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RESEARCH ARTICLE

Beating oxygen: chronic anoxia exposure reduces mitochondrial F_1F_O-ATPase activity in turtle (*Trachemys scripta*) heart

Gina L. J. Galli[1,2,*], Gigi Y. Lau[1] and Jeffrey G. Richards[1]

[1]Department of Zoology, The University of British Columbia, 6270 University Boulevard, Vancouver, BC, Canada, V6T 1Z4 and [2]Faculty of Life Sciences, University of Manchester, 2.4 Core Technology Facility, 46 Grafton Street, Manchester, M13 9NT UK

*Author for correspondence (gina.galli@manchester.ac.uk)

SUMMARY

The freshwater turtle *Trachemys scripta* can survive in the complete absence of O_2 (anoxia) for periods lasting several months. In mammals, anoxia leads to mitochondrial dysfunction, which culminates in cellular necrosis and apoptosis. Despite the obvious clinical benefits of understanding anoxia tolerance, little is known about the effects of chronic oxygen deprivation on the function of turtle mitochondria. In this study, we compared mitochondrial function in hearts of *T. scripta* exposed to either normoxia or 2 weeks of complete anoxia at 5°C and during simulated acute anoxia/reoxygenation. Mitochondrial respiration, electron transport chain activities, enzyme activities, proton conductance and membrane potential were measured in permeabilised cardiac fibres and isolated mitochondria. Two weeks of anoxia exposure at 5°C resulted in an increase in lactate, and decreases in ATP, glycogen, pH and phosphocreatine in the heart. Mitochondrial proton conductance and membrane potential were similar between experimental groups, while aerobic capacity was dramatically reduced. The reduced aerobic capacity was the result of a severe downregulation of the F_1F_O-ATPase (Complex V), which we assessed as a decrease in enzyme activity. Furthermore, in stark contrast to mammalian paradigms, isolated turtle heart mitochondria endured 20 min of anoxia followed by reoxygenation without any impact on subsequent ADP-stimulated O_2 consumption (State III respiration) or State IV respiration. Results from this study demonstrate that turtle mitochondria remodel in response to chronic anoxia exposure and a reduction in Complex V activity is a fundamental component of mitochondrial and cellular anoxia survival.

Key words: mitochondria, turtle, heart, proton conductance, anoxia, hypoxia, electron transport chain, oxidative phosphorylation.

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INTRODUCTION

Mitochondria are increasingly being recognised as the lynchpin in the progression of anoxia-/hypoxia-related cellular death, whereby O_2 deprivation leads to a chain of events within mitochondria, beginning with Ca^{2+} overload and reactive O_2 species (ROS) generation and culminating in cellular apoptosis and necrosis (Chen et al., 2007). Furthermore, O_2 limitations can transform a mitochondrion from an ATP producer to the dominant ATP consumer in the cell (St-Pierre et al., 2000a) as the F_1F_O-ATP synthase works in reverse, consuming ATP in an attempt to maintain membrane potential. As a result, ischemia-related research has focused its attention on the mitochondria and shown that pharmacological inhibition of electron transport chain proteins during periods of O_2 deprivation promote anoxic cell survival (Lesnefsky et al., 2004; Chen et al., 2006; Chen et al., 2007; Burwell et al., 2009). Interestingly, reduced mitochondrial oxidative capacity is associated with ischemic preconditioning (Nadtochiy et al., 2006) and the so-called 'hibernating myocardium' (Kelly et al., 2008), further supporting the critical role the mitochondria play in hypoxia-/anoxia-related disease in humans.

The majority of research into O_2-related mitochondrial dysfunction has focused on hypoxia-sensitive mammals as surrogates for the study of pathological conditions in humans. Largely ignored are the small numbers of air-breathing vertebrates that have evolved a natural tolerance to O_2 deprivation, which may provide valuable information about mitochondrial adaptations to hypoxic and anoxic conditions. Among the champions of anoxia-tolerant vertebrates are the freshwater turtles from the genera *Chrysemys* and *Trachemys*, which are capable of surviving for up to 5 months in complete anoxia at cold temperatures (Ultsch, 2006). An important component of this impressive anoxia tolerance is the turtles' ability to progressively suppress whole-animal metabolic rate to 10–20% of resting, aerobic values (Jackson, 1968; Herbert and Jackson, 1985) while also possessing impressive hepatic glycogen stores (Daw et al., 1967) and a unique strategy to buffer the metabolic protons produced from the heavy reliance on anaerobic metabolism for ATP production (Jackson, 2000). At the level of the mitochondria, anoxia exposure in *Trachemys scripta* has been shown to decrease protein synthesis in the heart (Bailey and Driedzic, 1996) while increasing the expression of cytochrome *c* oxidase (COX) and NADH-ubiquinone oxidoreductase genes (Cai and Storey, 1996; Storey, 2007). Functional studies on the brain of *T. scripta* have shown that the activation of mitochondrial K_{ATP} channels during anoxia limits Ca^{2+} entry into the mitochondria and decreases *N*-methyl-D-aspartate receptor (NMDAR) activity (Pamenter et al., 2008). While many studies have identified anoxia-induced molecular and biochemical modifications to turtle mitochondria, mitochondrial respiration in *Chrysemys picta* appears to be unaffected by chronic anoxia exposure (Birkedal and Gesser, 2004); however, a detailed analysis of the components of mitochondrial respiration in *T. scripta* has not yet been performed.

Mitochondrial function has been well studied in the European water frog, *Rana temporaria*, a hypoxia-tolerant frog that

overwinters under ice-covered ponds for periods of up to 9 months (Tattersall and Ultsch, 2008). Hypoxic survival in these frogs is associated with a severe depression in COX (mitochondrial Complex IV) and citrate synthase, which contribute to hypoxia-induced reductions in State III and State IV respiration (St-Pierre and Boutilier, 2001). In the same species, skeletal mitochondria subjected to anoxia exhibited a profound reduction in F_1F_O-ATPase synthase (Complex V) activity, which prevented this protein from running in reverse during anoxia and hydrolysing ATP (St-Pierre et al., 2000a). An understanding of how mitochondria from hypoxia- and anoxia-tolerant animals are modified in response to O_2 deprivations could illuminate potentially new and novel interventions to mitigate ischemic-related mitochondrial dysfunction in humans.

The objective of this study was to determine how mitochondria from the heart of the anoxia-tolerant vertebrate *T. scripta* are modified in response to long-term anoxia exposure. Specifically, we aimed to determine whether chronic anoxia exposure resulted in an inhibition of electron transport chain capacity, and if so, to determine what electron transport chain proteins or processes are modified. We chose to focus on mitochondria from the heart, because the heart must maintain function and circulation during long-term anoxia exposure to ensure substrate delivery and waste removal.

MATERIALS AND METHODS

Animals

Twenty-six red-eared slider turtles, *Trachemys scripta scripta* (Schoepff 1972) (425.2±41.6 g), were obtained from Niles Biological (Sacramento, CA USA) and transported by airfreight to the University of British Columbia. Turtles were allowed to recover from transport for at least 4 weeks and then were randomly divided into two groups: normoxic (N=13) and anoxic exposures (N=13). Normoxic turtles were housed in aquaria with shallow water (4 cm) in a temperature-controlled room (set to 5°C) for 4–5 weeks before experimentation. Anoxic turtles were acclimated to 5°C (as described above) for 4–5 weeks and then each individual animal was enclosed in a plastic chamber filled with water, which was then submerged in a large water-filled aquaria and sealed with a glass lid. Water within each chamber, and within the aquaria, was continuously bubbled with N_2. As a further precaution, access to the upper layer of water within each individual chamber was avoided by suspending a mesh below the surface of the chamber lid. The partial pressure of O_2 in water ($P_{W_{O_2}}$) was monitored continuously with a handheld O_2 meter (Oxyguard, Birkeroed, Denmark), and $P_{W_{O_2}}$ was never observed to exceed 0.3 kPa. All temperature and anoxia exposures occurred during winter months and turtles were fasted during the exposure period. Normoxic turtles were held at 5°C for an additional 2 weeks before experimentation, during which time they were fasted. All turtles were held under a 12 h:12 h light:dark photoperiod. All anoxia-exposed animals were found to be in a comatose state and unresponsive to tactile stimulation. All experimental protocols involving animals were approved by the University of British Columbia Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care.

Isolation of permeabilised cardiac fibres and mitochondria

In order to fully characterise the effects of chronic anoxia on mitochondrial function, we chose to work with both permeabilised cardiac fibres and isolated mitochondria. Permeabilised fibres were used to assess respiratory flux through the electron transport chain because these preparations leave the mitochondria intact within the cell cytoskeleton and they are believed to be the most physiologically relevant preparation. Isolated mitochondria were used for the determination of membrane potential, which cannot be performed on permeabilised fibres.

Protocols for permeabilising cardiac muscle and isolating mitochondria have been exhaustively reviewed (Kuznetsov et al., 2008). In brief, animals were decapitated and the heart was excised. A sample of ventricular tissue from each experimental group was frozen in liquid N_2 for metabolite analysis (see below) and stored at −80°C. The remaining ventricular tissue was placed into ice-cold skinning solution [in mmol l^{-1}: Ca-K_2-EGTA 2.8 (free concentration of Ca^{2+} 0.1 μmol l^{-1}), K_2-EGTA 7.2, Na^+ ATP 5.8, $MgCl_2$ 1.4, imidazole 20, taurine 20, potassium 2-(*N*-morpholino)ethanesulfonic acid (K-MES) 50, Na^+ phosphocreatine 15 mmol l^{-1}, dithiothreitol (DTT) 0.5, pH 7.1, adjusted with 5 mol l^{-1} KOH], and muscle pieces (2 mm in length) were cut from the spongy myocardium and gently separated into small cell bundles. Bundles were incubated for 30 min on an orbital shaker set at 30 r.p.m. in skinning solution containing 50 mg ml^{-1} saponin. The saponin concentration and the incubation time were chosen based on previous experiments on turtles (Galli and Richards, 2012). After permeabilisation, the fibre bundles were washed in respiration medium (in mmol l^{-1}: EGTA 0.5, $MgCl_2$ 1.4, taurine 20, KH_2PO_4 10, Hepes 20, BSA 1%, K-MES 60, sucrose 110, pH 7.1, adjusted with 5 mol l^{-1} KOH) for 10 min to remove all adenine nucleotides and phosphocreatine (PCr). This latter procedure was repeated twice and the bundles were then left in fresh respiration medium until use. All these preparative procedures were carried out at 4°C.

Mitochondria were isolated as described previously (Almeida-Val et al., 1994). The heart was dissected free of connective tissue and rinsed with ice-cold homogenisation buffer (250 mmol l^{-1} sucrose, 10 mmol l^{-1} Hepes, 1 mmol l^{-1} EGTA, 1% fatty-acid-free BSA, pH 7.4 at 4°C) and then minced into small pieces with scissors on ice in buffer. The tissue was then partially digested with 5 ml of trypsin (Type IV, Sigma-Aldrich, St Louis, MO, USA) for 5 min, resuspended in trypsin inhibitor (Type I-S, Sigma-Aldrich), left to settle, and then resuspended in 12 ml of fresh ice-cold buffer. The tissue solution was homogenised using three passes of a loose-fitting Teflon pestle at 100 rpm in a 12 ml glass mortar. The homogenate was centrifuged at 600 ***g*** for 10 min at 4°C in polycarbonate centrifuge tubes. The supernatant was then removed, filtered through cheesecloth and centrifuged again at 9000 ***g*** for 10 min at 4°C. The supernatant was removed and discarded, and the resulting pellet was washed with fresh buffer to remove the light 'fluffy' layer of the pellet (considered to be damaged mitochondria). The dark pellet was then resuspended in 12 ml of buffer and centrifuged again at 9000 ***g*** for 10 min at 4°C. Finally, the pellet was resuspended in 200 μl of fresh buffer and immediately analysed for protein content using the Bradford technique (Bradford, 1976) (Sigma-Aldrich). The mitochondrial suspension was kept on ice until assayed.

Respiration of mitochondria and cardiac fibres

Permeabilised cardiac fibre and mitochondrial respiration was measured with an Oroboros Oxygraph 2-k high-resolution respirometry system (Oroboros Instruments, Innsbruck, Austria). O_2 electrodes were calibrated daily with air-saturated respiration solution (in mmol l^{-1}: EGTA 0.5, $MgCl_2$ 1.4, taurine 20, KH_2PO_4 10, Hepes 20, BSA 1%, K-MES 60, sucrose 110, pH 7.1, adjusted with 5 mol l^{-1} KOH). Zero calibrations were achieved by injecting yeast into the experimental chambers. O_2 solubility in the assay medium was calculated as described previously (Gnaiger, 1983). Two identical respiration chambers (chamber A and chamber B) held at the same temperature were run in parallel for each

experimental run. Fibre bundles (wet mass 6–8 mg) or isolated mitochondria (0.3 mg protein ml^{-1}) were added to each chamber containing 2 ml of respiration medium. As respiration rates at 5°C in *T. scripta* permeabilised cardiac fibres can be difficult to detect (Galli and Richards, 2012) and lead to long experimental protocols and deterioration of preparations, all measurements of respiration rates were carried out at 13°C to help maximise our ability to detect treatment effects (normoxia *versus* anoxia) and complete protocols in a reasonable time frame. Preliminary analysis showed that changes in respiration rate with assay temperature are governed by a Q_{10} of 2 to 3 and differences between normoxic and anoxic exposure groups were similar when measured at 5, 13 or 21°C (data not shown). Thus, changes in temperature had no impact on the interpretation of our treatment effects.

Protocol for permeabilised fibre respiration

Respiratory flux through Complexes I, II and IV of the electron transport chain was measured in permeabilised fibres. A representative trace depicting the protocol, which was adapted from Kuznetsov et al. (Kuznetsov et al., 2008), can be seen in Fig. 1A. Pyruvate (5 mmol l^{-1}) and malate (0.25 mmol l^{-1}) were used as a carbon substrate and to spark the citric acid cycle, respectively. State III respiration was achieved through addition of saturating levels of ADP (1 mmol l^{-1}). Following ADP phosphorylation, rotenone (0.5 µmol l^{-1}) was added to inhibit Complex I, and succinate (10 mmol l^{-1}) was used to assess respiratory flux through Complex II. Antimycin A (5 µmol l^{-1}) was then added to block Complex II and flux through Complex IV was assessed by adding the electron donor tetramethyl-*p*-phenylene-diamine (TMPD, 0.5 mmol l^{-1}) and ascorbate (0.5 mmol l^{-1}). To avoid oxidation of TMPD, ascorbate was added prior to TMPD injection. Finally, to assess the functional integrity of the inner mitochondrial membrane, cytochrome *c* (10 µmol l^{-1}) was added to the chamber. At the end of experimentation, the respiration medium was removed from the respiration chamber and the fibres were retrieved and subsequently dried at 65°C for 1 h 30 min. Respiration rates are normalised to dry mass of the fibres.

Protocols for mitochondrial membrane potential and proton conductance measurements

The two respiration chambers containing the isolated mitochondria were randomly assigned to one of the following protocols.

Protocol 1: measurement of proton conductance

The kinetics of proton conductance was assessed by simultaneous measurements of O_2 consumption (described above) and proton-motive force, as previously described (Brown et al., 2007). Mitochondrial membrane potential was measured as an approximation of proton-motive force. Membrane potential was measured using the lipophilic cation tetraphenylphosphonium (TPP^+), whose uptake by mitochondria is membrane potential dependent. A TPP-selective electrode (Oroboros Instruments) was inserted into the respiratory chamber to measure external TPP concentration ([TPP^+]). Before addition of mitochondria, rotenone (2 µg ml^{-1}, dissolved in ethanol) and oligomycin (10 µg ml^{-1}, dissolved in ethanol) were added to the respiration chamber to inhibit Complex I and Complex V, respectively. The TPP electrode was then calibrated before each experiment by measuring the TPP-electrode output (in V) during three consecutive injections of 1 µmol l^{-1} TPP into the chamber, to make a final external [TPP^+] of 3 µmol l^{-1}. Once calibrated, mitochondria were injected into the chamber to a final concentration of 0.3 mg protein ml^{-1} at 13°C. Succinate (6 mmol l^{-1}) was then added to stimulate State II respiration, which is an approximation of State IV respiration. The kinetics of proton conductance was determined by inhibiting the substrate oxidation component stepwise by adding 0.5 mmol l^{-1} malonate (9–12 additions) and measuring the effect on membrane potential. After the final malonate addition, carbonyl cyanide-*p*-trifluoro-methoxyphenylhydrazone (FCCP; 1 µmol l^{-1}, dissolved in ethanol) was added to completely uncouple the mitochondria and

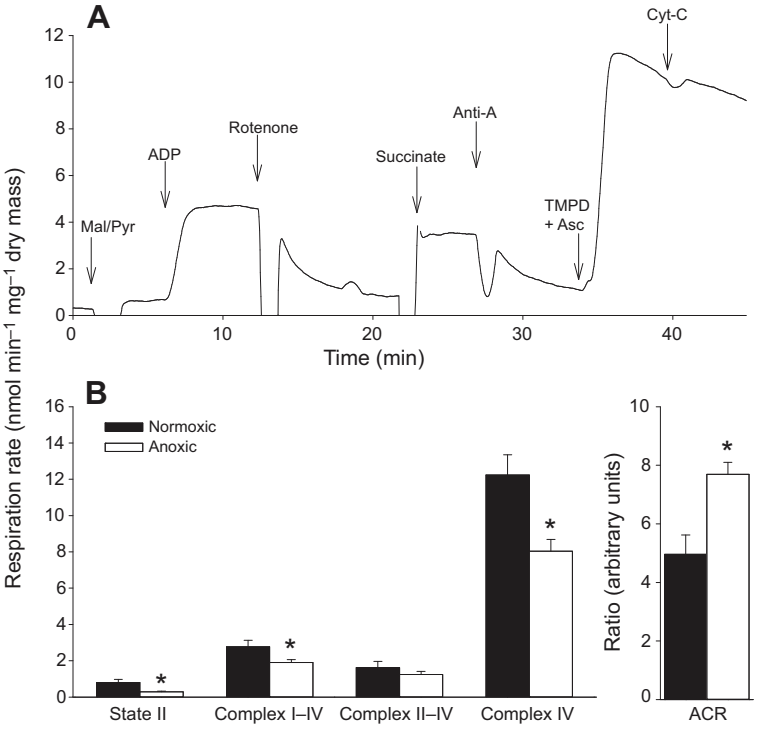

Fig. 1. The effect of chronic anoxia on respiratory parameters. Cardiac fibres were isolated from normoxia- (black bars; *N*=6) and anoxia-exposed (white bars; *N*=6) turtles held at 5°C. (A) Representative trace from a normoxia-exposed turtle showing experimental protocol (see Materials and methods for details). (B) Mean ± s.e.m. respiration rates under State II, non-phosphorylation conditions, as well as maximum respiration rates when supplied with substrates for Complex I (pyruvate and malate in the presence of saturating levels of ADP; Complexes I–IV), Complex II (succinate in the presence of saturating levels of ADP; Complexes II–IV) and Complex IV (TMPD in the presence of saturating levels of ADP; Complex IV). Acceptor control ratios (ACRs) were calculated as $(v_0+V_m)/v_0$, where V_m is respiration rate at saturating ADP concentrations (State III) and v_0 is the basal respiration rate of mitochondria before addition of ADP (State II). The responses depicted in the representative normoxic trace in A are very similar to the responses seen in the anoxic traces, except with lower overall respiration rates. Asterisks indicate a significant difference between normoxia- and anoxia-exposed animals (P<0.05, *t*-test).

determine the amount of electrode drift throughout the experiment. Membrane potential was calculated from the external [TPP^+] using a modified Nernst equation (Labajova et al., 2006):

$$\Delta\psi_m = a\log\left(\frac{([TTP^+]_{added} - [TPP^+]_{external})\times b}{v\times \text{mg protein}\times [TPP^+]_{external}}\right), \quad (1)$$

where a is a temperature-dependent coefficient (a=2.3RT/F, where R is the universal gas constant, T is absolute temperature and F is the Faraday constant), b is a binding constant used to correct for nonspecific binding of TPP, and v is the mitochondrial matrix volume.

The value of b was taken from Marcinkeviciute et al. (Marcinkeviciute et al., 2000), who calculated the constant in rat liver mitochondria incubated at 30°C. While other lipophilic cations are known to be temperature sensitive (Scaduto and Grotyohann, 1999), all of our membrane potential measurements were carried out at 13°C, so any differences observed between groups cannot be attributed to differential binding. The value of v was estimated at 0.001 ml mg^{-1} protein from values obtained from rat heart mitochondria (Das et al., 2003). This value has not been determined for turtle heart mitochondria and it is not known whether mitochondrial volume changes with anoxia, but the effect of changes in mitochondrial volume are negligible when TPP is used (Rottenberg, 1984).

Protocol 2: the effects of simulated acute anoxia on mitochondrial respiration and membrane potential

O_2 consumption and membrane potential were measured simultaneously while monitoring the effects of ADP-stimulated respiration in the presence and absence of O_2. Mitochondria were injected into the respiratory chamber with pyruvate (5 mmol l^{-1}) and malate (0.25 mmol l^{-1}) as substrates. ADP was injected to stimulate maximal respiration rate (250 μmol l^{-1}). Mitochondria were then left to enter State IV conditions (respiration following complete ADP phosphorylation) to consume all remaining O_2, thereby entering into an anoxic state. The Oxygraph system is a tight system that does not permit ambient air to readily leak into the chamber. However, there is a minute back-diffusion of O_2 from the polarographic sensors into the chamber at low [O_2] that is accounted for with background correction. Anoxic conditions were continued for 20 min, and then O_2 was reintroduced to the chambers to simulate anoxia/reoxygenation. As it was not possible to open the respiratory chambers while the TPP electrode was inserted, reoxygenation was achieved *via* injection of H_2O_2 and catalase. In preliminary experiments, this method was compared with simply opening the chamber to reoxygenate, and no differences between methods were observed in the mitochondrial response to anoxia/reoxygenation. To assess the effects of acute anoxia on ADP-stimulated respiration, mitochondria, which entered the anoxic condition in State IV, were given a second injection of ADP following reoxygenation. At the end of all experiments, oligomycin (2 μmol l^{-1}) was injected to block Complex V, followed by FCCP (1 μmol l^{-1}) to fully uncouple the mitochondria and assess TPP electrode drift.

Metabolite and enzyme analytical protocols

Tissue and mitochondria were extracted and assayed as previously described (Hassid and Abraham, 1957; Bergmeyer, 1983; Pörtner et al., 1990). Tissue was broken into small pieces under liquid N_2 with an insulated mortar and pestle and aliquots were transferred into 1.5 ml tubes and stored at −80°C. An aliquot of tissue was further ground under liquid N_2 into a fine power and used for the determination of pH_i with the methods of Pörtner et al. (Pörtner et al., 1990) and a thermostatted (21°C) Radiometer BMS3 Mk2 capillary microelectrode with a PHM84 pH meter (Radiometer, Copenhagen, Denmark). For the extraction of metabolites, 0.5–1 ml of ice-cold 1 mol l^{-1} $HClO_4$ was added to a microcentrifuge tube containing 50 to 100 mg of frozen tissue and the mixture was immediately homogenised on ice with a Polytron homogeniser (Kinematica, Bohemia, NY, USA) for 20 s followed by sonication for a further 20 s with a Kontes sonicator (Vineland, NJ, USA) set to its highest setting. A 200 μl aliquot of homogenate was then taken and frozen at −80°C for glycogen content measurement. The remaining homogenate was centrifuged at 10,000 ***g*** for 10 min at 4°C, and the supernatant was neutralised with 3 mol l^{-1} K_2CO_3. The neutralised extracts were assayed spectrophotometrically for ATP, CrP and lactate according to methods described by Bergmeyer (Bergmeyer, 1983). Total creatine (Cr) was measured on tissue extracts (Bergmeyer, 1983) after treatment with 1 mol l^{-1} KOH and found to be 7 μmol g^{-1} wet mass. Samples for glycogen analysis were thawed on ice, partially neutralised with 3 mol l^{-1} K_2CO_3, digested with amyloglucosidase, and measured for glucose as previously described (Hassid, 1957).

The activities of electron transport Complexes I, II, IV and V were measured in frozen ventricle. Briefly, ~80–100 mg of frozen tissue was weighted into a 2 ml tube and sonicated with three 10 s bursts in five volumes of ice-cold hypotonic medium (25 mmol l^{-1} K_2HPO_4, 5 mmol l^{-1} $MgCl_2$, pH 7.2). The resulting homogenate was then centrifuged at 600 ***g*** for 10 min and the supernatant was retained and centrifuged again at 600 *g* for 10 min. The supernatant from each sample was immediately frozen at −80°C and all four complexes were assayed on the first thaw. The maximal activity (V_{max}) was determined spectrophotometrically at 25°C with a VersaMax spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) in assay buffer (25 mmol l^{-1} K_2HPO_4, 5 mmol l^{-1} $MgCl_2$, 100 mmol l^{-1} KCl and 2.5 mg ml^{-1} BSA, pH 7.2). Rotenone-sensitive Complex I activity was monitored as a reduction of 5,5′-dithiobis(2-nitrobenzoic acid) (DCIP) at 600 nm over 5 min (assay buffer with 100 μmol l^{-1} DCIP, 0.2 mmol l^{-1} NADH, 65 μmol l^{-1} ubiquinone 2, 2 μg ml^{-1} antimycin A) in the absence or presence of 2 μg ml^{-1} rotenone. Complex II was also monitored as the reduction of DCIP over 5 min (assay buffer with 100 μmol l^{-1} DCIP, 20 mmol l^{-1} succinate, 65 μmol l^{-1} ubiquinone 2, 2 μg ml^{-1} antimycin A, and 2 μg ml^{-1} rotenone). For Complex IV activity, oxidation of reduced cytochrome *c* was monitored at 550 nm for 3 min (assay buffer with 0.6 mmol l^{-1} lauryl maltoside and 50 μmol l^{-1} reduced cytochrome *c*). Reduced cytochrome *c* was obtained by dialysing oxidised cytochrome *c* with ascorbate and 50 mmol l^{-1} Tris (pH 8.0). Oligomycin-sensitive Complex V activity was measured as oxidation of NADH (340 nm) for 5 min (5 mmol l^{-1} ATP, 2 mmol l^{-1} PEP, 0.2 mmol l^{-1} NADH, 3 U ml^{-1} lactate dehydrogenase and 3 U ml^{-1} pyruvate kinase) in the absence or presence of 0.5 μg ml^{-1} oligomycin. Extinction coefficients of DCIP, NADH and reduced cytochrome *c* were empirically determined to quantify V_{max} values for each enzyme complex. Enzyme activities were normalised to total soluble protein, which was quantified according to Bradford (Bradford, 1976).

Calculations and statistics

All reported respiration rates for permeabilised fibres and mitochondria are normalised to milligram of dry mass and protein, respectively. Acceptor control ratios (ACRs) were calculated as $(v_0+V_m)/v_0$, where V_m is the respiration rate at saturating ADP concentrations (State III), and v_0 is the basal respiration rate of mitochondria before addition of ADP (State II). Respiratory control

ratios (RCRs) in isolated mitochondria were calculated as the ratio between State III and State IV respiration rates. The ratio of ADP phosphorylated to atoms of O_2 consumed (ADP/O ratio) was calculated by making linear extrapolations of O_2 concentration under State III conditions after ADP titration, and at State IV after ADP depletion. The difference in O_2 concentrations at the intercepts is the total O_2 uptake (Gnaiger et al., 2000). To assess the rate of depolarisation or hyperpolarisation during anoxia and reoxygenation, respectively, mitochondrial membrane potential data were fit to a two-parameter exponential growth curve and differences between these curves were analysed using a one-way repeated-measures ANOVA.

Free cytosolic [ADP] ([ADP_{free}]) and [AMP] ([AMP_{free}]) were calculated from measured [ATP], [PCr] and intracellular pH (pH_i) values assuming equilibrium of the creatine kinase and adenylate kinase reactions, respectively, according to published protocols (Golding et al., 1995; Teague et al., 1996). Free [Cr] was estimated by subtracting [PCr] from total [Cr], which was determined on a separate group of turtles and was 7 μmol g^{-1} wet mass. The equilibrium constants for creatine kinase (K'_{CK}) and adenylate kinase (K'_{AK}) were corrected for experimental temperature, ionic strength, measured pH and free Mg^{2+} [assumed to be 1 mmol l^{-1} (Van Waarde et al., 1990)]. For our calculations, tissue [ATP], [PCr] and [free Cr] were converted from μmol g^{-1} wet mass to molar concentrations using a tissue water content of 0.6 ml g^{-1} wet mass (Farrell and Milligan, 1986), but final values for [ADP_{free}] and [AMP_{free}] are reported in μmol g^{-1} wet mass. Gibbs free energy of ATP hydrolysis ($\Delta fG'_{ATP}$) was estimated from pH_i, ATP, PCr and phosphate (P_i) concentrations as described earlier (Pörtner et al., 1996; Stecyk et al., 2009). Tissue P_i concentrations were taken directly from Stecyk et al. (Stecyk et al., 2009), who exposed *T. scripta* to a similar anoxic treatment. $\Delta fG'_{ATP}$ values are expressed as kJ mol^{-1} (more negative values indicate greater free energy available from the hydrolysis of ATP).

Statistical significance between O_2 exposure groups was assessed with a one- or two-factor repeated-measures ANOVA, as appropriate, and Student–Newman–Keuls *post hoc* tests. Statistical tests were performed using SigmaStat software (version 4; Systat Software, San Jose, CA, USA), and all data are reported as means ± s.e.m.

RESULTS

Effect of chronic anoxic exposure

Whole-animal and metabolic responses to anoxia

During 2 weeks of anoxia exposure at 5°C, the turtles were comatose with little or no signs of movement compared with the more active normoxic turtles. Upon dissection, hearts from both normoxic and anoxic turtles were spontaneously beating, although the anoxic group had a greatly reduced heart rate. Anoxic exposure had a dramatic effect on heart metabolism, with a 20-fold increase in [lactate], a 30-fold decrease in [glycogen], a decrease in pH and an 11-fold decrease in PCr (Table 1). Heart [ATP] from anoxic turtles decreased to 15% of normoxic values, while [ADP_{free}] and [AMP_{free}] remained unaffected by anoxia exposure, resulting in a decrease in heart [ATP]/[ADP_{free}] and $\Delta fG'_{ATP}$ (Table 1).

The effect of anoxia on cardiac fibre respiration

Fibres were in good condition throughout the protocol, as shown by the minimal effect of cytochrome *c* injection on the percentage change in respiration rate (normoxia exposed, 2.58±1.10%; anoxia exposed, 0.72±0.42%; no significant difference between experimental groups). ACRs of permeabilised cardiac fibres from *T. scripta* ranged between 4 and 8 (Fig. 1B), which is marginally higher than values previously reported for another species of freshwater turtle, *C. picta* (Birkedal and Gesser, 2004), which indicates that the fibres were in good condition. Two weeks of anoxia exposure led to a significant reduction in respiration rate in cardiac fibres supplied with substrates for Complex I (pyruvate + malate, State III respiration) and Complex IV (TMPD) (Fig. 1B). Similarly, State II respiration (with malate and pyruvate as substrates, but not ADP) was lower and the ACRs were higher in anoxia-exposed compared with normoxia-exposed turtles (Fig. 1B). There were no significant differences in respiration rates between fibres from normoxic and anoxic turtles when mitochondria were supplied with substrate for Complex II (succinate-stimulated respiration; Fig. 1B).

Citrate synthase and electron transport chain Complex activities

There were no significant differences in citrate synthase activity between normoxia- and anoxia-exposed turtle hearts (Fig. 2). Anoxia exposure had no effect on the activities of Complexes I, II and IV in isolated heart mitochondria; however, anoxia exposure led to a large reduction in Complex V activity compared with normoxia-exposed animals (Fig. 2).

Proton conductance

In order to determine whether proton conductance was affected by 2 weeks of anoxia exposure, we isolated mitochondria and measured the effects of changes in membrane potential on rotenone- and oligomycin-inhibited State IV respiration (Fig. 3). When proton conductance data were fit with two-parameter exponential growth curves, no significant differences were found (P=0.726) in the shape

Table 1. Metabolites from *Trachemys scripta* heart exposed to normoxia and 2 weeks of anoxia

	Normoxia exposed (N=6)	Anoxia exposed (N=11)
Lactate (nmol g^{-1} WM)	0.06±0.06[a]	20.60±1.10[b]
Glycogen (μmol glycosyl units g^{-1} WM)	254.4±24.0[a]	8.5±4.5[b]
ATP (μmol g^{-1} WM)	2.7±0.1[a]	0.4±0.1[b]
PCr (μmol g^{-1} WM)	3.3±0.9[a]	0.3±0.1[b]
pH	7.24±0.02[a]	6.97±0.02[b]
ADP_{free} (nmol g^{-1} WM)	38.7±14.1[a]	38.6±9.8[a]
AMP_{free} (μmol g^{-1} WM)	1.1±0.7[a]	6.8±3.1[a]
[ATP]/[ADP_{free}]	114.1±33.6[a]	15.4±3.8[b]
$\Delta fG'_{ATP}$ (kJ mol^{-1})	–57.9±1.7[a]	–47.0±1.1[b]

PCr, phosphocreatine; $\Delta fG'_{ATP}$, Gibbs free energy of ATP hydrolysis; WM, wet mass of tissue.

Within a row, values that share the same superscript letter are not significantly different (P>0.05). For the calculation of ADP_{free}, AMP_{free} and $\Delta fG'_{ATP}$, free creatine was calculated by subtracting PCr from total creatine (estimated to be 7 μmol g^{-1} WM) Metabolites were measured spectrophotometrically at room temperature.

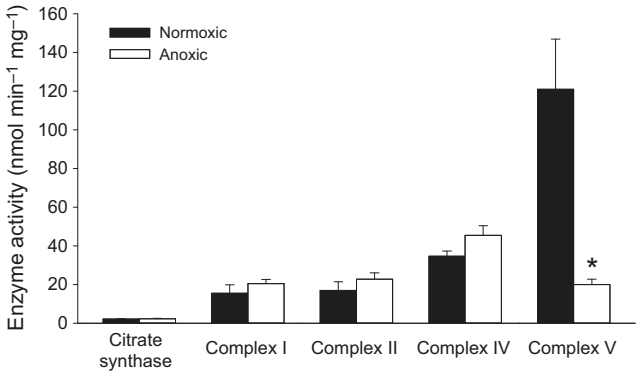

Fig. 2. The effect of chronic anoxia on the activity of citrate synthase and Complexes I, II, IV and V in normoxia- (black bars; *N*=6) and anoxia-exposed (white bars; *N*=6) turtle heart mitochondria. Data are means ± s.e.m. Asterisks indicate a significant difference between normoxia- and anoxia-exposed animals (*P*<0.05).

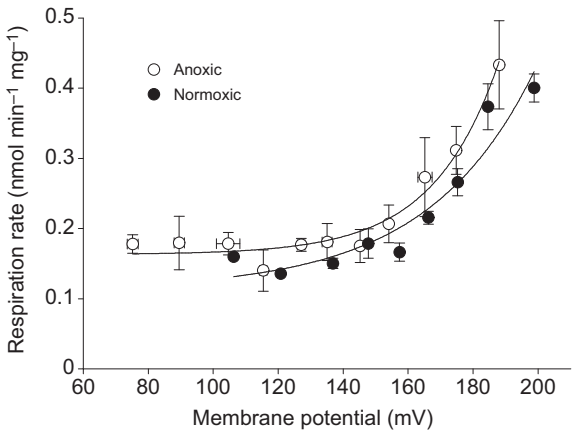

Fig. 3. The effect of chronic anoxia on the kinetics of proton conductance. Normoxia- (black circles, *N*=5) and anoxia-exposed (white circles, *N*=5) turtle heart mitochondria were subjected to stepwise changes in respiration rate and membrane potential by injecting increasing amounts of malonate (1 mmol l^{-1} steps with a final concentration of 16 mmol l^{-1}). Values are means ± s.e.m. See Results for statistical analysis.

of the normoxic (b=0.019±0.003, R^2=0.85) or anoxic curves (b=0.018±0.002, R^2=0.80).

Effect of acute anoxia exposure and reoxygenation

In order to determine whether the lower Complex V activity observed in the anoxic turtles' hearts helps to protect the mitochondria, we measured respiration rates and membrane potential simultaneously in isolated mitochondria before and after an acute (20 min) anoxia/reoxygenation protocol (Fig. 4). Isolated mitochondria from both groups of turtles were in good condition, as attested by high RCR values (normoxia exposed, 7.0±1.1; anoxia exposed, 9.9±1.1), which are greater than those previously reported for another species of freshwater turtle, *C. picta* (Almeida-Val et al., 1994).

Respiration rates and membrane potential prior to acute anoxia

State II respiration rates (with malate and pyruvate as substrates, but no added ADP) were not different between mitochondria isolated from normoxia-exposed turtles and those isolated from anoxia-exposed turtles (Fig. 5A). In agreement with the cardiac fibre data (Fig. 1), State III and IV respiration rates in isolated mitochondria respiring on malate and pyruvate were higher in normoxia-exposed turtles than in anoxia-exposed turtles (Fig. 5A). ADP/O ratios under these conditions were significantly greater in mitochondria from normoxia-exposed (2.5±0.32, N=5) compared with anoxia-exposed turtles (1.74±0.08, N=6; P<0.05). In both experimental groups, membrane potential depolarised in response to ADP addition (250 μmol l^{-1}) and returned to pre-ADP levels once mitochondria entered State IV conditions (Fig. 5B).

Respiration rates and membrane potential during anoxia and reoxygenation

During 20 min of acute anoxia, respiration rates could not be measured and membrane potential depolarised (Figs 5, 6). There were no significant differences between the anoxia- and normoxia-exposed groups in the magnitude or the rate of depolarisation of membrane potential during anoxia (Fig. 5B, Fig. 6B). Following reoxygenation, respiration rate of mitochondria from the anoxia-exposed turtles returned to pre-anoxic levels and there were no significant differences between State IV respiration rates before or after the acute anoxia exposure (Fig. 5A, Fig. 6A). However, in mitochondria from the normoxia-exposed turtles, State IV respiration rates were dramatically increased and membrane potential remained depolarised immediately upon reoxygenation (Figs 5, 6). While this effect was transient and diminished after 6–7 min, State IV respiration rates in the normoxic mitochondria following acute anoxia exposure stabilised at values that were significantly higher than the pre-anoxic respiration rates (Fig. 5A, Fig. 6A).

Respiration rates after anoxia/reoxygenation

A second application of ADP after anoxia/reoxygenation caused an increase in respiration rate and a depolarisation in membrane potential in both exposure groups that was not significantly different to values from pre-anoxic ADP stimulation (Fig. 5). The addition of 2 μmol l^{-1} oligomycin, to block Complex V, dramatically reduced mitochondrial respiration rates compared with State III conditions and eliminated the difference in State IV respiration rates between mitochondria from anoxia- and normoxia-exposed turtles (Fig. 5A). Throughout the protocol, no significant differences were found between membrane potential in normoxia- and anoxia-exposed animals (Fig. 5B, Fig. 6B).

DISCUSSION

Freshwater turtles are well known for their remarkable ability to survive months of anoxia exposure at cold temperatures (Ultsch, 2006). A critical component of this impressive anoxia tolerance is the continued function of the heart (Herbert and Jackson, 1985). However, contraction of the anoxic turtle heart is supported entirely by ATP derived from substrate-level phosphorylation (glycolysis and PCr hydrolysis), as evidenced by a large decrease in ventricular glycogen and PCr, and accumulation of lactate and H^+ (Table 1). In order to match cellular energy demands to the reduced capacity for ATP production, cardiac power output is reduced. Indeed, during anoxia at 5°C, heart rate and cardiac power output of *T. scripta* decreases progressively over the first 3 days and stabilises at values that are 5 and 20% of normoxic values, respectively (Stecyk et al., 2004). The exact mechanisms behind the downregulation of cardiac function are still being elucidated (Stecyk et al., 2009), but anoxic-induced alterations in metabolites may be involved.

The dramatic decreases in cardiac [ATP]/[ADP_{free}] and $\Delta_f G'_{ATP}$ (Table 1) observed during anoxia exposure have been postulated

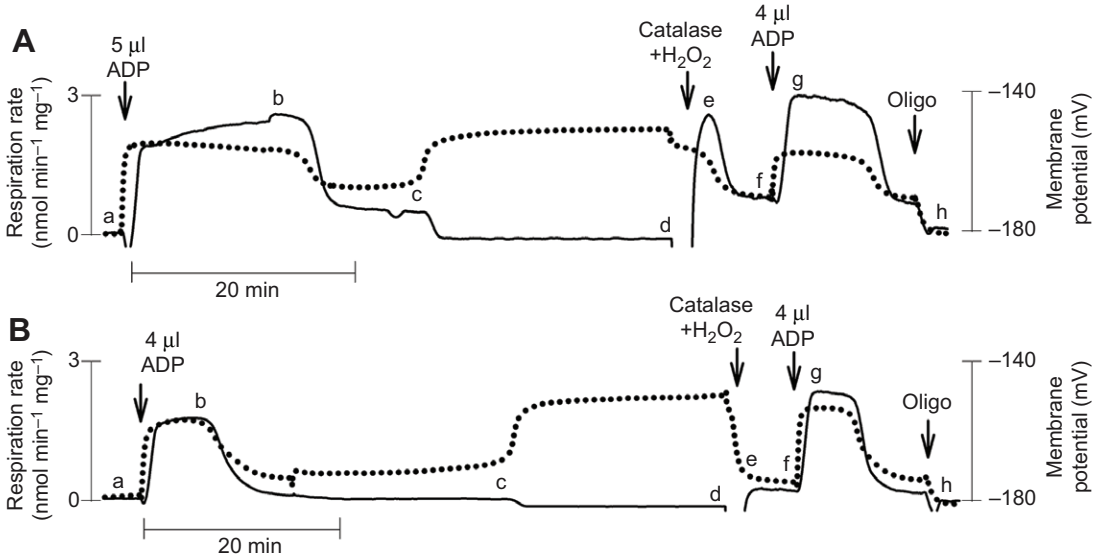

Fig. 4. Original traces from (A) a normoxia-exposed turtle and (B) an anoxia-exposed turtle of the effect of acute anoxia/reoxygenation on mitochondrial respiration (solid lines) and membrane potential (dotted lines). Letters indicate time points at which measurements were taken: (a) State II respiration rates with malate and pyruvate as substrates (no added ADP); (b) State III respiration rates with malate and pyruvate as substrates with saturating levels of ADP; (c) State IV respiration rates; (d) anoxia; (e) reoxygenation; (f) State IV respiration following anoxia/reoxygenation; (g) State III respiration following anoxia/reoxygenation; and (h) respiration rate following oligomycin injection. Averages for these time points are given in Fig. 5. Note different time scales for A and B.

to be contributing factors in the anoxic decline in heart rate and aortic blood flow (Stecyk et al., 2009). Elevated P_i, intracellular acidosis and a reduction in $\Delta fG'_{ATP}$ are known to depress cardiac function directly through a reduction in Ca^{2+} sensitivity, a diminished activation of actin-myosin cross-bridges, and a lowering of the phosphorylation potential (Bers, 2001). In this way, alterations in myocardial energetic status directly reduce cardiac energy demand, which extends the time the heart can continue to work during anoxia. Overall, in the case of cold (5°C)-acclimated turtles where autonomic control of the heart is inhibited (Stecyk et al., 2009), cardiac function is mainly controlled by alterations in cellular energy status.

The mitochondrion plays a central role in the regulation of cellular energy status (Benard et al., 2010) and disruption of mitochondrial

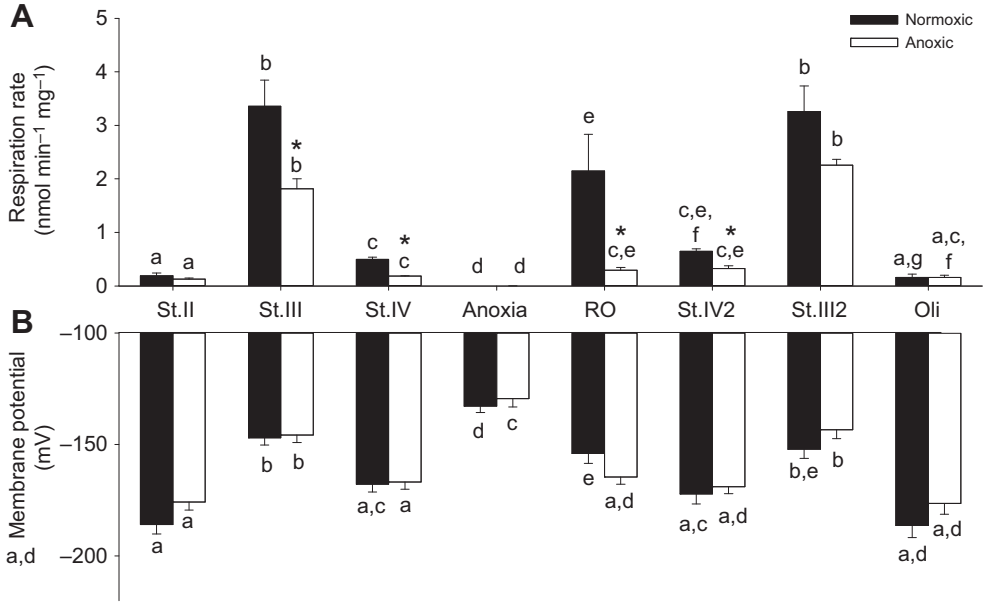

Fig. 5. Averages for the effect of acute anoxia/reoxygenation on mitochondrial respiration and membrane potential. (A) Respiration rate for normoxia- (black bars; *N*=6) and anoxia-exposed (white bars; *N*=6) turtle heart mitochondria. (B) Mitochondrial membrane potential. St.II, State II respiration rates with malate and pyruvate as substrates (no added ADP); St.III, State III respiration rates with malate and pyruvate as substrates with saturating levels of ADP; St.IV, State IV respiration rates; RO, reoxygenation; St.IV2, State IV respiration following anoxia/reoxygenation; St.III2, State III respiration following anoxia/reoxygenation; St.IV2, State IV respiration following anoxia/reoxygenation; Oli, respiration rate following oligomycin injection. Asterisks denote a significant difference between anoxia- and normoxia-exposed mitochondria. Within the same O_2 exposure group, values that share a common letter are not significantly different (*P*>0.05, *t*-test).

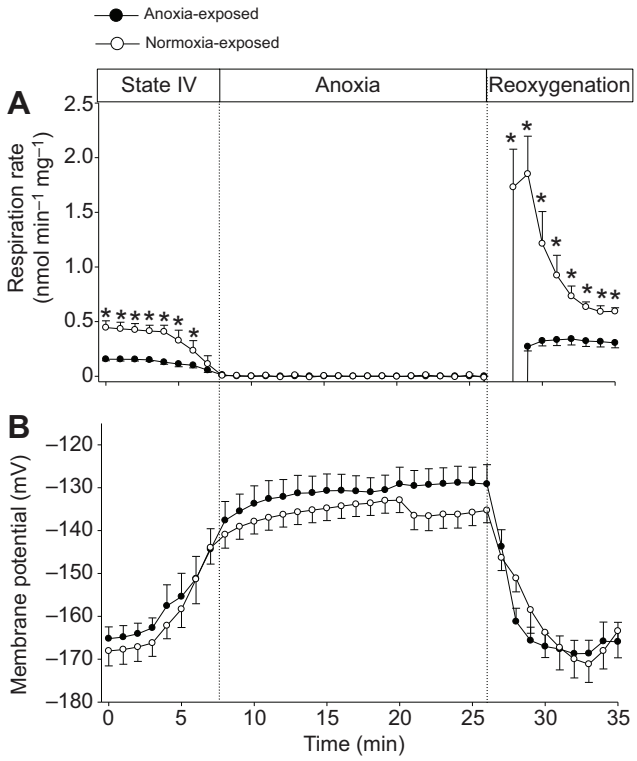

Fig. 6. Mitochondrial respiration rate and membrane potential during acute anoxia/reoxygenation. Respiration (top panel) rate and membrane potential (bottom panel) in normoxia- (black circles; *N*=6) and anoxia-exposed (white circles; *N*=6) turtle heart mitochondria during simulated acute anoxia/reoxygenation. Asterisks denote a significant difference between anoxia- and normoxia-exposed mitochondria (*P*>0.05, *t*-test).

function can lead to potentially devastating cellular effects. As a result, the turtle mitochondrion, which must endure months of complete anoxia every year, is not only inherently tolerant of anoxic conditions, but has also evolved the capacity to shut down oxidative phosphorylation through a reduction in Complex V activity (the F_1F_O-ATP synthase).

Complex V activity is reduced by long-term anoxia exposure in turtles

Long-term anoxia exposure in *T. scripta* led to a downregulation of respiratory capacity in the turtle heart as shown by the significant reductions in non-phosphorylating (State II) and phosphorylating (ADP-stimulated) respiration in permeabilised fibres when electrons were donated to Complexes I and IV (Fig. 1). These results obtained using permeabilised fibres were corroborated by analysis in isolated mitochondria, where both State III (with malate and pyruvate as substrates) and State IV respiration rates were significantly lower in mitochondria isolated from anoxic turtles compared with normoxic turtles (Figs 5, 6). These isolated mitochondrial data, along with the similar citrate synthase activities in both groups (Fig. 2), indicate that the changes in respiration seen in the permeabilised fibres is not due to anoxia-induced changes in mitochondrial volume density, but is rather due to a modification to the mitochondrion itself. Indeed, our enzymatic analysis points to a significant reduction in Complex V activity as the mechanism behind the anoxia-induced downregulation of respiratory flux. Similar decreases in Complex V activity have been documented in skeletal muscle of frogs exposed to anoxic conditions (St-Pierre and Boutilier, 2001) and reduced Complex V activity has been implicated in the cardioprotective effects of anoxic/ischemic preconditioning (Murphy and Steenbergen, 2011) and the so-called 'hibernating myocardium' (Page et al., 2008). The mechanisms behind this reduction in Complex V activity are not known, but in preconditioning models it has been proposed that Complex V is directly inhibited either through the binding of the inhibitory factor (Vander Heide et al., 1996) or through S-nitrosylation (Sun et al., 2007). These results are in contrast to the results observed in hypobaric hypoxia-exposed rats, where mitochondrial properties were not changed, but mitochondrial density increased (Costa et al., 1988); however, the present study used complete anoxia *versus* hypoxia, therefore it is not surprising that the results differ. Whatever the mechanism is for a reduction in Complex V activity, its beneficial effects on the anoxic cell are clear.

Reductions in Complex V activity prevent anoxia-induced ATP consumption

In mammals, O_2 limitations transform a mitochondrion from an ATP producer to the dominant ATP consumer in the cell (St-Pierre et al., 2000a). This occurs because anoxia prevents electron flow through the electron transport chain and the associated movement or consumption of protons from the mitochondrial matrix. Under these conditions, Complex V will run in reverse, consuming ATP to power the movement of protons out of the mitochondrial matrix in an attempt to maintain membrane potential (Rouslin, 1983; Jennings et al., 1991; Duchen, 1999; St-Pierre et al., 2000a). Dissipation of the mitochondrial membrane potential is known to initiate a cascade of events culminating in cellular apoptosis or necrosis (Gottlieb, 2011). The reverse proton pumping by Complex V under anoxic conditions however, only postpones the inevitable collapse of membrane potential because under these conditions the mitochondrion becomes the dominant ATP consumer in the cell and quickly exhausts the limited ATP reserves in anoxia.

The anoxia-induced reduction in Complex V activity in turtles prevents it from running in reverse, limiting anoxia-induced ATP consumption. This is evidenced by the fact that when State IV mitochondria were exposed to our acute anoxia trials (Fig. 4), there was a large increase in respiration rate immediately upon reoxygenation after the 20 min anoxia exposure in mitochondria isolated from normoxic turtles, which was absent in mitochondria isolated from anoxic turtles (see RO in Fig. 5A). This increase in respiration rate was transient, lasting 6–7 min, at which time respiration rates returned to similar State IV conditions as seen before the acute anoxia trials (see St.IV2 in Fig. 5A). These data indicate that during the 20 min acute anoxia period, mitochondria from normoxic turtles were consuming ATP at a faster rate than those from anoxic turtles, yielding high mitochondrial ADP levels and resulting in a stimulation of respiration, to values that approach State III conditions (Fig. 5A, Fig. 6A). The reduction in Complex V activity in turtles serves to prevent the anoxia-induced consumption of ATP that dominates in anoxia-exposed mammalian systems (Chen et al., 2007; Burwell et al., 2009).

Despite the dramatic differences in Complex V activity between normoxia- and anoxia-exposed turtle hearts during the acute anoxia trial, we do not see equally dramatic differences in mitochondrial membrane potential. In theory, the acute anoxia-induced reversal of Complex V in mitochondria from normoxic turtle hearts should have resulted in a higher membrane potential than that seen in mitochondria from our anoxic turtles. At the very least, during the onset of acute anoxia exposure, one would expect there to be an accelerated decline in membrane potential in mitochondria from the anoxic turtles, which

would be curtailed in mitochondria from normoxic turtles as Complex V functions in reverse. This was not the case in the present study. The rate of decline in membrane potential during the onset of anoxia was similar between both groups and membrane potential stabilised at values that were not statistically different between the two groups (Fig. 6), although the mitochondria from the normoxic group were slightly hyperpolarised relative to the anoxic group. These results are in contrast to those reported in mammalian heart mitochondria, where an inhibition of Complex V with oligomycin resulted in an accelerated rate of decline in mitochondrial membrane potential (Ruiz-Meana et al., 2006). The question remains, however, how is membrane potential preserved at similar levels during acute anoxia exposure in turtle mitochondria that differ in Complex V activity?

Mitochondrial membrane remodelling has been shown to occur in other organisms (Stuart et al., 1998; Casey et al., 2002; Nadtochiy et al., 2006; Kelly et al., 2008; Quarrie et al., 2011) in response to metabolic challenges, but the responses vary considerably. In aestivating snails, for example, mitochondrial membrane remodelling results in a decrease in proton leak (Stuart et al., 1998), which facilitates the maintenance of membrane potential during aestivation. Proton leak is the basal or induced leak of protons across the inner mitochondrial membrane that results in partial dissipation of the membrane potential and uncoupling of substrate oxidation from ATP synthesis (Divakaruni and Brand, 2011). Our analysis of proton conductance under State IV conditions (Fig. 3) suggests that long-term anoxia exposure does not result in a remodelling of the mitochondrial membrane to reduce proton leak in turtles. In mammalian models, enhanced proton leak during ischemic events has been noted, and it has been proposed that a mild uncoupling of the mitochondria lowers ROS generation and may prevent mitochondrial Ca^{2+} overload (Chen et al., 2007). This strategy has also been documented in mammalian models of ischemia tolerance (Nadtochiy et al., 2006; Kelly et al., 2008). However, not all mammalian studies agree with this conclusion. Neonatal cardiomyocytes from rats have been shown to downregulate proton leak following exposure to moderate levels of hypoxia (0.5% O_2) (Casey et al., 2002). Despite the considerable variation in the response of mitochondrial membrane remodelling among mammalian models, the anoxia-tolerant turtle does not modify proton leak to help preserve membrane potential during anoxia.

It is perhaps not surprising that there are differences between turtles and mammalian systems with respect to the effect of O_2 deprivation on proton leak, because the inner mitochondrial membrane of ectotherms is generally less leaky to protons than in homeothermic endotherms (Brookes et al., 1998; St-Pierre et al., 2000b). These differences in proton leak are primarily attributed to differences in the phoshopholipid composition of the inner mitochondrial membrane (Brand et al., 1991; Brookes et al., 1998). As a result, the inherent 'tightness' of the ectothermic mitochondrial membrane may serve to limit proton leak during anoxia, even in the highly depolarised mitochondria, and alleviate the need for membrane remodelling as seen in endotherms. Membrane depolarisation during acute anoxia exposure may serve as the signal for the reverse functioning of Complex V, which is prevented in mitochondria from anoxic turtles because of a downregulation of the protein.

Effects of acute anoxia/reoxygenation on turtle mitochondria

Acute anoxia/reoxygenation or ischemia/reperfusion (even of the order of 1–20 min) can cause irreversible damage to *in vitro* mammalian cardiac mitochondria. Anoxia and reoxygenation decreases the rate of ADP-stimulated O_2 consumption, increases State IV respiration, inhibits ATP production, damages the electron transport chain and disrupts mitochondrial integrity (Du et al., 1998; Ozcan et al., 2001; Ascensão et al., 2006). In contrast, turtle mitochondria, even those without prior anoxia exposure, were able to endure 20 min of anoxia followed by reoxygenation without any impact on subsequent ADP-stimulated State III or State IV respiration (Fig. 5). Of course, in contrast to mammalian preparations, the present experiments were carried out at 13°C [as opposed to room temperature (Du et al., 1998; Ascensão et al., 2006) or 30°C (Ozcan et al., 2001)], which may afford some protection against anoxia/reoxygenation injury. However, we performed the same experiment on turtle heart mitochondria at 37°C and found no effect of anoxia/reoxygenation on respiratory capacity (data not shown).

Neonatal and newborn mammalian hearts are also resistant to O_2 deprivation and this resistance has recently been linked with mitochondrial function (Ostadal et al., 1999; Drahota et al., 2005; Milerova et al., 2010). Mitochondria from developing mammals differ structurally and functionally from their adult counterparts (Milerova et al., 2010) and numerous properties have been linked with anoxic resistance, including: a heavy reliance on anaerobic glycolysis rather than aerobic respiration, a Ca^{2+}-insensitive mitochondrial permeability transition pore, a high mitochondrial membrane potential and a higher resistance to swelling (Ascensão et al., 2009). Interestingly, ectothermic cardiomyocytes and their mitochondria are structurally more similar to neonatal rather than adult mammals (Birkedal et al., 2006; Galli et al., 2006). This suggests that the turtle may share the neonatal mitochondrial specialisations outlined above, which would confer anoxia resistance. It is also possible that suppression of ROS (Milton et al., 2007), high antioxidant defences (Willmore and Storey, 1997) and activation of mitochondrial ATP-activated K^+ channels (Pamenter et al., 2008) may protect the turtle cardiomyocyte from acute oxidative damage. Obviously, identifying the properties that confer acute anoxia resistance in turtle mitochondria is an area of research rife with biomedical implications.

Concluding remarks

The present study has uncovered a mechanism in turtle mitochondria that appears to be fundamental to surviving long periods of anoxia. By dramatically reducing the activity of Complex V (the F_1F_O-ATPase), the turtle mitochondrion inhibits oxidative metabolism, limits the reverse function of the F_1F_O-ATPase, and thereby preserves ATP supplies and possibly limits ROS production. Interestingly, this strategy has been observed in mammalian models of hypoxia tolerance and has been used in clinical settings to improve cardiac function following an anoxic or ischemic insult. This suggests that fundamental mechanisms have been conserved throughout the animal kingdom to endure periods without O_2. Importantly, turtle heart mitochondria stand apart from mammalian mitochondria as they endure 20 min of anoxia and reoxygenation without any conspicuous injury. For these reasons, the turtle is shown to be a novel and promising model organism to reveal drug targets for O_2-related diseases of the heart.

AUTHOR CONTRIBUTIONS

G.L.J.G. and J.G.R. conceived and designed the overall experiment. G.L.J.G. conducted all of the experiments except for the enzymatic analysis measurements, which were carried out by G.Y.L. Interpretation, analysis and drafting of the article were carried out by all authors.

COMPETING INTERESTS

No competing interests declared.

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