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Mitochondria from the systemic heart of Pacific hagfish (*Eptatretus stoutii*) are insensitive to one hour of anoxia followed by reoxygenation

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ABSTRACT

Pacific hagfish (*Eptatretus stoutii*) are an ancient agnathan vertebrate known to be anoxia tolerant. To study their metabolic organization and the role of the mitochondria in anoxia tolerance we developed a novel protocol to measure mitochondrial function in permeabilized cardiomyocytes and how this is affected by one hour of anoxia followed by reoxygenation. When measured at 10 °C the mitochondria had a respiration rate of 2.1 ± 0.1pmol/s/mg WW during OXPHOS with saturating concentrations of glutamate, malate, and succinate. This is comparatively low compared to other ectothermic species. The functional characteristics of the mitochondria were quantified with mitochondrial control ratios. These demonstrated that proton leak contributed to just under 50% of the oxygen flux, with the remainder going towards ATP phosphorylation. Finally, when the preparations were exposed to an anoxia-reoxygenation protocol there was no difference in respiration compared to that of a heart sample from the same animal maintained under normoxia for the same time. When Complex I alone or Complex I and II were stimulated following one hour of anoxia there was no decline in oxygen flux observed. However, if Complex II was activated alone there was a significant decline in respiration. This decrease was however also observed in the mitochondria maintained in normoxia for one hour. In conclusion, Pacific hagfish cardiac mitochondria demonstrated a low rate of oxygen consumption, a loosely coupled electron transfer system, and a resistance to one hour of anoxia.

1. Introduction

The hagfish (Class Myxini) are one of two surviving groups of the ancient agnathans, with lampreys being the other. These fish occupy a basal position in vertebrate phylogeny and as a result, are of particular relevance when working to understand the evolution of physiological and biochemical processes of early vertebrates. Hagfish have featured in multiple examinations of vertebrate evolution, including the cardiovascular system (Farrell, 2007; Stephenson et al., 2017), neurology (Bullock et al., 1984; Ota et al., 2007; Khonsari et al., 2009), and osmolarity (Bellamy and Jones, 1961; Sardella et al., 2009; Currie and Edwards, 2010; Baker et al., 2015). One focus of recent studies is the ability of some hagfish species to survive significant periods of anoxia. For example, Cox et al. (2010, 2011) have demonstrated that the Pacific hagfish (*Eptatretus stoutii*) can survive up to 36 h of anoxia. As such, hagfish are the most primitive vertebrates known to survive extended anoxia, with the other known anoxia tolerant groups being found in more derived groups such as elasmobranchs, teleosts, and reptiles (Renshaw et al., 2002; Lefevre and Nilsson, 2023). Compared to these species the anoxia tolerance of hagfish is comparatively understudied. It may be significantly rooted in their extraordinarily low metabolic rate, the lowest known in vertebrates. However, energy sparing through a low metabolic rate is likely not the complete story. While all hagfish that have had their metabolic rates examined have been found to have very low energy requirements, not all of these can tolerate anoxia. Respirometry measures taken in resting, post-absorptive animals found the oxygen consumption of the New Zealand hagfish (*Eptatretus cirrhatus*) is 6.93 ± 0.23 mL O_2 kg^{-1} h^{-1} at 11 °C (Forster, 1990) while that of the Pacific hagfish is 19.7 ± 2.3mL O_2 kg^{-1} h^{-1} at 10 °C (Cox et al., 2011), however, the New Zealand species is more sensitive to hypoxia than the Pacific species (Davison et al., 1990; Forster et al., 1992). If low metabolism was the main predictor of anoxia tolerance in hagfish, it would be predicted that the New Zealand hagfish, with the lower oxygen needs, would be more resilient to low oxygen not less. Therefore, more factors than just metabolism are likely to contribute to the anoxia tolerance observed in some hagfish species.

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Beyond just overall energy demand, the metabolic organization of hagfish may contribute to survival in hypoxia or anoxia. In some hagfish, anaerobic metabolism accounts for a far larger proportion of the energy supply than in more derived vertebrates (Hansen and Sidell, 1983). Measurement of the activity of multiple enzymes in the cardiac ventricle of Atlantic hagfish (*Myxine glutinosa*) suggests a carbohydrate based metabolism with high glycolytic capacity and low aerobic capacity (Hansen and Sidell, 1983). For example, the ratio of pyruvate kinase to cytochrome *c* oxidase, an indicator of anaerobic to aerobic capacity, is 5.6 times higher in the ventricle of Atlantic hagfish than in the ventricle of Atlantic cod (*Gadus morhua*) (Hansen and Sidell, 1983) In addition, the oxygen tension in the systemic and portal hearts of the Atlantic hagfish have been reported to be 12.3 ± 1.7 and 11.0 ± 1.6 mmHg respectively (Hansen and Sidell, 1983). Importantly, the poisoning of these hearts with cyanide and azide, inhibitors of mitochondrial function, did not significantly impact their function while inhibiting glycolysis with iodoacetate did impair heart function even in aerobic conditions (Hansen and Sidell, 1983). Therefore, the high capacity for anaerobic metabolism and reduced dependence on aerobic energy production in some hagfish species enables their hearts to function in conditions of severe hypoxia or even anoxia as the anaerobic capacity is greater than its energy requirements (Gillis et al., 2015).

While the energy requirements of some hagfish organs may be met by anaerobic metabolism, they are not a fully anaerobic species, they still require oxygen for their long-term survival. Like all vertebrates, hagfish utilize mitochondria to generate ATP aerobically via the electron transport system (ETS). Mitochondria are also involved in cellular signaling and regulating apoptosis, and their dysfunction plays a role in various pathologies (Raha and Robinson, 2000; Murphy, 2009; Jomova et al., 2023). Relevant to our investigations of hagfish anoxia tolerance, mitochondria are central to the deleterious consequences of anoxia-reoxygenation. During anoxia the ETS is unable to maintain the proton motive force (pmf) without oxygen to act as the final electron receptor. As a result, the mitochondria may become ATP consumers as ATP synthase runs in reverse to maintain the pmf against proton leak (St-Pierre et al., 2000). This can be mitigated in some species by the inhibition of ATP synthase, allowing the pmf to drop to a lower steady state that is less costly to maintain (St-Pierre et al., 2000). Whether or not hagfish employ such a strategy is currently unknown. The effects of anoxia on the mitochondria also have consequences during reoxygenation where mitochondrial dysfunction can result in a surge in reactive oxygen species (ROS) leading to widespread oxidative damage that may result in apoptosis or necrosis (Chouchani et al., 2014, 2016). While the specific sources of ROS during reoxygenation are still under debate it is likely that an unbalancing of the ETS during anoxia creates conditions that favour ROS production when oxygen returns. A proposed pathway for how mitochondria produce excessive ROS is that succinate, a Complex II substrate, accumulates during anoxia while the ETS is nonfunctional. This enlarged succinate pool drives increased proton transfer upon the resumption of ETS function with reoxygenation. Additionally, during anoxia the pool of ADP is steadily depleted. The unavailability of ADP reduces the functional capacity of ATPase, limiting its ability to relieve the pmf through ATP phosphorylation. Upon reoxygenation the combination of high succinate and low ADP creates a hyperpolarized pmf. This state is more favourable for reverse electron flow (RET) through Complex I which forms superoxide ($O_2^{\cdot-}$). (Chouchani et al., 2014, 2016). The mitochondria of the anoxia tolerant freshwater turtle, the red-eared slider (Trachemys scripta) have been investigated for strategies to prevent the surge of ROS during reoxygenation (Bundgaard et al., 2018; Bundgaard et al., 2019a). These authors suggest that an intense suppression of metabolism protects the turtle mitochondria from the accumulation of succinate and depletion of ADP during anoxia, mitigating the unbalancing of the mitochondria that increased ROS production (Bundgaard et al., 2019b). Experiments with isolated mitochondria have shown that the addition of ADP does reduce hydrogen peroxide production during reoxygenation by allowing for the dissipation of the pmf (Bundgaard et al., 2019a). The anoxia tolerant epaulette shark (*Hemiscyllium ocellatum*) employs similar adaptations, utilizing hypometabolism during anoxia to conserve energy and reduce succinate accumulation while inhibiting Complex I and II function during reoxygenation to mitigate ROS production (Devaux et al., 2019). These adaptations are reviewed in Hawrysh et al. (2022) and Lefevre and Nilsson (2023).

The goals of this study were to examine mitochondrial function in the systemic heart of the Pacific hagfish to increase our understanding of its energy metabolism and how it is affected by anoxia reoxygenation. To accomplish this, we developed a novel protocol for measuring the oxygen respiration of Pacific hagfish mitochondria in permeabilized cardiac muscle. The contribution of different electron transfer complexes was investigated by comparing different substrate choices. The function of the mitochondria was further investigated by measuring various mitochondrial control ratios that quantify the coupling efficiency of the oxidative phosphorylation system. The tolerance of the mitochondria to anoxia was investigated by comparing the respiration rate of mitochondria that had been exposed to one hour of normoxia or one hour of anoxia followed by reoxygenation.

2. Materials and methods

2.1. Animal collection and housing

Pacific hagfish were collected in Barkley Sound, British Columbia, Canada with baited Korean cone traps at a depth of 100 m. Animals were then transported to the Bamfield Marine Sciences Center, British Columbia, Canada and held in outdoor covered flowthrough seawater tanks. Hagfish were held for less than one month between capture and experimental sampling, during which they were not fed. The sex and reproductive state of Pacific hagfish cannot be readily determined externally. Therefore, these traits were not considered in our experimental protocol. Hagfish were selected at random for use in mitochondrial assays. Measurements of citrate synthase were performed using hagfish held at the University of Guelph that were being utilized for other experiments. These fish were held at 10 °C in 32 ppt water in a recirculating system. Animals were fed to satiation once per week and starved for two weeks prior to sampling. All procedures were conducted in accordance with the University of Guelph's Animal Care Committee (AUP#3958), the Bamfield Marine Science Center's Animal Care Committee (AUP#RS22–02) and Canadian Council for Animal Care.

2.2. Preparation of permeabilized cardiac tissue

All hagfish were euthanized with a sharp blow to the cranium followed by decapitation. The ventricle of the systemic heart was removed and placed in ice cold biopsy preservation solution (BIOPS) solution (2.77 mM CaK_2-EGTA, 7.23 mM K_2-EGTA, 5.77 mM Na_2-ATP, 6.56 mM $MgCl_2$, 20 mM Taurine, 15 mM Na_2-Phosphocreatine, 20 mM Imidazole, 0.5 mM dithiothreitol, 50 mM MES hydrate, 750 mM sucrose, pH: 7.1). This BIOPS recipe was modified from the published methods with the addition of 750 mM sucrose to match the osmolarity of the hagfish (Fontana-Ayoub and Gnaiger, 2016). The mass of the ventricles ranged from 24.3 mg to 104.0 mg with a mean mass of 61.6 ± 3.6 mg. The ventricle was divided into two halves on the sagittal plane and the muscle fibers were teased apart with a fine tipped probe under a dissecting microscope. The separated fibers were then placed into a BIOPS solution containing 5 g/L saponin solution (Doerrier et al., 2018). The fibers were mixed on a shake plate for 30 min on ice to permeabilize the tissue. The tissues were then rinsed in 3 × 10 min washes of MiRO5 solution (0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM lactobionic acid, 20 mM Taurine, 10 mM KH_2PO_4, 20 mM HEPES, 750 mM sucrose, 1 g/L BSA, pH: 7.1). The tissues were then stored on ice until they were moved to an O2K Oxygraph (Oroboros Instruments, Innsbruck, Austria) for the measurement of mitochondrial respiration. All chemicals used were

acquired from Millipore Sigma (Burlington, USA).

2.3. Measurement of mitochondrial respiration

Mitochondrial respiration was measured in an Oroboros Oxygraph (Innsbruck, Austria) at 10 °C with the two halves of each heart being measured simultaneously in the two chambers of the oxygraph. Protocols followed the SUIT (substrate, uncoupler, inhibitor, titration) method. The substrates used were as follows. Leak respiration was stimulated with 1 mM glutamate, 1 mM malate, and 1 mM succinate. Oxidative phosphorylation (OXPHOS) was stimulated with 0.1 mM ADP. The integrity of the outer mitochondrial membrane was tested with the addition of 10 μM cytochrome *c*, with an average percent increase of 10.5 ± 1.5%. Maximal respiration was achieved by uncoupling the mitochondria with 2 μM carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP). Finally, the mitochondrial respiration was inhibited by the addition of 7.5 μM antimycin A, 1.5 μM rotenone, and 15 mM malonate. The oxygen flux of the treatments was normalized to the wet weight in milligrams of each ventricle section.

2.4. Respiration of the N and S mitochondrial pathways

The relative contribution of electron transport complexes was investigated by providing different Krebs cycle substrates to oxidative phosphorylation. The N pathway refers to electrons transferred by Complex I receiving protons through NADH. The S pathway refers to the activity of Complex II receiving protons from $FADH_2$. Finally, the NS pathway refers to the combined activity of both Complex I and Complex II. Contributions to the proton motive force from outside of the electron transport system were not investigated in this study. The respiration of the N pathway was measured with the addition of 1 mM glutamate and 1 mM malate to stimulate Complex I. The respiration of the S pathway was measured with the addition of 1 mM succinate and 1.5 μM rotenone to stimulate Complex II and inhibit Complex I. The combined respiration of the NS pathway used all the substrates, 1 mM glutamate, 1 mM malate, and 1 mM succinate to stimulate both complexes. All mitochondrial pathway treatments received the same concentration of ADP to stimulate the OXPHOS state.

2.5. Calculation of mitochondrial control ratios

Control ratios that assign a quantitative value to aspects of mitochondrial function were calculated using different respiratory states. For the purposes of these calculations, respiration was divided into three categories. The Leak state (L) was measured when Krebs cycle substrates were provided but ADP was absent. Oxygen consumption in this state was considered to be only what was required to maintain the proton motive force against nonspecific proton leak. The OXPHOS state (P) was measured when the mitochondria were provided with Krebs cycle substrates and a saturating concentration of ADP. Oxygen consumption in this state is what is required to sustain the phosphorylation of ADP. Finally, the Excess state (E) was measured when the mitochondria were fully uncoupled through the addition of CCCP. This state is considered the maximum rate of respiration attainable by the mitochondria without any limitation by the phosphorylation system. The selected mitochondrial control ratios were calculated using the following equations (Gnaiger, 2020).

Respiratory Acceptor Control Ratio: This value is defined as the ratio of OXPHOS oxygen flux over Leak oxygen flux. It is related to the P – L Control Efficiency but has a range of 1.0 to infinity. It was calculated with the equation, P/L.

E – P Control Efficiency: This value is an expression of the limitation of respiration that is exerted by the phosphorylation system. It was calculated with the equation, (E – P)/E.

E – L Coupling Efficiency: This value is an expression of the strength of coupling in the oxidative phosphorylation system. A value of 0.0 indicates an uncoupled system while a value of 1.0 indicates a fully coupled system. It was calculated with the equation, (E – L)/E.

P – L Control Efficiency: This value is an expression of the proportion of OXPHOS capacity that is associated with proton leak and is also an indication of coupling strength. A value of 0.0 indicates an uncoupled system while a value of 1.0 indicates a fully coupled system. It was calculated with the equation, (P – L)/P.

Net P/E Control Ratio: This value expresses the OXPHOS capacity, corrected for Leak rate, as a fraction of the total respiratory capacity. It was calculated with the equation, (P – L)/E.

2.6. Anoxia exposure of mitochondria

The anoxia exposure of the mitochondria was performed based on the following timeline. Leak respiration was achieved through the addition of 1 mM glutamate and 1 mM malate for the CI treatment. The addition of 1 mM succinate and 1.5 μM rotenone for the CII treatment. The addition of 1 mM glutamate, 1 mM malate, and 1 mM succinate for the CI + CII treatment. Oxidative phosphorylation was stimulated with 0.1 mM ADP. Once the rate of OXPHOS had stabilized we introduced a gentle nitrogen flow into the respiration chamber to remove oxygen from the medium. Anoxia took approximately 15–20 min to develop and was verified using the Oxygraph's internal oxygen sensor. The normoxic controls were performed simultaneously with the other ventricle section from the same heart. The controls followed the same timeline with the respiration chamber being left open for one hour to maintain oxygen at air saturated levels. Post-anoxia respiration was measured following the stabilization of oxygen concentration in the chamber. A representative trace of the CI + CII anoxia exposure experiment is shown in Fig. 1.

2.7. Measurement of citrate synthase activity

The enzyme activity of citrate synthase was quantified as an indicator of mitochondrial content in homogenates of the systemic heart, liver, and white muscle of Pacific hagfish. These measurements on tissue samples from hagfish held at the University of Guelph, Canada, were preformed using a citrate synthase activity kit from Cayman Chemical (Ann Arbor, USA). This colourimetric assay was performed at 25 °C and measured the production of Coenzyme A by the citrate synthase reaction. The activity was measured as the change in absorbance at 412 nm every 30 s for a total period of 20 min. A microplate reader (SpectraMax Plus, Molecular Devices, San Jose, USA) was used to make these measurements and the linear area of the absorbance plot was used to calculate activity. The protein content of the homogenates was determined with a bicinchoninic acid (BCA) protein assay (Fisher Scientific, Waltham, USA) and used to normalize the enzyme activity.

2.8. Statistical analysis

The data was tested for normal distribution with a Shapiro-Wilk normality test. The data was normally distributed so no transformations were applied, and parametric tests were used. The respiration rates of each treatment were compared before and after the one hour of normoxia or anoxia with an unpaired two-sided *t*-test ($\alpha < 0.05$). The change in respiration between normoxic and anoxic treatments was compared by taking the slope change of each treatment and comparing the normoxic and anoxic treatments using an unpaired two-sided t-test ($\alpha < 0.05$). Statistical analysis was performed in R Studio version 4.3.2.

3. Results

3.1. N and S linked mitochondrial respiration

The N linked respiration, when only Complex I was activated, of the mitochondria had a P state oxygen flux of 1.87 ± 0.2 pmol/s/mg WW ($n = 8$). The S linked respiration, when only Complex II was activated, the

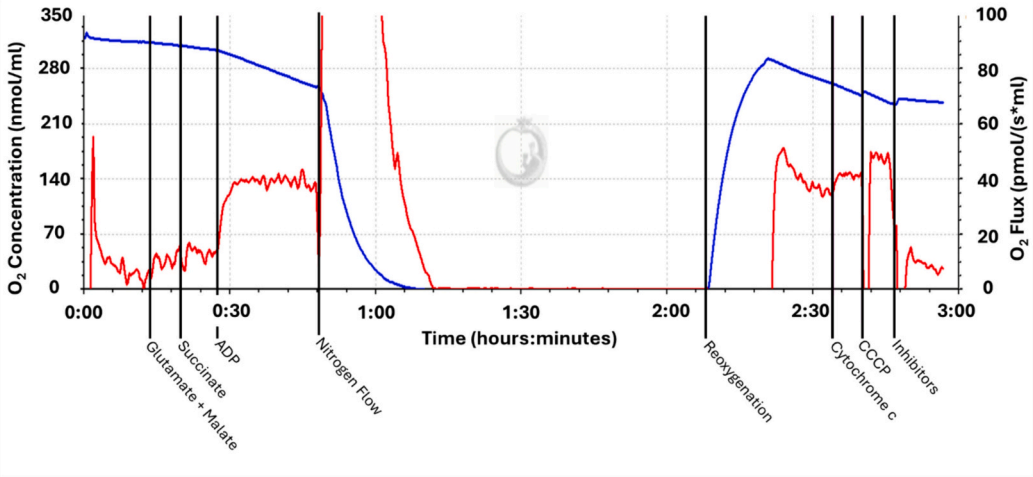

Fig. 1. Representative trace of the Oxygraph data from one of our replicates. In the figure the blue line is the measured O_2 concentration and the red line is O_2 flux. This trace is taken from the Complex I + Complex II treatments with anoxia exposure. This trace demonstrates the timeline of our experiments with the addition of substrates 1 mM glutamate, 1 mM malate, 1 mM succinate. The Leak rate (L) was measured following the addition of all substrates. Oxidative phosphorylation was stimulated with 0.1 mM ADP and the OXPHOS rate (P) was measured. Anoxia was achieved by a gentle nitrogen flow into the respiration chamber. The mitochondria were reoxygenated following one hour. The integrity of the outer mitochondrial membrane was tested with the addition of 10 μM cytochrome *c*, Mitochondria were uncoupled with the addition of 2 μM CCCP and the excess rate (E) was recorded. Finally, the mitochondrial respiration was inhibited by the addition of 7.5 μM antimycin A, 1.5 μM rotenone, and 15 mM malonate to achieve the background respiration. The Complex I treatment followed the same timeline with only glutamate and malate added. The Complex II treatment consisted of the addition of succinate and 1.5 μM rotenone prior to ADP. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mitochondria had a P state oxygen flux of 1.64 ± 0.1 pmol/s/mg WW (n = 8). Finally, the NS respiration, when both Complex I and Complex II were activated the mitochondria had a P state oxygen flux of 2.15 ± 0.2 pmol/s/mg WW (n = 8) (Fig. 2).

3.2. Mitochondrial control ratios

The mitochondrial control ratios were calculated with the Leak, OXPHOS, and Excess states of the mitochondria that were in a state of NS respiration, the mean respirations were used to calculate each ratio. The mean Leak oxygen flux was 1.03 ± 0.05 pmol/s/mg WW (n = 8). The mean OXPHOS respiration rate was 2.09 ± 0.1 pmol/s/mg WW (n = 8). The mean uncoupled oxygen consumption was 2.2 ± 0.17 pmol/s/mg WW (n = 8). The calculated respiratory control ratios are listed in Table 1.

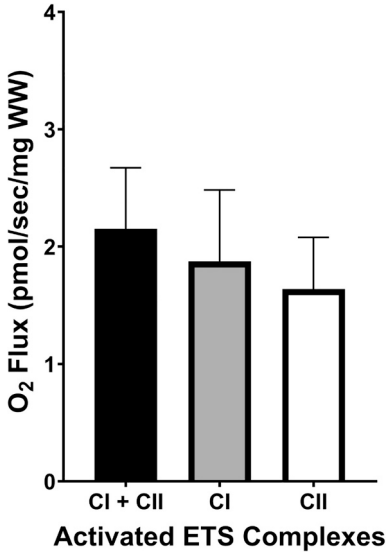

Fig. 2. OXPHOS respiration of Pacific hagfish (*Eptatretus stoutii*) cardiac mitochondria, maintained in normoxia, with different combinations of Krebs cycle substrates. CI + CII; 1 mM glutamate, 1 mM malate, 1 mM succinate, and 0.1 mM ADP. CI; 1 mM glutamate, 1 mM malate, and 0.1 mM ADP. CII; 1 mM succinate, 1.5 μM rotenone, and 0.1 mM ADP. N = 8, with an N equaling the heart of a single hagfish. CI, Complex 1; CII, Complex 2. ETS; electron transport system. Data used was taken from the Time = 0 h part of our experiment.

3.3. Effects of anoxia exposure on mitochondrial respiration

The mitochondria were exposed to one hour of anoxia or normoxia with each of the three substrate treatments. Oxygen consumption during OXPHOS was measured once before the anoxia exposure, Time = 0 h, and once after the exposure, Time = 1 h. When only Complex I was provided with substrates the respiration rate declined from 1.95 ± 0.18 pmol/s/mg WW before anoxia to 1.58 ± 0.14 pmol/s/mg WW after anoxia (Fig. 3). This change in respiration was not statistically significant ($p = 0.16$, $\alpha \leq 0.05$). The normoxic control also lacked a significant change ($p = 0.38$, $\alpha \leq 0.05$). The rate of decline between normoxia and anoxia treatments were not significantly different ($p = 0.34$, $\alpha \leq 0.05$) (Table 2). When Complex II alone was stimulated the oxygen flux changed from 1.42 ± 0.12 pmol/s/mg WW before anoxia to 0.92 ± 0.11 pmol/s/mg WW after one hour of anoxia (Fig. 3). This difference was statistically significant ($p = 0.014$, $\alpha \leq 0.05$). However, the rate of oxygen consumption also significantly declined when the mitochondria were exposed to one hour of normoxia ($p = 0.004$, $\alpha \leq 0.05$). The rate of

Table 1
Respiratory control ratios of Pacific hagfish (*Eptatretus stoutii*) mitochondria in permeabilized cardiac fibers measured in normoxia.

Control Ratio	E – P Control Efficiency	E – L Coupling Efficiency	P – L Control Efficiency	Net P/E Control Ratio	Respiratory Control Ratio
Equation	(E – P)/E	(E – L)/E	(P – L)/P	(P – L)/E	P/L
Value	0.02 ± 0.06	0.52 ± 0.04	0.49 ± 0.02	0.49 ± 0.04	2.05 ± 0.1

Ratios were calculated with the mean oxygen flux of mitochondria during leak (L), oxidative phosphorylation (P), and uncoupled (E) respiration states. Values are unitless ratios and presented as mean ± SE. *N* = 8, with an N equaling the heart of a single hagfish.

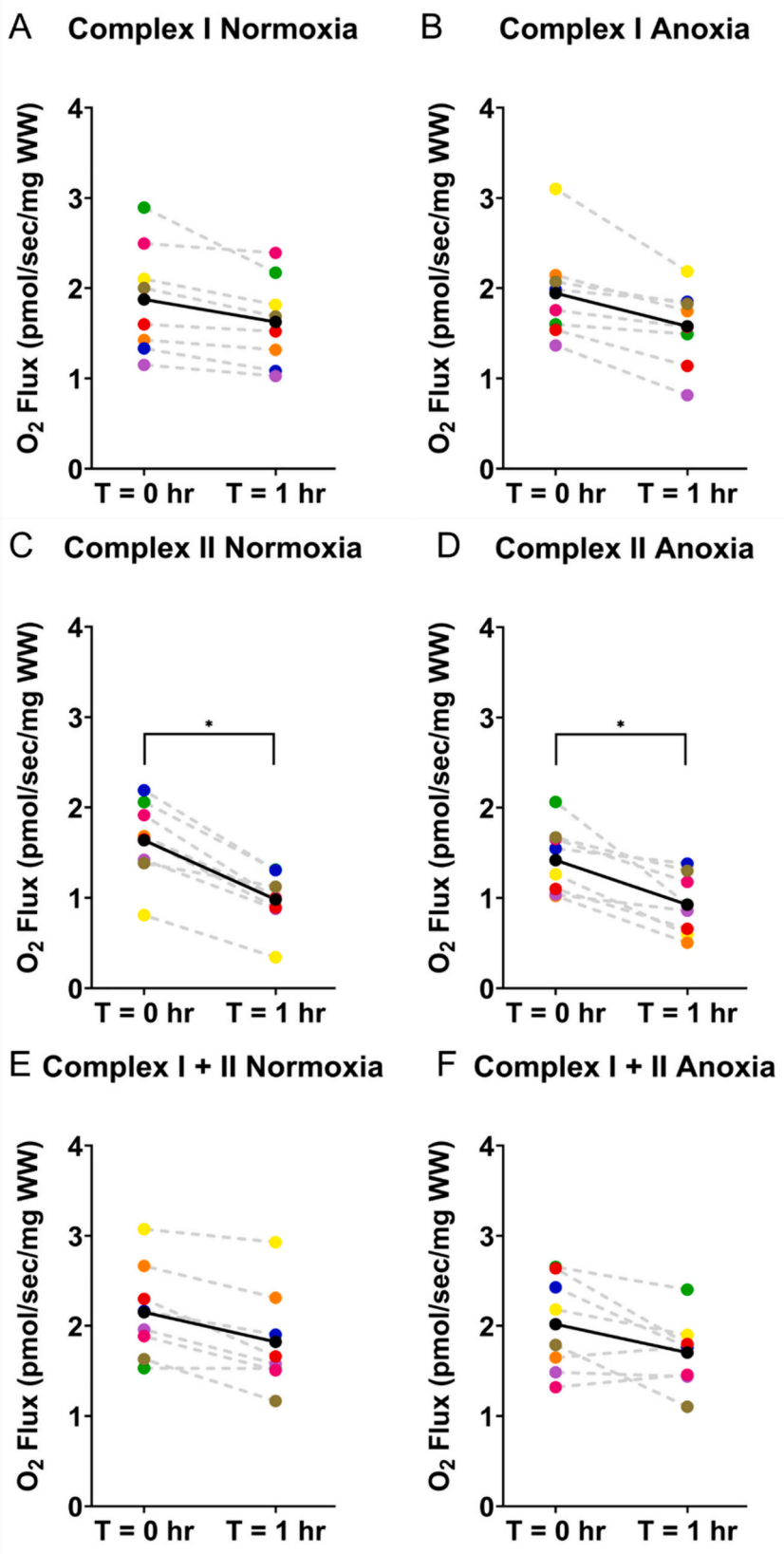

Fig. 3. The influence of one hour of normoxia or anoxia on the OXPHOS respiration of Pacific hagfish (*Eptatretus stoutii*) cardiac mitochondria under different Krebs cycle substrate conditions. Panels A and B) Complex I; 1 mM glutamate and malate. Panels C and D) Complex II; 1 mM succinate and 1.5 μM rotenone. Panels E and F) Complex I + II; 1 mM glutamate, malate, and succinate. Data is presented with each individual replicate represented as a unique coloured circle. Grey dashed lines indicate the slope of oxygen flux between $T = 0$ h and $T = 1$ h. The black line is the mean respiration at T = 0 h and T = 1 h along with the slope change between the means. Asterix indicates significant difference between Time = 0 h and T = 1 h of each treatment group. T, Time.

Table 2
Mean change in OXPHOS respiration of Pacific hagfish (*Eptatretus stoutii*) cardiac mitochondria following exposure to either one hour of normoxia or one hour of anoxia.

Mitochondrial Substrates	Glutamate, Malate, ADP	Succinate, ADP	Glutamate, Malate, Succinate, ADP
1 Hour Normoxia	−0.24 ± 0.07	−0.65 ± 0.07	−0.33 ± 0.31
1 Hour Anoxia	−0.37 ± 0.09	−0.49 ± 0.1	−0.31 ± 0.13
P Value	0.345	0.25	0.926

Ratios were calculated by subtracting the mitochondrial respiration at Time = 1 h by the respiration at Time = 0 h from our anoxia exposure experiments. Values are represented as the mean ± SE. $N = 8$, with an N equaling the heart of a single hagfish.

decline experienced by both normoxia and anoxia exposed mitochondria was not statistically different from each other ($p = 0.25$, $\alpha \leq 0.05$) (Table 2). Finally, when both Complex I and Complex II were stimulated, the respiration rate of the mitochondria changed from 2.02 ± 0.17 pmol/s/mg WW before anoxia to 1.7 ± 0.13 pmol/s/mg WW after anoxia (Fig. 3). This decline in respiration was not significant ($p = 0.19$ $\alpha \leq 0.05$). The normoxic treatment also did not show a significant decline in oxygen consumption $p = 0.24$, $\alpha \leq 0.05$). As with the previous treatments there was no difference in the rate of decline of each exposure group ($p = 0.92$, $\alpha \leq 0.05$) (Table 2).

3.4. Mitochondrial content of heart, liver and white muscle

The activity of citrate synthase was measured as an indicator of mitochondrial content. The systemic heart had an activity of 0.142 ± 0.022 μmols/min/mg protein ($n = 3$). The liver had a citrate synthase activity of 0.025 ± 0.002 μmols/min/mg protein (n = 3). The white muscle had an activity of 0.050 ± 0.002 μmols/min/mg protein (n = 3) (Table 3).

4. Discussion

This investigation represents, to our knowledge, the first time mitochondrial function has been measured in a hagfish species. We found that cardiac mitochondria had an exceptionally low rate of metabolic consumption with respiration being highest when both Complex I and Complex II were activated. Complex I alone had a respiration rate equal to 91% of the maximal rate while Complex II alone had a respiration rate that was 73% the combined rate. This non additive relationship between the electron transfer complexes is commonly observed in mitochondrial function (Gnaiger, 2020). Exposing the mitochondria to one hour of anoxia followed by reoxygenation had no significant effect on their rate of oxygen consumption and this rate was not different from controls exposed to normoxia for one hour. These results suggest mitochondria from the systemic heart of Pacific hagfish can tolerate anoxia exposure without significant deleterious effects on their post-anoxic function.

Table 3
Citrate synthase activity in the systemic heart, liver and white muscle of Pacific hagfish (*Eptatretus stoutii*) maintained in normoxia.

Tissue	Enzyme Activity (μmols/min/mg protein)
Systemic Heart	0.14 ± 0.02
Liver	0.03 ± 0.001
White Muscle	0.05 ± 0.002

Citrate synthase activity was measured as of μmols of Coenzyme A produced per minute, normalized to mg of protein. Values shown are mean activities ± SE. $N = 3$, with an N equaling the heart of a single hagfish.

4.1. Functional characteristics of heart mitochondria

In the current study, the measured respiratory activity of the heart mitochondria is comparatively low compared to that of other vertebrate species. For example, the maximum oxygen flux in the hagfish heart mitochondria is 5% that previously measured in liver mitochondria from the sea lamprey (*Petromyzon marinus*), another agnathan species (Leblanc et al., 1995). Additionally, the respiration rate of mitochondria measured in the current study is approximately 1.6% that measured in mitochondria from the rainbow trout (*Oncorhynchus mykiss*) heart at the same temperature, (Michaelsen et al., 2021). The measured rates of citrate synthase also suggests that the hagfish heart has a comparatively low mitochondrial capacity. For example, the rate of this enzyme in the hagfish heart was one third that measured in heart samples of European perch (*Perca fluviatilis*) at 16 °C, (Pichaud et al., 2019).

In hagfish, the systemic heart is a venous heart with no coronary circulation and this species lives in an environment where oxygen is often limited. This suggests therefore that the heart normally functions at comparatively low oxygen tensions. Previous work has established that the hagfish heart has low energy requirements with a comparatively low aerobic capacity, Hansen & Sidell, (1983) have described it as being "anaerobically geared" (Forster, 1990; Hansen and Sidell, 1983). Altogether data from the current study suggest that the Pacific hagfish heart has a low concentration of mitochondria with a small capacity for oxidative phosphorylation. While this comparatively low aerobic capacity reflects the low energy requirements of the heart, it also suggests limited energetic investment in cellular pathways for aerobic energy production. Finally, comparison of the citrate synthase activities in the heart, liver and white muscle in the current study, suggests that the mitochondrial content of the white muscle and liver are 35% and 20%, respectively, that of the systemic heart. This suggests that the heart has a greater aerobic capacity then these other tissues.

Mitochondrial function can be broken down into component characteristics by quantifying the mitochondrial control ratios (Gnaiger, 2020). First, the respiratory control ratio (RCR), measured as the OXPHOS oxygen consumption over the LEAK flux, was calculated as 2.05 in the Pacific hagfish. Comparatively, Michaelsen et al. (2021) working on mitochondria from the rainbow trout heart reported an RCR of 10.0, four times higher than the hagfish. This indicates that the trout mitochondria are more tightly coupled and has a higher capacity for ATP synthesis than that of the hagfish. The tightness of the coupling in the mitochondria can be further quantified by the E – L coupling efficiency and P – L control efficiency ratios. This is done by determining the proportion of the maximum oxygen flux and OXPHOS flux that is made up by proton leak. The leak rate of oxygen flux represents the oxygen consumption that is required to maintain the proton motive force against the non-specific leak across the mitochondrial membrane. For both ratios a value of 0.0 indicates an uncoupled system, where the membranes provide no resistance to proton leak, such a state would indicate heavily damaged mitochondria. A value of 1.0 in both ratios would indicate a fully coupled system, where none of the protons transported by the ETS escape across the membrane. We found that the cardiac mitochondria had an E – L coupling efficiency ratio of 0.51 ± 0.04 and a P – L control efficiency ratio of 0.5 ± 0.02, indicating that just under 50% of the oxygen flux of the mitochondria is accounted for by proton leak that does not contribute to ATP production. Additionally, the net P/E control ratio quantifies the OXPHOS capacity corrected for leak as a proportion of total oxygen flux. The Pacific hagfish had a net P/E control ratio of 0.49 ± 0.04, demonstrating that around 50% of the oxygen flux is accounted for by OXPHOS. Comparatively, work by Michaelsen et al., (2021) demonstrates that mitochondria from the rainbow trout heart had a E – L coupling efficiency ratio of 0.922 and a P – L control efficiency of 0.900, indicating a much higher efficiency, where only 10% of the proton motive force is lost to leak. The inefficiency of the hagfish mitochondria, as indicated by this analysis, could be reflective of the lack of investment in the mitochondria, or it

could serve a protective effect.

In our experimental protocol, the ETS substrates were saturated, pushing the proton motive force to its maximum. A hyperpolarized proton motive force can have deleterious effects as the greater resistance to proton flux can cause an increase in ROS production. Given the low capacity of the hagfish mitochondria to utilize the proton motive force for ATP production it may be preferable to bleed off the excess protons through leak, rather than having them contribute to ROS generation. The final ratio calculated was the E – P control efficiency ratio, this ratio quantifies the degree of control the phosphorylation system exerts over oxygen flux. A higher ratio indicates a larger difference between the uncoupled flux and OXPHOS flux. Indicating that the ETS can translocate protons at a faster rate than they can be utilized by the ATP phosphorylation system. The cardiac mitochondria had an E – P control efficiency of 0.02 ± 0.06, indicating that the phosphorylation system exerts very little control over the oxygen flux as the addition of an uncoupler resulted in a minor increase in oxygen flux. Once again, we can compare these results to recently generated values for mitochondria from the rainbow trout heart which had a E – P control efficiency of 0.22. This suggests that an approximately 20% increase in oxygen flux when these mitochondria were fully uncoupled (Michaelsen et al., 2021) and that the ETS of the hagfish mitochondria have less excess capacity to translocate protons than those from the more derived rainbow trout. Taken together these control values suggest that Pacific hagfish mitochondria are loosely coupled and have a low capacity in their electron transport complexes and phosphorylation system. We might therefore predict that the hagfish mitochondria would have a lower density of ETS components in their inner mitochondrial membrane compared to more active species that invest more into their mitochondria.

4.2. The influence of one hour anoxia on mitochondrial function

The hagfish mitochondria in this study appeared to be relativity insensitive to one hour of anoxia exposure followed by reoxygenation. In the treatments where the mitochondria were provided with substrates for Complex I alone or for both Complex I and II, there was no significant effect of anoxia. Both the normoxic and anoxic chambers showed small non-significant declines in respiration. The exception to this was when the mitochondria were only provided with succinate as a metabolic substrate. This substrate treatment had a significant decrease (40–50%) in respiration over one hour in both the normoxic control and anoxia treatment. As there was a similar decrease in respiration in mitochondria provided with only succinate regardless of the availability of oxygen, we can conclude that the loss of function was not due to the anoxia exposure. An alternative explanation is that this decline may be due to the accumulation of oxaloacetate by the Kreb's cycle which can be a potent inhibitor of Complex II function (Gnaiger, 2020). This pathway is known to be a potential confounding factor in mitochondrial experiments (Gnaiger, 2020). For this reason, it is common practice to supplement succinate with rotenone when Complex I substrates are not provided. Rotenone inhibits Complex I which would otherwise regenerate the NAD^+ required to generate oxaloacetate from malate (Gnaiger, 2020). Rotenone was used in the current study, but it is not known how effectively it inhibited Complex I and prevented the accumulation of oxaloacetate. This is speculation and further investigation is required to establish why a decline in respiration was only observed when succinate was provided as the sole fuel. Regardless of the potential confounding factors that effected the Complex II treatment, there was no observed difference between the normoxic and anoxic mitochondria with any substrate combination. Therefore, we are still able to conclude that one hour of anoxia does not have substantial effects on Pacific hagfish mitochondrial function.

Studies of other anoxia tolerant species provide an example of how anoxia's effects on the mitochondria may be mitigated. For example, work by Duffy et al. (2023) demonstrates that 5 min of anoxia at 37 °C followed by reoxygenation caused the respiration rate of rat liver mitochondria to decrease by 80%, but that similar treatment of mitochondria from torpid thirteen lined ground squirrels (*Ictidomys tridecemlineatus*) had no effect on respiration. It is suggested that the mitochondria of these ground squirrels are protected during hibernation by the suppression of mitochondrial activity (Duffy et al., 2023). Specifically, the inhibition of Complex I may reduce the rate of ROS production via reverse electron flow (RET) through Complex I. While suppressing Complex II may reduce the perturbance of the proton motive force by succinate accumulation (Mathers et al., 2017; Mathers and Staples, 2019). Adaptations to protect against mitochondrial disfunction have also been studied in the freshwater turtle (*Trachemys scripta elegans*) (Bundgaard et al., 2018; Bundgaard et al., 2019a; Galli and Richards, 2012). As in hibernating ground squirrels, mitochondrial function in turtles acclimated to cold temperatures is reduced via the suppression of multiple complexes of the electron transport system (Galli and Richards, 2012; Bundgaard et al., 2018; Bundgaard et al., 2019a). In both the squirrels and turtles, suppressing mitochondrial function likely serves the purpose of attenuating the deleterious effects of low oxygen on the mitochondria (Hawrysh et al., 2022; Lefevre and Nilsson, 2023).

Where the models discussed above differ from the Pacific hagfish, is that they experience hypoxia or anoxia as part of seasonal hibernation where they and their mitochondria become acclimatized to low temperatures and have lower energy demands than normal. The Pacific hagfish instead experience hypoxia or anoxia while burrowed in the mud at the bottom of the ocean or feeding in the body cavities of deceased marine mammals (Martini, 1998). Their exposure is likely acute and without an opportunity for prior acclimatization. A species that is perhaps more comparable to the Pacific hagfish is the epaulette shark (Renshaw et al., 2002). This anoxia tolerant elasmobranch often experiences hypoxia or anoxia in coral reef tidal pools. An acute non-seasonal exposure such as this is perhaps more comparable to the circumstances in which the hagfish experience low oxygen, albeit at higher temperatures than the deep sea hagfish. Epaulette sharks demonstrate similar adaptations to the species we have covered above. They make use of hypometabolism to reduce their energy costs during anoxia and upon reoxygenation the epaulette shark inhibits mitochondrial function, suppressing the activity of Complex I and Complex II to mitigate the consequences of mitochondrial function during the critical period of reoxygenation (Devaux et al., 2019). That these adaptations are found in both mammals and elasmobranchs is a promising sign for their potential utilization in hagfish, however we cannot make that determination with our current data. The features of Pacific hagfish mitochondrial function characterized in this study could provide a form of passive protection to anoxia and achieve similar results to the acclimation seen in other species. While more active species need to shift their mitochondria into a state of reduced activity this appears to be the standard condition for at least some hagfish species (Gatrell et al., 2019; Hansen and Sidell, 1983). A comparatively high anaerobic capacity and limited investment in mitochondria may provide energy during anoxia while also minimizing the consequences of mitochondrial disfunction. The relatively loose coupling of the Pacific hagfish mitochondria may also function to reduce ROS production, providing an alternative route to RET for excess protons. Protective uncoupling of the mitochondrial membrane as a means of reducing ROS production as has been observed in mammals via the use of uncoupling proteins such as UCP1 (Oelkrug et al., 2010, 2014). For now, the specific mechanisms utilized in the hagfish mitochondria to tolerate anoxia reoxygenation remain speculation. Regardless, the current work increases our understanding of the cellular mechanisms involved in anoxia tolerance. Future research will further explore the details of Pacific hagfish mitochondrial function, how they operate, and how they survive without oxygen.

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CRediT authorship contribution statement

Maria A. Yutsyschyna: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Jared B. Shaftoe:** Writing – review & editing, Methodology, Investigation. **Todd E. Gillis:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no competing or financial interests.

Data availability

Data will be made available on request.

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