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RESEARCH ARTICLE

Feeding effects on liver mitochondrial bioenergetics of *Boa constrictor* (Serpentes: Boidae)

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ABSTRACT

Snakes are interesting examples of taxa that can overcome energy metabolism challenges, as many species can endure long periods without feeding, and their eventual meals are of reasonably large sizes, thus exhibiting dual extreme adaptations. Consequently, metabolic rate increases considerably to attend to the energetic demand of digestion, absorption and protein synthesis. These animals should be adapted to transition from these two opposite states of energy fairly quickly, and therefore we investigated mitochondrial function plasticity in these states. Herein, we compared liver mitochondrial bioenergetics of the boid snake *Boa constrictor* during fasting and after meal intake. We fasted the snakes for 60 days, and then we fed a subgroup with 30% of their body size and evaluated their maximum postprandial response. We measured liver respiration rates from permeabilized tissue and isolated mitochondria. From isolated mitochondria, we also measured Ca^{2+} retention capacity and redox status. Mitochondrial respiration rates were maximized after feeding, reaching an approximately 60% increase from fasting levels when energized with complex I-linked substrates. Interestingly, fasting and fed snakes exhibited similar respiratory control ratios and citrate synthase activity. Furthermore, we found no differences in Ca^{2+} retention capacity, indicating no increase in susceptibility to mitochondrial permeability transition, and no changes in mitochondrial redox state, although fed animals exhibited increases in the release of H_2O_2. Thus, we conclude that liver mitochondria from *B. constrictor* snakes increase respiration rates during the postprandial period and quickly improve the bioenergetic capacity without compromising redox balance.

KEY WORDS: Fasting, Specific dynamic action, Liver mitochondria, Calcium retention capacity, Mitochondrial permeability transition, Redox balance

INTRODUCTION

Mitochondria are complex and dynamic organelles present in eukaryotic cells responsible for energy production and cellular homeostasis. They play a fundamental role in balancing energetic homeostasis via intracellular signaling, apoptosis, and metabolism of amino acids, lipids, cholesterol, steroids and nucleotides, and their primary known function of oxidation of energetic substrates and ATP production (Duchen, 2000). This energy expenditure at the cellular level needs to be finely tuned to the varying availability of energy substrates from food resources and energetic demand from activities to allow better organismal performance. Animals can face challenges owing to environmental changes (such as seasonal scarcity of food), behavior or life-history traits, resulting in an increase in energy expenditure, such as for reproduction and migration. One basic regulation of energy expenditure depends on the control of oxidative phosphorylation (OXPHOS), as this process accounts for most of the whole-animal oxygen consumption and has a considerable effect on cellular respiration flux (Benard et al., 2006; Brown et al., 1990; Dejean et al., 2001; Rolfe and Brown, 1997).

Ambush-foraging snakes are commonly used as experimental model organisms because of their resistance to long periods of food deprivation and the magnitude of their physiological responses after feeding on large meals (Andrade et al., 2004; Lignot et al., 2005; McCue, 2007, 2008; McCue et al., 2012; Secor and Carey, 2016; Secor and Diamond, 1998, 2000; Starck and Beese, 2001; Wang and Rindom, 2021). These snakes survive exceptional long periods of fasting by employing different strategies for energy conservation, such as reducing metabolic rates, organ mass and activity, and control of the mobilization of fuel sources (McCue, 2007; McCue et al., 2012). In contrast, once fed, ambush-foraging snakes exhibit a remarkably increased metabolism, of comparatively higher magnitude than other animals (Secor and Diamond, 1998). The postprandial metabolic increment after meal intake termed specific dynamic action (SDA) (Kleiber, 1961) may last for several days, depending on temperature regime, and meal size and quality (Andrade et al., 2004; Cruz-Neto et al., 1999; Gavira and Andrade, 2013; Secor and Diamond, 1997). Such elevated metabolism after feeding is mostly, if not fully, fueled by aerobic metabolism. Thus, studies of the modulation of energy pathways involving oxidation of substrates ultimately leading to oxygen consumption and ATP production through the mitochondrial respiratory chain are essential to understand the regulation of metabolism at a cellular level.

In endothermic vertebrates, research has mainly focused on the mitochondrial effects of fasting, and studies conducted in mammals and birds report that food deprivation is accompanied by decreased mitochondrial respiration rates and increased rates of reactive oxygen species (ROS) production (Bourguignon et al., 2017; Dumas et al., 2004; Menezes-Filho et al., 2019; Roussel et al., 2019; Sorensen et al., 2006). Mitochondria unwittingly generate ROS as a by-product, and at low levels, ROS serve as redox signaling molecules, allowing adaptation to changes in environmental nutrients and the oxidative environment (Schieber and Chandel, 2014; Shadel and Horvath, 2015). However, excess ROS can

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exhaust the antioxidant system and promote damage to proteins, lipids and DNA, leading to oxidative stress (Hamanaka and Chandel, 2010). In species adapted to prolonged fasting, including mammalian hibernators, there seem to be mechanisms that allow the mitigation of oxidative stress (Ensminger et al., 2021). Nevertheless, although a robust body of literature exists for the physiological effects of fasting and feeding in snakes (McCue, 2008; Secor, 2009), knowledge of the optimization of metabolism at the subcellular level during periods of fasting or during the metabolic increment after meal intake is lacking (Butler et al., 2016).

We hypothesize that snakes will display mitochondrial plasticity, exhibiting an increase in the capacity for ATP generation during the postprandial period following the increase in energetic demand of digestion and absorption. To test this, we investigated the liver mitochondrial function and redox balance after 60 days of fasting and during the postprandial period in the ambush-foraging boid snake *Boa constrictor*. This neotropical snake feeds infrequently, surviving periods of fasting longer than 2 months (McCue and Pollock, 2008). This species can ingest large meals, exhibiting large increments in aerobic metabolic rate (Andrade et al., 2004; de Figueiredo et al., 2020; Toledo et al., 2003; da Mota-Araujo et al., 2021). As the liver plays a vital role in snake's metabolism, participating in the oxidation of triglycerides, the synthesis of cholesterol, lipoprotein and amino acids, and the control of blood sugar levels, it is relevant to assess the contribution of this organ to the overall energetic demand after meal intake. In boas, the liver exhibits increased mass (Secor and Diamond, 2000) and a larger volume of glycogen granules 2 days post-feeding (da Mota-Araujo et al., 2021). Thus, we compared mitochondrial liver bioenergetics of fasted and fed *B. constrictor*, evaluating mitochondrial respiration, Ca^{2+} retention capacity and mitochondrial redox status, as well as H_2O_2 release, NAD(P) redox state and aconitase activity.

MATERIALS AND METHODS

Reagents

We purchased the fluorescent probes Calcium Green™-5N and Amplex™ UltraRed from Thermo Fisher Scientific (Eugene, OR, USA) and dissolved them in deionized water and dimethyl sulfoxide (DMSO), respectively. All other chemicals were obtained from Sigma-Aldrich (St Louis, MO, USA). Stock solutions of respiratory substrates and nucleotides were prepared in a 20 mmol l^{-1} HEPES solution with the pH adjusted to 7.2 using KOH.

Animals

We obtained juvenile snakes *Boa constrictor* Linnaeus 1758 (N=9, body mass=152.0±16.0 g; total length=73.7±3.3 cm, mean±s.d.) from Centro de Recuperação de Animais Silvestres do Parque Ecológico do Tietê (CRAS, São Paulo, SP, Brazil). We housed the animals in individual boxes (56.4×38.5×20.1 cm, length×width×height) with venting holes in the lid, under natural light and temperature (25±2°C, mean±s.d.) with free access to water. Initially, we fed all animals with mice (*Mus musculus*) to standardize the beginning of the treatment (with the equivalent of 5% of their body masses). After, we kept all snakes in a fasting condition for 2 months. Then, we divided the snakes into two groups: fasting (N=5) and fed (N=4). We fed the snakes of the 'fed group' with mice accounting for 30% of their body mass and euthanized them 2 days after prey ingestion, which is usually when maximum oxygen consumption ($\dot{V}_{O_2,max}$) is achieved (peak SDA; Secor and Diamond, 2000; de Figueiredo et al., 2020). We performed all measurements at the Laboratory of Bioenergetics at Universidade Estadual de Campinas (UNICAMP), Campinas, SP, Brazil. We anesthetized the snakes with isoflurane and sectioned the medulla after cessation of reflexes. All experimental procedures were approved by the Local Committee for Ethics in Animal Experimentation (CEUA/UNICAMP: 5301-1/2019) and complied with the ARRIVE guidelines. The Brazilian Institute for Environment (SISBIO; number 69655-1) authorized the use of *B. constrictor*.

Permeabilized liver tissue

We rapidly removed a portion of the liver and immersed it in ice-cold BIOPS buffer [10 mmol l^{-1} Ca-EGTA buffer (2.77 mmol l^{-1} CaK_2EGTA, 7.23 mmol l^{-1} K_2EGTA, free concentration of calcium 0.1 mmol l^{-1}), 20 mmol l^{-1} imidazole, 50 mmol l^{-1} KCl 4-morpholinoethanesulfonic acid, 0.5 mmol l^{-1} dithiothreitol, 7 mmol l^{-1} $MgCl_2$, 5 mmol l^{-1} ATP, 15 mmol l^{-1} phosphocreatine, pH 7.1]. Then, we permeabilized liver samples of 8 to 10 mg tissue in ice-cold buffer containing saponin (0.5 mg ml^{-1}) for 30 min, and gently stirred and washed the samples with MIR05 medium (60 mmol l^{-1} potassium lactobionate, 1 mmol l^{-1} $MgCl_2$, 20 mmol l^{-1} taurine, 10 mmol l^{-1} KH_2PO_4, 20 mmol l^{-1} HEPES, 110 mmol l^{-1} sucrose, 1 g l^{-1} BSA, pH 7.1) at 4°C (Busanello et al., 2017; Kuznetsov et al., 2008). We dried the samples with filter paper and weighed them before respirometric measurements.

Mitochondrial isolation

We isolated liver mitochondria by tissue homogenization followed by conventional differential centrifugation (Ronchi et al., 2013). Briefly, we removed the liver, which we then finely minced and homogenized in an isolation medium containing 250 mmol l^{-1} sucrose, 1 mmol l^{-1} EGTA and 10 mmol l^{-1} HEPES buffer (pH 7.2) at 4°C. We centrifuged the homogenate for 10 min at 800 ***g***. Then, we centrifuged the collected supernatant at 7750 ***g*** for 10 min. We resuspended the resulting pellet in buffer containing 250 mmol l^{-1} sucrose, 0.3 mmol l^{-1} EGTA and 10 mmol l^{-1} HEPES buffer (pH 7.2) and centrifuged again at 7750 ***g*** for 10 min. We resuspended the final pellet containing liver mitochondria in an EGTA-free buffer at an approximate protein concentration of 60 mg ml^{-1}, quantified by the Bradford method using bovine serum albumin (BSA) as standard.

Mitochondrial oxygen consumption

We measured mitochondrial respiration by monitoring the rates of oxygen consumption using a high-resolution oxygraph (Oroboros Instruments, Innsbruck, Austria), equipped with a magnetic stirrer, in a temperature-controlled chamber maintained at 30°C for permeabilized tissue and 28°C for isolated mitochondria, according to standard protocols used for ectothermic animals in our laboratory. We suspended the permeabilized liver tissues in 2 ml of MIR-05 supplemented with 300 µmol l^{-1} EGTA and 5 mmol l^{-1} malate, 10 mmol l^{-1} pyruvate and 10 mmol l^{-1} glutamate. After measuring the basal O_2 consumption, respiration linked to OXPHOS was elicited by the addition of 400 µmol l^{-1} of ADP. Then, we added 1 µg ml^{-1} of oligomycin to cease the phosphorylation by ATP synthase (state 4_o), which reduces oxygen consumption. Finally, we titrated carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) until maximal electron transport system capacity, which occurred at a concentration of 800 nmol l^{-1}, eliciting maximal respiration rate ($\dot{V}_{max}$).

We suspended the isolated liver mitochondria (0.5 mg ml^{-1}) in 2 ml of standard reaction medium (125 mmol l^{-1} sucrose,

65 mmol l^{-1} KCl, 2 mmol l^{-1} KH_2PO_4, 1 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} HEPES buffer, with the pH adjusted to 7.2 with KOH) supplemented with 200 µmol l^{-1} EGTA and 1 mmol l^{-1} malate, 2.5 mmol l^{-1} pyruvate and 2.5 mmol l^{-1} glutamate, which generate NADH and feed electrons at respiratory complex I. We performed sequential additions of 300 µmol l^{-1} ADP, 1 µg ml^{-1} oligomycin and 100 nmol l^{-1} FCCP. For isolated mitochondria, we applied an additional protocol for the evaluation of the different mitochondrial complexes. We measured basal respiration with complex I-linked substrates (1 mmol l^{-1} malate, 2.5 mmol l^{-1} pyruvate and 2.5 mmol l^{-1} glutamate), followed by the addition of ADP and FCCP as described above, then we added 1 µmol l^{-1} rotenone to block complex I followed by the addition of 5 mmol l^{-1} succinate to stimulate complex II. Because the addition of 1 µmol l^{-1} antimycin A or 1 µmol l^{-1} myxothiazol were without effect on blocking complex III, we discarded the final addition of 1 mmol l^{-1} *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) plus 100 µmol l^{-1} ascorbate aimed for stimulation of complex IV.

Assessment of mitochondrial Ca^{2+} retention capacity

We suspended liver mitochondria (0.5 mg ml^{-1}) in a standard reaction medium supplemented with 10 µmol l^{-1} EGTA, 0.2 µmol l^{-1} of a calcium indicator (Calcium Green™-5N) and respiratory substrates (1 mmol l^{-1} malate, 2.5 mmol l^{-1} pyruvate and 2.5 mmol l^{-1} glutamate). We continuously monitored the fluorescence in a spectrofluorometer (Hitachi F-4500, Tokyo, Japan) at 28°C using excitation and emission wavelengths of 506 and 532 nm, respectively, and slit widths of 5 nm. We performed repeated pulses of $CaCl_2$ additions (60 µmol l^{-1}) after mitochondria were added to the system. We measured the amount of $CaCl_2$ added before the start of Ca^{2+} release by mitochondria into the medium as an index of the susceptibility to Ca^{2+}-induced mitochondrial permeability transition (MPT), confirmed by the assessment of MPT inhibition in the presence of 1 µmol l^{-1} cyclosporine A (CsA). We converted the raw fluorescence readings into Ca^{2+} concentration levels (expressed as µmol l^{-1}) according to the hyperbolic equation: $[Ca^{2+}]=K_d\times[(F-F_{min})/(F_{max}-F)]$, where K_d is the dissociation constant, F is any given fluorescence, F_{min} is the lowest fluorescence reading after addition of 0.5 mmol l^{-1} EGTA and F_{max} is the maximal fluorescence obtained after two sequential additions of 1 mmol l^{-1} $CaCl_2$. We performed these additions of EGTA and Ca^{2+} at the end of each trace. We experimentally determined a K_d of 26.8 µmol l^{-1} for the probe Calcium Green™-5N in the incubation condition, as previously described (Sartori et al., 2021).

Citrate synthase activity

We measured the catalytic activity of the enzyme citrate synthase in mitochondrial samples monitoring the conversion of oxaloacetate and acetyl-CoA to citrate and CoA–SH, and by measuring the formation of the colorimetric product thionitrobenzoic acid (TNB) at 412 nm and 37°C (Shepherd and Garland, 1969) on a microplate reader (Power Wave XS-2, Biotek Instruments, Winooski, VT, USA). We calculated the enzyme activity using the changes in absorbance after substrate (250 µmol l^{-1} oxaloacetate) addition to the assay buffer (10 mmol l^{-1} Trizma, pH 8.0) containing 50 µmol l^{-1} acetyl-CoA and 100 µmol l^{-1} DTNB.

Hydrogen peroxide (H_2O_2) release

We monitored the H_2O_2 released by isolated liver mitochondria by the conversion of Amplex™ UltraRed to fluorescent resorufin in the presence of horseradish peroxidase (HRP). We incubated the suspensions of mitochondria from fasting and fed snakes (0.5 mg ml^{-1}) in a reaction medium containing NAD-linked substrates (1 mmol l^{-1} malate, 2.5 mmol l^{-1} pyruvate and 2.5 mmol l^{-1} glutamate), 10 µmol l^{-1} Amplex™ UltraRed, 1 U ml^{-1} HRP and 30 U ml^{-1} superoxide dismutase (SOD). Additionally, we added 100 µmol l^{-1} phenylmethyl sulfonyl fluoride (PMSF) to inhibit the conversion of Amplex™ UltraRed by carboxylesterase independent of H_2O_2 (Miwa et al., 2016). We monitored the fluorescence over time with a temperature-controlled spectrofluorometer at 28°C (Hitachi F-4500, Tokyo, Japan) using excitation and emission wavelengths of 563 and 586 nm, respectively, and slit widths of 5 nm. For calibration, we added known amounts of H_2O_2 to the reaction medium with mitochondrial samples.

NAD(P) redox state

We suspended the isolated liver mitochondria (0.5 mg ml^{-1}) in a standard reaction medium supplemented with 200 µM EGTA, and 5 mmol l^{-1} succinate plus 1 µmol l^{-1} rotenone, and monitored the changes in the redox state of NAD(P) in a spectrofluorometer (Hitachi F-7100) at 28°C, using excitation and emission wavelengths of 366 and 450 nm, respectively, and slit widths of 5 nm. We used succinate and rotenone as substrates to prevent oxidation of NADH at complex I. Of note, only the reduced forms of NAD(P) exhibit a strong endogenous fluorescence signal. The peroxide-metabolizing system supported by NADPH was challenged with exogenous tert-butyl hydroperoxide (t-BOOH), an organic peroxide metabolized through the glutathione peroxidase/reductase system (Liu and Kehrer, 1996). As a reference, we added known amounts of NADH to the reaction medium in the absence of mitochondria.

Aconitase activity

We measured aconitase activity as the increase of fluorescence owing to the generation of NADPH from the reduction of $NADP^+$ by exogenous isocitrate dehydrogenase (IDH2). For this, the isocitrate was previously generated by aconitase (Gardner et al., 1994). Mitochondria (0.05 mg ml^{-1}) were added to 2 ml of medium (36 mmol l^{-1} Tris, 1 mmol l^{-1} sodium citrate, 25 mmol l^{-1} KH_2PO_4, 0.6 mmol l^{-1} $MnCl_2$, 0.05% Triton X-100, 0.2 mmol l^{-1} $NADP^+$, 0.5 U ml^{-1} IDH2, pH 7.4) at 37°C. The fluorescence was monitored over time in a spectrofluorometer (Hitachi F-7100) operating with slit widths of 5 nm and using 340 and 450 nm as the excitation and emission wavelengths, respectively. We calculated the specific enzyme activity using a calibration curve with known amounts of NADPH.

Statistical analyses

We tested for data normality and homoscedasticity using the Shapiro–Wilk and Barlett's *K*-squared tests, respectively, using the R package. For variables that met the assumptions of parametric tests, we performed two-tailed unpaired *t*-tests for independent samples for comparison between fasted and fed snakes. Whenever data failed the premises, we compared the groups using Mann–Whitney tests. We performed all analyses in Prism GraphPad software v. 7.1. We present the results as individual data and bars representing means and s.e.m., assuming a significance level of 0.05.

RESULTS

Oxygen consumption of liver permeabilized tissue and isolated mitochondria

Wet liver mass did not differ between fasting (3.2±0.2 g) and fed snakes (3.0±0.2 g). Oxygen levels started at approximately

220 nmol ml^{-1} and were reduced to a minimum level of approximately 150 nmol ml^{-1} for permeabilized fibers and 100 nmol ml^{-1} for isolated mitochondria.

Liver permeabilized tissue from fed snakes exhibited 30% higher $\dot{V}_{max}$ than fasting snakes (unpaired *t*-test, P=0.0086; Fig. 1A). Citrate synthase activity of liver permeabilized tissue did not differ between the groups (unpaired *t*-test, P<0.5; Fig. 1B). For isolated mitochondria, fed snakes exhibited 40%, 58% and 64% higher respiration rates supported by complex I-linked substrates at basal, OXPHOS and state 4$_o$, respectively, compared with fasting snakes (*t*-test, $P\leq0.05$; Fig. 2A,B). Mitochondrial $\dot{V}_{max}$ stimulated with NAD-linked substrates was 53% higher in the fed group (*t*-test, $P\leq0.05$), while mitochondrial $\dot{V}_{max}$ stimulated with succinate as substrate for complex II was not different between fasting and fed snakes (*t*-test, P=0.19; Fig. 2C). *Boa constrictor* mitochondria were insensitive to antimycin A and myxothiazol for blockade of complex III. Mitochondrial respiratory control ratios and citrate synthase activity did not differ between groups (Mann–Whitney, P=0.45; Fig. 2D,E).

Assessment of mitochondrial Ca^2 retention capacity

Ca^{2+} retention capacity was evaluated by sequential additions of Ca^{2+} pulses (Fig. 3A,B) to the medium. Mitochondria of fasting snakes were able to take and retain 264±67 nmol Ca^{2+} mg^{-1} protein, which was not different from the 465±79 nmol Ca^{2+} mg^{-1} protein retained by fed snakes (*t*-test, P>0.05; Fig. 3C). With the presence of CsA, both groups of snakes similarly increased resistance to MPT opening, 1140±35 nmol Ca^{2+} mg^{-1} protein in the fasting group versus 900±173 nmol Ca^{2+} mg^{-1} protein in the fed group (Mann–Whitney, P=0.30; Fig. 3C).

Mitochondrial redox status

H_2O_2 released from liver mitochondria of fed snakes (76±16 pmol min^{-1} mg^{-1} protein) was 2-fold higher than from fasting snakes (37±3 pmol min^{-1} mg^{-1} protein) (Mann–Whitney, P=0.02; Fig. 4A,B). There was no difference in the NADPH-dependent capacity to metabolize peroxide in fasting versus fed snakes (*t*-test, P=0.16; Fig. 4C,D). We found no changes in aconitase activity: in fasting animals, the activity was 56±8 mU mg^{-1} protein and in fed animals, 68±13 mU mg^{-1} protein (Fig. 4E,F).

DISCUSSION

The present study revealed that *B. constrictor* liver mitochondria exhibited profound energetic changes in response to a meal intake. After feeding, mitochondrial respiration rates from *B. constrictor* were increased in comparison to unfed snakes. Mitochondria are dynamic structures, undergoing fusion and fission processes, and changes in number, morphology and distribution, depending on the developmental, physiological and environmental conditions (Mishra and Chan, 2016). Notwithstanding, the capacity to shift liver mitochondrial profiles 2 days after meal intake in boas was remarkable, bringing attention to the underlying mechanisms and the potential effects on mitochondria from other tissues directly or indirectly involved in the digestion and absorption processes.

Respiration rates from liver permeabilized fibers are in agreement with those from isolated mitochondria. However, in permeabilized fibers, we only observed significant differences between fasting and fed boas regarding the maximal respiration rate. That is because the permeabilized tissues contain higher intraindividual variability owing to heterogeneity of the subsampled tissue, in contrast to the more homogeneous suspensions obtained from the isolation procedure (Kuznetsov et al., 2002). In liver isolated mitochondria, we observed remarkable increases in respiration rates related to basal, oxidative phosphorylation (OXPHOS or state 3), and $\dot{V}_{max}$ with NAD-linked substrates of fed snakes in comparison to fasted snakes.

The increases in mitochondrial respiration rates should reflect the meal size, time spent fasting and the moment of post-feeding sampling. For example, varying periods of fasting in *B. constrictor* did not change the total energetic cost of digestion. However, it changed the temporal profile of the postprandial response (de Figueiredo et al., 2020). The increase in mitochondrial respiration rates seems to be fueled by NAD-linked substrates because we did not see differences in $\dot{V}_{max}$ between fasting and fed snakes when using substrates that feed electrons to complex II. Indeed, upregulation of genes for respiratory complex I, among other genes related to OXPHOS, was reported during digestion in snakes (Duan et al., 2017). Unfortunately, mitochondrial function studies in snakes are scarce. Interestingly, a recent study found that low temperature can impact coupling and efficiency in liver mitochondria of the snake *Natrix natrix*, but only when respiration is driven by succinate as the respiratory substrate (Dubinin et al., 2019), indicating that different sources of stimulus can impact mitochondrial function distinctly in snakes.

Other interesting findings were that feeding did not influence the quantity or quality of isolated mitochondria of fasting and fed *B. constrictor* liver because citrate synthase activity and respiratory

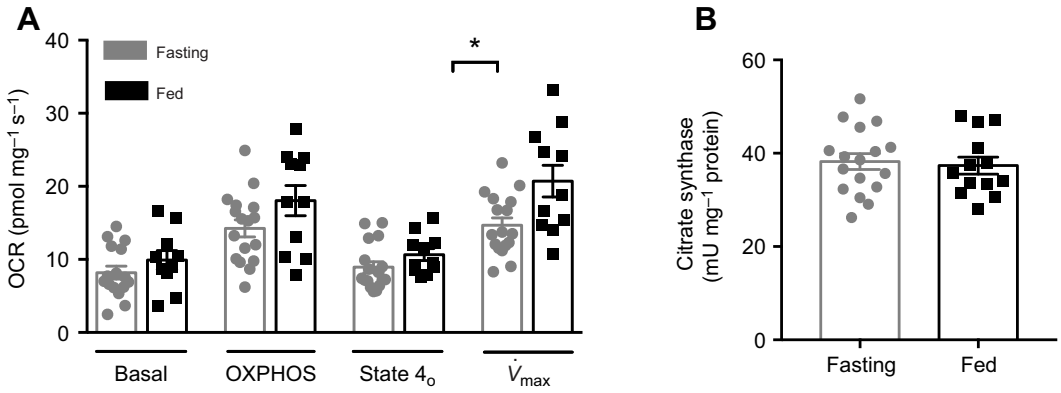

Fig. 1. Feeding increased maximal respiration rate in liver permeabilized tissue from *Boa constrictor* snakes. (A) Oxygen consumption rate (OCR) of liver permeabilized tissue in the presence of 5 mmol l^{-1} malate, 10 mmol l^{-1} pyruvate and 10 mmol l^{-1} glutamate as substrates (basal), after additions of 400 µmol l^{-1} ADP (OXPHOS), 1 µg ml^{-1} oligomycin (state 4$_o$) and 0.8 µmol l^{-1} FCCP ($\dot{V}_{max}$). OCR is expressed per gram wet mass of tissue. (B) Citrate synthase activity. Bars denote means±s.e.m.; N=17 fasting, N=11 fed; each dot represents an independent experiment; *P<0.01, *t*-test.

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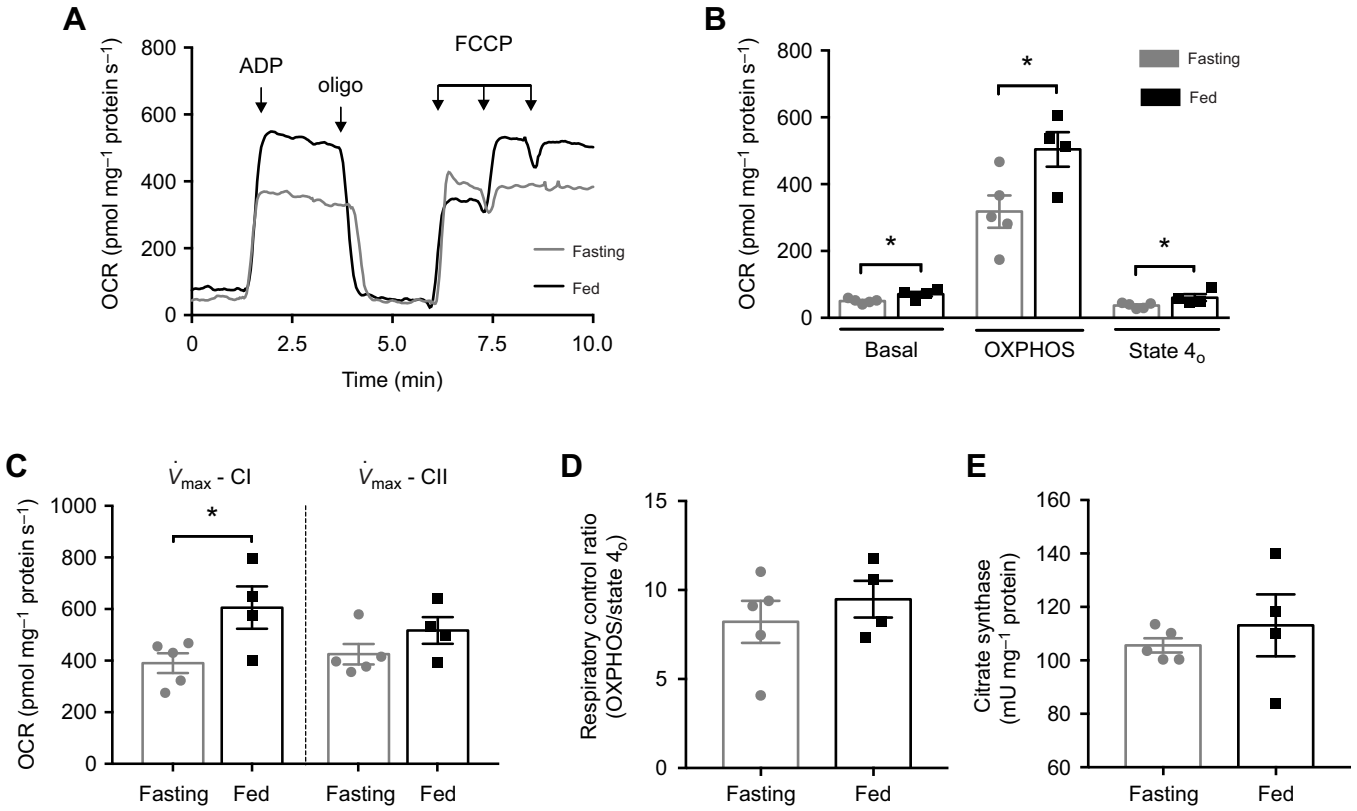

Fig. 2. Feeding increased *B. constrictor* basal respiration, oxidative phosphorylation capacity, state 4_o and $\dot{V}_{max}$ in liver isolated mitochondria energized with complex I-linked substrates. (A) Representative traces of oxygen consumption rate (OCR) of fasting and fed snakes, in the presence of 1 mmol l^{-1} malate, 2.5 mmol l^{-1} pyruvate and 2.5 mmol l^{-1} glutamate as substrates, with additions of 300 µmol l^{-1} ADP, 1 µg ml^{-1} oligomycin (oligo) and 50 nmol l^{-1} FCCP, where indicated by the arrows. (B) Quantification of OCR per mg mitochondrial protein; *$P \leq 0.5$, *t*-test. (C) Maximal respiration rate ($\dot{V}_{max}$) stimulated with complex I- and complex II-linked substrates; *$P \leq 0.5$, *t*-test. (D) Respiratory control ratios (OXPHOS/state 4_o). (E) Citrate synthase activity. Data are presented as means±s.e.m., and each dot represents individual data (N=5 fasting, N=4 fed).

control ratios were maintained similarly. Mitochondria from fasting boas exhibited lower respiratory rates in all measured states, following the low resting energetic demand of the species (de Figueiredo et al., 2020; Stuginski et al., 2018). The capacity to also exhibit a lower respiration rate after ATP synthase blockade with oligomycin (state 4_o), which can be used as an indirect measurement of proton leak rate, could also be considered an important contributing factor towards energy saving in the liver (Brand et al., 1993). However, measurements of mitochondrial efficiency (i.e. ATP/O, as the efficiency with which mitochondria convert oxygen into ATP) should be considered in future studies to better characterize the energy conservation at fasting states. For example, in long-fasting birds, the mitochondrial energy efficiency between ATP production and O_2 consumption was increased (Bourguignon et al., 2017; Monternier et al., 2015, 2017; Roussel et al., 2019). In contrast, studies showed that fasting mammals exhibited compromised mitochondrial efficiency during food deprivation periods (Brown and Staples, 2011; Menezes-Filho et al., 2019). Further investigations could also determine the mechanism of the insensitivity to complex III inhibitors in snakes, which suggests that the ubiquinol–cytochrome *c* oxidoreductase complex exhibits a different molecular structure, as this outcome was also observed in *Bothrops alternatus* (Ogo et al., 1993) and *Python regius* (Bundgaard et al., 2020). Interestingly, snakes were claimed to show high levels of modifications in proteins involved in aerobic metabolism, especially in the complex IV, cytochrome *c* oxidase subunit I (Castoe et al., 2008).

The mitochondrial Ca^{2+} retention capacity is a proxy for evaluating MPT susceptibility, a phenomenon characterized by the Ca^{2+}-dependent opening of a non-specific pore in the inner mitochondrial membrane. MPT affects the structure and function of mitochondria, which is ultimately related to cell death by apoptosis or necrosis and to many pathological conditions (Vercesi et al., 2018). The amount of Ca^{2+} that leads to overload, thus triggering MPT, varies with the source and conditions of mitochondria and the presence of protectors or inducers acting on the still debated pore constitutional units (Kowaltowski et al., 2001). MPT can be sensitized by oxidative stress and oxidized NADPH ($NADP^+$) (Castilho et al., 1995; Vercesi et al., 1988; Zago et al., 2000), as excess ROS increase oxidation of protein thiols and promotes disulfide bonds and cross-linked protein aggregation in the inner mitochondrial membrane (Castilho et al., 1995; Fagian et al., 1990; Valle et al., 1993; Vercesi, 1984). Unlike mice mitochondria, which showed a higher susceptibility to MPT at fasting (Menezes-Filho et al., 2019), snakes exhibited no significant differences in mitochondrial Ca^{2+} retention in response to feeding, despite the tendency of lower Ca^{2+} retention capacity at fasting. CsA reversed this tendency, although maintaining the lack of significant differences.

Regarding the redox status, liver mitochondria from fed *B. constrictor* exhibited higher rates of H_2O_2 release after ingestion of a meal than after fasting. Results from the literature should be compared with caution as the differences depend on the substrates, the respiratory state measured, the technique used, and the capacity of H_2O_2 scavenging (Munro and Pamenter, 2019). In birds, it was

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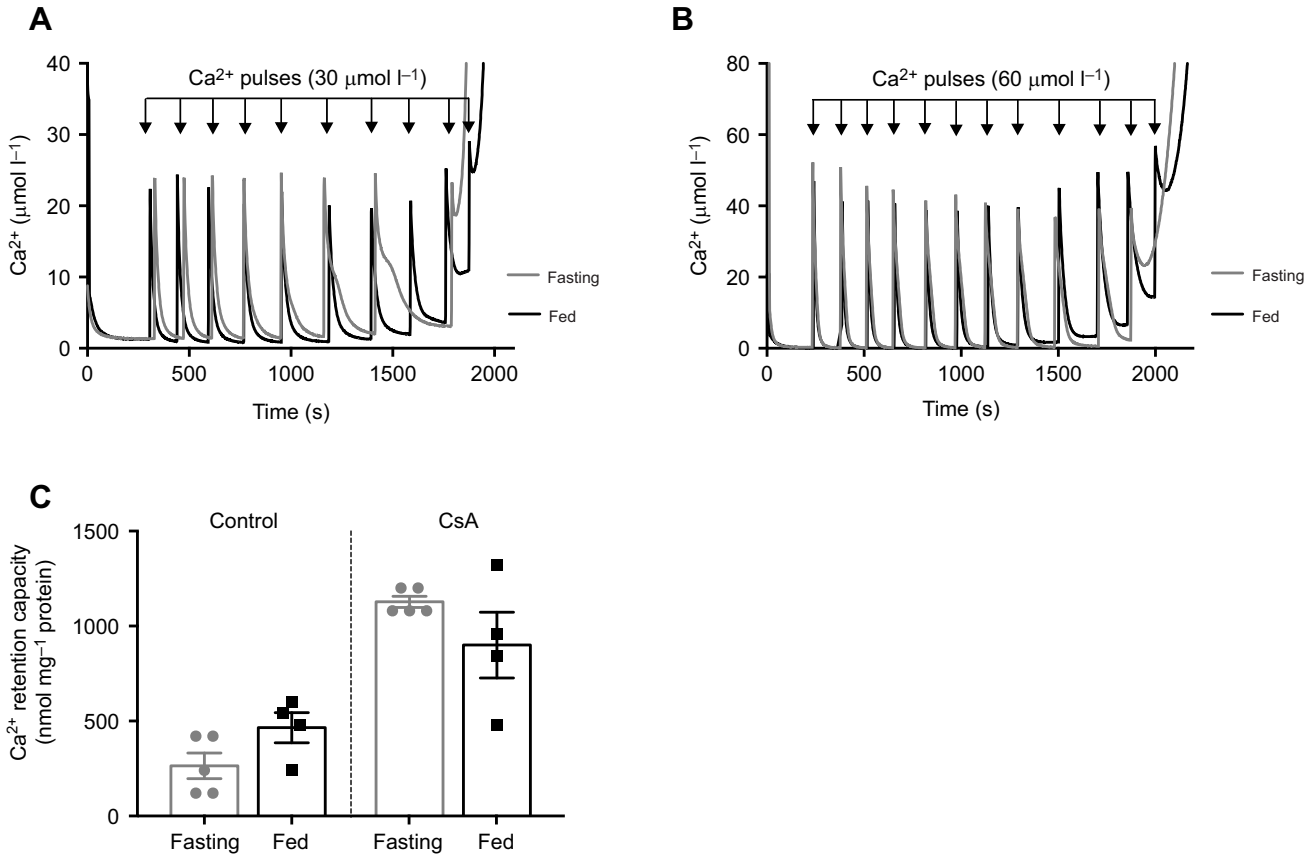

Fig. 3. Feeding did not affect mitochondrial Ca^{2+} retention capacity in *B. constrictor* snakes. After addition of mitochondria to the system, Ca^{2+} retention capacity was accessed by consecutive additions of Ca^{2+} pulses until Ca^{2+}-induced Ca^{2+} release as the consequence of the MPT opening. Representative traces are depicted (A) in the control condition, Ca^{2+} pulses of 30 μmol l^{-1}, and (B) in the presence of cyclosporine A (CsA), Ca^{2+} pulses of 60 μmol l^{-1}. (C) Amount of Ca^{2+} retained in each condition before the onset of permeability transition. Data are presented as means±s.e.m., and each dot represents individual data (*N*=5 fasting, *N*=4 fed).

also found that liver mitochondria released higher rates of H_2O_2 at a basal state after feeding than after fasting, using NAD-linked substrates and succinate (Roussel et al., 2019). In contrast, fasting brown trout (*Salmo trutta*) exhibited higher *in vivo* levels of H_2O_2 when subjected to fasting than when feeding continuously for 2 weeks (Salin et al., 2018). Rat liver mitochondria also exhibited higher levels of H_2O_2 in the 72 h fasting group compared with a control, overnight fasting group, measured at phosphorylation state with succinate as a substrate (Sorensen et al., 2006). The reduced H_2O_2 of fasting boas may be due to the low energetic demand during fasting in snakes (Ensminger et al., 2021) and may be related to the remarkable capacity of metabolic regulation in such animals (McCue, 2007). Nevertheless, a 2-month fasting period in *B. constrictor* may not be sufficient to induce detrimental effects in mitochondria. Ambush-hunting snakes were shown to possess lower metabolic rates than active foraging snakes that feed more frequently (Stuginski et al., 2018), meaning that the energetic costs could be sustained for long periods using stored energy reserves. For example, the rattlesnake *Crotalus durissus* was shown to endure 12 months of food deprivation with slow body mass loss and no changes in resting V_{O_2} (Leite et al., 2014). We did not observe differences in the redox status of NAD(P), indicating that the reducing power used for the antioxidant system was similar in both fasting and fed conditions. We also did not find differences in aconitase activity, a redox-sensible enzyme whose activity is reversibly decreased by oxidants (Sadek et al., 2002; Scandroglio et al., 2014). Supporting evidence showed an increased antioxidant defense in digesting snakes, as genes encoding antioxidant enzymes such as catalase, peroxiredoxin, glutathionine transferase and heat shock protein were shown to be upregulated in digesting pythons (Duan et al., 2017). Our results suggest that the increased H_2O_2 released by mitochondria from fed boas did not compromise the mitochondrial redox balance.

Studies are increasingly showing that ROS generation is not essentially connected to damage, with demonstrations that ROS can act as signaling molecules, playing an essential role in the crosstalk from mitochondria and nucleus to maintain cell homeostasis (Shadel and Horvath, 2015). Of note, in mammals, there are remarkable differences between an acute fasting event and chronic fasting regimes as intermittent fasting or caloric restriction interventions. In both intermittent fasting and caloric restriction, there is growing evidence that chronic recurrent fasting regimes improve defenses against oxidative stress and repair damaged molecules (de Cabo and Mattson, 2019). In liver mitochondria from rodents, caloric restriction did not affect respiration rates but reduced ROS generation when energized with complex I-linked substrates and protected against MPT opening (Lambert et al., 2004; López-Torres et al., 2002; Menezes-Filho et al., 2017). Similar adaptive mechanisms can be potentially operative in *B. constrictor*, which is adapted to recurrent fasting regimes. Nevertheless, more studies could be performed to carefully evaluate the contrasting effects of transient beneficial ROS and harmful sustained elevated ROS levels in response to a fasting–feeding transition in snakes with different feeding strategies.

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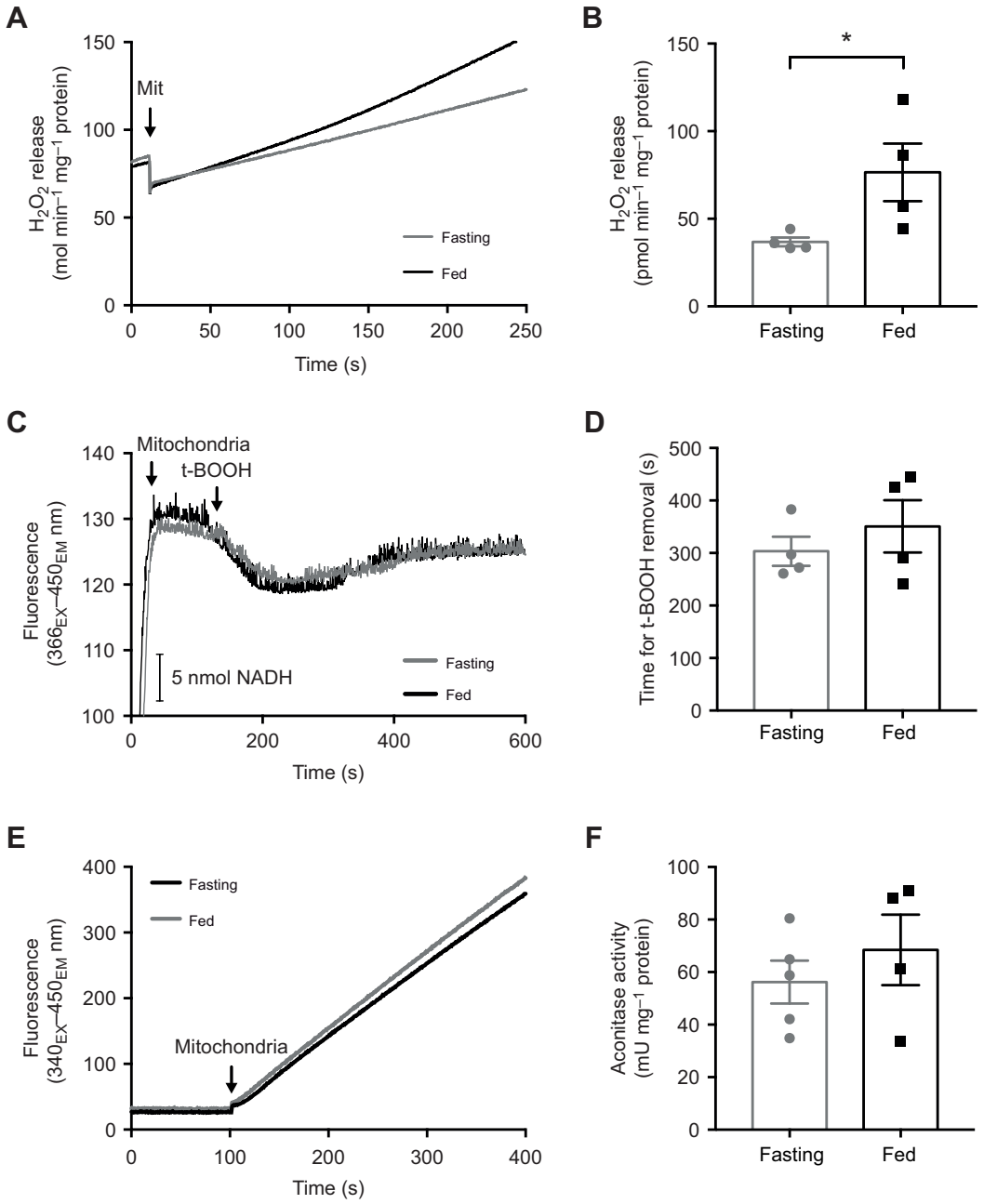

Fig. 4. Liver mitochondria from fed individuals exhibit higher rates of H_2O_2 release but no evidence of oxidative stress in comparison to fasting individuals. (A) Representative traces of H_2O_2 release assayed by the Amplex™ UltraRed probe in fasting (grey line) and fed (black line) liver mitochondria during resting respiratory state supported by complex I substrates. (B) H_2O_2 release in the basal state (*N*=4; **P*=0.03, Mann–Whitney test). (C) Representative traces of the endogenous fluorescence of NAD(P) in the reduced state monitored over time in mitochondria incubated in standard reaction medium supplemented with succinate and rotenone in the absence of exogenous ADP. (D) Time spent to recover the reduced state of NAD(P) following the addition of 2.5 μmol l^{-1} organic peroxide (t-BOOH) load. The recovery time was used to indirectly estimate the rate of peroxide removal in each group (*N*=4) and the ability of mitochondria to scavenge peroxide. (E) Representative traces of the increase of endogenous fluorescence of NADPH in the presence of isocitrate dehydrogenase and $NADP^+$ following oxidation of isocitrate formed by aconitase. (F) Aconitase activity (*N*=5 fasting, *N*=4 fed). Data are presented as means±s.e.m., and each dot represents individual data.

Concluding remarks

In summary, our results show that liver mitochondria of *B. constrictor* possess postprandial effects, exhibiting a rapid shift of mitochondrial bioenergetics towards higher respiration rates and OXPHOS supported by complex I-linked substrates, demonstrating the plasticity of mitochondrial function in snakes. Furthermore, our results show that the adaptation in mitochondrial function of boas might play a vital role in the fasting and feeding transition and be pivotal in organismal fitness by affecting animal performance.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: H.R.M.A.., M.R.S., A.L.C.; Methodology: H.R.M.A., M.R.S., C.D.C.N.; Formal analysis: H.R.M.A., M.R.S.; Investigation: H.R.M.A., M.R.S., C.D.C.N.; Resources: M.R.S., C.D.C.N., J.E.C.; Writing - original draft: H.R.M.A., M.R.S.; Writing - review & editing: H.R.M.A., M.R.S., J.E.C., A.L.C.; Supervision: J.E.C., A.L.C.; Project administration: J.E.C., A.L.C.; Funding acquisition: A.L.C.

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Data availability
Data are available in Dryad (Sartori et al., 2021): doi:10.5061/dryad.xpnvx0kgn

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