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Low production of mitochondrial reactive oxygen species after anoxia and reoxygenation in turtle hearts

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Summary statement

Low rates of mitochondrial $O_2^{\cdot-}$ production are responsible for lack of tissue damage upon reoxygenation after anoxia in turtles, likely due to low succinate accumulation and low degradation of adenine nucleotides.

Abstract

Extremely anoxia-tolerant animals, such as freshwater turtles, survive anoxia and reoxygenation without sustaining tissue damage to their hearts. In contrast, for mammals, the ischemia-reperfusion (IR) injury that leads to tissue damage during a heart attack is initiated by a burst of superoxide ($O_2^{\cdot-}$) production from the mitochondrial respiratory chain upon reperfusion of ischemic tissue. Whether turtles avoid oxidative tissue damage because of an absence of mitochondrial superoxide production upon reoxygenation, or because the turtle heart is particularly protected against this damage, is unclear. Here, we investigate whether there was an increase in mitochondrial $O_2^{\cdot-}$ production upon the reoxygenation of anoxic red-eared slider turtle hearts *in vivo* and *in vitro*. This was done by measuring the production of H_2O_2, the dismutation product of $O_2^{\cdot-}$, using the mitochondria-targeted mass-spectrometric probe *in vivo* MitoB, while in parallel assessing changes in the metabolites driving mitochondrial $O_2^{\cdot-}$ production succinate, ATP and ADP levels during anoxia and H_2O_2

consumption and production rates of isolated heart mitochondria. We found that there was no excess production of *in vivo* H_2O_2 during 1 h of reoxygenation in turtles after 3 h anoxia at room temperature, suggesting that turtle hearts most likely do not suffer oxidative injury after anoxia because their mitochondria produce no excess $O_2^{\cdot-}$ upon reoxygenation. Instead, our data support the conclusion that both the low levels of succinate accumulation and the maintenance of ADP levels in the anoxic turtle heart are key factors in preventing the surge of $O_2^{\cdot-}$ production upon reoxygenation.

Introduction

Anoxia-tolerant freshwater turtles regularly experience reoxygenation after hypoxia following a dive (Williams and Hicks, 2016) and after anoxia following winter hibernation (Herbert and Jackson, 1985; Ultsch and Jackson, 1982), without sustaining appreciable tissue damage (Bundgaard et al., 2018; Wasser et al., 1992). Isolated turtle hearts are also exceptionally tolerant of anoxia and reoxygenation, maintaining function (Bundgaard et al., 2018; Milton et al., 2007; Wasser et al., 1992). This is remarkable, since reperfusion of hypoxic or ischemic mammalian tissue with oxygenated blood causes a burst of mitochondrial $O_2^{\cdot-}$ production, which initiates a cascade of oxidative damage that leads to extensive ischemia-reperfusion (IR) injury (Kowaltowski et al., 2009). This phenomenon has been extensively studied in mammals as it can occur clinically, for example following removal of a blood clot with current treatments for heart attack or stroke, or as a consequence of surgical procedures, such as organ transplantation (Martin et al., 2019; Murphy and Steenbergen, 2008; Yellon and Hausenloy, 2007). The ability of turtles, and other anoxia-tolerant animals, to survive anoxia and reoxygenation without sustaining oxidative tissue damage has been described as one of the three core adaptations that underly anoxia tolerance (Bickler and Buck, 2007). In turtles, this tolerance has been attributed to 1) relatively high levels of antioxidant enzymes in turtles (Willmore and Storey, 1997a) that could effectively scavenge $O_2^{\cdot-}$ and H_2O_2 (Bickler and Buck, 2007; Galli and Richards, 2014; Hermes-Lima and Zenteno-Savín, 2002; Hermes-Lima et al., 2015), and/or 2) absence or suppression of the generation of $O_2^{\cdot-}$ by the mitochondrial respiratory chain in turtles upon reoxygenation after anoxia (Bundgaard et al., 2019a; Milton et al., 2007; Nayak et al., 2009; Pamenter et al., 2007).

Hypothesis 1), that high antioxidant levels are responsible for the absence of oxidative damage in turtles is based on the observation that antioxidant systems are relatively highly expressed in turtle tissues compared to other ectotherms and similar to mammals, based on

comparisons of values reported in the literature (Hermes-Lima and Zenteno-Savín, 2002; Hermes-Lima et al., 2015; Rice et al., 1995; Willmore and Storey, 1997a). However, no formal comparison have been made in parallel between species, and the activities of the antioxidant enzymes glutathione peroxidase, catalase, superoxide dismutase, and glutathione reductase in the heart all decrease by 20-50% when turtles are exposed to anoxia for 20 h at 5°C (Willmore and Storey, 1997a; Willmore and Storey, 1997b). This is paradoxical if antioxidant defences are responsible for preventing oxidative damage upon reoxygenation.

Instead, in support of hypothesis 2) our previous work has shown that the central factors that drive excess production of mitochondrial $O_2^{\cdot-}$ and H_2O_2 after ischemia and reperfusion in mammals might not be present to the same extent in turtles, even after prolonged anoxia. The mechanism that is thought to drive $O_2^{\cdot-}$ and H_2O_2 production during reperfusion involves accumulation of succinate and depletion of adenine nucleotides during ischemia (Chouchani et al., 2014; Murphy, 2009). This leads to a highly reduced mitochondrial ubiquinone pool and an elevated proton motive force, which together, upon the return of oxygen, cause reverse electron transport (RET) at complex I that drives extensive production of $O_2^{\cdot-}$ and H_2O_2 (Fig. 1). Isolated cardiac turtle mitochondria can produce $O_2^{\cdot-}$ and H_2O_2 by RET when incubated *in vitro* with excess succinate (Bundgaard et al., 2019b). It has previously been shown that turtles do accumulate some succinate in the heart with anoxia (Buck, 2000; Bundgaard et al., 2019b; Hochachka et al., 1975). However, the level of succinate accumulated appears to be 2-3 orders of magnitude lower in the turtle after anoxia than after ischemia in the mouse (Bundgaard et al., 2019b). Furthermore, in contrast to mammals (Martin et al., 2019), turtles are able to maintain adenine nucleotide levels during anoxia (Galli et al., 2013; Stecyk et al., 2009), partly because they are able to enter rapid metabolic depression with anoxia and switch to anaerobic glycolysis (Bickler and Buck, 2007; Jackson, 2002). In particular, maintenance of ADP may enable dissipation of the mitochondrial proton motive force through activation of the F_0F_1- ATP synthase, thereby preventing RET upon reperfusion. Together, these findings suggest that the conditions for extensive ROS production upon reperfusion may not be present after anoxia in turtles. Consistent with this, the production of mitochondrial H_2O_2 *in vitro* does not increase with anoxia and reoxygenation in turtle brain slices or neuronal cultures (Milton et al., 2007; Nayak et al., 2009; Pamenter et al., 2007). However, whether mitochondrial H_2O_2 production occurs in the turtle heart *in vivo* upon anoxia followed by reoxygenation is not known.

Here, for the first time, we set out to measure changes in the production of mitochondrial $O_2^{\cdot-}$ and H_2O_2 *in vivo* during reoxygenation of an anoxia-tolerant animal, the

anoxia-tolerant red-eared slider turtle (*Trachemys scripta*). To do this we used the mass-spectrometric probe MitoB which specifically measures the *in vivo* levels of H_2O_2 within mitochondria that are formed from the dismutation of $O_2^{\cdot-}$. The use of MitoB has previously been validated in *Drosophila melanogaster* (Cochemé et al., 2011; Cochemé et al., 2012), mice (*Mus musculus*) (Chouchani et al., 2013; Chouchani et al., 2014; Logan et al., 2014b; Lopez-Fabuel et al., 2016) and brown trout (*Salmo trutta*) (Salin et al., 2015; Salin et al., 2017; Salin et al., 2018), and can thus be used in a wide range of animal models and experimental settings (Logan et al., 2014a; Murphy, 2016). We hypothesized that turtles do not produce a burst of $O_2^{\cdot-}$ upon reoxygenation after anoxia, so that the MitoP/B ratio would be unchanged upon reoxygenation. A corollary is that this may arise due to the limited accumulation of succinate and/or preservation of ADP levels during anoxia that prevent RET upon reoxygenation. We tested these hypotheses by measuring the MitoP/B ratio *in vivo* and *in vitro* while in parallel assessing changes in succinate and ATP and ADP levels during anoxia. We found no elevation in MitoP/B upon reoxygenation after anoxia in turtle hearts *in vitro* and *in vivo* and that this was associated with far lower changes in succinate and ADP during anoxia compared to mammalian tissues. Thus, a major aspect of the ability of anoxia-tolerant turtle to tolerate hypoxia is the lack of ROS production upon reoxygenation, which is due in large part to the lack of dramatic changes in succinate and ADP during anoxia.

Materials and methods

Chemicals

All chemicals were purchased from Sigma-Aldrich. MitoB (3-hydroxybenzyl)triphenylphosphonium bromide, was dissolved in 60°C ethanol to a concentration of 5 mM and diluted to 0.5 mM with saline (0.9% NaCl/H_2O w/v). MitoB is rapidly distributed to the mitochondria when administered to animals (Cochemé et al., 2012; Logan et al., 2014a). MitoB is converted to MitoP by reaction with H_2O_2. The amount of MitoP generated is thus proportional to the amount of H_2O_2, the concentration of MitoB, and the exposure time. To eliminate the dependence on the concentration of MitoB, MitoP can be expressed as the MitoP/MitoB ratio. The MitoP/MitoB ratio then reflects the levels of H_2O_2 and can be used to assess relative changes in H_2O_2 in the mitochondria. Importantly, as the reaction of MitoB with H_2O_2 is 10^7 times slower than the other peroxidase reactions of the mitochondria, MitoB does not affect physiological H_2O_2 levels (Cochemé et al., 2012; Salin et al., 2017). Oligomycin, an inhibitor of F_0F_1-ATP synthase (complex V) was used as 5 mg/mL stock in ethanol, further diluted to 0.25 mg/mL in saline.

Animals

Male and female red-eared slider turtles (*Trachemys scripta elegans*) were purchased from NASCO (Fort Atkinson, WI, USA) and kept for several months before being used for experiments. Turtles were kept at 25°C in fresh water aquaria with free access to basking platforms on a 12:12 light:dark cycle and fed three times a week with dry pellets and raw krill in either Manchester University, UK or Aarhus University, DK. MitoB, oligomycin and pentobarbital were all injected into the supravertebral venous sinus with a 23-gauge syringe (Mans, 2008). A total of 32 turtles were used in this study, and the specific details about the experimental groups are given in the descriptions below. All procedures in Manchester were carried out in accordance with The Animals (Scientific Procedures) Act, 1986. Local ethical approval was granted by The University of Manchester Animal Welfare Ethical and Review Board, and all experiments were conducted within the UK Home Office laws for animal care and experimentation. All experiments in Denmark were conducted in accordance to the laws for animal care and experimentation in Denmark under the permit 2015-15-0201-00544.

Experimental procedure – *in vivo* anoxia and reoxygenation

Turtles (average body weight per group in parantheses ± SEM) were injected with 1 nmol MitoB per g body weight (nmol/g) as ~0.2% of body weight (v/w) of a 0.5 mM solution (equal to ~500-700 µL), left to equilibrate for 5 min in shallow water and then exposed to either 3 h anoxia / 1 h reoxygenation (320.5±15.4 g), 3 h anoxia (293.7±21.8 g) or 4 h normoxia (324.7±21.5 g) (n=5 for all groups). Anoxic exposure was conducted in water-filled aquaria, continuously bubbled with nitrogen gas to keep the oxygen tension <2%, monitored by a EcoSense ODO200 oxygen meter. To prevent pulmonary ventilation, turtles were kept submerged by a metal mesh to prevent surface breathing. Normoxic and reoxygenating turtles were kept in tanks with shallow water (~2 cm), allowing them easy access to air breathing. Experiments were conducted at 21°C. Turtles were euthanised by an overdose of pentobarbital (50 mg/kg), injected into the supravertebral sinus. When the corneal reflex was gone (after ~5 min), the turtles were beheaded by guillotine and the brain was destroyed by freezing in liquid nitrogen. Anoxic turtles were prevented from breathing by pressing their heads into their shells before removing them from the water and keeping them pressed in until decapitation after anaesthesia. Tissues were sampled as described below in the section *Tissue sampling*.

Experimental procedure – *in vivo* inhibition of F_0F_1-ATP synthase during reoxygenation

We assessed the effect of inhibition of F_0F_1-ATP synthase (complex V) with oligomycin during reoxygenation on *in vivo* H_2O_2 production. We compared the MitoP/B ratio after 3 h anoxia and 1h reoxygenation in turtles injected with 0.5 mg/kg oligomycin (n=4, 315.5±18.3 g) or vehicle (n=4, 345.5±23.6 g). Following the same procedure as described above in the *Experimental procedure – in vivo anoxia and reoxygenation* section, we injected 0.25 mg/mL oligomycin into the supravertebral venous sinus with a 23 gauge syringe to a final concentration of 0.5 mg/kg oligomycin. To inject the drug, the heads of the comatose anoxic turtles were pressed into the shell and the nostrils were covered with a wet paper tissue to prevent them from breathing when briefly removed from the aquaria. The anoxic turtles remained comatose during handling and after transfer back to the anoxic aquaria. In parallel, a control group was injected with vehicle. After a total of 3h anoxia, turtles were reoxygenated for 1h and tissues were sampled as described in the section *Tissue sampling*.

Experimental procedure – *in vitro* inhibition of F_0F_1-ATP synthase during reoxygenation

Next, we tested the effect of inhibiting F_0F_1-ATP synthase on H_2O_2 production in turtle heart strips, based on the procedure described previously (Joyce et al., 2014; Overgaard et al., 2005). Six turtles (424.5±59.7 g) were injected with MitoB into the supravertebral sinus. It was allowed to distribute within the turtle for 15 min, before euthanasia with pentobarbital as described above. The ventricle was quickly dissected out and placed in ice-cold turtle Ringer (100 mM NaCl, 25 mM $NaHCO_3$, 1 mM NaH_2PO_4, 2.5 KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$ and 10 mM glucose). The ventricle was then cut into ~1x1x10 mm slices from base to apex, avoiding areas with connective tissue. Six strips were mounted on glass rods connected to force transducers with surgical silk thread and immersed in turtle Ringer kept at 21°C bubbled with 98% air, 2% CO_2 (normoxia) delivered by a gas-mixing pump (Wösthoff, Bochum, Germany) which resulted in pH 7.6-7.8. The Ringer buffer was not exchanged during the experiment to mimic ischemic conditions. The strips were mounted in two different setups; two strips were mounted on a 305C Dual-Mode muscle lever connected to a 701C electrical stimulator (Aurora Scientific, ON, Canada) and four were mounted in separate setups connected to force transducers (Statham UC 2, Oxnard, CA, USA) and stimulated with Grass SD 9 stimulators (Quincy, MA, USA) connected to platinum electrodes. The two setups were used in parallel to be able to investigate all six experimental

treatments in each turtle. The length of the strips and stimulation intensity from the stimulators were then adjusted to give a maximal isometric force output and the strips were allowed to equilibrate for 30 min in normoxia. The strips were then exposed to four treatments, which were randomised between chambers; **1)** 90 min normoxia (normoxic control), **2)** 30 min normoxia, followed by 60 min anoxia (anoxic control), **3)** 60 min anoxia (98% N_2, 2% CO_2)/30 min reoxygenation (A/R) or **4)** A/R with 10 µM oligomycin (A/R + omy). Oligomycin was added 15 min before reoxygenation to allow the drug to equilibrate with the heart strip. After the experiment, the strips were removed from the setup, frozen in liquid N_2 and stored at -80°C until analysed for MitoP/B, [ATP], [ADP] and [succinate]. The efficiency of oligomycin inhibition of complex V was assessed in a fifth strip exposed to 10 µM oligomycin in turtle Ringer for 30 min in a 2 mL eppendorf placed in a heating block at 25°C. The strip was frozen in liquid nitrogen and stored at -80°C until assayed for F_0F_1-ATP synthase activity.

Tissue sampling

For collecting tissues after *in vivo* experiments, after confirming absence of all reflexes (corneal reflex, limb withdrawal, and head and jaw tone) following pentobarbital administration, the plastron was cut open with a bone-saw. The heart ventricle (~1 g) was removed and all connective tissue dissected away. The blood and tissues from anoxic turtles had the characteristic dark colour of deoxygenated haemoglobin and myoglobin, indicating that they were only minimally reoxygenated before sampling of tissues. All samples were snap-frozen in liquid nitrogen and stored at -80°C before further experiments.

Succinate content

Frozen heart ventricle samples were weighed into Precellys tubes prefilled with ceramic beads (CK28-R; Stretton Scientific Ltd., Derbyshire, UK), and 25 µl/mg extraction solution (30% acetonitrile, 50% methanol and 20% water) was added together with 100 pmol internal standard ($^{13}C_4$-succinate (Sigma-Aldrich)). Samples were lysed using a Precellys®24 tissue homogeniser (Bertin Corp, Rockville, MD 20850, USA. 6500 rpm, 15 seconds x 2) and centrifuged twice (17,000 x g, 10 min, 4°C), retaining the supernatant at each step. The supernatant was transferred to autosampler vials and stored at -80°C until liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis on a LCMS-8060 mass spectrometer (Shimadzu, UK) with a Nexera X2 UHPLC system (Shimadzu, UK) as

described previously (Prag et al., 2022) and quantified from a standard curve run under the same conditions. Briefly, samples were separated with a SeQuant ZIC-HILIC column (MerckMillipore, UK) with a ZIC-HILIC guard column. A flow rate of 200 µL/min was run with buffer A: 10 mM ammonium bicarbonate and buffer B: 100% acetonitrile, and run gradient of 0–0.1 min, 80% MS buffer B; 0.1–4 min, 80–20% B; 4–10 min, 20% B, 10–11 min, 20–80% B; 11–15 min, 80% B. The instrument was run in negative ion mode with multiple reaction monitoring and spectra were recorded using LabSolutions software (Shimadzu, UK).

ATP/ADP assay

The ATP and ADP content of heart ventricle samples were determined using a bioluminescence-based luciferase assay (Strehler, 1974), as described in detail elsewhere (Bundgaard et al., 2019b). Briefly, frozen tissue samples were homogenised in perchloric acid (3% v/v $HClO_4$, 2 mM Na_2EDTA, 0.5% Triton X-100), and samples and standards were then pH neutralised with 2 M KOH, 2 mM Na_2EDTA and 50 mM MOPS. For ADP measurements, neutralised sample was mixed with 250 uL ATP sulfurylase assay buffer (20 mM Na_2MoO_4, 5 mM GMP, 0.2 U ATP sulfurylase (New England Biolabs), in buffer 100 mM Tris-HCl, 10 mM $MgCl_2$, pH 8.0), to degrade ATP, incubated for 30 min at 30°C and then heat-inactivated for 5 min at 100°C. Samples and standards were then diluted in buffer (100 mM Tris, 2 mM Na_2EDTA, 50 mM $MgCl_2$, pH 7.75) in luminometer tubes. To convert ADP to ATP, pyruvate kinase (6U/mL) and phosphoenolpyruvate (100 mM) was added to one set of samples for ADP measurements and incubated in the dark for 30 min at 25°C. ATP was measured by bioluminescence using an AutoLumat LB-953-Plus luminometer (Berthold, Germany) by addition of luciferase/luciferin solution (7.5 mM DTT, 0.4 mg/ml BSA, 1.92 µg luciferase/ml, 120 µM D-luciferin), delivered via auto injection, protected from light and measured for 1 min.

ROS production with MitoB

In vivo mitochondrial H_2O_2 -production was measured as the ratio between the oxidised product MitoP and the mitochondria-targeted H_2O_2-probe MitoB (MitoP/B) by LC-MS/MS, as described in detail elsewhere (Cochemé et al., 2012). Briefly, ~100 mg of frozen tissue was homogenised in 60% (v/v) acetonitrile and 0.1% (v/v) formic acid in HPLC-grade H_2O and the homogenate was spiked with internal standards (100 pmol d_{15}-MitoB and 50 pmol

d_{15}-MitoP kindly provided by Richard C. Hartley, University of Glasgow, UK) to generate standard curves before centrifugation and re-extraction of the pellet. Standard curves were prepared in parallel with analysis of the samples. The supernatants were filtered, dried and re-dissolved in 20% (v/v) acetonitrile and 0.1% (v/v) formic acid in HPLC-grade H_2O. The samples were then centrifuged and transferred to 1.5 mL autosampler vials for LC-MS/MS determination of MitoP and MitoB.

Complex V activity assay

Complex V activity was assayed as the oligomycin-sensitive decrease in NADH in the presence of rotenone and KCN in turtle heart homogenate as described in detail previously (Bundgaard et al., 2019a). Briefly, ~50 mg tissue was homogenised by bullet blending at 50Hz for 10 min at 4°C in a Thermo tissue lyzer in 25 mM potassium phosphate buffer, pH 7.2, and homogenate was added to 100 mM Tris, 50 mM KCl, 2 mM MgCl2, 0.2 mM EDTA, pH 8.0, with 1 mM phosphoenolpyruvate, 2.5 mM ATP, 2 U/mL lactate dehydrogenase and 4 U/mL pyruvate kinase. Background NADH oxidation was prevented by addition of 10 µM rotenone, 300 nM antimycin A and 2 mM KCN.

Aconitase activity

Aconitase is an enzyme in the citric acid cycle and the mitochondrial matrix isoform is very susceptible to oxidation by $O_2^{\cdot -}$ and thus serves as a sensitive tissue marker of oxidative damage (Gardner et al., 1994). Total aconitase activity (i.e. both cytosolic and matrix isoforms) was assayed based on the coupled enzyme assay described by Gardner et al. (1994), where aconitase converts citrate to isocitrate coupled with the decarboxylation to α-ketoglutarate with the reduction of NADP to NADPH by isocitrate dehydrogenase. Turtle hearts were homogenised (by bullet blending at 50Hz for 10 min at 4°C in a Thermo tissue lyzer) in 50 mM Tris-HCl buffer pH 7.4 with 0.6 mM $MnCl_2$. 20 µM dl-fluorocitrate was added to the isolation buffer to protect the oxidation sensitive iron-sulfur cluster. Aconitase activity was assayed immediately in 50 mM Tris-HCl buffer pH 7.4 with 0.6 mM $MnCl_2$, 5 mM sodium citrate, 0.2 mM NADP and 0.4 U/mL type IV isocitrate dehydrogenase from porcine heart as the appearance of NADPH at 340 nm at 25°C on a microplate reader (SpectraMax® iD3, Molecular Devices, California, USA) using ε_{340} NADPH = 6.22 x 10^3 M^{-1} cm^{-1}. Background rate was measured in the presence of 100 µM dl-fluorocitrate.

In vitro H_2O_2 production and consumption

The *in vitro* capacity for H_2O_2 consumption and production was assessed on isolated mitochondria from turtles (n=3, 233±44.4 g), green iguanas (n=3, 310±42.4 g) and mice (n=3, 26.3±2.9 g) hearts. For turtle and green iguana, animals were anaesthetized with 50 mg/kg pentobarbital injected into the supravertebral sinus or tail vein, respectively. Mice were killed by cervical dislocation. Heart tissue samples were stored in STE buffer (250 mM sucrose, 5 mM TRIS and 1 mM EGTA, pH 7.4 with 0.5% (w/v) fatty-acid free bovine serum albumin (BSA)) on ice for ~30 min before mitochondrial isolation. Mitochondria were isolated by differential centrifugation as described in detail elsewhere (Bundgaard et al., 2019). Briefly, tissue was homogenised in a Potter-Elvehjem homogeniser on ice in STE buffer, and the homogenate was then centrifuged at 700g for 5 min at 4°C. The supernatant was then filtered through cheese cloth and centrifuged twice at 10,000g for 10 min at 4°C, before the final pellet was resuspended in STE without BSA. Protein content was determined with a BCA assay using BSA as a standard.

H_2O_2 consumption

H_2O_2 consumption was measured based on the method described by (Munro et al., 2016). Isolated mitochondria were added to a 1 mL Eppendorf containing respiration buffer (70 mM sucrose, 220 mannitol, 2 mM EGTA, 5 mM $MgCl_2$, 5 mM $KH_2PO_4^-$, 10 mM HEPES, pH 7.4 with 0.3% (w/v) BSA on the day) equilibrated to 21°C (on an eppendorf thermomixer) with 2.5 µM H_2O_2 and either 1) no substrate, 2) 2.5 mM malate, 10 mM glutamate and 1 mM ADP (Mal/Glu/ADP), 3) 20 mM succinate. After 0, 2.5, 5, 7.5 and 10 min a 180 µL aliquot was removed and mixed with 20 µL quenching solution (250 µM Amplex UltraRed, 50 U/mL HRP and 250 U/mL superoxide dismutase) in a black 96-well microplate to convert all H_2O_2 remaining in the solution to water and producing the flurorescent product resorufin. After 10 min, fluorescence was measured at 544 nm excitation/590 nm emission on a microplate reader (SpectraMax® iD3, Molecular Devices, California, USA). H_2O_2 consumption was standardized with a standard curve of 0, 1 and 2.5 µM H_2O_2 in respiration buffer which was prepared in parallel.

H_2O_2 production

Mitochondrial H_2O_2 production was measured as previously described (Bundgaard et al., 2019) on an Oroboros O2K high-resolution respirometer fitted with a FluoO2K module with

filters specific for Amplex UltraRed (Oroboros Instruments, Innsbruck, Austria). The machines were calibrated with respiration buffer equilibrated with air at 21°C every day. Mitochondria (62.5 µg/mL) were added to each of the two chambers, followed by the components for the Amplex UltraRed assay (10 µM Amplex UltraRed, 5 U/mL horseradish peroxidase and 25 U/mL superoxide dismutase) and the fluorescent signal was calibrated with injections of 0.1 µM H_2O_2. Two protocols were run in parallel to match the conditions for the H_2O_2 consumption assay. In one chamber, 2.5 mM malate and 5 mM glutamate were added to initiate non-phosphorylating, leak respiration, before addition of 1 mM ADP to initiate phosphorylating respiration. Cytochrome c (10 µM, equine) was then injected to assess the integrity of the outer membrane, which did not elicit an increase in respiration rate above 10% in any experiment. In the other chamber, 20 mM succinate was injected to initiate maximal H_2O_2 production via RET, before addition of 0.5 µM rotenone and 1 mM ADP to stimulate phosphorylating respiration.

Statistics

Significant differences were determined by one- or two-way ANOVA with Tukey's or Sidak's multiple comparisons test for normally distributed data (Prism 9, Graphpad Software, San Diego, CA, USA) and experimental groups were considered significantly different when $p < 0.05$. Statistical comparisons and P-values are given in the results section below. All data are shown as means±SEM, and significant differences $p < 0.05$ are marked by *.

Results

We assessed mitochondrial ROS production *in vivo* in the hearts of red-eared slider turtles (*Trachemys scripta elegans*) under three conditions; normoxia, anoxia, and reoxygenation after anoxia (Fig. 2A). To see if reoxygenation of anoxic tissues led to an increase in the *in vivo* production of mitochondrial $O_2^{\cdot -}$ we measured the proportion of its dismutation product H_2O_2 using the conversion of MitoB to MitoP. The MitoP/MitoB ratio in hearts from turtles exposed to anoxia and reoxygenation was similar to that from from turtles exposed to anoxia alone, and both were lower than the ratio in hearts from normoxic controls (Fig. 2B, one-way ANOVA, $P<0.001$). This result is consistent with the lack of O_2 as substrate for $O_2^{\cdot -}$ generation during anoxia, and thus lowered H_2O_2 production. When controlling for the different durations of anoxia and reoxygenation (Fig. 2A), the rate of MitoB to MitoP conversion was not elevated in turtles exposed to reoxygenation after anoxia compared to

anoxia alone (Fig. 2C, Tukey's multiple comparisons test, P=0.9949). The activity of the $O_2^{\cdot-}$-sensitive citric acid cycle enzyme aconitase was also not different between the experimental groups (Fig. 2D, one-way ANOVA, P=0.8919), suggesting that it was not differentially inactivated by $O_2^{\cdot-}$. Together, these findings show that there is no excessive production of mitochondrial $O_2^{\cdot-}$ in the turtle heart upon reoxygenation after anoxia *in vivo*.

In mammalian hearts, the key factors driving excessive production of mitochondrial $O_2^{\cdot-}$ upon reperfusion are thought to be accumulation of succinate and depletion of adenine nucleotides (Chouchani et al., 2014). So, we next assessed whether these factors change during anoxia in the turtle hearts. We found a minor apparent ~2-fold elevation in [succinate] upon anoxia to 10±2.3 ng/mg, that did not achieve significance (Tukey's multiple comparisons test, P=0.2647), and which was restored to normoxic levels upon reoxygenation (Fig. 3A). This contrasts dramatically with the ~10-fold elevation in succinate in the mouse heart upon ischemia to ~400 ng/mg (Chouchani et al., 2014; Martin et al., 2019). There were no significant changes in [ATP] and [ADP] in the turtle heart upon anoxia (Fig. 3B, two-way ANOVA, P=0.6022). The ATP/ADP ratio (Fig. 3C) did decrease significantly in the turtle heart upon anoxia compared to normoxia (Tukey's multiple comparisons test, P=0.0486), probably due to inactivation of oxidative phosphorylation. The ATP/ADP ratio was restored after 1 h reoxygenation. The lack of change in [ATP] and [ADP] contrast markedly with the dramatic decreases in these upon ischemia in the mouse heart (Chouchani et al., 2014; Martin et al., 2019). Together, these results suggest that there are major metabolic differences in the turtle heart during anoxia compared to the hearts of mammals, and that the key drivers of mitochondrial $O_2^{\cdot-}$, production upon reoxygenation do not accumulate during anoxia in turtles. This feature underlies the lack of oxidative damage to their hearts upon reoxygenation after anoxia.

Next, we wanted to test whether the turtles' ability to maintain ADP during anoxia was solely responsible for preventing mitochondrial $O_2^{\cdot-}$, production upon reoxygenation. The presence of ADP upon reoxygenation would allow the mitochondrial proton motive force to be dissipated through the action of the F_0F_1-ATP synthase and thus prevent mitochondrial $O_2^{\cdot-}$ production by RET. To test this hypothesis, we assessed the effect of inhibition of the F_0F_1-ATP synthase on mitochondrial $O_2^{\cdot-}$ production after anoxia and reperfusion *in vivo*. To do this, we injected anoxic turtles with the F_0F_1-ATP synthase inhibitor oligomycin 15 min prior to reoxygenation (Fig. 4A), which led to a significant decrease in F_0F_1-ATP synthase activity (Fig. 4B, student's t-test, P<0.001). The [ADP] did

not change significantly (Fig. 4C, Sidak's multiple comparisons test, P=0.9936), while [ATP] (Fig. 4C) and the ATP/ADP ratio (Fig. 4D) were significantly lower in oligomycin-treated turtles compared to controls (Sidak's multiple comparisons test and student's t-test, P=0.005 and P=0.0326, respectively). This suggests that the lower ATP levels with oligomycin was due to further degradation to purine nucleotide degradation products. Interestingly, [ATP] and the ATP/ADP ratio were around 2-fold lower in turtle hearts with oligomycin than in controls after 1h of reoxygenation (Fig. 4C,D), similar to the fold-reduction in anoxia (Fig. 3B,C), which suggests that turtles are able to maintain 50% of normal ATP levels by glycolysis, even during normoxia. We found no difference in the MitoP/B ratio (Fig. 4E, student's t-test, P=0.8531), nor total aconitase activity (Fig. 4F, student's t-test, P=0.1840) upon reoxygenation of oligomycin-treated turtles compared to controls. This suggests that maintenance of ADP levels is not solely responsible for preventing excessive mitochondrial $O_2^{\cdot-}$ production in turtles after anoxia and reoxygenation, but that other factors, such as the low accumulation of succinate during anoxia, may also contribute.

Anoxia *in vivo* in the turtle differs from ischemia in mammals, because in ischemia there is no blood flow while in anoxia there is efflux of lactate from the heart, enabling glycolysis to continue (Gruszczyk et al., 2022). Thus, metabolism may differ between the two systems. To assess this, we injected turtles with MitoB *in vivo* and then, to mimic ischemia, we prepared heart slices and exposed the heart strips to anoxia and reoxygenation *in vitro* (Fig. 5A). The MitoP/B ratio did not vary significantly between the experimental groups (Fig. 5B, one-way ANOVA, P=0.9890), and while [succinate] was slightly higher in the anoxic *in vitro* incubation compared to normoxia (Fig. 5C, Tukey's multiple comparisons test, P=0.1189), ATP concentration (Fig. 5D) and ATP/ADP ratios (Fig. 5E) decreased during anoxia compared to control (Sidak's and Tukey's multiple comparisons test, P=0.1658 and P=0.2345, respectively). However, these small, non-significant changes were similar to those *in vivo* in the anoxic turtle (Fig. 2). We repeated these experiments in the presence of oligomycin (Fig. 5A), which inhibited the activity of the F_0F_1-ATP synthase (Fig. 5G, student's t-test, P<0.001), but this did not affect the outcome (Figs. 5B-E). Thus, even under conditions mimicking ischemia, with or without artificial inhibition of the F_0F_1-ATP synthase with oligomycin, the metabolic changes are similar to anoxia *in vivo* and turtle heart tissue does not produce excessive $O_2^{\cdot-}$ after anoxia and reoxygenation. The relative force of contraction of the heart strips recovered after anoxia and reoxygenation was similar in all groups (Fig. 5F). This suggests that there was no functional effect of anoxia and reoxygenation on turtle hearts regardless of the presence of oligomycin.

Finally, we tested whether the antioxidant capacity is higher in turtle mitochondria compared to a mammal (mouse) or an anoxia-intolerant reptile (green iguana) (Fig. 6). We found that isolated turtle heart mitochondria did not scavenge H_2O_2 more effectively than heart mitochondria from mice or green iguanas under conditions for state III respiration (malate, glutamate and ADP) or reverse electron transfer (succinate alone) (Sidak's multiple comparisons test, P=0.6774, P=0.0893, P=0.7982, P=0.0894, respectively). Moreover, all three animals tested produced H_2O_2 to a similar degree when incubated with succinate alone (two-way ANOVA, P=0.3091), showing that both turtles and green iguana mitochondria have the capacity to produce H_2O_2 by reverse electron transfer *in vitro*. It just seems to be the case that at least turtle hearts are able to avoid producing H_2O_2 *in vivo*.

Discussion

Turtles are able to return from complete anoxia without sustaining oxidative tissue damage to their hearts (Bickler and Buck, 2007; Bundgaard et al., 2018; Milton et al., 2007; Wasser et al., 1992), in contrast to mammals which encounter severe oxidative damage after ischemia and reperfusion (Martin et al., 2019; Murphy and Steenbergen, 2008; Yellon and Hausenloy, 2007). In this study, we investigated whether turtles avoid oxidative damage to their hearts because they do not produce excessive $O_2^{\cdot-}$ and H_2O_2 after anoxia and reoxygenation *in vivo*, and whether this is because they are able to avoid the metabolic changes that are thought to drive reperfusion damage in mammals.

We show that there is no increase in the MitoP/B ratio (Fig. 2B,C) or decrease in the $O_2^{\cdot-}$-sensitive marker enzyme aconitase (Fig. 2D) in turtle hearts after 3h anoxia and 1h reoxygenation *in vivo*, which shows that there is no burst of mitochondrial H_2O_2 and $O_2^{\cdot-}$ production upon reoxygenation of anoxic turtle hearts. In comparison, extensive H_2O_2-production is observed in mouse hearts exposed to 30 min ischemia at 37°C and 15 min of reperfusion. This is reflected in ratios of ~0.125 MitoP/B per hr after 30 min ischemia and 15 min reperfusion, compared to ~0.0125 MitoP/B per hr after 45 min of normoxia (Chouchani et al., 2014), and in a ~50% decrease in aconitase activity in mice with ischemia and reperfusion (Bulteau et al., 2005; Chouchani et al., 2014; Gardner, 2002). Absence of mitochondrial $O_2^{\cdot-}$ in the turtle heart *in vivo* is supported by the findings in turtle brain slices and neuronal cell cultures where there was no excessive production of H_2O_2 after exposure to anoxia and reoxygenation *in vitro* (Milton et al., 2007; Nayak et al., 2009; Pamenter et al., 2007). Furthermore, studies have found that other downstream cellular markers of oxidative stress, such as lipid peroxidation (Willmore and Storey, 1997a) and the GSH/GSSG ratio

(Willmore and Storey, 1997b), only increase slightly or not at all in turtle tissues after anoxia and reoxygenation *in vivo*. This is in keeping with the remarkable tolerance of turtle hearts to maintain function after *in situ* anoxia or ischemia and reoxygenation (Bundgaard et al., 2018; Wasser et al., 1992). Together, this shows that turtle hearts avoid oxidative reoxygenation damage because there is no burst of mitochondrial $O_2^{\cdot-}$ upon reoxygenation after anoxia.

We next considered how mitochondria are thought to produce $O_2^{\cdot-}$ upon reperfusion. Electrons delivered from accumulated succinate led to a highly reduced ubiquinone pool, and low levels of F_0F_1-ATP synthase substrate (ADP) prevent dissipation of the proton motive force through ATP synthesis. Together, this leads to RET from accumulated succinate to complex I where superoxide is produced (Fig. 1) (Chouchani et al., 2014; Murphy, 2009). Here, we show that the factors driving $O_2^{\cdot-}$ and H_2O_2 in mammals, succinate accumulation and ADP depletion, are largely unaffected in the turtle heart after anoxia *in vivo* (Fig. 3) compared to those in the ischemic mammalian heart where oxidative damage can occur (Chouchani et al., 2014; Martin et al., 2018). Anoxic incubation of heart strips also caused a modest, but insignificant increase in [succinate] and transient decrease in ATP levels (Fig. 5). Even longer exposure (14-28 days) to anoxia at 4-5°C also only cause a modest, albeit statistically significant increase in succinate levels in turtles (Bundgaard et al., 2019b) and in the closely related freshwater turtle *Chrysemys picta* (Buck, 2000), suggesting that while succinate accumulates in the turtle heart, it never reaches the high levels driving oxidative damage after ischemia in mammalian hearts.

Similarly, while there was a significant ~50% decrease in the ATP/ADP ratio with both *in vivo* and *in vitro* anoxia, this was restored to normoxic levels in the group with 1h reoxygenation (Fig. 3 and 5). Only in the experimental groups which were treated with oligomycin did ATP levels stay low after reoxygenation (Fig. 4 and 5), consistent with a lower ATP production when F_0F_1-ATP synthase is inhibited. This was associated with little loss of adenine nucleotides during anoxia in turtle hearts. This is consistent with the fact that ADP is not degraded in turtles hearts with prolonged anoxia (Bundgaard et al., 2019b; Galli et al., 2013; Kelly and Storey, 1988; Stecyk et al., 2009) and reflects the turtles' remarkable ability to maintain energy balance during anoxia (Bickler and Buck, 2007; Bundgaard et al., 2019b; Jackson, 2002; Stecyk et al., 2009). It likely also reflects the difference between ischemia, where absence of blood flow not only decreases oxygen levels but also causes acidification and accumulation of other end products (Gruszczyk et al., 2022), and whole-animal anoxia, where the circulation remains active to remove end-products and provide metabolic fuel. Together, this shows that the factors necessary to drive excessive production

of $O_2^{\cdot-}$ and H_2O_2 in the mammalian heart upon reperfusion after ischemia are not present after anoxia in turtle hearts.

We considered three possible explanations for these findings, 1) that succinate levels never accumulate to levels that are high enough to drive harmful $O_2^{\cdot-}$ and H_2O_2 production or, 2) that turtle mitochondrial antioxidant capacity is so high that excess production of $O_2^{\cdot-}$ and H_2O_2 would be instantly buffered under these conditions, and therefore not be reflected in an increase MitoP/B ratio. In support of 1), we have shown here that even with artificial inhibition of F_0F_1-ATP synthase, [succinate] is never high enough to drive extensive production of $O_2^{\cdot-}$ and H_2O_2 in turtle heart tissue (Fig. 3, 4 and 5), and therefore that preservation of ATP levels alone is not enough to explain why turtles avoid oxidative reoxygenation damage. In this study, we were unable to elevate succinate levels to those observed in mammals using turtle tissue, but previous work in isolated mitochondria demonstrated that turtle mitochondria exposed to mM concentrations of succinate do generate ROS via RET (Bundgaard et al., 2019b). This highlights the importance of the turtles' ability to avoid extensive succinate accumulation during anoxia. This likely reflects the turtle's ability to rapidly enter metabolic depression when they encounter anoxia and their ability to shift their energy metabolism to anaerobic glycolysis, sequestering accumulated lactate in their shells (Jackson, 2002). Succinate may also be excreted from the cardiomyocytes during anoxia via the monocarboxylate transporter 1 (Prag et al., 2020), although succinate did not accumulate in anoxic heart strips (Fig. 5) and does not accumulate in the blood with prolonged anoxia in turtles (Buck, 2000).

Furthermore, we tested hypothesis 2) and showed that the antioxidant capacity of isolated turtle mitochondria was comparable to that in mouse but higher than green iguana mitochondria (Fig. 6), suggesting that turtle heart mitochondria do not have an exceptionally high antioxidant capacity compared to mammals or other reptiles. Thus, while antioxidants may contribute to regulation of mitochondrial ROS levels *in vivo*, high levels of antioxidants are unlikely to be the main mechanism that limits ROS production upon reoxygenation compared to mammals.

Finally, the absence of reoxygenation damage even in the presence of oligomycin in turtle hearts could be due to 3) that there are other intrinsic aspects of turtle metabolism and mitochondrial physiology that contribute to prevent $O_2^{\cdot-}$ and H_2O_2 production upon reoxygenation as well as low succinate accumulation and preservation of ADP levels. This could include activation of uncoupling factors of the inner mitochondrial membrane, e.g. via uncoupling proteins or K_{ATP}-channels (Pamenter et al., 2008; Staples and Buck, 2009), direct

regulation of the electron transport chain complexes via post-translational modifications such as S-nitrosation of complex I (Bundgaard et al., 2018; Chouchani et al., 2013) or phosphorylation of complex II (Mathers et al., 2016), or through regulation of the highly stable supercomplex interactions between complex I and III in turtles (Bundgaard et al., 2019a). Decreases in mitochondrial respiration rate (Bundgaard et al., 2019a; Galli et al., 2013; Pamenter et al., 2016) and capacity for H_2O_2 production (Bundgaard et al., 2019a; Milton et al., 2007) with prolonged anoxia in turtles further points to intrinsic regulation of mitochondrial physiology with anoxia.

In conclusion, we show that low rates of mitochondrial $O_2^{\cdot -}$ production is the main factor responsible for the absence of tissue damage upon reoxygenation in turtles. This is likely due to a low total accumulation of succinate and the lack of degradation of adenine nucleotides during anoxia in the turtles. These factors would remove the potential for RET to occur upon reoxygenation in anoxia-tolerant turtles.

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Competing interests

No competing interests declared.

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Figures

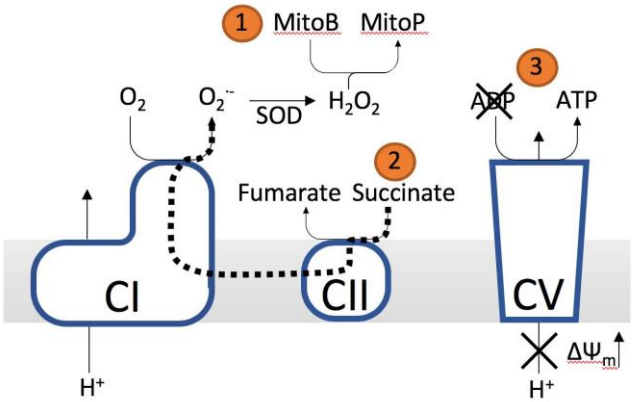

Fig. 1. Model of the main mechanistic elements driving reverse electron transfer in the mitochondria during reoxygenation. Electrons from oxidation of accumulated succinate moves in the reverse direction from CII to CI due to a high proton motive force ($\Delta\Psi_m$), set by inhibition of CV due to depletion of the substrate ADP. The direction of the electron flow is marked with the dashed line. We investigated the conditions of this mechanism in turtles exposed to anoxia and reoxygenation by assessing 1) *in vivo* H_2O_2 production with MitoB, 2) succinate accumulation and 3) ADP and ATP concentrations and the effect of inhibition of CV on the H_2O_2 production. CI: complex I (NADH dehydrogenase), CII: complex II (succinate dehydrogenase), CV: complex V (F_0F_1-ATP synthase), SOD: superoxide dismutase.

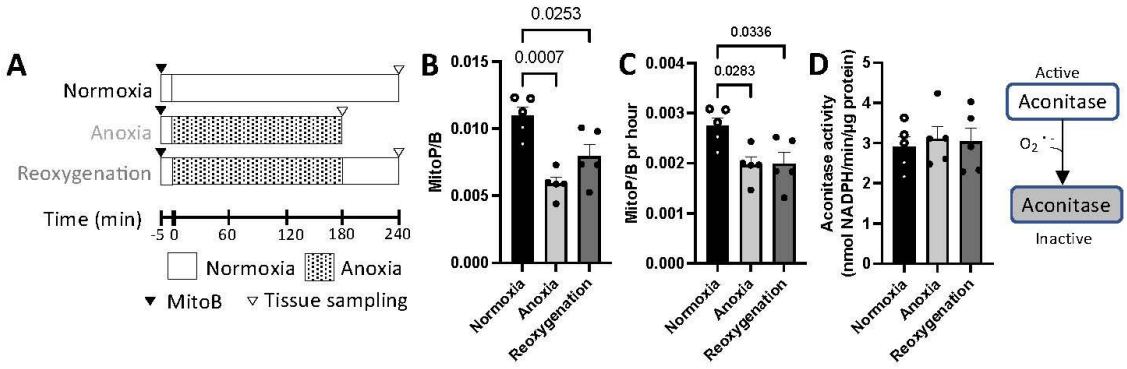

Fig. 2. *In vivo* ROS production in turtle hearts after anoxia and reoxygenation. **A)** Experimental groups. Turtles were injected with 1 nmol/g MitoB and allowed to equilibrate in normoxia for 5 min, before exposing turtles to 4 h normoxia, 3 h anoxia, or 3 h anoxia followed by 1 h reoxygenation in normoxia. **B)** *In vivo* H_2O_2 production measured by MitoP/B ratio and **C)** MitoP/B ratio pr hour in turtle hearts. **D)** Total aconitase activity as a measure of oxidative damage. Data are means±SEM, n=5. Significantly different P-values are shown, for statistical details see Results section.

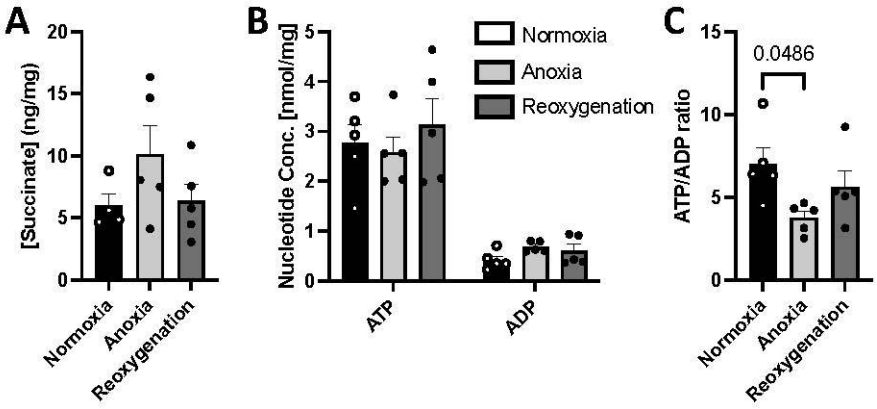

Fig. 3. A) Absolute succinate concentration in turtle hearts. The statistical differences between the anoxia and normoxia groups and the anoxia and reoxygenation groups were p=0.2647 and p=0.2875, respectively. **B)** Absolute adenine nucleotide concentration and, **C)** ATP/ADP ratio in turtle hearts. Experimental groups as described in Fig. 2, data are means±SEM, n=5. Significantly different P-values are shown, for statistical details see Results section.

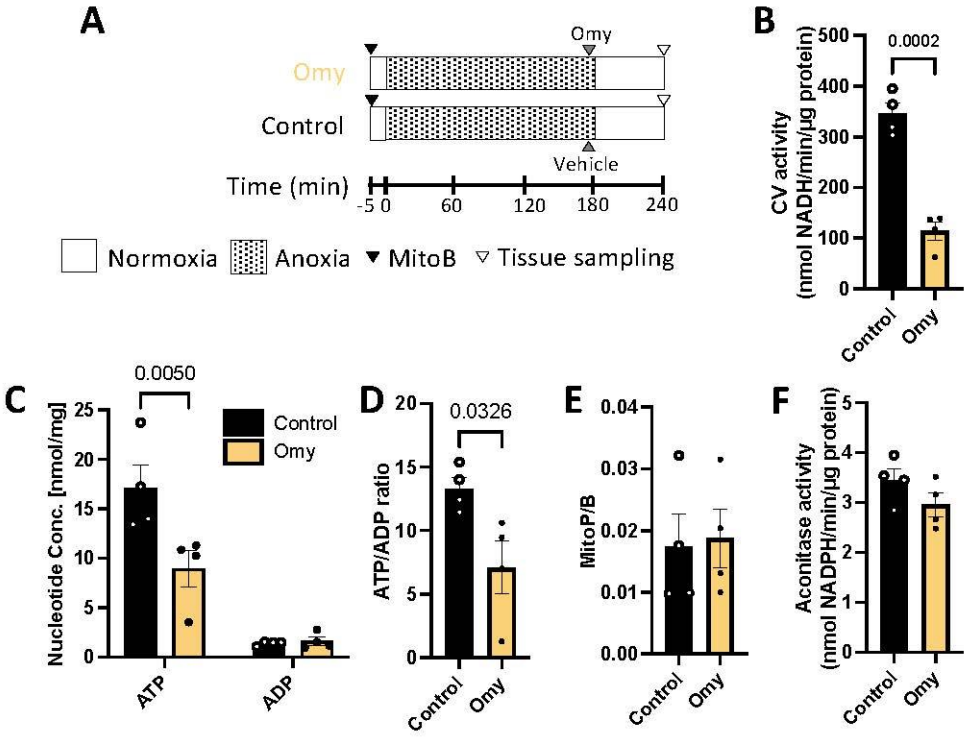

Fig. 4. Inhibition of F_0F_1-ATP synthase with oligomycin does not increase *in vivo* ROS production in turtle hearts after anoxia and reoxygenation. **A)** Experimental groups. Turtles were injected intravenously with 1 nmol/g MitoB and allowed to equilibrate in normoxia for 5 min, before exposing turtles to 3 h anoxia followed by 1 h reoxygenation in normoxia. Turtles were injected intravenously with 0.5 mg/kg oligomycin or vehicle 15 min before reoxygenation. **B)** Oligomycin sensitive F_0F_1-ATP synthase (complex V, CV) activity of turtle heart homogenate. **C)** Absolute adenine nucleotide concentration and **D)** ATP/ADP ratio. **E)** *In vivo* H_2O_2 production measured by MitoP/B ratio, **F)** Total aconitase activity as a measure of oxidative damage. Data are means±SEM, n=4. Significantly different P-values are shown, for statistical details see Results section.

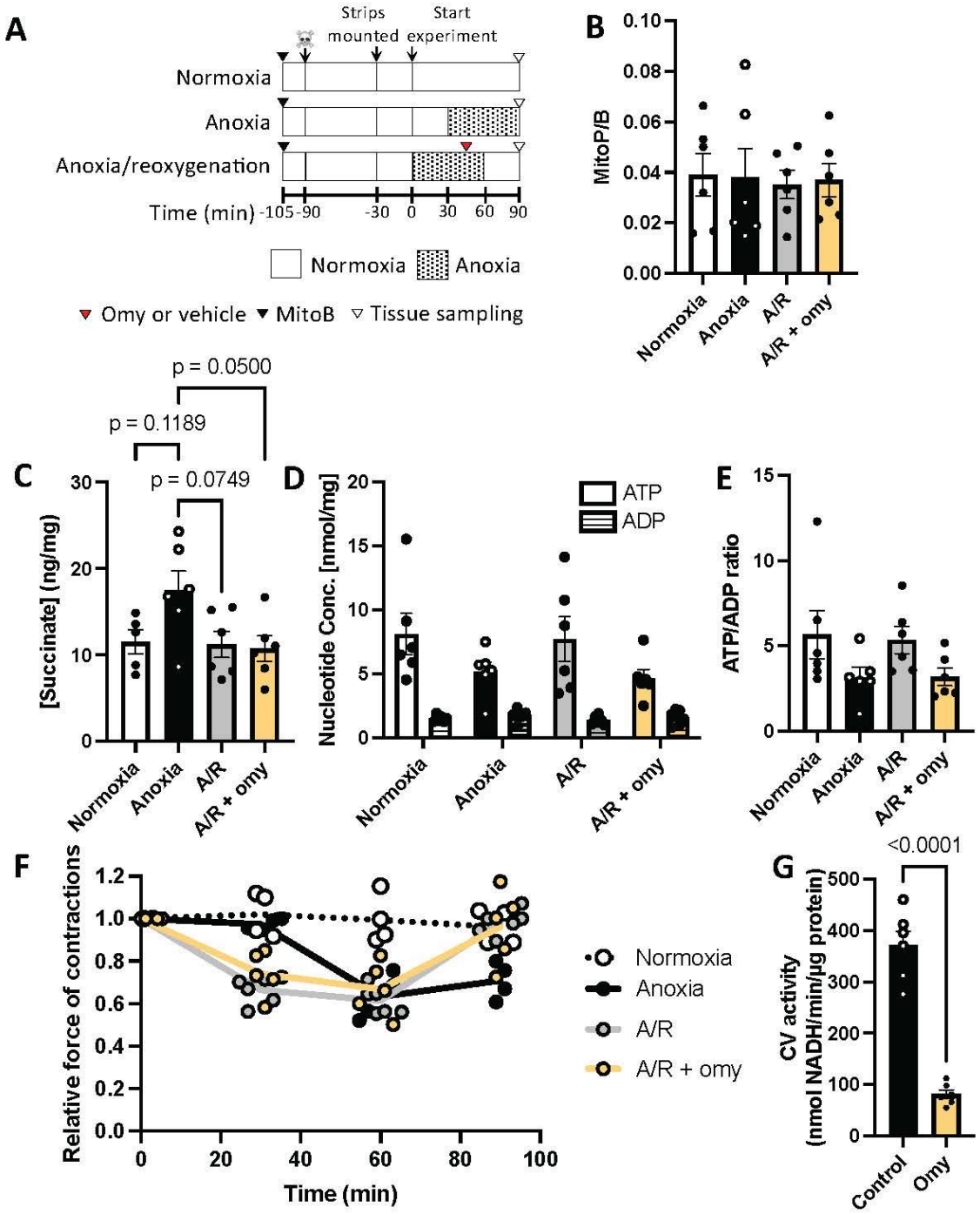

Fig. 5. *In vitro* incubation of turtle heart strips with the F_0F_1-ATP synthase inhibitor oligomycin does not increase ROS production measured by MitoB/P. **A)** Experimental groups. Turtles were injected intravenously with MitoB and allowed to equilibrate for 15 minutes before euthanasia and mounting of heart strips on force transducers. After stabilisation of heart strips in turtle Ringer at 25°C, they were exposed to either *1)* 90 min

normoxia (normoxic control), *2)* 30 min normoxia, followed by 60 min anoxia (anoxic control), *3)* 60 min anoxia (98% N_2, 2% CO_2)/30 min reoxygenation (A/R), *4)* A/R with 10 µM oligomycin. Oligomycin was added after 45 min of anoxia as indicated. **B)** MitoB/P ratio, **C)** absolute succinate concentration, **D)** absolute adenine nucleotide concentration and **E)** ATP/ADP ratio of turtle heart strips. **F)** Relative force of contraction of heart strips during the experiment. **G)** ATP synthase activity of a heart strip incubated with 10 µM oligomycin in parallel to the experimental strips for 30 min. Data are means±SEM, n=6. Significantly different P-values are shown, for statistical details see Results section.

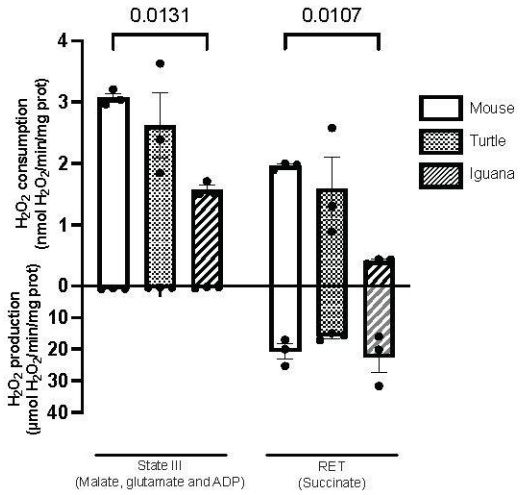

Fig. 6. *In vitro* H_2O_2 consumption and production rates in isolated mitochondria from mouse, turtle and green iguana. H_2O_2 consumption rates were measured by an Amplex UltraRed microplate based fluorescent assay and H_2O_2 production rates were measured with an Oroboros O2K respirometer fitted with a FluoO2K module with filters specific for Amplex UltraRed. State III respiration was measured with malate, glutamate and ADP and reverse electron transfer (RET) was measured with succinate alone. Data are means±SEM, n=3. Significantly different P-values are shown, for statistical details see Results section.