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The air-breathing Alaska blackfish (*Dallia pectoralis*) suppresses brain mitochondrial reactive oxygen species to survive cold hypoxic winters

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ABSTRACT

The Alaska blackfish (*Dallia pectoralis*) is the only air-breathing fish in the Arctic. In the summer, a modified esophagus allows the fish to extract oxygen from the air, but this behavior is not possible in the winter because of ice and snow cover. The lack of oxygen (hypoxia) and near freezing temperatures in winter is expected to severely compromise metabolism, and yet remarkably, overwintering Alaska blackfish remain active. To maintain energy balance in the brain and limit the accumulation of reactive oxygen species (ROS), we hypothesized that cold hypoxic conditions would trigger brain mitochondrial remodeling in the Alaska blackfish. To address this hypothesis, fish were acclimated to warm (15 °C) normoxia, cold (5 °C) normoxia or cold hypoxia (5 °C, 2.1–4.2 kPa; no air access) for 5–8 weeks. Mitochondrial respiration, ADP affinity and H_2O_2 production were measured at 10 °C in isolated brain homogenates with an Oroboros respirometer. Cold acclimation and chronic hypoxia had no effects on mitochondrial aerobic capacity or ADP affinity. However, cold acclimation in normoxia led to a suppression of brain mitochondrial H_2O_2 production, which persisted and became more pronounced in the cold hypoxic fish. Overall, our study suggests cold acclimation supresses ROS production in Alaska blackfish, which may protect the fish from oxidative stress when oxygen becomes limited during winter.

1. Introduction

Teleost fishes have successfully inhabited some of the most diverse and extreme environments on earth. Polar species are particularly well-adapted and exhibit a wide variety of novel traits that allow them to survive sub-zero temperatures and severe fluctuations in oxygen availability (Feller and Gerday, 1997; Guderley, 2004; Verde et al., 2006). The Alaska blackfish (*Dallia pectoralis*) is a particularly interesting example, as this species is the only known air-breathing fish to inhabit the Arctic (Campbell and Lopéz, 2014; Caspers, 1976; Jordan and Evermann, 1897; Lefevre et al., 2014; Stecyk et al., 2020). A modified esophagus allows the blackfish to extract oxygen from the air (Crawford, 1971, 1974) which is particularly useful during summer months when temperatures are relatively high (12-15 °C) and the lakes become progressively hypoxic due to dense vegetation and poor mixing (Blackett, 1962). Indeed, laboratory studies have shown the Alaska blackfish increases air-breathing under hypoxic conditions, which may help to support the energetic demands of foraging and reproduction (Lefevre et al., 2014). However, air-breathing is no longer possible when winter arrives because the lakes become covered in thick layers of ice and snow. The barrier also prevents oxygen diffusion into the water column, which causes severe aquatic hypoxia (9% to 75% air saturation, or ~1.9 to 15.8 kPa; (Haynes et al., 2014; Lefevre et al., 2014; Leppi et al., 2016)). This predicament exposes the blackfish to a near freezing (<5 °C), oxygen-limited environment, which represents a significant metabolic challenge. When these conditions are replicated acutely in the laboratory, blackfish $\dot{M}O_2$ is reduced by ~30–50%, suggesting that aquatic respiration is not capable of sustaining metabolic processes in winter conditions (Lefevre et al., 2014).

Another remarkable trait of the Alaska blackfish is their ability to remain active when acclimatized or acclimated to cold, hypoxic conditions and restricted from air-breathing. In winter, when ambient conditions become untenable, wild Alaska blackfish migrate through the shallow drainage ditches that connect the numerous lakes and ponds of the Arctic tundra to seek out less hypoxic and/or deeper unfrozen refugia (Haynes et al., 2014; Leppi et al., 2016). The fish can also be caught by baited hook and line from waters covered with more than a meter of ice, with temperatures of between 2 and 4 °C and with P_{O2}

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ranging from 0.8 kPa at depth to 3.6 kPa (Lefevre et al., 2014). In captivity, 5 °C-acclimated Alaska blackfish chronically exposed to aquatic hypoxia (6–8 weeks at 6.3–8.4 kPa) and restricted from air-breathing actively feed and exhibit behavioral aggression towards conspecifics (Haynes et al., 2014; Lefevre et al., 2012; Stecyk et al., 2020). In aggregate, the findings suggest that this species has evolved metabolic adaptations to mitigate the effects of cold hypoxia and remain active.

Maintaining activity levels in cold hypoxic environments is metabolically challenging because a reduction in temperature and oxygen availability profoundly compromises mitochondrial ATP production by oxidative phosphorylation. Briefly, electrons derived from carbohydrates, fats, and proteins are transferred to the mitochondrial electron transport system (ETS), where they move through a series of complexes and finally bind to oxygen at complex IV. The energy released from the transfer of electrons is utilized by complexes I, III, and IV to pump protons against their electrochemical gradient and establish a proton-motive force that drives ATP production through complex V (the F1Fo ATP-synthase) (Papa et al., 2012). Although most electrons are transferred to complex IV, a small proportion slip from the ETS and bind directly to molecular oxygen to produce superoxide (Brand, 2016). When oxygen becomes limited, the ETS is inhibited and electron slip becomes more common, leading to reduced ATP production and the overproduction of ROS (Solaini et al., 2010). Although cold temperatures reduce metabolic demand which can protect the mitochondria from hypoxia, they can further compromise ATP production because rates of biochemical reactions and electron transport are reduced. In this way, the combination of cold temperatures and hypoxia can lead to tissue and organ failure unless animals make compensatory adjustments to maintain energy balance (Guderley, 2004; Guderley and Johnston, 1996).

Previous work has shown that the Alaska blackfish modifies its cardiac and metabolic physiology in order to maintain activity in cold hypoxic water (Kubly and Stecyk, 2015, 2019; Lefevre et al., 2014; Shiels et al., 2022; Stecyk et al., 2020). A reduction in temperature from 15 °C to 5 °C in normoxic Alaska blackfish causes a combination of down-regulatory, cold-compensatory and acute and perhaps direct responses to temperature, including; reduced ventricular L-type Ca^{2+} current density (Kubly and Stecyk, 2015), prolongation of action potential duration and ventricular relaxation (Kubly and Stecyk, 2019; Stecyk et al., 2020), enhanced inotropic responsiveness to adrenergic stimulation (Kubly and Stecyk, 2019), and the persistence of an impressive capability to increase metabolic rate 5- to 8-fold at cold temperature (Lefevre et al., 2014). Similarly, chronic hypoxic submergence at 5 °C elicits a shortening of action potential duration (Stecyk et al., 2020) and a remodeling of ventricular myocyte intracellular Ca^{2+} transient (Shiels et al., 2022). In addition to these adaptive responses, Alaska blackfish may remodel metabolic pathways to sustain ATP production. Indeed, cold acclimation in some cold-active teleosts leads to a compensatory increase in mitochondrial content, enzymatic activity of ETS complexes, citrate synthase activity, mitochondrial efficiency and oxidative capacities per milligram of protein (Bouchard and Guderley, 2003; Dos Santos et al., 2012; Ekström et al., 2017; St-Pierre et al., 1998; van den Thillart and Modderkolk, 1978; Wodtke, 1981; Yan and Xie, 2015), and reviewed in (Guderley, 2004). These mechanisms work together to increase the capacity of mitochondria to produce more ATP at low temperatures. In contrast to cold temperature, strategies to survive hypoxia in vertebrates often involve metabolic rate suppression to lower ATP demand and limit the production of ROS. However, results from teleosts are sparse and variable, with some species showing a reduction in mitochondrial content and oxidative capacity (shovelnose ray shark heart (*Glaucostegus typus*) and tench skeletal muscle (*Tinca tinca*) (Hickey et al., 2012b; Johnston and Bernard, 1982)), and others showing no differences (epaulette shark heart (*Hemiscyllium ocellatum*), snapper heart (*Pagrus auratus*) and killifish liver (*Fundulus heteroclitus*) (Cook et al., 2013; Du et al., 2016b; Hickey et al., 2012b), or tissue-specific effects (goldfish, *Carassius auratus L.* (Farhat et al., 2021)). Furthermore, all these studies have been conducted at acclimation temperatures >13 °C, and to the best of our knowledge, the effects of hypoxia on mitochondria from an arctic species has not been characterized. Indeed, the metabolic priorities during seasonal fluctuations may be different in temperate arctic fish, especially in species that remain active.

In addition to mitochondrial function, very little is known about the combined effects of cold temperature and hypoxia on ROS production in teleosts. Mitochondrial H_20_2 production in hypoxia-tolerant *H. ocellatum* (Hickey et al., 2012b) and *F. heteroclitus* (Du et al., 2016b) are reduced by hypoxia acclimation, and cold-acclimated salmon produce less H_20_2 than their warm-acclimated counterparts (Gerber et al., 2021). Clearly, pathways involved in mitochondrial ROS production are plastic and affected by environmental adaptation, but like respiratory capacity, the combined effects of cold temperature and hypoxia on ROS production have not been measured in an arctic cold-active species.

In light of the lack of information on temperate teleosts from the Arctic, we investigated the effects of cold acclimation (15 °C to 5 °C) under normoxic and hypoxic conditions on mitochondrial respiration and ROS production in the brain of the Alaska blackfish. We chose to study brain tissue because very little is known about seasonal remodeling of mitochondria in this tissue, and we expected it would be important in a cold-active species to maintain nervous co-ordination and motor conduction. We hypothesized that brain mitochondrial aerobic capacity would be increased to support energetic demands in fish acclimated to cold normoxia and cold hypoxia, and H_20_2 production would be suppressed to manage ROS accumulation.

2. Materials and methods

2.1. Experimental animals and exposure conditions

Animals were collected under appropriate Alaska Department of Fish and Game permitting (SF-2016-30d) and the University of Alaska Anchorage (UAA) Institutional Animal Care and Use Committee approved all procedures (852,440, 852,441 and 852,442). A total of twenty Alaska blackfish (*Dallia pectoralis*) of both sexes were utilized (body mass of 22.9 ± 5.0 g, ranging from 8.4 to 48.6 g). Fish were captured with minnow traps from Duck Hunter's Training pond (Palmer, AK, USA) in summer and transported to the UAA vivarium. Fish were maintained indoors under a 12 h:12 h light:dark photoperiod in two 300 L (190x40x40 cm WxHxD) fiberglass aquaria containing recirculating, dechlorinated and aerated water. Holding temperature initially matched natural habitat temperature (10–12 °C).

After one week of being held at 10–12 °C, fish were randomly assigned to two temperature acclimation groups: "warm" (15 °C) or "cold" (5 °C). Water temperature was increased by 0.5 °C per day to 15 °C or lowered by 1 °C per day to 5 °C (regulated using a Teco-TR20 heater/cooler system (Senkor Group, Inc., Terrell, TX, USA)) and then maintained for 5 weeks (Fig. S1). During this time, the water was aerated and the fish had access to atmospheric air. At the conclusion of the temperature acclimation period, the cold-acclimated fish were randomly assigned to be maintained in cold (5 °C) normoxia (5 N fish) or acclimated to cold hypoxia without air access (5H fish), whereas warm-acclimated fish continued to be maintained in warm (15 °C) normoxic conditions (15 N fish). The exposures occurred in parallel and sampling did not occur until the conclusion of the 5-week cold, hypoxia acclimation period to avoid an acclimation duration x temperature treatment effect (Fig. S1).

5H fish were exposed to chronic hypoxic submergence following procedures previously described (Stecyk et al., 2020). Briefly, 5H fish were denied access to atmospheric O_2 by the placement of an impenetrable grate (1 cm^2) 10 cm below the water surface, and water P_{O2} was progressively lowered (by ~3–4 kPa every 3 days) and then maintained between 2.1 kPa and 4.2 kPa. The final level of hypoxia was selected to represent the lower limits of the range of dissolved oxygen levels (~1.9

to 15.8 kPa) that Alaska blackfish experience in winter in their natural environment (Leppi et al., 2016). Water P_{O2} was measured and maintained at appropriate levels using a one-channel oxygen regulator system (Loligo Systems, Tjele, Denmark) that regulated the bubbling of the water with 100% N_2. The system was calibrated daily following the manufacturer's protocol and confirmed once or twice a day using a fibre optic FDO 925 oxygen probe and Multi 3410 m (WTW, Weilheim, Germany).

2.2. Animal husbandry

Animal husbandry followed protocols previously detailed (Shiels et al., 2022). Briefly, fish were fed bloodworms ad libitum bi-daily throughout the temperature acclimation and experimental exposure periods, but food was withheld 24 h prior to experimental measurements. Water changes occurred weekly to maintain levels of nitrite, nitrate, and ammonium below recommended levels (Tetra EasyStrips, Tetra, Blacksburg, VA, USA). For 5 N and 5H fish, the fresh water added to the tanks was pre-chilled to 5 °C to ensure minimal temperature fluctuation. Also, to prevent 5H fish from gaining air access and/or experiencing increased water oxygen levels, water changes were accomplished with as little disturbance to the water column as possible, without lowering the water level below the level of the submerged grating and with the water pre-bubbled with 100% N_2 to match the oxygen level within the tank.

2.3. Isolation of brain homogenates

Fish were euthanized with an overdose of buffered tricaine methanesulfonate (MS-222; 1.0 g l^{-1} + 1.0 g l^{-1} $NaHCO_3$; Sigma Aldrich, St. Louis, MO, USA; temperature and oxygen concentration of the MS-222 solution matched that of the acclimation condition of the fish) and the brain was dissected, weighed and cleared of any connective tissue. Homogenates were prepared by adapting previous protocols for heart tissue (Hellgren et al., 2021). Briefly, the brain tissue was rinsed with ice-cold phosphate buffered saline and minced into small pieces with a razor blade. The tissue was resuspended in ice-cold respiration media (EGTA 0.5 mM, $MgCl_2$ 3 mM, K-MES 60 mM, KH_2PO_4 10 mM, HEPES 20 mM, sucrose 110 mM and 1% BSA) and homogenized (IKA Ultra-turrax T25). The homogenate was filtered with nylon mesh (1cm^2) and 0.19 ± 0.02 mg ml^{-1} was injected into each of the two chambers of an Oroboros Oxygraph-2 K for measurement of mitochondrial respiration and H_2O_2 production (see below). The rest of the homogenate was frozen at −80 °C for analysis of protein and citrate synthase activity.

2.4. Mitochondrial respiration and H_2O_2 production

Respiration and H_2O_2 production were measured simultaneously with an Oxygraph O_2-k high-resolution respirometry system (Oroboros Instruments GmbH, Innsbruck, Austria), fitted with an O_2k-fluorescence LED2-module. Two identical respiration chambers were run in parallel for each experiment and held at a common intermediate test temperature (10 °C) with saturating levels of oxygen ([O_2] remained above 100 nm ml^{-1} throughout the protocol). This experimental design allowed us to reveal the effects of acclimation on mitochondrial respiration and ROS production, because any differences between the experimental groups at a common test temperature and saturating levels of oxygen indicate a chronic change, rather than acute. Oxygen electrodes were calibrated every morning with air-saturated respiration medium. To measure H_2O_2 production, 10 μM Amplex® UltraRed and 1 U/ml horseradish peroxidase (HRP) were added to each chamber. Amplex® UltraRed oxidizes in the presence of H_2O_2 and forms resorufin, using HRP as a catalyst. Amplex® UltraRed was excited at 563 nm and emission was read at 587 nm. 5 U/ml superoxide dismutase (SOD) was also added to the chambers to convert any extramitochondrial superoxide ($O_2^{\cdot-}$) to H_2O_2. At the beginning of each experiment, brain homogenates (9.7–29.4 μg protein ml^{-1}) were added to each chamber containing 2 ml of respiration medium, and the Amplex UltraRed signals were calibrated with known quantities of exogenously added H_2O_2.

2.5. Substrate inhibitor protocols

2.5.1. Protocol 1: Aerobic capacity and H_2O_2 production

We measured three main variables commonly used to assess mitochondrial aerobic capacity. Firstly, leak respiration rate (Leak) is the amount of oxygen used to compensate for proton leak across the mitochondrial inner membrane. Secondly, we measured OXPHOS respiration rate, which is the maximum rate of ADP-stimulated respiration and is a measure of mitochondrial ATP production. Lastly, we used a protonophore, Carbonyl cyanide-*4*-(trifluoromethoxy)phenylhydrazone (FCCP), to uncouple mitochondrial respiration and measure the maximal respiration rate of the ETS, otherwise known as maximum electron transfer (ET) capacity. We measured these three parameters with different substrate combinations using a standard substrate inhibitor titration protocol (SUIT protocol), designed according to Pesta and Gnaiger (2012).

An original trace of a representative SUIT protocol is presented in Fig. 1. First, pyruvate (5 mM), malate (2 mM), and glutamate (10 mM) were added to achieve leak respiratory state with complex-I (CI) substrates in the absence of adenylates ($Leak_{CI}$). When O_2 consumption was stable, saturating ADP (5 mM) was injected to activate oxidative phosphorylation with either CI substrates ($OXPHOS_{CI}$). Succinate (10 mM) was then added to assess respiration in the presence of CI and CII substrates ($OXPHOS_{CI+CII}$). In some experiments, succinate (10 mM) was also added prior to ADP to measure leak in the absence of adenylates with complex I and II substrates ($Leak_{CI+CII}$). To uncouple mitochondria and assess ET with CI and CII substrates (ET_{CI+CII}), FCCP was titrated to a

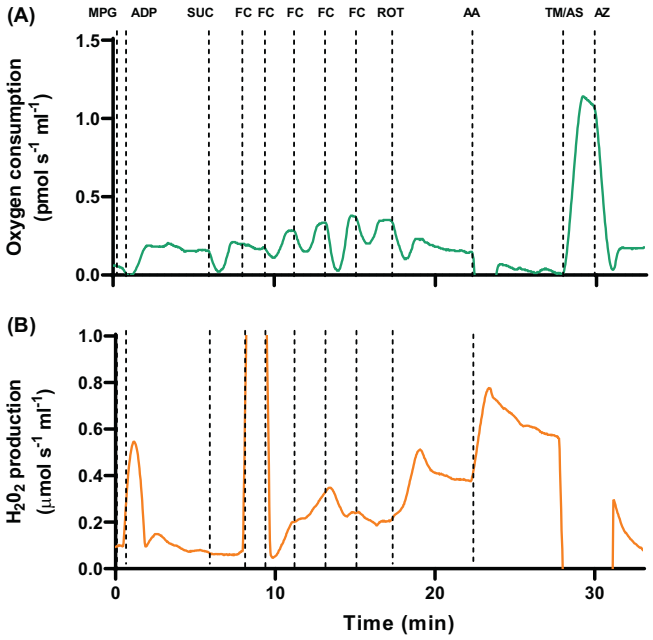

Fig. 1. Original trace of simultaneous measurement of mitochondrial oxygen consumption and H_2O_2 production in Alaska blackfish brain mitochondria. Data is taken from brain mitochondria that was isolated from a warm-acclimated (15 N) fish. Mitochondria were added to the chamber and a range of substrates and inhibitors were injected to investigate the electron transport system (see Materials and Methods for full details). Abbreviations: MPG, malate, pyruvate and glutamate; ADP, adenosine diphosphate; SUC, succinate; FC, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP, this was titrated until respiration reached a plateau); ROT, rotenone; AA, antimycin-A; TM, *N*,*N*,*N*,*N*-tetramethyl-*p*-phenylenediamine (TMPD); AS, ascorbate; AZ, azide.

final concentration of 0.1–0.3 μM. Next, the CI inhibitor rotenone (0.5 μM) was added to assess ET_{CI+CII}, with CII substrates only. To block the ETS and assess residual non-mitochondrial O_2 consumption (ROX), the complex-III (CIII) inhibitor antimycin-A (2.5 μM) was added. To assess complex-IV (CIV) activity in isolation, the electron donor *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD; 0.5 mM) was added in combination with ascorbate (2 mM) to avoid autooxidation of TMPD. Lastly, the CIV inhibitor sodium azide (50 mM) was added to assess background non-mitochondrial O_2 consumption from the addition of TMPD. Values for mitochondrial respiration and H_2O_2 production were normalized to the homogenate protein content (Bradford assay, see below).

2.5.2. Protocol 2: Determination of the apparent affinity of ADP stimulated respiration

Pyruvate (5 mM), malate (0.25 mM) and succinate (10 mM) were used to provide maximum substrate stimulation with complex I and II substrates. ADP was then added in steps from 0.5 μM to 500 μM and respiration rate was monitored at each [ADP]. A one-site saturation Michael–Menten non-linear regression model was used to describe the effects of changes in [ADP] on homogenate respiration rate and to determine the apparent affinity (K_m) of ADP stimulated respiration.

2.6. Protein content and citrate synthase analysis

Protein content of brain homogenates was assessed using the Quick start Bradford dye reagent kit (Bio-Rad laboratories, Watford, UK) and reading absorbance at 550 nm (BioTek Synergy HTX multimode reader). We also measured citrate synthase activity as an estimate of mitochondrial volume density (Larsen et al., 2012), as previously described (Galli et al., 2013). Briefly, homogenates were centrifuged at 600*g* for 10 min and resuspended in assay buffer that contained 50 mM Tris·HCl, pH 8.0. The maximal enzymatic activity (V_{max}) was monitored in the presence or absence of oxaloacetate by the appearance of 5-thio-2-nitrobenzoic acid as a result of the reaction of free acetyl-CoA with 5,5′-dithiobis(2-nitrobenzoic acid) at 412 nm over a 10-min incubation period (assay buffer with 0.5 mM oxaloacetate, 0.3 mM acetyl-CoA, 0.15 mM 5,5-dithiobis-2-nitrobenzoic acid). Unfortunately, two citrate synthase samples were damaged during processing (one 15 N and one 5H). However, the remaining samples were run in duplicate within the same plate, and a separate technical replicate was also obtained. Enzyme activities were normalized to total soluble protein, which was quantified according to the Bradford assay.

2.7. Statistical analysis

To estimate mitochondrial efficiency, the OXPHOS coupling efficiency ratio (1-L/P) was calculated as $1\text{-}Leak_{T,CI}/OXPHOS_{CI}$. The OXPHOS control ratio (P/E ratio) was calculated as $OXPHOS_{CI+CII}/ET_{CI+CII}$. Results are reported as means ± SEM (n represents number of fish) and statistical analysis was performed using GraphPad Prism 9.2 (GraphPad Sofware, San Diego, CA, USA). Data were first checked for normality by generating quantile-quantile plots, and given that some data were not normally distributed, all data were log transformed before performing a one-way ANOVA with a Tukey's multiple comparison test. For citrate synthase analysis, a nested one-way ANOVA was performed to take into account the technical replicate. Differences were considered statistically significant when $P < 0.05$.

3. Results

Brain mitochondrial preparations were of high quality, as attested by good OXPHOS-coupling efficiency ratios (0.74 ± 0.03) and respiratory control ratios (4.09 ± 0.39) with CI substrates (malate, pyruvate and glutamate). Furthermore, mitochondrial oxygen consumption and H_2O_2 production responded to substrates and inhibitors in the expected manner (Pesta and Gnaiger, 2012) (Fig. 1).

While citrate synthase activity was significantly elevated in hypoxia acclimated fish compared to the warm acclimated group (Fig. 2), there were no significant differences in aerobic capacity (Fig. 3A-G), coupling efficiency (Fig. 3H-J) or ADP affinity (Fig. 4) between any of the three experimental groups. However, compared to warm acclimated normoxic fish, mitochondrial H_20_2 production was significantly supressed in cold acclimated normoxic and cold acclimated hypoxic Alaska blackfish in $LEAK_{CI}$ and $ETSC_{II}$ states, as well as antimycin A treatment (Fig. 5A, E and F). This effect was also evident in the $OXPHOS_{CI}$ state in the cold and hypoxia acclimated group (Fig. 5B).

4. Discussion

The Alaska blackfish is the only air-breathing fish in the Arctic, and one of few species that remain active in cold hypoxic waters. Previous studies have shown that the Alaska blackfish maintains activity in cold hypoxic water by modifying its cardiac and metabolic physiology (Kubly and Stecyk, 2015, 2019; Lefevre et al., 2014; Shiels et al., 2022; Stecyk et al., 2020). Here we show that physiological plasticity is also evident in the brain; cold acclimation from 15 °C to 5 °C in normoxia led to a suppression of H_20_2 production, which persisted and became more pronounced in fish chronically exposed to 5 °C and aquatic hypoxia (restricted air access). However, mitochondrial aerobic capacity was similar between all three experimental groups when measured at the common temperature of 10 °C. These results suggest Alaska blackfish brain mitochondria prioritize ROS management over ATP production during winter, which presumably protects the brain from oxidative stress and cellular dysfunction.

4.1. Cold acclimation under normoxic and hypoxic conditions lead to a suppression of mitochondrial H_20_2 production

Despite the ecological implications, very little is known about the effects of thermal acclimation or chronic hypoxia on mitochondrial ROS production in fishes. Although cold temperatures are associated with lower mitochondrial respiration rates which should reduce ROS production, cold acclimation in fishes often leads to an increase in membrane unsaturated fatty acid content (Cossins et al., 1977; Hazel, 1995). The increase in unsaturation is an adaptive response which maintains membrane fluidity at cold temperatures, but unfortunately this also renders the membranes more sensitive to lipid peroxidation (Guderley, 2004). The problem can also be exacerbated by the well-known increase in mitochondrial content and capacity that occurs with cold acclimation

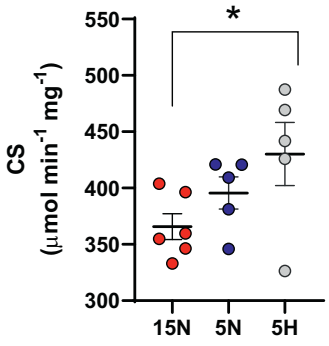

Fig. 2. Effect of cold and hypoxic acclimation on Alaska blackfish brain citrate synthase activity. Citrate synthase activity was measured in Alaska blackfish brain from warm normoxic (15 N, red circles, $n = 6$), cold normoxic (5 N, blue circles, n = 6) and cold hypoxic (5H, grey circles, $n = 5$) acclimations. Statistical significance was assessed with a nested one-way ANOVA, followed by Tukey's post-hoc tests. Values were considered significant when $P < 0.05$, which are denoted by asterisks (*). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

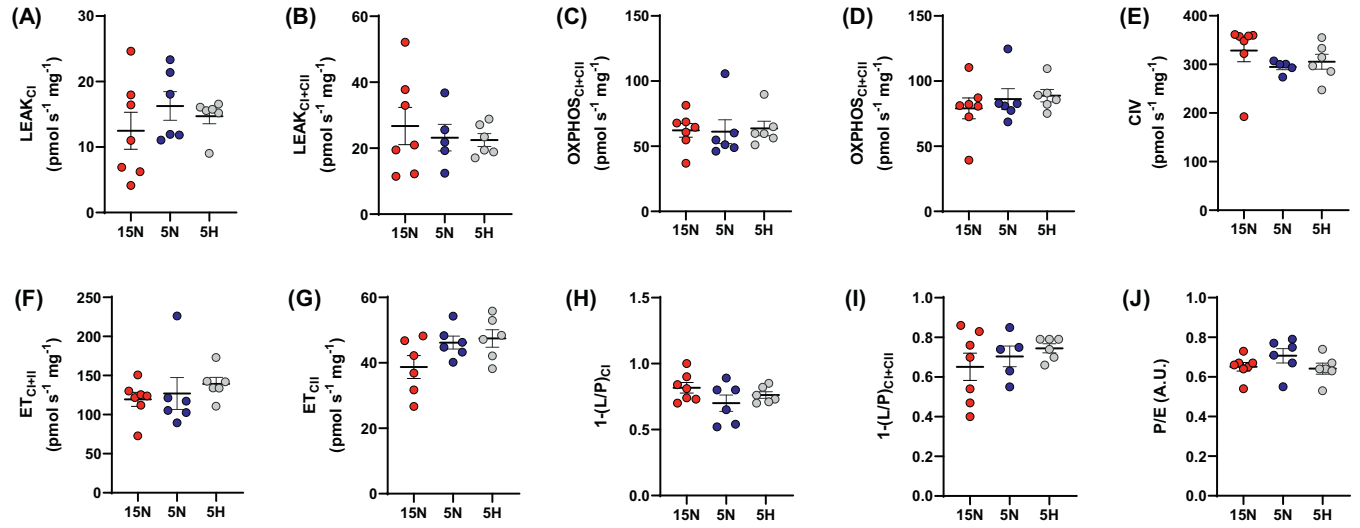

Fig. 3. Effect of cold and hypoxic acclimation on Alaska blackfish mitochondrial O_2 consumption. Mitochondrial O_2 consumption was measured in Alaska blackfish from warm normoxic (15 N, red circles, $n = 7$), cold normoxic (5 N, blue circles, n = 6) and cold hypoxic (5H, grey circles, n = 6) acclimation conditions. Each panel represents a different respiratory state. (A) Leak respiration with substrates for complex I, in the presence of adenylates ($Leak_{CI}$). (B) Leak with substrates for complex I and II ($Leak_{CI+CII}$). (C) Oxidative phosphorylation, with substrates for complex I ($OXPHOS_{CI}$). (D) OXPHOS with substrates for complexes I and II ($OXPHOS_{CI+CII}$). (E) Respiration through complex IV (CIV). (F) Electron-transfer capacity, with substrates for complexes I and II (ET_{CI+CII}). (G) ET with substrates for complex II (ET_{CII}). (H) oxidative efficiency control ratio with substrates for complex I ($1\text{-}L/P_{CI}$), (I) 1-L/P with complex I and II substrates ($1\text{-}L/P_{CI+CII}$). (J) phosphorylation efficiency ratio (P/E). Statistical significance was assessed with one-way ANOVA, followed by Tukey's post-hoc tests. Values were considered significant when $P < 0.05$, which are denoted by asterisks (*). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

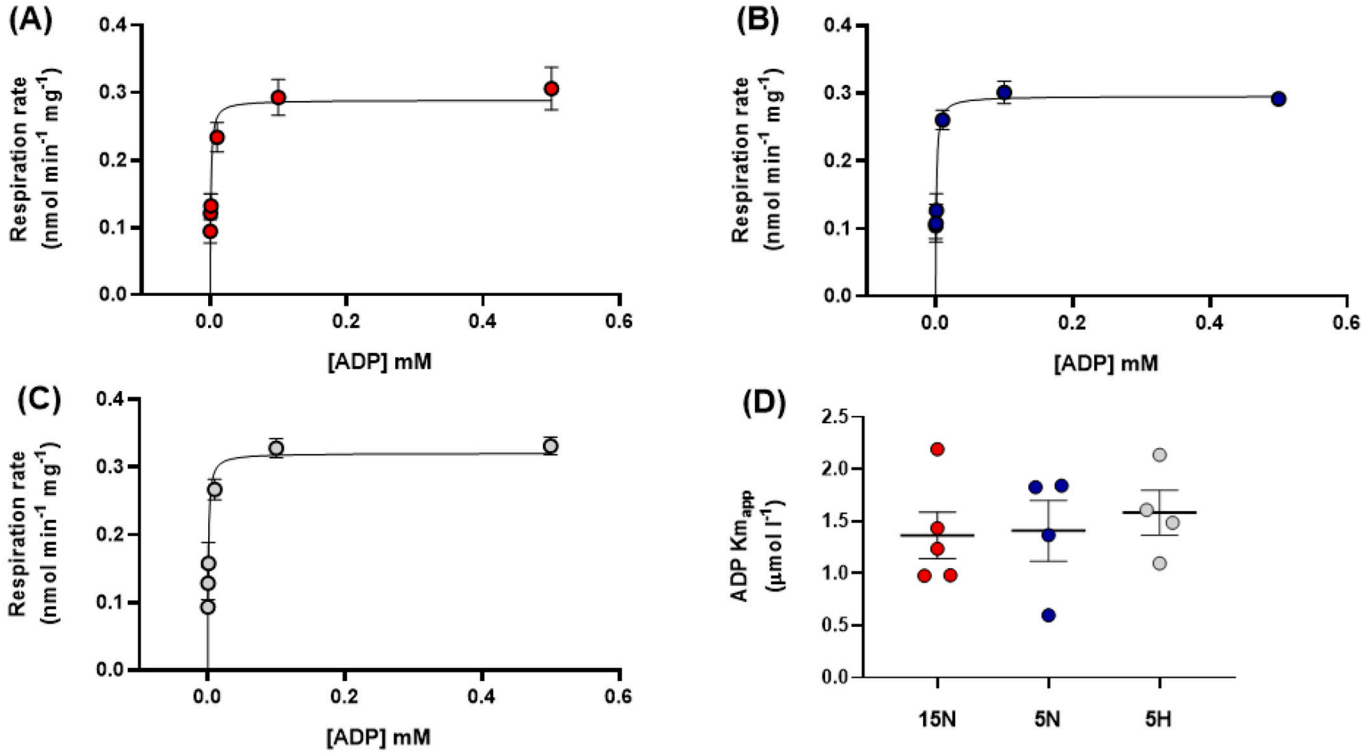

Fig. 4. Effect of temperature and hypoxia acclimation on the apparent affinity for total ADP of Alaska blackfish brain homogenates. ADP dependent respiration was determined in warm acclimated normoxic blackfish (15 N, red circles; Panel A), cold acclimated normoxic blackfish (5 N, blue circle; Panel B), and cold hypoxic acclimated blackfish (5H, grey circles; Panel C). For each experimental group, ADP concentration giving half maximal respiration rate (ADP K_m) was determined by fitting a one-site saturation Michael–Menten non-linear regression curve to the ADP curves (Panel D, values are mean ± SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(see below), which is expected to increase mitochondrial ROS production. Therefore, we expected that cold acclimated normoxic Alaska blackfish may modify mitochondrial properties to avoid oxidative stress. Indeed, here we show that acclimation form 15 °C to 5 °C in normoxia caused a suppression of H_20_2 production in Alaska blackfish brain mitochondria, and that this effect was still apparent and more

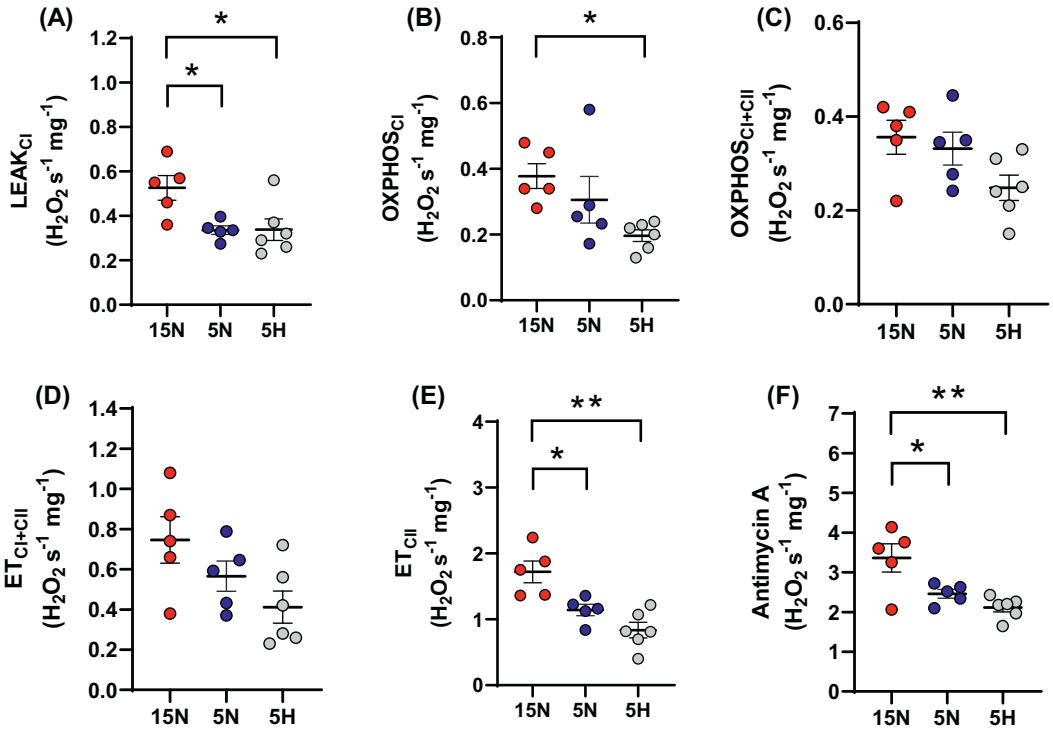

Fig. 5. Effect of cold and hypoxic acclimation on Alaska blackfish mitochondrial H_2O_2 production. Mitochondrial H_2O_2 production was measured in blackfish from warm normoxic (15 N, red circles, n = 7), cold normoxic (5 N, blue circles, n = 6) and cold hypoxic (5H, grey circles, n = 6) acclimations. Each panel represents a respiratory state. (A) Leak respiration with substrates for complex I, in the absence of adenylates (Leak$_{N,CI}$). (B) Oxidative phosphorylation, with substrates for complex I (OXPHOS$_{CI}$). (C) Oxidative phosphorylation with substrates for complexes I and II (OXPHOS$_{CI+CII}$). (D) Electron-transfer capacity, with substrates for complexes I and II (ET$_{CI+CII}$). (E) Electron-transfer capacity, with substrates for complex II (ET$_{CII}$). (F) Electron-transfer capacity in the presence of antimycin-A. Statistical significance was assessed with one-way ANOVA, followed by Tukey's post-hoc tests. Values were considered significant when $P < 0.05$, which are denoted by asterisks (*). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

pronounced when cold acclimated fish were exposed to chronic hypoxia (Fig. 5). Therefore, our data suggests the management of ROS is a priority for overwintering Alaska blackfish, and suppression of mitochondrial H_2O_2 production may protect the brain from oxidative stress. Indeed, for vertebrate species that experience prolonged periods of oxygen deprivation during the winter months, such as the crucian carp (*Carassius carassius*) and freshwater turtles of the genus *Trachemys and Chrysemys*, cold acclimation in normoxia serves as an important cue for the priming of physiological processes for winter anoxia survival (Hochachka, 1986; Jackson, 2000; Stecyk, 2017).

Very few studies have attempted to quantify the effects of thermal acclimation on mitochondrial ROS production in fish tissue, and those that have are contradictory. A recent study in Atlantic salmon (*Salmo salar*) showed that 12 °C acclimated fish had significantly higher rates of cardiac mitochondrial ROS production than 20 °C acclimated fish (Gerber et al., 2020). This finding contrasts with studies on *F. heteroclitus* liver and temperate wrasse (*Notolabrus celidotus*) heart which found no effect of cold acclimation on H_2O_2 production across a range of acute temperatures (Chung and Schulte, 2015; Iftikar et al., 2015). With regards to hypoxia, *F. heteroclitus* and the doublespot acara (*Aequidens pallidus*) had suppressed liver mitochondrial H_2O_2 production after hypoxic acclimation (Du et al., 2016a; Heinrichs-Caldas and de Almeida-Val, 2021), but there were no changes in the flag cichlid (*Mesonauta festivus*) (Heinrichs-Caldas and de Almeida-Val, 2021). Lastly, 24 h of hypoxia in ocean quahog (*Arctica islandica*) led to an increase in hepatopancreas ROS production in the LEAK state, whereas it was suppressed in the OXPHOS state (Steffen et al., 2021). These studies highlight the large variation in redox responses that are evident in fish acclimating to different temperatures and oxygen regimes. It is difficult to identify an emerging pattern with such a small number of studies, but it is likely that differences may relate to the species habitat, activity level or tissue type.

While we did not directly investigate the mechanism for the suppression of mitochondrial H_2O_2 production in the Alaska blackfish brain, our experimental protocol allows us to speculate. For example, it seems unlikely that the suppression is related to complexes I or II, because the reduction was evident in the presence of rotenone and antimycin A, which block the CI_Q and $CIII_{Qi}$ ROS sites, respectively (Brand, 2016). In the presence of these inhibitors, ROS production commonly occurs at the $CIII_{Qo}$ site, which is a major source of oxidative stress in the postischemic mammalian heart (Chen and Zweier, 2014). Nevertheless, pharmacological inhibition of the $CIII_{Qo}$ site (e.g. with myxothiazol or stigmatellin) would be necessary to confirm its involvement because other sites proximal to CIII can contribute to ROS production under these conditions (Brand, 2016). Alternatively, other factors may explain our results, including differential antioxidant profiles. Indeed, several studies have shown acclimation to cold temperature and/or hypoxia increases enzymatic activity of antioxidants in the brain of many fish, including superoxide dismutase and catalase (Du et al., 2016a; Johannsson et al., 2018; Lushchak et al., 2005; Malek et al., 2004; Tseng et al., 2011). However, this response is not ubiquitous, and antioxidant responses are highly species and tissue dependent (Leveelahti et al., 2014). Clearly, further research is necessary to confirm the site or source of suppressed H_2O_2 production in the cold hypoxic Alaska blackfish.

4.2. *Cold acclimation under normoxic or hypoxic conditions had no effect on mitochondrial aerobic capacity or ADP affinity*

A large body of work has shown that cold acclimation leads to skeletal and cardiac muscle mitochondrial remodeling in cold-active species (reviewed in (Guderley, 2004)). The most common response to

cold temperature is an increase in mitochondrial volume density which serves to enhance aerobic capacity (per mg tissue) and maintain ATP production in the face of temperature-induced reductions in enzymatic reactions. This response has been found in a large range of cold-tolerant temperate species, including stickleback (*Gasterosteus aculeatus*), European eel (*Anguilla anguilla L.*), *C. auratus* and *C. carassius* (Dos Santos et al., 2012; Egginton and Johnston, 1984; Egginton and Sidell, 1989; Johnston and Maitland, 1980; Orczewska et al., 2010; Tyler and Sidell, 1984). In addition to increasing the surface area of mitochondria, cold acclimation can increase the oxidative capacity of mitochondria by enhancing enzymatic activity of ETS complexes, as seen in European carp (*Cyprinus carpio*), stickleback (*Pungitius pungitius and G. aculeatus*), chain pickering (*Esox niger*), rainbow trout (*Oncorhynchus mykiss*) and *F. heteroclitus* (Grim et al., 2010; Guderley and Foley, 1990; Guderley et al., 1994; Itoi et al., 2003; Kleckner and Sidell, 1985; Kraffe et al., 2007)). As expected, these modifications are associated with higher rates of mitochondrial respiration (per mg of tissue or protein) at a common test temperature (Dos Santos et al., 2012; Guderley and Johnston, 1996; Kraffe et al., 2007). However, in the present study, we found cold acclimated normoxic Alaska blackfish had similar rates of brain mitochondrial respiration as warm acclimated fish, when tested at a common temperature (Fig. 3). This result aligns well with previous work that found that total oxygen consumption and in vivo heart rate of 5 °C and 15 °C acclimated Alaska blackfish varied with a Q_{10} of ~2, which suggests no thermal compensation, but rather that the fish allows metabolic rate to be influenced by the acute and perhaps direct effects of temperature (Lefevre et al., 2014; Stecyk et al., 2020). Therefore, if we apply these Q_{10} effects to our data, we can expect Alaska blackfish mitochondrial respiration rates (and therefore ATP production) to be approximately 50% lower at 5 °C than at 15 °C.

There may be several reasons for the lack of thermal compensation of mitochondrial aerobic capacity in cold acclimated Alaska blackfish. Firstly, the lack of compensation may reflect a trade-off between ATP production and ROS management. For example, increasing mitochondrial aerobic capacity will enhance ATP production at cold temperatures, but this will come at the cost of greater proton leak which will be energetically costly, as well as increased electron slip which will lead to higher levels of ROS production (Guderley, 2004). This trade-off has been suggested to explain the incomplete compensation of mitochondrial aerobic capacities in *O. mykiss* during seasonal acclimatization (Guderley, 2004). Alternatively, the vast majority of studies investigating mitochondrial remodeling in fish have focused on skeletal or cardiac muscle, so our results may represent tissue-specific differences. However, while some studies have shown brain mitochondria are insensitive to thermal acclimation (e.g. catfish (Yan and Xie, 2015), others have shown cold temperature leads to an increase in mitochondrial oxidative capacity (*O. mykiss*; (Evans et al., 1962)) and enzymatic activity (*C. auratus* and *E. niger* (Caldwell, 1969; Kleckner and Sidell, 1985)).

Similar to cold acclimation, exposure to chronic hypoxia at 5 °C had no effects on Alaska blackfish mitochondrial aerobic capacity despite an increase in citrate synthase activity. Indeed, mitochondrial respiration rates were similar between all experimental groups, and across all respiratory states, when tested at a common temperature (Fig. 3). Previous work has shown acute hypoxia (~2.5 kPa for 6 h) reduces oxygen consumption in cold acclimated Alaska blackfish, indicating that they were unable to extract sufficient oxygen from the water to maintain standard metabolic rate. Given that the level of hypoxia in our study was below the Alaska blackfish critical oxygen tension (P_{Crit}, ~5 kPa), it can be assumed that the fish was hypoxemic and the mitochondria were oxygen deprived, which imposes significant limitations on ATP generation and the overproduction of ROS (Richards, 2009). To compensate for these problems, previous work has shown chronic hypoxia leads to a down regulation of mitochondrial density and capacity in a wide variety of vertebrates, including mammals (reviewed in (Murray and Horscroft, 2016)), reptiles (Bundgaard et al., 2019; Galli et al., 2013), amphibians (St-Pierre et al., 2000; Storey et al., 2021) and fish (e.g. zebrafish (*Danio rerio*), *G. typus*, mudsuckers (*Gillichthys mirabilis*) and *C. auratus* (Gracey et al., 2001; Hickey et al., 2012a; Meer et al., 2005). While it might seem counterintuitive to reduce aerobic capacity when oxygen is limited, this strategy is thought to limit ROS production (Murray and Horscroft, 2016), similar to cold acclimation. Nevertheless, several fish species do not show any change in mitochondrial respiration in response to hypoxia (including sablefish (*Anoplopoma fimbria*), *P. auratus*, *F. heteroclitus* and *H. ocellatum* (Cook et al., 2013; Du et al., 2016a; Gerber et al., 2019; Hickey et al., 2012a)), and *C. carassius* actually increase mitochondrial density (Johnston and Bernard, 1984). These species-specific differences in response to hypoxia likely relate to different habitats and activity levels, and the associated trade-off between ATP production and ROS generation. Given that we found a suppression of ROS in the cold hypoxic Alaska blackfish (see below), our data suggests this fish may prioritize low levels of ROS in hypoxia at the expense of reduced ATP production.

While we saw no change in mitochondrial respiration with cold or hypoxic acclimation, we found an increase in citrate synthase activity in hypoxic acclimated fish, suggesting that mitochondrial content was elevated. Interestingly, compared to their red-blooded relatives, icefish that lack hemoglobin have an increased mitochondrial density without any change in aerobic capacity; this adaptation is thought to enhance oxygen diffusion across the membrane by reducing intracellular oxygen diffusion gradients (O'Brien and Mueller, 2010). In these species, cristae density is similar to the red-blooded icefish, which explains the similar respiration rates despite the greater mitochondrial volume. Clearly, it is necessary to confirm and expand on the citrate synthase findings in the present study with electron microscopy imaging of Alaska blackfish mitochondrial morphology.

5. Conclusions and perspectives

Taken together, our data suggests the management of ROS is a priority for overwintering Alaska blackfish. The lack of metabolic compensation alongside the active suppression of H_20_2 production may protect the brain from oxidative stress, but this could come at a cost of reduced ATP production. This could be a problem for a cold-active species which needs to maintain nervous co-ordination and motor conduction. Nevertheless, it is possible that glycolytic energy production is upregulated to maintain energy balance in the overwintering Alaska blackfish. In this respect, it would be interesting to measure the effects of cold temperature and hypoxia exposure on glycolytic enzymes and markers of metabolic status (e.g. ATP/ADP ratio, lactate production and Gibbs free energy of ATP hydrolysis). Future work should also investigate the seasonal changes in mitochondrial substrate preference of Alaska blackfish, because hypoxia causes the brain to switch from carbohydrate to lipid metabolism in goldfish (Farhat et al., 2021). Lastly, the laboratory acclimations that we used in this study are not fully reflective of the cues that Alaska blackfish experience between seasons (e.g. changes in photoperiod). These environmental signals may be important for mitochondrial remodeling and should be considered in future studies.

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Author contributions

Conceptualization: J.A.W·S., H.A.S, E.W, C.S.C., G.G.; Investigation: J.A.W·S., C.S.C., G.G.; Formal analysis: G.G.; Writing - original draft: G.G.; Writing - review & editing: J.A.W.S, H.A.S, E.W, C.S.C., G.G.; Project administration: J.A.W.S.; Funding acquisition: J.A.W.S.

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Data sharing

Stored in repository: The data that support the findings of this study are openly available in figshare at DOI: https://doi.org/10.6084/m9.figshare.19513999

Declaration of Competing Interest

We are not aware of any conflict of interest arising from this work.

Data availability

Data will be made available on request.

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