroxidase and 1 µM Amplex red fluorescent dye using a fluorescence spectrophotometer (SFM-25, Kontron Instrument) at excitation and emission wavelengths of 560 nm and 584 nm, respectively. The fluorescent signal was calibrated using a standard curve obtained after successive addition of H_2O_2 (up to 35 pmol). Amounts of H_2O_2 release were corrected from background rate of product formation in the absence of exogenous substrate as described previously (Voituron et al. 2017). Free radical electron leak (ROS/O ratio) was calculated as the fraction (%) of basal non-phosphorylating oxygen consumption that is reduced into H_2O_2 at the respiratory chain instead of H_2O at the cytochrome-c oxidase. Similarly, the oxidative cost of mitochondrial ATP synthesis was calculated from the ratio of H_2O_2 generation under phosphorylating state divided by the corresponding rate of ATP synthesis (ROS/ATP ratio).

Statistical analyses

All statistical analyses were performed with linear models in R 3.3.2 (R Development Core Team 2016). We first checked the effects of treatment groups on change in plasma corticosterone levels from before the end of the experiment with a linear model fitted with the *lm* package (Venables and Ripley 2002). We included initial plasma corticosterone level

and body mass as covariates in this model. Next, repeated measurements (body mass and SMR) were analyzed with mixed-effects linear models in the *lme* package. Dependence among repeated measurements was accounted using random intercept model where the intercept of the model is allowed to vary randomly among individuals. A compound symmetry covariance structure that includes within-subject correlated errors was used and the random term was always kept in the models. This model assumes that individuals react in the same way to treatments and provides information about intra-class correlations and therefore consistency of inter-individual differences. In addition, all models included fixed effects of experimental treatments, treatment exposure groups (categorical variable) and their interactions. Since SMR increases exponentially with body mass, we log-transformed SMR prior to the analyses and included log-transformed body mass as a covariate in the analyses. The normality and homogeneity of variance of residuals was systematically checked in the full models and was found to be satisfactory. In a third set of analyses, we compared final measurements of liver mass and mitochondria functioning across treatment groups with a linear model including fixed effects of experimental treatments, treatment exposure groups and their interactions. In all models, the significance of fixed effects was tested with type III F statistics using the *Anova* procedure in the *car* package. We removed non-significant variables ($\alpha = 0.05$) one by one using a backward elimination procedure. Whenever significant differences were found among treatment groups, we used Tukey's procedure to conduct post hoc tests (pairwise comparisons between the experimental groups) with the *lsmeans* package (Lenth 2016). Results are presented as mean ± standard error unless otherwise stated.

Results

Corticosterone supplementation increases body and liver masses

Before the experiment, animals in the different groups were not significantly different in terms of SVL, body mass and body condition, SMR and initial plasma corticosterone levels among treatment groups (ANOVA, all $P > 0.66$). Analysis of intra-individual change in plasma corticosterone levels confirmed that treated groups exhibited higher concentration following CORT treatment (treatment: $F_{2,44} = 71.6$, $P < 0.0001$; exposure group: $F_{2,44} = 6.49$, $P = 0.003$, see supplementary data). On average, plasma corticosterone levels were 154.8 ng.mL^{-1} (± 18.3) higher in treated than in control lizards, similar to previous effects reported with the same protocol (Voituron et al. 2017). In addition, treated lizards exhibited a significant increase of body mass after 21D treatment (+ 17%), whereas the body mass of control lizards did not change significantly over time (treatment × exposure group: $F_{3,94} = 18.13$, $P < 0.0001$) (Table 1). Total liver mass (treatment × exposure group: $F_{2,43} = 8.24$, $P = 0.0009$) and relative liver mass (g of liver per 100 g body mass; liver mass controlled for variation in body mass; treatment × exposure group: $F_{2,42} = 8.33$, $P = 0.0009$) differed also between treatments after 11 days of exposure only. There was a significant increase of liver mass at D11 and D21 in treated lizards relative to controls (Table 1).

Corticosterone supplementation and whole organism oxygen consumption

Overall, SMR are non-significantly different between treatments apart for a slight, close to significant decrease in SMR from treated lizards relative to controls at D21 (Table 1; treatment × exposure group: $F_{3,92} = 2.69$, $P = 0.05$; Student's *t* test at D21: $P = 0.07$). However, when controlling for the positive relationship between individual body mass and individual SMR (log–log slope = 0.67 ± 0.13; $F_{1,90} = 25.78$, $P < 0.0001$), the best fit model indicated that mass-corrected SMR differences over time between treatments ranged from a small positive difference for CORT at D3, followed by a return to values before treatment at D11, and a significant decrease in mass-corrected SMR from treated lizards at D21 (treatment × exposure group: $F_{3,90} = 5.01$, $P = 0.003$). These results can be interpreted as evidence that corticosterone-treated lizards maintained relatively similar whole organism SMR despite their increase in body mass during the 21-day exposure period, thus had lower mass-corrected SMR through time.

Corticosterone supplementation and liver mitochondrial functions

The treatment affected mitochondrial activities with contrasted effects between the rates of ATP synthesis and oxygen consumption over the course of the study (Table 1 and Fig. 2, see Table S1 for statistical details). The rate of ATP synthesis was significantly lower in the corticosterone treatment irrespective of treatment exposure. In contrast, both basal and phosphorylating rates of oxygen consumption did not change after 3 days of treatment, then were significantly lower in 11-day treated lizards compared to control animals, and eventually returned to same levels than in controls at day 21 (Table 1). Consequently, mean values of the mitochondrial coupling efficiency (ATP/O ratio) were significantly lower after 3 days of corticosterone treatment, but were not significantly different from control values after 11 and 21 days of treatment (treatment × exposure group: $F_{2,41} = 4.14$, $P = 0.02$, see Fig. 2).

Table 1 Effects of exogenous treatment of corticosterone (CORT: 3×10^6 ng.ml^{-1} of sesame oil; CONT: only sesame oil) during 3, 11 and 21 days in *Zootoca vivipara*

Morphological and metabolic traits	Treatment	Day 0	Day 3	Day 11	Day 21
Body and liver mass					
Body mass (g)	CONT	2.00 ± 0.03 [a]	2.01 ± 0.04 [a]	1.95 ± 0.04 [a]	2.03 ± 0.04 [a]
	CORT	2.00 ± 0.04 [a]	2.02 ± 0.04 [a]	2.06 ± 0.04 [a]	**2.26 ± 0.06 [b]**
Liver mass (mg)	CONT		66.13 ± 3.04 [a]	**56.12 ± 2.18 [b]**	**57.12 ± 1.68 [b]**
	CORT		60.25 ± 2.40 [a]	69.12 ± 4.34 [b]	**71.78 ± 2.44 [b]**
Relative liver mass (% of BW)	CONT		3.07 ± 0.15 [a]	**2.64 ± 0.10 [b]**	**2.71 ± 0.09 [b]**
	CORT		2.88 ± 0.12 [a]	**3.30 ± 0.19 [b]**	**3.46 ± 0.09 [b]**
SMR (J.min^{-1})					
Whole-organism SMR	CONT	6.70 ± 0.19[a]	7.00 ± 0.16[a]	**7.30 ± 0.22[b]**	7.26 ± 0.32[ab]
	CORT	6.69 ± 0.17[a]	7.29 ± 0.18[ab]	**7.58 ± 0.22[b]**	**6.96 ± 0.20[a]**
Muscle fibers oxygen consumption (pmol O_2.s^{-1}.mg^{-1} fresh mass)					
Maximal respiration	CONT		19.92 ± 1.59[a]	19.01 ± 0.70[a]	20.53 ± 0.85[a]
	CORT		19.26 ± 1.75[a]	20.97 ± 0.93[a]	20.54 ± 1.54[a]
Phosphorylating respiration	CONT		15.24 ± 1.07[a]	14.18 ± 0.71[a]	15.55 ± 0.57[a]
	CORT		15.18 ± 1.37[a]	15.0 ± 0.85[a]	16.29 ± 1.41[a]
Basal respiration	CONT		4.80 ± 0.25[a]	4.96 ± 0.20[a]	**5.23 ± 0.09[b]**
	CORT		5.05 ± 0.34[a]	4.99 ± 0.32[a]	**6.11 ± 0.34[b]**
Liver mitochondrial oxygen consumption and ATP fluxes (nmol ATP/min.mg protein or nmol O.min^{-1}.mg protein^{-1})					
ATP synthesis	CONT		37.21 ± 4.39[a]	42.06 ± 6.08[a]	44.43 ± 7.52[a]
	CORT		**27.0 ± 2.72[b]**	**21.87 ± 1.94[b]**	**35.09 ± 4.43[b]**
Phosphorylating respiration	CONT		21.35 ± 2.35[ab]	24.94 ± 3.39[a]	22.34 ± 2.90[ab]
	CORT		21.10 ± 2.44[ab]	**12.40 ± 1.18[b]**	20.32 ± 2.40[ab]
Basal respiration	CONT		3.85 ± 0.44[a]	3.93 ± 0.39[a]	**2.33 ± 0.41[b]**
	CORT		3.92 ± 0.45[a]	**2.12 ± 0.19[b]**	**1.99 ± 0.22[b]**
Liver mitochondrial ROS production (pmol H_2O_2/min.mg protein)					
Basal state	CONT		167.69 ± 22.4[a]	129.86 ± 13.05[a]	164.86 ± 27.92[ab]
	CORT		181.80 ± 30.6[a]	**63.92 ± 8.85[b]**	**97.57 ± 7.35[b]**
Phosphorylating state	CONT		50.84 ± 6.83[ab]	42.87 ± 4.58[ab]	57.24 ± 10.72[a]
	CORT		60.26 ± 11.5[a]	**20.64 ± 3.13[b]**	**29.97 ± 4.14[b]**

The metabolic traits studied include whole organism standard metabolic rates (SMR), muscle fibers and mitochondrial liver oxygen consumptions, liver mitochondrial ATP and ROS production. Data are reported as mean ± SE (n = 8–9 per group). Significant differences among groups from Tukey post hoc tests of the best model comparing CONT and CORT-treated individuals (see main text) are specified with different letters and bold

The rates of mitochondrial reactive oxygen species production under both basal non-phosphorylating and active phosphorylating states were not significantly altered after 3 days of glucocorticoid treatment but exhibited between 40 and 50% decrease in treated lizards relative to controls after 11 and 21 days (Table 1, see Table S1 for statistical details). Overall, the electron leak defined by the ROS/O ratio was not significantly changed by the glucocorticoid treatment neither in basal non-phosphorylating state (treatment × exposure group: $F_{2,41} = 2.09$, $P = 0.14$; treatment: $F_{1,43} = 0.13$, $P = 0.82$; exposure group: $F_{2,44} = 10.21$, $P = 0.0002$) nor in active phosphorylating state (treatment × exposure group: $F_{2,41} = 1.81$, $P = 0.18$; treatment: $F_{1,43} = 0.89$, $P = 0.35$; exposure group: $F_{2,44} = 2.26$, $P = 0.12$). In contrast, the oxidative cost of ATP synthesis (ROS/ATP ratio) was marginally affected by hormonal treatment, being higher in the 3-day treated group than in the control group, but returning to the level of control groups after 11 and 21 days of treatment (treatment × exposure group: $F_{2,41} = 3.02$, $P = 0.06$; Fig. 3).

Corticosterone supplementation and muscle mitochondria functioning

Whatever the state of activation (basal non-phosphorylating, phosphorylating and maximal), cellular oxygen consumption of lizard muscle was not different between

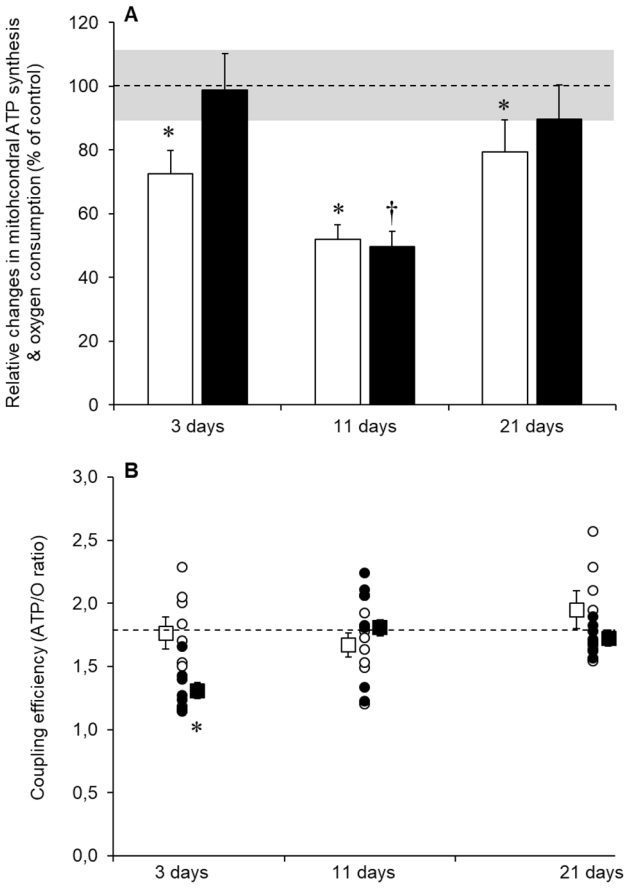

Fig. 2 Effect of 3, 11 and 21 days of exogenous corticosterone treatment (3×10^6 ng.ml^{-1}) on liver metabolism in *Zootoca vivipara*. **A** Phosphorylating mitochondrial oxygen consumption (black bars) and ATP production (white bars); the values are represented as % of control values (±SE represented by the horizontal gray-shaded rectangle). **B** Mitochondrial efficiency (ATP/O) in function of treatment time. Circles represent individuals' data and squares represent means for each group. White represent controls while black represent treatment groups. * and † Significantly different from the corresponding control

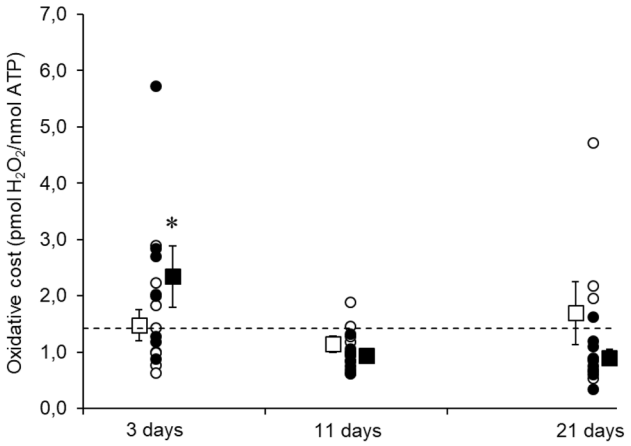

Fig. 3 Effect of 3, 11 and 21 days of exogenous corticosterone treatment (3×10^6 ng.ml^{-1}) on oxidative costs of an ATP production in *Zootoca vivipara*. The dotted horizontal line represent the values of control lizards. Circles represent individuals' data while squares represent the mean of each group. White represent controls while black represent treatment groups. Values are means ± SE. for 8–9 animals

control and treated individuals neither at 3, 11 days nor at 21 days of treatment (Table 1 and Table S1).

Discussion

This study aimed at unraveling the time-dependent mechanisms linking corticosterone and metabolism at different levels of organization (whole organism, cellular and mitochondrial) in an ectothermic organism. The data presented here provide first clear evidence of GC-dependent regulation of the liver mitochondrial functionality in a time-dependent manner leading to a restoration of O_2 consumption and ATP production after 21 days of corticosterone increase. Indeed, short-term GC supplementation induced a significant decrease of mitochondrial ATP synthesis without changes in oxygen consumption and H_2O_2 release, thus causing a lower efficiency (ATP/O) and a higher oxidative cost of an ATP molecule production (ROS/ATP). In contrast, longer-term GC supplementation induced a significant decrease of mitochondrial H_2O_2 release with mitochondrial oxidative phosphorylation characteristics to returning to the same level as controls. McEwen and Wingfield (2003) defined the allostatic state as the "*altered and sustained activity levels of the primary mediators,* e.g., *glucocorticosteroids, that integrate physiology and associated behaviors in response to changing environments and challenges*". The present data show that liver mitochondria decrease first their efficiency and then their O_2 consumption and ATP production but finally restore these two mitochondrial fluxes in response to GC elevation after 21 days. In addition, the data also suggest a tissue-dependent response, with corticosterone treatment affecting functioning of mitochondria in the liver but not that of skeletal muscles. Interestingly, changes in metabolism of mitochondria in vitro were not reflected by variation in the whole organism metabolism in vivo.

The present data expand results of a previous study on this species (Voituron et al. 2017) providing the time dynamics of functional regulation of the mitochondrial oxygen consumption, ATP and H_2O_2 productions under GC secretion. In response to the glucocorticoid supplementation, the liver mitochondria first lowered its ATP synthesis rate without changing its oxygen consumption (Fig. 2). This first mitochondrial response could be explained by a combination of negative GCs effects on the activity of F_0F_1-ATPase (e.g., Morin et al. 2000). Consequently, the cellular ATP/AMP ratio is modified triggering a cascade of genes regulation

involved in mitochondrial respiratory chain expression and function (Teperino et al. 2010; Mouchiroud et al. 2014). This could explain the mitochondrial pattern characterized by low values of all fluxes reflecting a diminished activity of the respiratory chain at 11 days of treatment. Interestingly, this mitochondrial hypometabolism restored both the efficiency (ATP/O) and oxidative cost of an ATP molecule production (ROS/ATP). Altogether, these elements are in line with scenarios of downregulation of the respiratory chain subunits (Pandya et al. 2004) and of the activity of substrate oxidation reported after glucocorticoids treatment in rodents (Roussel et al. 2004; Arvier et al. 2007). The nearly total recovering of both oxygen and ATP after 21 days of treatment associated with lower ROS release might be ascribed to de novo mitogenesis (Jornayvaz and Shulman 2010). Indeed, glucocorticoids can induce higher expression of key nuclear genes that are required to produce new mitochondria (Psarra and Sekeris 2011). Such time-dependent response in terms of ATP production associated with lower H_2O_2 production may explain why male common lizards showed lower superoxide dismutase activity in corticosterone-treated individuals after 21 days (Cote et al. 2010). These results are however not congruent with data on endotherms in which chronically elevated GC levels accelerate aging and reduce lifespan (Schoenle et al. 2018), thus imposing fitness costs potentially through oxidative balance (Costantini 2011).

All these mitochondrial adjustments occurred without modification of whole organism metabolism (present study; Voituron et al. 2017). Even if only two tissues were tested in the study, the data thus strongly suggest that whole organism oxygen consumption cannot be used as an accurate proxy for neither ATP production nor ROS release by tissue-specific mitochondria (Salin et al. 2015). However, the GC effect on whole organism metabolism, an important parameter of the total energy budget, still remains an open question since it has been demonstrated that chronic increase of corticosterone slightly increases metabolic rates at rest in pregnant female common lizards (Meylan et al. 2010) when other lizard species reduce their total oxygen consumption rate when exposed to an increase in corticosterone (Miles et al. 2007; Durant et al. 2008). Nevertheless, the restoration of O_2 consumption and ATP production together with lower ROS release observed in vivo might constitute a proximal explanation of the increase in survival of male common lizards with corticosterone enhancement (Meylan and Clobert 2005; Cote et al. 2006).

Even if oxygen consumption between tissues has been assessed at different levels of organization, the data suggest that muscles and liver showed differential response patterns. This statement would be wrong only if mitochondrial content in muscles strongly increase during acute and mild-time stress that has never been reported and not congruent with mitogenesis dynamic (Jornayvaz and Shulman 2010). The data available on mammals and birds are puzzling with no muscular effect in rodents or chickens under dexamethasone (Dumas et al. 2003; Roussel et al. 2004; Jiao et al. 2018) or muscle wasting mediated, in part, by GR-dependent transactivation of genes that drive myocyte atrophy (Patel et al. 2014). In addition, the muscular impact of GC is dose-dependent with moderate or transient exposure to GCs enhancing muscle performance (Caruso et al. 2014) or preparing muscle of birds to long distance migration (Pradhan et al. 2019). Even if muscles of common lizards do not exhibit a mitochondrial response, muscles could be involved as amino-acids source for liver neo-glucogenesis under corticosterone.

Conclusion and perspectives

This study demonstrates that liver mitochondrial energetics of ectotherms is directly influenced in a time-dependent manner by exposure to higher plasma concentrations of circulating GCs. After 21 days exposure to GCs, the liver mitochondria exhibit a different interplay between O_2 consumption, ATP production and H_2O_2 release compared to before the GCs exposition. The coupling between these three mitochondrial processes is thus plastic and differentially regulated leading to a critical period when stress is acute (low ATP production with high H_2O_2 release), an intermediate period when efficiency returns to the initial levels but with low respiratory chain activity, and finally a complete "recovery" to the initial fluxes and ratios. This time-dependent relationship between GC and mitochondria in ectotherms needs to be taken into account to better understand mechanisms that ensure and drive the flow of energy during physiopathological responses (e.g., Rohleder 2012) but also toward adaptive processes.

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Data availability The datasets generated during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest There is no conflict of interest to declare.

Ethical approval The present investigation was carried out according to the ethical principles of the Préfecture de Seine-et-Marne under agreement A77-341-1.

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