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RESEARCH ARTICLE

Taurine depletion impairs cardiac function and affects tolerance to hypoxia and high temperatures in brook char (*Salvelinus fontinalis*)

Toni-Anne M. Dixon[1], Emma-Lee M. Rhyno[1], Nir El[1], Samuel P. McGaw[1], Nathan A. Otley[1], Katya S. Parker[1], Elena C. Buldo[1], Claire M. Pabody[1], Mireille Savoie[2], Amanda Cockshutt[3], Andrea J. Morash[2], Simon G. Lamarre[4] and Tyson J. MacCormack[1,*]

ABSTRACT

Physiological and environmental stressors can cause osmotic stress in fish hearts, leading to a reduction in intracellular taurine concentration. Taurine is a β-amino acid known to regulate cardiac function in other animal models but its role in fish has not been well characterized. We generated a model of cardiac taurine deficiency (TD) by feeding brook char (*Salvelinus fontinalis*) a diet enriched in β-alanine, which inhibits cardiomyocyte taurine uptake. Cardiac taurine levels were reduced by 21% and stress-induced changes in normal taurine handling were observed in TD brook char. Responses to exhaustive exercise and acute thermal and hypoxia tolerance were then assessed using a combination of *in vivo*, *in vitro* and biochemical approaches. Critical thermal maximum was higher in TD brook char despite significant reductions in maximum heart rate. *In vivo*, TD brook char exhibited a lower resting heart rate, blunted hypoxic bradycardia and a severe reduction in time to loss of equilibrium under hypoxia. *In vitro* function was similar between control and TD hearts under oxygenated conditions, but stroke volume and cardiac output were severely compromised in TD hearts under severe hypoxia. Aspects of mitochondrial structure and function were also impacted in TD permeabilized cardiomyocytes, but overall effects were modest. High levels of intracellular taurine are required to achieve maximum cardiac function in brook char and cardiac taurine efflux may be necessary to support heart function under stress. Taurine appears to play a vital, previously unrecognized role in supporting cardiovascular function and stress tolerance in fish.

KEY WORDS: β-Alanine, Heart rate, Critical thermal maximum, Hypoxic bradycardia, Isolated perfused heart, Regulatory volume decrease, Osmorespiratory compromise

INTRODUCTION

In fish, environmental or physiological stressors that increase oxygen (O_2) demand or reduce O_2 supply have the potential to disturb the osmotic homeostasis of tissues. Exercise, high temperatures and hypoxia, for example, are associated with enhanced branchial perfusion (Gonzalez and McDonald, 1992; Holeton and Randall, 1967; Onukwufor and Wood, 2020), which in freshwater fish comes at the expense of ion loss (Wood and Eom, 2021). Osmotic homeostasis in tissues can also be disrupted if O_2 supply is insufficient to meet demand; in hypoxic or ischemic cardiac muscle, the accumulation of anerobic end products can increase intracellular osmolality by >50 mmol l^{-1} (Steenbergen et al., 1985; Tranum-Jensen et al., 1981). A decrease in extracellular osmolality or increase in intracellular osmolality leads to an influx of water, cell swelling and the activation of regulatory volume decrease mechanisms in cardiomyocytes. The subsequent efflux of ions and intracellular osmolytes, the most important being the β-amino acid taurine (Vislie, 1980a,b; Vislie and Fugelli, 1975), restores osmotic balance, normalizes cell volume and prevents dysfunction (Chara et al., 2011). Taurine is the most abundant free amino acid in cardiac muscle, reaching concentrations of 45–67 mmol l^{-1} in salmonids (Fugelli and Vislie, 1982; Gates et al., 2022; Gras et al., 1982). In both mammals and cephalopods, its actions extend beyond that of just an osmolyte, as it exhibits cardioprotective properties and regulates heart function and metabolism (MacCormack et al., 2016; Schaffer et al., 2010, 2014). Taurine likely functions similarly in fish (Gates et al., 2022; Henry and MacCormack, 2018), but its contribution to supporting cardiovascular function and stress tolerance is essentially unknown.

The maintenance of aerobic performance under thermal stress is tied to a fish's capacity to increase cardiac output ($\dot{Q}$) proportionally with temperature (Eliason and Anttila, 2017; Farrell, 2009). There is no clear consensus on the mechanism(s) responsible for dictating upper thermal maxima in fish (Portner and Knust, 2007; Jutfelt et al., 2018), but delivery of oxygen to the brain plays an important role (Andreassen et al., 2022). In salmonids, stroke volume (V_S) does not increase with acute warming because of limitations in venous return (Sandblom and Axelsson, 2007), so $\dot{Q}$ is constrained by the capacity to increase heart rate (f_H) with temperature. In inactive salmonids, upper thermal tolerance correlates with maximum heart rate ($f_{H,max}$; Farrell, 2009), which is set by the thermal sensitivity of electrical excitability in the heart (Haverinen and Vornanen, 2020). The Na^+ current (I_{Na}) responsible for overcoming the resting K^+ current (I_K) and depolarizing the cardiomyocyte to generate an action potential is carried by inward Na^+ flux through voltage-gated Na^+ channels on the sarcolemmal membrane (Vornanen et al., 2002). I_K rises with temperature and I_{Na} must increase in concert to trigger an action potential. I_{Na} is more thermally sensitive than I_K in salmonids, which leads to atrio-ventricular blockade and arrhythmia as temperatures approach the animal's upper lethal limit (Haverinen and Vornanan, 2020). I_{Na}

[1]Department of Chemistry and Biochemistry, Mount Allison University, Sackville, NB, Canada, E4L 1E4. [2]Department of Biology, Mount Allison University, Sackville, NB, Canada, E4L 1E4. [3]Department of Chemistry, Saint Francis Xavier University, Antigonish, NS, Canada, B2G 2W5. [4]Departement de Biologie, Université de Moncton, Moncton, NB, Canada, E1A 3E9.

*Author for correspondence (tmaccormack@mta.ca)

A.C., 0000-0003-3024-2687; A.J.M., 0000-0001-6161-9999; S.G.L., 0000-0001-7038-4626; T.J.M., 0000-0002-6080-9413

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kinetics can be affected by a variety of factors in ventricular myocytes, including taurine. Electrophysiology studies have illustrated that taurine has significant but variable effects on I_{Na} in vertebrate cardiomyocytes (Sada et al., 1996, and references therein), the mechanisms behind which are still unclear. In isometrically contracting ventricular muscle preparations, maximum pacing frequency was significantly lower in taurine-deficient rainbow trout hearts (Gates et al., 2022), suggesting that taurine may be necessary to support $f_{H,max}$ and aerobic performance at high temperatures in salmonids.

In addition to affecting I_{Na}, taurine influences multiple excitation–contraction coupling mechanisms to control cardiac Ca^{2+} flux and promote survival under hypoxia in mammalian hearts (Satoh and Sperelakis, 1998). A decline in the ATP/Mg-ADP ratio under hypoxia activates sarcolemmal ATP-sensitive K^+ (K_{ATP}) channels to promote inward K^+ flux, which hyperpolarizes cardiomyocytes and decreases force production to reduce ATP demand in the face of reduced supply. Ischemia reduces cardiac taurine content by close to half in dogs and a similar efflux is evident in perfused rat hearts (Nathan and Crass, 1982) and primary cardiomyocytes (Ren et al., 2021). Taurine inhibits K_{ATP} channel activity, so its efflux at the onset of hypoxia releases channel inhibition to hasten the protective effects of channel activation (Tricarico et al., 2000). Cardiac K_{ATP} channels are activated by anoxia (Paajanen and Vornanen, 2002), shorten action potential duration (Cameron et al., 2003), either enhance or depress contractility, depending on the species (MacCormack and Driedzic, 2002; MacCormack et al., 2003), and contribute to cardiomyocyte hypoxia survival in fish (Chen et al., 2005), but their regulation by taurine has not been studied. If taurine efflux contributes to the activation of K_{ATP} in fish, cardiac hypoxia tolerance may be compromised if taurine levels are reduced by a prior stress. Species-specific or intraspecific differences in cardiac taurine levels (see Gates et al., 2022) could also contribute to variations in hypoxia tolerance via this mechanism.

To fuel cardiac function, aerobic ATP production via the mitochondrial electron transport system (ETS) is enhanced at elevated temperatures and highly regulated under hypoxia. Taurine plays an important role in supporting ETS function by regulating the production of mitochondrial proteins through its interactions with a leucine codon (UUG). For amino acids encoded by a UUG, taurine conjugation in the wobble position of $tRNA^{Leu(UUR)}$ is required for normal decoding and post-transcriptional modification (Kirino et al., 2004). Taurine deficiency interferes with this conjugation and contributes to the reduced expression of several mitochondrial proteins, with subsequent impairment of ETS function in rat cardiomyocytes (Jong et al., 2010, 2012). Taurine is thus important in supporting aerobic ATP production in the ETS and may also contribute to the regulation of oxidative stress through the same mechanism. Taurine-deficient rat cardiomyocytes exhibit multiple markers of oxidative stress but recover fully when taurine levels are normalized (Jong et al., 2012). As the thermal sensitivity of ETS function may contribute to heart failure in fish at high temperatures (Christen et al., 2018; Iftikar and Hickey, 2013), characterizing taurine's contribution in this respect will further clarify the mechanisms underlying thermal tolerance.

The aquaculture industry is decreasing its reliance on taurine-rich fish meal in commercial feeds and replacing it with protein sourced from plants, which have insufficient taurine content to support optimal growth (Gaylord et al., 2006). Climate change is also increasing the frequency and severity of environmental stressors (Diaz and Breitburg, 2009; Jenny et al., 2016; O'Reilly et al., 2015; Reid et al., 2018) and causing ecosystem-scale shifts that may impact the availability of taurine-rich prey for wild fish (Doubleday et al., 2016; Oke et al., 2020). The importance of taurine in supporting mammalian cardiac function is well established, but little is known about its functional role in fish hearts, where concentrations are likely to be far more dynamic. Our aim was to characterize the significance of intracellular taurine in supporting cardiac function under acute exercise, and thermal and hypoxic stress in a salmonid. We focused on brook char (*Salvelinus fontinalis*), which are a relatively eurythermal salmonid (Durhack et al., 2021) that are still sensitive to acute thermal (Benfey et al., 1997; Chadwick et al., 2015; Morrison et al., 2020; Stitt et al., 2014) and hypoxic (Graham, 1949) stressors and that are vulnerable to climate change-induced habitat loss (Meisner, 1990). The physiological effects of acute taurine supplementation differ from the effects of changes in intracellular taurine (Schaffer et al., 2014), so we generated an *in vivo* model of chronic cardiac taurine deficiency using a β-alanine-enriched feed (Gates et al., 2022). β-Alanine is a structural analog of taurine that competitively inhibits taurine uptake through its transporter, Tau-T, reducing cardiomyocyte taurine levels (Rasmussen et al., 2016). We hypothesized that cardiac taurine deficiency would reduce maximum cardiac function, impair mitochondrial energy metabolism, and decrease whole-animal tolerance to acute exercise, high temperature stress and hypoxia. To test these hypotheses, we assessed maximum cardiac function *in vitro* using isolated perfused working hearts exposed to severe hypoxia and reoxygenation and studied mitochondrial metabolism and protein expression. Exercise, hypoxia and thermal tolerance were characterized through exhaustive chase, time to loss of equilibrium (LOE) and critical thermal maximum (CT_{max}) tests, and heart function was examined *in vivo* using electrocardiography. We demonstrate that cardiac taurine deficiency impairs maximum cardiac function, alters mitochondrial metabolism and causes substantial changes in acute thermal and hypoxia tolerance. Taurine appears to play a vital, previously unrecognized role in supporting cardiovascular function and environmental stress tolerance in fish.

MATERIALS AND METHODS

Animals and experimental diet

Male and female brook char, *Salvelinus fontinalis* (Mitchill 1814), were reared at the University of New Brunswick, Canada, before transfer to the Harold Crabtree Aqualab facility at Mount Allison University. Fish were housed in 750 l tanks receiving partially recirculating, filtered and aerated freshwater at 16±0.5°C (total system volume of 2400 l) for at least 2 weeks prior to experimentation. Aerobic scope is maximized in brook char in this temperature range (Durhack et al., 2021; Graham, 1949). Animals were fed once daily to satiation on one of two experimental diets: control brook char were fed 3 mm VITA salmonid chow (EWOS, Surrey, BC, Canada) while taurine-deficient (TD) brook char were fed the same chow vacuum-coated with 5% by mass β-alanine. Inhibition of taurine transport and promotion of taurine efflux are the only known cardiovascular effects of β-alanine supplementation (Allo et al., 1997). Animals were maintained on their respective diets for a minimum of 4 weeks prior to experimentation. Cardiac taurine levels declined to a minimum after 4 weeks in rainbow trout fed a 3% β-alanine-enriched diet and remained there for at least 8 weeks (Gates et al., 2022). Fish were fasted for 24 h before experimentation. All experiments were performed in accordance with the guidelines provided by the

Canadian Council on Animal Care and approved by the Mount Allison University Animal Care Committee (protocol 101873 and 103144).

Tissue sampling and hematology

In experiments where tissue samples were collected, fish were lethally anesthetized [300 mg l^{-1} tricaine methanesulfonate (TMS) and 600 mg l^{-1} $NaHCO_3$] in a darkened chamber containing aerated water. Blood was sampled from a caudal vessel using a heparinized syringe and placed on ice until tissue sampling was complete. Fish were killed by severing the spinal cord and samples of heart and brain were collected and rapidly frozen in liquid nitrogen. For fish in the time-to-exhaustion experiment (see below), the first gill arch was then removed and transferred to ice-cold glutaraldehyde fixative and stored at 4°C for subsequent scanning electron microscopy imaging. An aliquot of blood was aspirated into a capillary tube and centrifuged at 500 ***g*** to determine hematocrit. Hemoglobin concentration was measured with a HB201 meter (Hemocue AB, Ängelholm, Sweden) and concentration was corrected for use with fish blood (Clark et al., 2008). The remaining blood was then transferred to a microcentrifuge tube, centrifuged at 500 ***g*** for 5 min at 4°C, and the plasma collected and frozen in liquid nitrogen. Plasma osmolality was assessed in triplicate in thawed samples using a vapor pressure osmometer (VAPRO 5520, Wescor Inc., Logan, UT, USA). All other tissue samples were stored at −80°C until analysis.

In vivo stress tolerance and cardiac function

Time to exhaustion

Control (n=8; mean±s.e.m. body mass 124.3±12.0 g) and TD brook char (n=8; 205.7±23.8 g) were transferred into an opaque 30 l cylindrical tank containing aerated holding tank water at 16°C. Following a 5 min acclimation period, individual fish were manually chased to exhaustion until no longer responsive to physical manipulation. Time to exhaustion was recorded before the fish was removed from the system, euthanized, and tissues sampled as described above.

Acute hypoxia tolerance and f_H

The acute hypoxia tolerance of control (n=8; body mass 502.0 ±127.7 g) and TD brook char (n=8; 456.3±163.3 g) was assessed by measuring time to LOE following acute exposure to an oxygen partial pressure (P_{O_2}) of 2.1 kPa. Fish were removed from their housing tanks and tested 4 at a time in 100 l tanks at 16°C. Following a 24 h acclimation period in fully aerated water, P_{O_2} was decreased at a rate of 0.32 kPa min^{-1} to a minimum of 2.1 kPa by bubbling nitrogen gas into the tanks using a WitroxCTRL oxygen control system (Loligo Systems, Copenhagen, Denmark). A timer was started when P_{O_2} reached 2.1 kPa and the time to LOE for individual fish was recorded. Fish were then immediately removed from the tank, weighed and transferred to an aerated recovery tank at 16°C. Once the animal regained equilibrium and resumed normal swimming behaviors, it was returned to its housing tank. No mortality occurred during or after the hypoxia challenge and fish were not reused for any subsequent experiment described within.

Resting f_H was measured in conscious control (n=8, body mass 562.3±58.1 g) and TD brook char (n=6, 521.3±37.8 g) during exposure to moderate hypoxia and reoxygenation. Fish were anesthetized in 150 mg l^{-1} TMS and 300 mg l^{-1} $NaHCO_3$, before being transferred to a soft foam table. The gills were then continuously flushed with a maintenance dose of anesthetic (83 mg l^{-1} TMS, 166 mg l^{-1} $NaHCO_3$) in aerated freshwater maintained at 16°C. To record the electrocardiogram (ECG), platinum subdermal needle electrodes were inserted just under the skin overlying the heart. Fish were revived in anesthetic-free water and allowed to recover for 18–24 h in a perforated PVC pipe within a 30 l tank of continuously filtered and aerated freshwater maintained at 16±0.1°C. The pipe allowed animals some freedom of movement while preventing electrode leads from becoming tangled. The tank was subsequently bubbled with nitrogen to reduce P_{O_2} to 12.5 kPa for 30 min, then 8.3 kPa for 30 min, before aeration was reintroduced and P_{O_2} maintained at 20.8 kPa for 90 min. Fish were then removed from the system, euthanized and tissues sampled as described above. The ECG signal was filtered and amplified using a BioAmp interfaced to a Powerlab 4/26 data acquisition system (ADInstruments Inc., Colorado Springs, CO, USA) and f_H was monitored continuously throughout the trial.

Resting blood pressure

A dorsal aortic cannulation was performed according to Axelsson and Fritsche (1994) on control (n=6; body mass 561±99 g) and TD brook char (n=4; 706.3±62.7 g) to measure blood pressure (P_A). Following cannulation, fish were revived and transferred into a perforated PVC pipe within a 30 l tank of continuously filtered and aerated freshwater maintained at 16±0.1°C. The cannula was attached to an MLT844 physiological pressure transducer (MEMSCAP, Durham, NC, USA) interfaced through a bridge amplifier to a PowerLab 4/26 data acquisition system and Lab Chart 8 software (ADInstruments Inc.). The transducer was calibrated to a static water column daily and the cannula flushed with 2 cannula volumes of heparinized (50 U ml^{-1}) saline (in mmol l^{-1}: 143 NaCl, 2.88 $CaCl_2$, 0.90 $MgSO_4$, 3.35 KCl, 2.25 NaH_2PO_4, 5.50 $NaHCO_3$ and 10 Hepes, pH 7.8) every 12 h to prevent blood clot formation. Fish were allowed to recover overnight for approximately 18 h before measurement of P_A, recorded as the average value obtained over a representative 1 min interval.

Acute thermal tolerance

The acute thermal tolerance of control (n=7; body mass 391.1 ±109.1 g) and TD brook char (n=7; 446.5±189.0 g) was examined by determining their CT_{max} following established protocols (Becker and Genoway, 1979; Gallant et al., 2017). Though not always predictive of survival (Strowbridge et al., 2021), CT_{max} is a common metric of acute thermal sensitivity in fish that has been linked with more ecologically relevant indicators of thermal tolerance (Åsheim et al., 2020; Sunday et al., 2011). Fish were removed from their housing tanks and tested individually in a 60 l glass aquarium containing continuously aerated tank water at 16°C. Following a 5 min acclimation period, water temperature was increased at 0.3°C min^{-1} using a DYNEO DD heating immersion circulator (Julabo, Seelbach, Germany). CT_{max} was recorded as the temperature when LOE occurred. Upon reaching CT_{max}, fish were immediately removed, weighed and transferred to a recovery tank at 16°C. Animals were returned to their housing tanks once they regained the ability to maintain equilibrium and no mortality occurred during or after the CT_{max} challenge. Fish were not reused for any subsequent experiment described within.

Arrhenius breakpoint temperature test

Cardiac sensitivity to acute thermal stress was assessed in a separate group of anesthetized control (n=8; body mass 147.9±9.9 g) and TD brook char (n=8; 213.9±21.4 g) using Arrhenius breakpoint temperature (ABT) tests (Casselman et al., 2012; Gilbert and

Farrell, 2021; Hardison et al., 2021; Schwieterman et al., 2022). Fish were anesthetized as described above and the gills and body continuously flushed with a maintenance dose of anesthetic (83 mg l^{-1} TMS, 166 mg l^{-1} $NaHCO_3$) in vigorously aerated freshwater maintained at 16°C. Preliminary experiments determined that 83 mg l^{-1} TMS was the minimum dose required to maintain anesthesia for the duration of the test in this species. Fish were fitted with ECG electrodes as described above. $f_{H,max}$ was stimulated by intraperitoneal injection of isoproterenol (4 µg kg^{-1}) and atropine (1.2 mg kg^{-1}) in saline (composition described in 'Resting blood pressure', above) to maximally activate β-adrenoreceptors and inhibit muscarinic acetylcholine receptors, respectively. ABT tests began when f_H stabilized 25–30 min after drug injection.

The temperature of the water flushing the gills and body of the animal was continuously monitored with a thermocouple (t-type probe, ADInstruments Inc.) and was increased at a rate of 5°C h^{-1} using a DYNEO DD immersion circulator (Julabo). The ECG signal was collected as above and $f_{H,max}$ was monitored continuously throughout the trial. Water temperature was increased until arrhythmias were noted and $f_{H,max}$ declined considerably. Fish were then euthanized, and tissues sampled as above.

In vitro cardiac function: isolated perfused working hearts

Cardiac function was assessed *in vitro* using isolated perfused working heart preparations. Brook char were anesthetized, weighed, the spinal cord severed, and the heart carefully removed into ice-cold saline (in mmol l^{-1}: 143 NaCl, 2.88 $CaCl_2$, 0.90 $MgSO_4$, 3.35 KCl, 2.25 NaH_2PO_4, 5.50 $NaHCO_3$, 10 Hepes, 5.0 glucose, pH 7.8) bubbled with 99.5% O_2/0.5% CO_2. Taurine (0.7 mmol l^{-1}) was included in the saline to mimic plasma taurine levels in salmonids (Gras et al., 1982). Adrenaline (3.0 nmol l^{-1}) was included to maintain cardiac tonus (Shiels and Farrell, 1997) and was renewed every 30 min to account for photodegradation. The heart was supported by a Teflon mesh platform and mounted in a constant-pressure perfusion system (Clow et al., 2004) with stainless steel input and output cannulas in the sinus venosus and ventral aorta, respectively. Perfusate temperature was maintained at 16±0.1°C using a recirculating water bath connected to the preload and afterload reservoirs. Input (P_{in}) and output (P_{out}) pressures were corrected for tubing resistance and set to 15 and 25 cmH_2O (where 1 cmH_2O is ∼98 Pa), respectively. *In vivo* filling pressures in salmonids are close to zero (Altimiras and Axelsson, 2004) so the P_{in} used here is not physiologically relevant. Our previous work on rainbow trout (Gates et al., 2022) showed subtle impacts of taurine deficiency on cardiac contractility under relatively extreme conditions (e.g. high levels of extracellular Ca^{2+} and rapid pacing frequencies). Our aim in the current study was to assess maximum cardiac function, so P_{in} was set to 15 cmH_2O to achieve maximum V_S in order to stretch the myocardium to stimulate peak tension development. A P_{out} of 25 cmH_2O was chosen based on preliminary measurements of resting dorsal aortic blood pressure. *In vivo*, P_{out} in the ventral aorta is ∼30% higher than in the dorsal aorta (Bushnell and Jones, 1992), so the P_{out} used in the current experiments is sub-physiological. P_{out} was measured from a cannula side arm at the level of the heart using an MLT844 pressure transducer (MEMSCAP) calibrated to a static water column before use. Output flow was measured with an ultrasonic flow probe (PE2XL, Transonic Systems Inc., Ithaca, NY, USA) interfaced to a T403 flow meter (Transonic Systems Inc.). The hearts did not maintain a regular, spontaneous f_H and net $\dot{Q}$ was highly variable as atrial and ventricular contractions were not coordinated. Hearts were therefore stimulated at 60 beats min^{-1} using square-wave pulses (50 V, 10 ms duration) from a stimulator (SD9, Grass Technologies Inc., Warwick, RI, USA) connected to the input and output cannulas, which served as positive and negative electrodes. Stimulation restored coordination of atrial and ventricular contractions and stabilized $\dot{Q}$. The pressure transducer and flow meter were interfaced to a Powerlab 8/26 data acquisition system (ADInstruments) and data were monitored using Lab Chart 8 software. $\dot{Q}$ was determined from the mean of the pulsatile flow data and the mass of the animal and f_H was verified from the same data using the cyclic measurements function in Lab Chart. V_S was calculated by dividing $\dot{Q}$ by f_H. The rate of output pressure development by the heart, or the contractility index ($+dP/dt$), was determined as the mean rise in pressure over time for an individual beat and the data represent the average of at least 3 representative beats. Cardiac power output (PO) was calculated using $\dot{Q}$, peak P_{out}, P_{in} and ventricular mass as previously described (Speers-Roesch et al., 2013).

Basic cardiac function was first assessed in hearts from control (n=8; body mass 652.4±10.9 g) and TD brook char (n=8; 585.7 ±48.1 g) perfused with oxygenated saline. Hearts were allowed to stabilize in the system for a minimum of 20 min before $\dot{Q}$, f_H, V_S,$+dP/dt$ and PO were recorded. A maximum pacing frequency test was then performed, where stimulation frequency was increased in 12 beats min^{-1} increments every 30 s until arrhythmias were noted or f_H no longer followed stimulation frequency. Hearts were then removed from the system, and the ventricle isolated, blotted dry, weighed and frozen in liquid nitrogen for subsequent biochemical analyses.

Hearts from a separate group of control (n=8; body mass 637.6 ±40.5 g) and TD brook char (n=8; 724.6±46.0 g) were used to assess function during exposure to acute severe hypoxia and reoxygenation. Hearts were mounted in the system and perfused with oxygenated saline for a minimum of 20 min to stabilize. The perfusate was then immediately switched to saline equilibrated and continuously bubbled with 99.5% N_2/0.5% CO_2. Function was monitored for 20 min under severe hypoxia before the perfusate was switched back to oxygenated saline. Recovery was monitored for 20 min before experiments were terminated and hearts were removed from the system and processed as above.

Mitochondrial function

Tissue samples were obtained from the apex of the ventricle in control (n=8; body mass 500.4±32.1 g) and TD brook char (n=8; 454.7±47.4 g) and immersed in 2 ml cold BIOPS buffer (in mmol l^{-1}: 2.77 CaK_2EGTA, 7.23 K_2EGTA, 5.77 Na_2ATP, 6.56 $MgCl_2 \cdot 6H_2O$, 20 taurine, 20 imidazole, 0.5 dithiothreitol, 50 MES hydrate, 15 Na_2 phosphocreatine, 50 sucrose, pH 7.1 at 0°C). Fiber bundles were pulled from the tissue using fine forceps and immediately transferred to fresh BIOPS buffer with 50 µg ml^{-1} saponin in a 6-well culture plate and shaken on ice for 30 min to permeabilize the tissue. Fibers were then washed in 2 ml of MiRO5 mitochondrial respiration media (in mmol l^{-1}: 0.5 EGTA, 3 $MgCl_2$, 60 lactobionic acid, 20 taurine, 10 KH_2PO_4, 20 Hepes, 110 D-sucrose, with 1 g l^{-1} fatty acid-free BSA, pH 7.1 at 30°C) for 15 min on ice. Fibers were blotted dry and weighed before use in respiration assays. Fiber bundles weighed between 1 and 3 mg. We recognize that the concentration of taurine in the respiration media was sub-physiological, but we aimed to assess function using established protocols to facilitate comparisons with existing studies.

Respirometry followed the protocol described by Iftikar and Hickey (2013). Duplicate fiber samples were added to paired

chambers filled with 2 ml of MiRO5 media in an Oroboros O2k FluoRespirometer (Oroboros Instruments, Innsbruck, Austria). ETS function throughout oxidative phosphorylation (OXPHOS) was assessed using a substrate–uncoupler–inhibitor titration (SUIT) protocol. SUIT protocol chemicals were prepared in advance according to the manufacturer's recommendations (Oroboros O2k-Procedures 2016) and added to the chambers in the following order: Complex I (CI) substrates malate (1 mmol l^{-1}), glutamate (10 mmol l^{-1}) and pyruvate (5 mmol l^{-1}) were first added to initiate the Kreb's cycle and measure the baseline 'LEAK' state II respiration through CI when phosphorylation of ADP is not occurring. ADP (5 mmol l^{-1}) was then added to initiate OXPHOS to measure state III respiration, 'OXP I'. Cytochrome *c* (10 µmol l^{-1}) was added to evaluate the integrity of the outer mitochondrial membrane. Succinate (10 mmol l^{-1}) was then added to stimulate $FADH_2$ production and enable electrons to enter the ETC via Complex II (CII) as well as CI, 'OXP I–II'. Carbonyl cyanide *P*-(trifluoromethoxy) phenylhydrazone (FCCP, 0.5 µmol l^{-1}) was then added to uncouple the mitochondria; FCCP was added in 1 µl increments to a maximum of 5 µl until O_2 flux stopped increasing, giving electron transport capacity, 'ET'. Rotenone (0.5 µmol l^{-1}) was added to inhibit CI function and reveal the O_2 consumption rate of CII-fueled OXPHOS, 'OXP II'. Antimycin A (2.5 µmol l^{-1}) was added to inhibit electron flow to Complex III (CIII) and decrease O_2 consumption. *N*,*N*,*N*',*N*'-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD, 0.5 mol l^{-1}) and ascorbate sodium salt (2 mmol l^{-1}) were added simultaneously to measure the steady-state activity of cytochrome *c* oxidase, where ascorbate functioned to keep TMPD in its reduced state. Finally, sodium azide (0.1 mol l^{-1}) was added to cease all complex function to measure the residual background O_2 consumption.

Throughout the titration protocol, O_2 flux (pmol O_2 s^{-1} ml^{-1}) at each stage was calculated using O2k DatLab software (Oroboros Instruments). The respiratory control ratio (RCR) was calculated as the ratio of OXP I to LEAK respiration rates. The maximal rate of Complex IV (CIV) O_2 consumption, 'CCO', was calculated as the steady-state rate following the addition of TMPD and ascorbate, minus the background rate of O_2 consumption after sodium azide addition.

Gill morphology

Fixed gill arches from fish in the time-to-exhaustion experiment were prepared as previously described (Callaghan et al., 2016) and imaged with a JSM-5600 scanning electron microscope (SEM; JOEL USA, Peabody, MA, USA) operating at 10 kV and a 48 mm working distance. Morphometric measurements were manually acquired using in-house image analysis software (dmfMeasure, Mount Allison University Digital Microscopy Facility) and ionocytes were identified using established criteria (Dymowska et al., 2012). To avoid bias, samples were coded prior to processing and analyzed without knowledge of their respective experimental treatment group.

Biochemical analyses

Taurine

Frozen tissue was homogenized in 10 volumes of 1.5 mol l^{-1} perchloric acid (PCA) using Kimble Kontes Teflon pestles (DWK Life Sciences, Millville, NJ, USA) and a wand-type sonicator (F60 Sonic Dismembrator, ThermoFisher Scientific, Waltham, MA, USA). The homogenate was then diluted with 2 PCA volumes of double distilled H_2O (ddH_2O), neutralized with 0.5 PCA volumes of 3 mol l^{-1} $KHCO_3$, and centrifuged at 3000 ***g*** for 5 min at 4°C. Taurine was quantified in supernatants by high-performance liquid chromatography (HPLC) using a 1200 series HPLC with a diode array detector (Agilent, Santa Clara, CA, USA). The column was a 150×4.6 mm ACE Equivalence C18 column (ACE Equivalence, EQV-5C18-1546). The method was run with 0, 1, 10 and 20 mmol l^{-1} taurine standards that were processed in the same manner as tissue samples using a mobile phase composed of a mixture of 20 mmol l^{-1} phosphate buffer at pH 7.2 and 45:45:10 methanol:acetonitrile:ddH_2O. Samples were mixed with 0.4 mol l^{-1} borate buffer (pH 10.2) and derivatized in-needle using a mixture of *o*-phthaldialdehyde and 2-mercaptoethanol before injection onto the column.

Lactate and malondialdehyde

Plasma samples from the chase to exhaustion and *in vivo* hypoxia exposure of the ECG equipped fish experiment were thawed on ice, mixed with 5 volumes of 6% PCA, and centrifuged at 10,000 ***g*** at 4°C. Lactate was quantified in triplicate in supernatants by following the reduction of NAD^+ to NADH at 340 nm in the reaction catalyzed by lactate dehydrogenase. Lactate concentration was determined by referencing to a standard curve of L-lactate. Malondialdehyde (MDA) concentration was measured in triplicate in isolated perfused working heart preparations using a commercially available assay kit (MDA-586, Oxisresearch, Berlingame, CA, USA). In both cases, absorbance was measured in a SpectraMax 190 microplate spectrophotometer (Molecular Devices, San Jose, CA, USA).

Protein quantification by western blotting

Cytochrome *c* oxidase subunit 3 (MT-COX3), a core subunit of CIV in the ETC, was quantified. The protein sequences for MT-COX3 from *Homo sapiens*, *Mus musculus*, *Rattus norvegicus* and *Danio rerio* were obtained and compared with the target sequence of commercially available antibodies using Jalview version 2 (Waterhouse et al., 2009). An unconjugated polyclonal antibody for MT-COX3 (MBS9708822, MyBioSource, San Diego, CA, USA) was chosen that targeted a highly conserved sequence between key active sites.

Control (n=5; body mass 537.1±125.8 g) and TD brook char (n=5; 433.6±149.7 g) were removed from their housing tanks, euthanized as above, and the ventricle sampled and rapidly frozen in liquid nitrogen. Samples (20–30 mg) were thawed and placed into bead-filled tubes. Each sample was solubilized in 300 µl 1× protein extraction buffer composed of 4× protein solubilization buffer, ddH_2O and 50× Pefabloc (Bioshop, Burlington, ON, Canada) (final concentration 1×). Samples were disrupted 3 times for 60 s each at 6.5 m s^{-1} in a FastPrep-24 5G benchtop homogenizer (MP Biomedicals, Irvine, CA, USA) with 60 s on ice between treatments. Samples were then centrifuged at 16,000 ***g*** for 5 min and the supernatants were removed and re-centrifuged at 16,000 ***g*** for 2 min. Total protein content was measured in triplicate using a bicinchoninic acid protein assay (ThermoFisher Scientific) and compared with standards of bovine gamma globulin following established protocols (Bonisteel et al., 2018).

MT-COX3 samples were prepared at 3 µg total protein content with dithiothreitol (DTT) and Novex Bolt LDS sample buffer (4×, Life Technologies, Carlsbad, CA, USA). Samples were heated at 70°C for 5 min and centrifuged briefly before loading into Bolt 4–12% Bis-Tris Plus acrylamide gels (Invitrogen, Waltham, MA, USA). Protein migration was compared with a NovexSharp Pre-Stained ladder and MagicMark XP western standard

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(Invitrogen). Blots were electrophoresed at 200 V for 30 min in 1× MES SDS running buffer and then transferred to PVDF membranes at 20 V for 60 min in 1× Bolt transfer buffer (Life Technologies). Following transfer, PVDF membranes were blocked in 2% Amersham ECL blocking solution (Cytiva, Marlborough, MA, USA) for 1 h at room temperature with gentle agitation. Blots were then incubated with the MT-COX3 primary antibody (1:1000 dilution in 2% ECL blocking solution) for 1 h with gentle agitation. After incubation, membranes were washed with 1× TBS-T once for 15 min and 3 times for 5 min. Membranes were then incubated with a 1:1500 dilution of goat anti-rabbit IgG HRP secondary antibody (Bio-Rad, Hercules, CA, USA) in 2% ECL blocking solution for 1 h with gentle agitation. The same wash protocol was repeated following this incubation period. The PVDF membranes were then incubated with a 1:1 chemiluminescent solution of Luminol (Amersham ECL Select, Cytiva) and peroxide and visualized with a VersaDoc Molecular MP 5000 Imager (Bio-Rad). Quantity One 1-D analysis software (Bio-Rad) was used to process the images and associated ImageLab software was used to quantify the visualized bands.

Data analysis and statistics

Statistical analyses were performed using Prism 9 Software (GraphPad, San Diego, CA, USA). For most endpoints, unpaired *t*-tests were used to compare between treatment groups or conditions. Non-parametric Mann–Whitney *U*-tests were used when variances differed between treatment groups, as assessed using an *F*-test. Two-way ANOVA with or without repeated measures, as appropriate, were used to compare f_H in *in vivo* hypoxia exposures and functional and biochemical parameters in perfused hearts exposed to hypoxia and reoxygenation. In all instances, normality was assessed with either a Shapiro–Wilk or Kologorov–Smirnov test to ensure test assumptions were satisfied. When significant interactions were detected in two-way ANOVA, the effect of treatment group at a specific point was compared using an unpaired *t*-test. *P*-values of less than 0.05 were considered significant in all cases. Box and whisker plots show the range and median values, and all other data are expressed as means±s.e.m.

RESULTS

In fish sampled directly from their holding tank, heart taurine concentration was more variable (P=0.035) and 21% lower in TD brook char than in controls (P=0.006; Fig. 1). Brain taurine levels were half those in heart and identical across treatment groups (P=0.924). Plasma taurine concentration was 1.4±0.1 µmol ml^{-1} and did not differ between treatment groups (P=0.879). Relative ventricular mass did not differ between control and TD brook char (0.088±0.002% and 0.090±0.004%, respectively; n=26 for both groups). Qualitatively, TD brook char fed well and appeared healthy, but were more aggressive during feeding than control fish, consistent with previous observations of TD rainbow trout (Gates et al., 2022).

Exhaustive exercise

Mean body mass was significantly higher in TD brook char (P=0.009) used in manual chase tests but there was no correlation between body mass and time to exhaustion (P=0.725) and the latter did not differ between treatment groups (P=0.662; Fig. 2A). Heart taurine concentration decreased significantly following the exhaustive chase in control (P=0.002) but not in TD brook char (P=0.934; Fig. 2B,C). Resting plasma lactate levels were relatively high in both treatment groups compared with previous measurements in this species (Morrison et al., 2020), suggesting the animals were experiencing some level of stress prior to chasing. Lactate levels did not change following the exhaustive chase in control brook char (P=0.127) but were significantly lower following the exhaustive chase in TD animals (P=0.047; Fig. 2D,E).

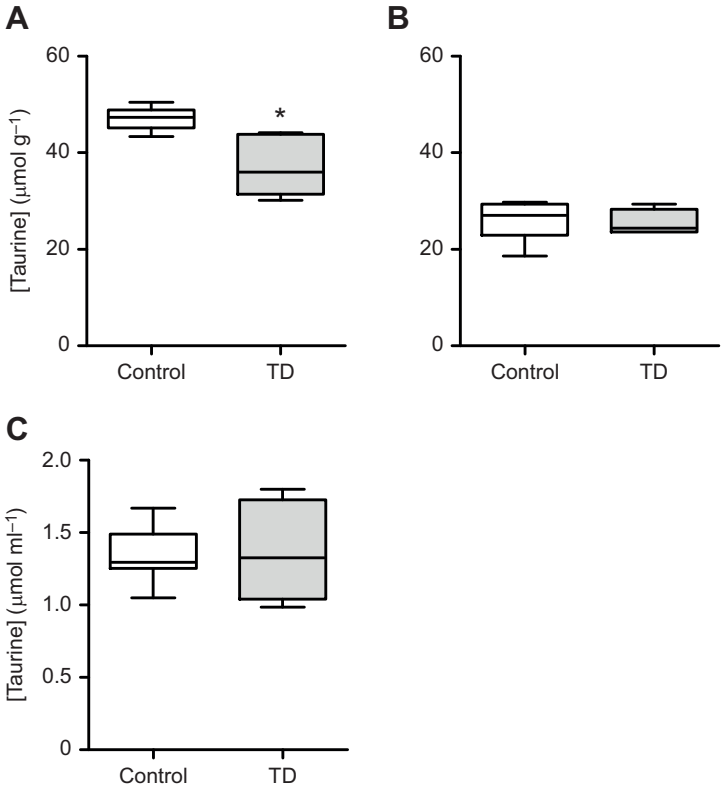

Fig. 1. Taurine concentration in control and taurine-deficient (TD) brook char. (A) Heart (n=7 control, n=5 TD), (B) brain (n=7 control, n=4 TD) and (C) plasma (n=8 for both groups) taurine concentration. Asterisk indicates a significant difference between treatment groups.

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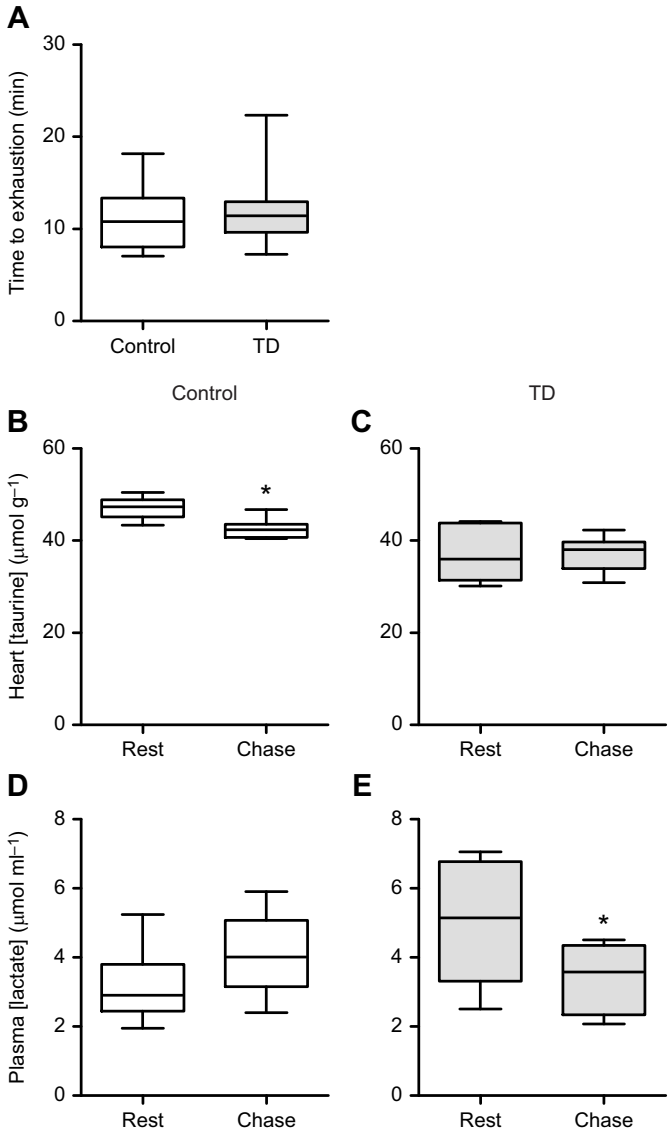

Fig. 2. Response to exhaustive exercise in control and TD fish. (A) Time to exhaustion during an exhaustive chase (n=8 for both groups). (B–D) Heart taurine and plasma lactate concentrations in control (B,D) and TD brook char (C,E) sampled at rest or following an exhaustive chase (n=5–8). Asterisk indicates a significant difference between rest and chase group.

Response to hypoxia

Control brook char exposed to moderate hypoxia and reoxygenation exhibited heart taurine concentrations of 39.7±3.5 µmol g^{-1} (Fig. 3A), 15.5% lower than those in resting fish sampled directly from their holding tank (Fig. 2B). In TD fish exposed to the same stressor, heart taurine content was essentially identical to that measured in resting TD brook char (41.2±4.0 versus 42.6 ±0.8 µmol g^{-1}, respectively, Figs 3A and 2C). Plasma taurine concentration was significantly less variable (P=0.038) and lower in TD brook char following hypoxia and reoxygenation (P=0.005) (Fig. 3B). With respect to f_H, two-way repeated measures ANOVA identified significant interactions between oxygenation state and treatment group when either absolute f_H or the change in f_H (Δf_H) between oxygenation conditions was examined (Fig. 3C–E). Data were thus split, and the effect of treatment group was tested under each individual oxygenation state using unpaired t-tests. Under normoxic conditions, resting f_H was lower in conscious TD brook char than in controls (P=0.043). Control fish exhibited a typical bradycardia after 30 min at a P_{O_2} of 8.33 kPa, decreasing f_H by 49%. In contrast, f_H fell by only 24% in TD brook char, significantly less than in controls (P=0.010). Both recovered to a similar extent following reoxygenation (P=0.133). There were no correlations between either heart or plasma taurine concentration and f_H under any of the conditions tested (data not shown). In uninstrumented fish, time to LOE under acute hypoxia (P_{O_2} 2.10 kPa) varied from 17 to 135 min in control fish (Fig. 3F), which is similar to the range observed for Atlantic salmon under identical conditions (Anttila et al., 2013). Time to LOE was significantly lower (P=0.003) and far less variable (P<0.0001) in the TD brook char, ranging from 5 to 22 min. Under normoxic conditions, dorsal aortic blood pressure did not significantly differ (P=0.525) between control and TD brook char (34.3±1.8 and 36.4±2.7 cmH_2O, respectively), but the sample size was limited for TD fish so results should be interpreted with caution. Hematocrit, hemoglobin concentration, mean corpuscular hemoglobin concentration (MCHC), plasma lactate and plasma osmolality were measured in ECG-instrumented fish following hypoxia and reoxygenation and no differences were detected between treatment groups (Table S1).

Response to thermal stress

Control brook char exhibited a CT_{max} of 29.8±0.9°C (Fig. 4A, which is comparable to previous measurements on this species (Benfey et al., 1997; Carline and Machung, 2001; Morrison et al., 2020). CT_{max} was significantly higher by 1°C in TD brook char (P=0.017). In the ABT test, TD brook char were larger than control animals (214 versus 148 g, respectively) but there were no significant relationships between body mass and any of the parameters derived from the test (data not shown). Prior to atropine and isoproterenol injection, f_H was 84.4±1.4 beats min^{-1} in anesthetized fish and it increased to 98.5±1.4 beats min^{-1} 20 min post-injection; there were no significant differences between treatment groups at either point (data not shown). Temperature at maximum $f_{H,max}$ was more variable in TD animals (P=0.002; Fig. 4B,C) but there was no significant difference between treatment groups (P=0.328; Fig. 4F). Maximum $f_{H,max}$ was lower in TD brook char by close to 20 beats min^{-1} (P=0.006; Fig. 4E). The temperature at which arrythmias appeared (T_{arr}) was more variable in TD brook char (P=0.011) but it did not differ between treatment groups (P=0.204; Fig. 4G). The ABT was higher than the optimum temperature for aerobic scope in brook char (~15°C; Durhack et al., 2021; Graham, 1949) and was not affected by TD (P=0.138; Fig. 4H). Following the ABT test, heart taurine levels were significantly more variable in TD brook char (P=0.045) but there were no differences between treatment groups (P=0.505; Fig. 4D). Taurine levels in control fish sampled after the ABT test were identical to those in controls sampled directly from their holding tank, whereas in TD brook char, they were approximately 10 µmol g^{-1} higher than in TD fish sampled from their holding tank. There were no significant correlations between heart taurine content and any of the endpoints assessed in the ABT test (data not shown).

Gill morphology

SEM imaging revealed no differences in gill morphology or cell type composition between control and TD brook char (Table S2).

In vitro heart function

Under initial oxygenated conditions, $\dot{Q}$ and V_S did not differ, but PO was significantly lower in TD brook char (Fig. 5A–C). After a 20 min exposure to severe hypoxia, $\dot{Q}$, V_S and PO declined

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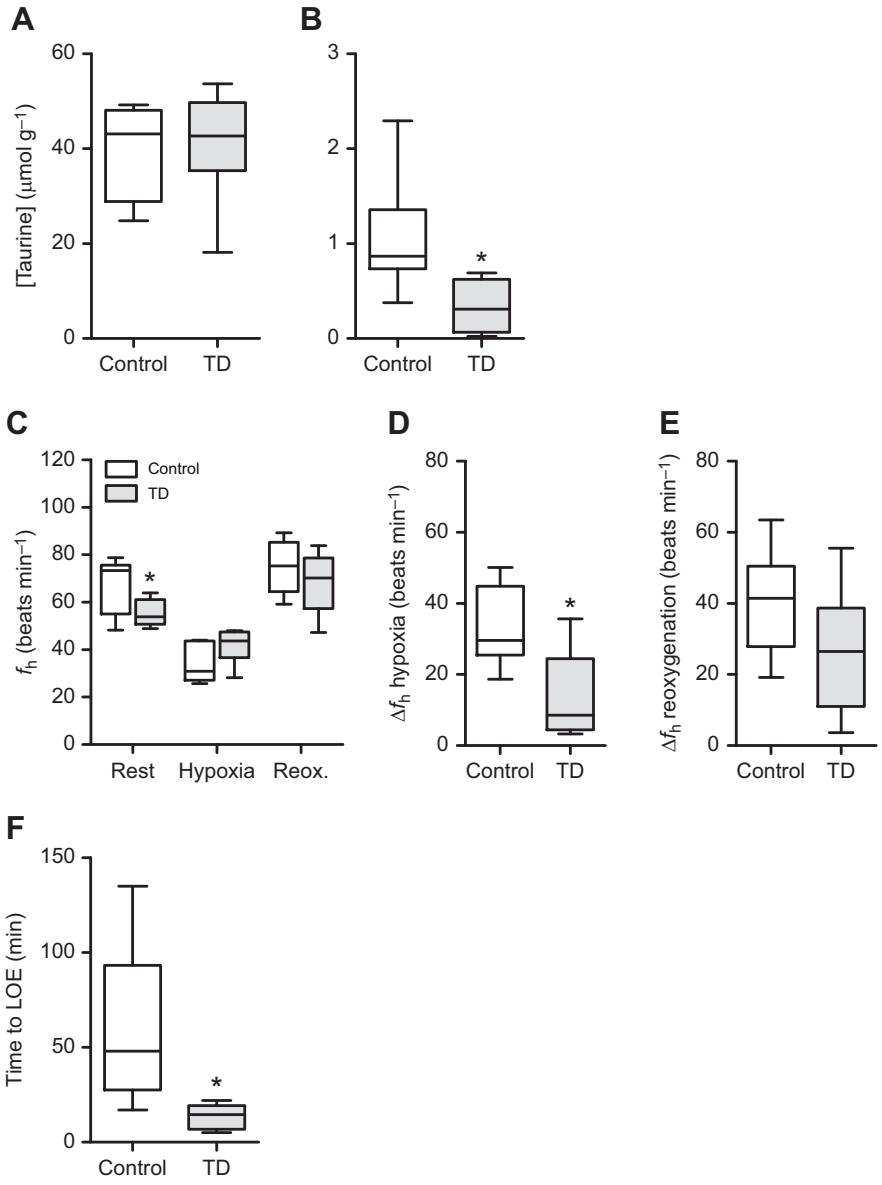

Fig. 3. Response to hypoxia in control and TD fish. (A,B) Taurine concentration in the heart (A) and plasma (B) following 30 min at a P_{O_2} of 8.33 kPa and 1 h reoxygenation (n=8 for both groups). (C) Heart rate (f_H) under normoxia (Rest), hypoxia and reoxygenation (Reox.), (D) the decrease in f_H following 30 min at a P_{O_2} of 8.33 kPa and (E) the increase in f_H 1 h following reoxygenation (n=8 control, n=6 TD). (F) Time to loss of equilibrium (LOE) following acute transfer to severe hypoxia (P_{O_2} 2.10 kPa) in control and TD brook char (n=8 for both groups). Asterisks indicate significant differences between control and TD treatment groups.

significantly in both treatment groups and did not recover following 20 min of reoxygenation. In TD fish, $\dot{Q}$ and V_S fell by close to 60%, significantly more than in control animals, which exhibited a 25% decrease. There was no effect of diet treatment on +dP/dT, but there was a significant effect of oxygenation state; +dP/dT declined to an equal extent in the two treatment groups under severe hypoxia and failed to recover following reoxygenation (Fig. 5D). Maximum pacing frequency was consistent at 120 beats min^{-1} (data not shown) and no differences were noted between control and TD hearts (P=0.629). Taurine levels were approximately 10 μmol g^{-1} higher in both control and TD perfused hearts than in hearts sampled directly from the animals. Taurine content in oxygenated perfused hearts did not significantly differ from that in hearts exposed to hypoxia and reoxygenation in either control or TD fish (P=0.316 and 0.375, respectively; Fig. 5E,F).

MDA content was quantified in isolated perfused hearts exposed to oxygenated conditions only and hearts that were exposed to severe hypoxia and reoxygenation (Fig. 6). A significant interaction was detected between treatment group and oxygenation state (P<0.0001). In oxygenated hearts, taurine levels were higher in control than in TD fish (P<0.0001) but levels were similar between groups in hearts exposed to severe hypoxia and reoxygenation (P=0.136). In both control and TD brook char, MDA levels were significantly lower in hearts exposed to severe hypoxia and reoxygenation than in those that had been perfused with oxygenated saline only (P<0.0001 and P=0.027, respectively).

Mitochondrial function and protein expression

In permeabilized cardiac muscle fiber experiments, RCRs were significantly lower in TD brook char than in controls (P=0.029; Fig. 7), but there were no significant differences in any of the other functional parameters assessed. Western blots revealed a 39% decrease in the expression of the mitochondrial COX3 subunit in TD brook char (P=0.008 Wilcoxon signed rank test).

DISCUSSION

Taurine deficiency is associated with a variety of negative effects on cardiac function and metabolism in mammalian models (Schaffer et al., 2014), but little is known about the physiological functions of taurine in fish, where concentrations are likely to change far more

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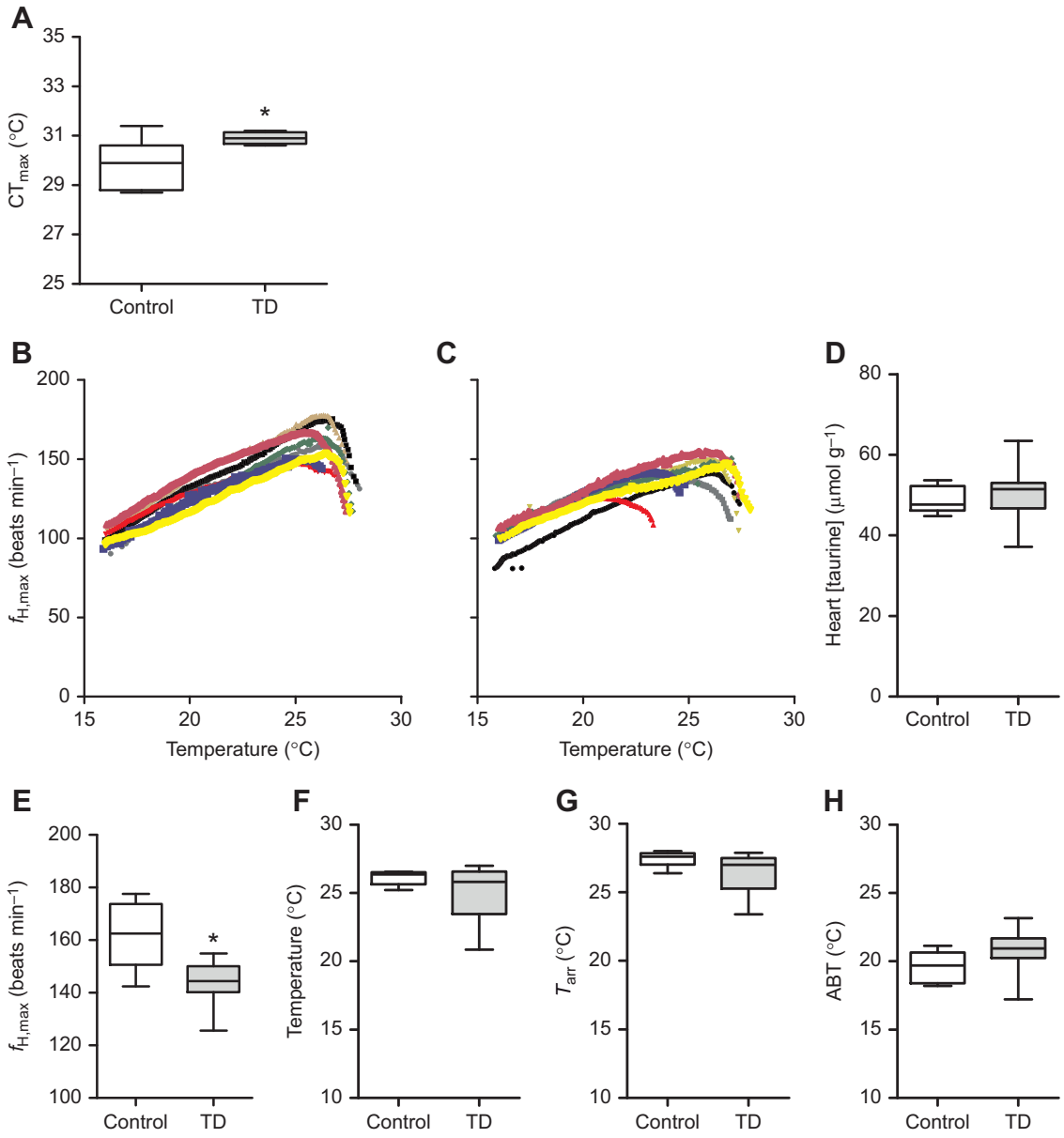

Fig. 4. Response to thermal stress of control and TD fish. (A) Critical thermal maximum (CT_{max}; n=8 for both groups). Note that CT_{max} and ABT tests were carried out on different animals. (B,C) Relationships between maximum heart rate ($f_{H,max}$) and temperature for control (B) and TD animals (C), respectively. (D) Heart taurine concentration following the ABT test. (E–H) Maximum $f_{H,max}$ (E), temperature at maximum $f_{H,max}$ (F), temperature at which arrythmias first appeared (T_{arr}; G) and Arrhenius breakpoint temperature (ABT; H) were derived from the data in B and C. Significant differences between treatment groups are indicated by an asterisk.

frequently. Our results demonstrate that reducing cardiac taurine levels by just 10 µmol g^{-1} (~20%) significantly affects whole-animal sensitivity to acute thermal and hypoxic stress, impairs heart function and influences energy metabolism.

Exercise-induced taurine efflux is impaired in TD brook char

We previously postulated that the osmotic stress associated with strenuous exercise may lead to cardiac taurine efflux (Gates et al., 2022) and we confirmed that in the current study. Cardiac taurine levels decreased significantly in control brook char following an exhaustive chase. No change in cardiac taurine was evident in TD brook char following the same chase, suggesting the mechanisms responsible for taurine efflux are ineffective at low intracellular taurine concentrations. Taurine efflux mechanisms have not been fully characterized in fish and appear to be species specific but are likely to involve multiple membrane proteins, including Tau-T (Chara et al., 2011; Schaffer et al., 2010). At lower intracellular taurine concentrations, the gradient across the sarcolemmal membrane may be insufficient to promote efflux through one or more of these proteins. Impeding taurine efflux under a putative osmotic stress should promote cardiomyocyte swelling and impact heart function. Any such reduction in the capacity of the heart to deliver O_2 is unlikely to affect time to exhaustion in this test, where swimming will be fueled largely by anaerobic metabolism. Control fish showed a non-significant increase in plasma lactate after chasing, while it decreased in TD fish. Plasma lactate levels were high in both treatment groups prior to chasing, complicating the interpretation of this data. Plasma lactate concentrations following

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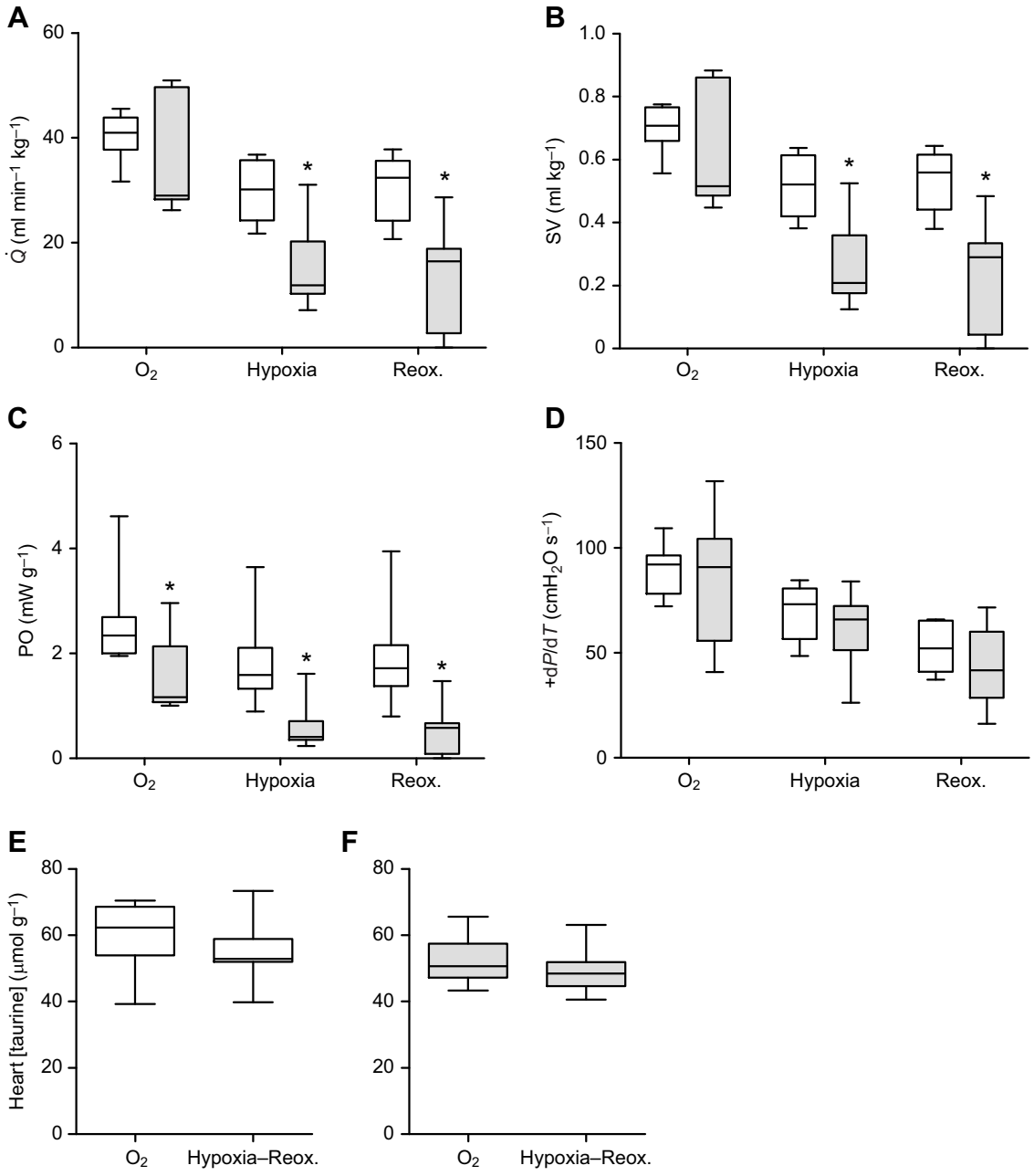

Fig. 5. *In vitro* heart function in control and TD fish. (A) Cardiac output ($\dot{Q}$), (B) stroke volume (V_S), (C) power output (PO) and (D) maximum rate of output pressure development (+dP/dT) under oxygenated conditions (O_2) or following severe hypoxia and reoxygenation (Reox.) (n=8 for both groups). (E,F) Taurine concentrations in perfused hearts from control (E) and TD brook char (F) under oxygenated conditions (O_2) and following hypoxia–reoxygenation. Significant differences between treatment groups are denoted with an asterisk.

hypoxia and reoxygenation were also marginally lower in TD fish than in controls (Table S1). The heightened sensitivity to hypoxia suggests systemic O_2 delivery is not enhanced in TD animals, but changes in mitochondrial function (see below) could increase their capacity for lactate oxidation and reduce plasma levels as a result.

Taurine deficiency increases CT_{max} but decreases $f_{H,max}$

Aerobic performance at high temperatures is dependent upon maximum cardiac function (Eliason and Anttila, 2017) and this is limited by $f_{H,max}$ in salmonids (Gamperl and Driedzic, 2009). Arrythmias, and the resulting interruptions in blood flow, likely contribute to LOE and eventual death at temperatures ≥CT_{max}. Despite exhibiting a lower maximum $f_{H,max}$ in ABT tests, CT_{max} was higher in TD brook char, implying they are less sensitive to thermal stress. T_{arr} was unaffected in TD brook char so the decline in maximum $f_{H,max}$ is probably unrelated to changes in the cardiac Na^+ current, which is responsible for triggering high temperature arrythmias in fish (Haverinen and Vornanen, 2020; Haverinen et al., 2021).

We expected taurine levels to decline under thermal stress in response to the activation of anaerobic metabolism and a putative increase in intracellular osmolality, but this was not observed. Cardiac taurine concentration was maintained in control fish subjected to the ABT test and increased by ∼10 µmol g^{-1} in TD

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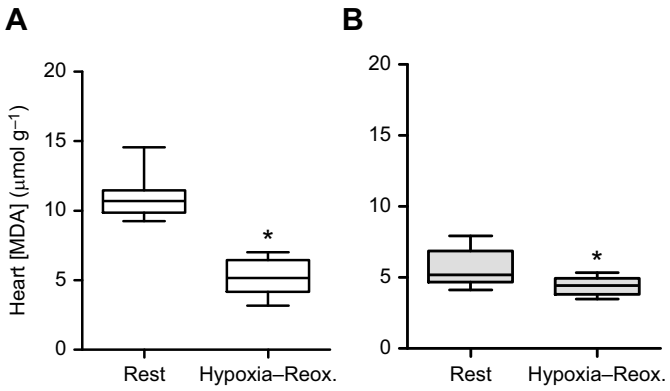

Fig. 6. Heart malondialdehyde (MDA) concentration in control and TD fish. MDA concentration was measured in ventricular muscle tissue of isolated hearts from control (A) and TD brook char (B) under normoxia (Rest) or following severe hypoxia and reoxygenation (n=8 for both groups). Significant differences between treatment groups are denoted with an asterisk.

animals (Fig. 4D). Salmonids have some capacity to synthesize taurine from cysteine (Yokoyama et al., 1997, 2001) and it is possible that synthesis is stimulated at elevated temperatures or by activation of β-adrenoreceptors. If TD brook char enhanced the expression of these pathways to compensate for a lack of cellular uptake, taurine production may increase disproportionately in these animals under acute thermal stress. Morrison et al. (2020) showed that plasma osmolality increased by ∼14 mOsm kg^{-1} in 15°C-acclimated brook char following CT_{max} testing. TD brook char may exhibit an increase in plasma osmolality not seen in control fish, leading to the difference in cardiac taurine content.

Maximum pacing frequency was maintained in TD hearts *in vitro* but rates were lower than those seen *in vivo*, likely because isolated hearts were working at maximum $\dot{Q}$ and their β-adrenoreceptors were not fully activated, unlike in the ABT test. Cardiac TD may only affect maximum f_H *in vivo* by changing pacemaker function or through regulation by neural and/or humoral effectors. This interpretation is supported by the observation that resting f_H was lower and the hypoxic bradycardia diminished in conscious TD brook char at their acclimation temperature of 16°C (Fig. 3). In anesthetized fish, basal f_H and the response to atropine and isoproterenol did not differ between treatment groups but the experiment did not quantify the individual cholinergic and adrenergic contributions to f_H so its interpretive value is limited.

Taurine deficiency may also affect maximum f_H by influencing other excitation–contraction coupling mechanisms. Isometrically contracting ventricular muscle strips from TD rainbow trout exhibited a lower maximum pacing frequency (Gates et al., 2022) and supplemental extracellular taurine tended to increase maximum pacing frequency in similar preparations from killifish (*Fundulus heteroclitus*; Henry and MacCormack, 2018). In the latter study, taurine's effects on contractility were sensitive to ryanodine, an inhibitor of Ca^{2+} release from the sarcoplasmic reticulum (SR). No differences in contractility, as quantified by +dP/dT, were evident between control and TD brook char hearts paced at 60 beats min^{-1} in this study, but impacts may be magnified at high temperatures and/or high f_H. The importance of SR Ca^{2+} cycling generally increases with pacing frequency (Henry and MacCormack, 2018; Shiels and Farrell, 1997; Shiels and Galli, 2014). If SR function was significantly impaired in TD brook char, cardiac contractility, and hence $\dot{Q}$, would be reduced at $f_{H,max}$ and animals would be more sensitive to acute thermal stress, which was not the case. CT_{max} was higher in TD brook char, suggesting either that $\dot{Q}$ was unaffected or that taurine deficiency increased thermal tolerance via an unrelated mechanism. Direct measurements of $\dot{Q}$ *in vivo* and additional electrophysiological and pharmacological studies would be useful in clarifying the precise mechanisms underlying the influence of taurine deficiency on CT_{max}.

Cardiac mitochondrial function is compromised at high temperatures in Arctic char (*Salvelinus alpinus*), a close relative of brook char, and this may contribute to the development of arrythmias and heart failure near CT_{max} (Christen et al., 2018). Mitochondrial respiration was relatively uncoupled in permeabilized cardiac muscle fibers from TD brook char, but overall function was not impacted. If aerobic ATP production was compromised in TD animals, it should result in an inability to maintain cardiomyocyte membrane potential and manifest as a decrease in T_{arr}, which was not the case. The observation of lower plasma lactate levels in TD fish following the exhaustive chase suggests that TD either enhances aerobic or inhibits anaerobic ATP production. In either case, effects were modest and best serve to demonstrate that energy metabolism is impacted to some degree by TD, as it is in mammals (De La Puerta, 2010; Ito et al., 2015) and cephalopods (MacCormack et al., 2016).

Taurine deficiency reduces hypoxia tolerance by impairing cardiac function

At the whole-animal level, TD brook char exhibited a dramatic increase in sensitivity to acute hypoxia exposure. Gill morphology and gross metrics of blood oxygen carrying capacity did not differ in TD animals, so tolerance was not limited by a reduction in branchial oxygen uptake efficiency unless hemoglobin O_2 affinity was greatly reduced. The magnitude of the hypoxic bradycardia was lower in TD brook char, likely to offset reductions in V_S and protect $\dot{Q}$. Changes in vascular resistance that augment V_S in salmonids (Gamperl and Driedzic, 2009) may also be enhanced, but the profound reduction in time to LOE (77%) indicates that compensatory mechanisms were ineffective in TD brook char. Hypoxia is detected by chemoreceptors at the gill and bradycardia is mediated by the release of acetylcholine onto the cardiac pacemaker by the vagal nerve (Farrell, 2007). A reduction in acetylcholinergic tonus on the pacemaker could increase f_H under hypoxia to defend , but it would come at the expense of contractility, myocardial oxygen supply and demand, and the filling time required to enhance V_S (Farrell, 2007). This response may be mediated by a reduction in either vagal signaling, acetylcholine receptor density or sensitivity, acetylcholinesterase activity or gill chemoreceptor sensitivity, any of which may be impacted by taurine deficiency.

In perfused heart experiments, V_S was 55% lower in TD hearts under severe hypoxia than in controls, and function did not recover upon reoxygenation. f_H, preload and afterload were not altered under severe hypoxia *in vitro* as they would be in the whole animal so the overall impact on V_S and $\dot{Q}$ *in vivo* is unknown. Despite the large reduction in V_S relative to controls, ventricular power output and +dP/dT were similar in control and TD hearts under severe hypoxia. The cellular mechanisms responsible for the loss of *in vitro* cardiac function under severe hypoxia in TD fish relative to controls remain to be elucidated. We have demonstrated that taurine's effects on +dP/dT in a perfused cephalopod heart mirror its effects on maximum isometric tension development in muscle preparations from the same species (MacCormack et al., 2016). In TD rainbow trout hearts, isometric tension development was less sensitive to

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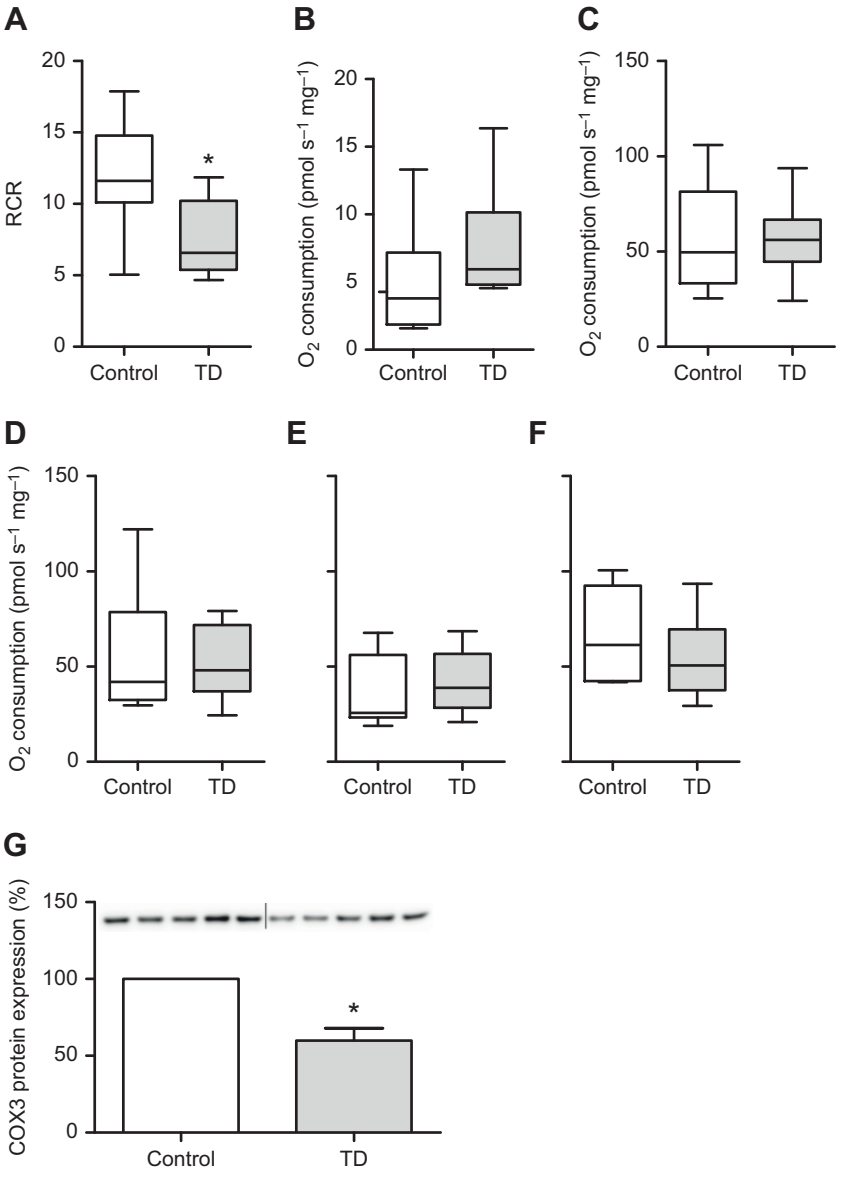

Fig. 7. Mitochondrial function in permeabilized ventricular muscle fibers from control and TD brook char. (A) Respiratory control ratio (RCR), (B) LEAK respiration (B), (C) electron transport capacity (C), and complex I (OXP I; D), complex II (OXP II; E) and cytochrome *c* oxidase fueled O_2 flux (CCO; F) (n=8 for both groups). (G) Relative mitochondrial COX3 protein expression and associated western blot images in ventricular tissue (n=5 for both treatment groups). Asterisks denote significant differences between treatment groups.

Ca^{2+} and lower at all contraction frequencies, although differences were small (Gates et al., 2022). This pattern is far more pronounced in highly TD rat hearts (60–75% less taurine) (Eley et al., 1994; Lake et al., 1990) and is caused by a decrease in the quantity of contractile elements in the muscle (Eley et al., 1994). It is plausible that cardiac contractility was impaired to some degree in TD brook char but +dP/dT was not sufficiently sensitive to detect it under the conditions tested. As noted earlier, some effects of acute taurine supplementation on cardiac contractility in fish are sensitive to ryanodine (Henry and MacCormack, 2018). Taurine influences SR function by two main mechanisms: it promotes the phosphorylation of phospholamban to augment SR Ca^{2+}-ATPase activity and it enhances ryanodine receptor activity to promote Ca^{2+}-induced Ca^{2+} release (Brittsan and Kranias, 2000; Ramila et al., 2015; Schaffer et al., 1994; Steele et al., 1990). The SR can contribute significantly to excitation–contraction coupling in salmonid cardiomyocytes when β-adrenoreceptors are stimulated (Cros et al., 2014), as they were here. *In vitro* pharmacological studies suggest the SR plays only a minor role in contractility under severe hypoxia (MacCormack et al., 2003) but compensation by sarcolemmal Ca^{2+} transport mechanisms may mask important functional effects (Shiels and Galli, 2014). It seems doubtful, however, that taurine would have such a profound impact on SR function that a 21% reduction in its concentration would cause V_S to collapse and that the effect would only manifest during severe hypoxia.

The severe hypoxia-specific effect of TD on V_S indicates a possible link to changes in ATP production. Taurine inhibits sarcolemmal K_{ATP} channels (Park et al., 2004; Tricarico et al., 2000) and the rapid efflux of taurine under hypoxia (Nathan and Crass, 1982) associated with rising intracellular osmolality (Schaffer et al., 2014; Steenbergen et al., 1985) promotes K_{ATP} channel activation to protect the heart. Impairing ATP production activates K_{ATP} channels in fish cardiomyocytes, but the associated current magnitude is lower than it is in mammalian cells (Paajanen and Vornanen, 2002). Despite this, K_{ATP} channel activation under hypoxia is sufficient to shorten cardiac action potential duration and improve cardiomyocyte survival in goldfish (Cameron et al., 2003; Chen et al., 2005). K_{ATP} channels influence anoxic cardiac contractility in fish, although the effects are species specific (MacCormack and Driedzic, 2002; MacCormack et al., 2003). With

lower intracellular taurine concentrations, the magnitude of taurine efflux under severe hypoxia will decrease and this may curtail or delay K_{ATP} channel activation and compromise heart function. Heart taurine content was 15% lower in control brook char following hypoxia and reoxygenation *in vivo* but it was unchanged in TD fish, suggesting hypoxia-induced efflux did not occur in the latter group. This interpretation is supported by the observation that plasma taurine levels were also higher in control brook char following hypoxia and reoxygenation, likely as a result of tissue efflux into the blood. Taurine efflux may be necessary to support heart function under oxygen limitation, at least in hypoxia-sensitive species such as brook char. More robust species that avoid the accumulation of anaerobic end products under hypoxia by suppressing their aerobic metabolic demands may not rely on this mechanism.

In isolated perfused hearts, taurine concentration was slightly lower in both treatment groups following severe hypoxia and reoxygenation, but the differences were not significant. Notably, taurine content was ∼10 µmol g^{-1} higher in hearts from both treatment groups *in vitro*. This may reflect the slightly higher osmotic strength of the perfusate relative to the plasma (∼305 versus 295 mosmol kg^{-1}, respectively), which would trigger either water efflux from the heart or taurine uptake from the saline, which contained a physiological concentration of taurine (0.7 µmol ml^{-1}). How this may have impacted function is unclear but given the potency of taurine's effects on some endpoints examined within, it is worthy of consideration.

Taurine deficiency did not induce oxidative stress in the heart, which can contribute to the loss of cardiac function during and after hypoxia and reoxygenation (Schaffer et al., 2014). Oxidative phosphorylation was somewhat uncoupled in mitochondria from TD hearts compared with that in controls and this may have reduced reactive oxygen species production by the ETS. In mammalian models, taurine deficiency reduces the translation of proteins with leucines encoded by UUG codons, as the associated $tRNA^{Leu(UUR)}$ requires sufficient taurine for effective decoding and post-transcriptional modification (Shaffer and Kim, 2018). The genes for CI subunits ND5 and ND6 contain multiple UUG codons and these proteins are under-expressed in TD mammals, causing impaired ETS function and an increase in reactive oxygen species production (Jong et al., 2012). We were unable to reliably quantify the expression of ND5, but the CIV subunit COX3 in brook char contains a UUG encoded leucine (L116; gene ID 808537) and its expression was reduced in TD fish. O_2 flux through CIV did not differ in TD fish, but changes in the expression of other mitochondrial proteins may underlie the decrease in RCR and lipid peroxidation. Taurine also has membrane-stabilizing effects (Huxtable, 1992), but it is unlikely that just a 20% reduction in concentration would be sufficient to impact the structure of the inner mitochondrial membrane enough to alter RCR.

Conclusions

We have demonstrated that a modest reduction in cardiac taurine content dramatically affects whole-animal and cardiac responses to acute thermal and hypoxic stress, revealing a powerful and previously uncharacterized regulator of cardiac function in fish. Cardiac taurine levels change in response to intracellular and extracellular osmotic disturbances and the capacity to deal with such challenges varies greatly between species and even within a single species. Interspecific and intraspecific differences in cardiac taurine handling may help to explain, at least to some extent, why some species of fish are able to maintain heart function under stress while others cannot. Further characterizing taurine's regulatory roles and the conditions under which cardiac taurine levels change will undoubtedly improve our understanding of how cardiovascular function limits stress tolerance in fish.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: T.-A.M.D., E.-L.M.R., S.P.M., N.A.O., K.S.P., A.J.M., S.G.L., T.J.M.; Methodology: T.-A.M.D., E.-L.M.R., N.E., S.P.M., N.A.O., K.S.P., E.C.B., C.M.P., M.S., A.C., A.J.M., S.G.L., T.J.M.; Software: T.J.M.; Validation: M.S., T.J.M.; Formal analysis: T.-A.M.D., E.-L.M.R., N.E., S.P.M., N.A.O., K.S.P., E.C.B., M.S., A.C., A.J.M., S.G.L., T.J.M.; Investigation: T.-A.M.D., E.-L.M.R., N.E., S.P.M., N.A.O., K.S.P., E.C.B., C.M.P., M.S., A.C., A.J.M., S.G.L., T.J.M.; Resources: N.A.O., K.S.P., A.C., A.J.M., S.G.L., T.J.M.; Data curation: T.-A.M.D., E.-L.M.R., N.E., S.P.M., N.A.O., K.S.P., E.C.B., M.S., A.J.M., S.G.L., T.J.M.; Writing - original draft: T.-A.M.D., E.-L.M.R., N.E., S.P.M., N.A.O., K.S.P., A.J.M., T.J.M.; Writing - review & editing: T.-A.M.D., E.-L.M.R., N.E., S.P.M., E.C.B., C.M.P., M.S., A.C., A.J.M., S.G.L., T.J.M.; Visualization: T.-A.M.D., E.-L.M.R., N.E., S.P.M., N.A.O., K.S.P., E.C.B., M.S., A.C., A.J.M., S.G.L., T.J.M.; Supervision: C.M.P., A.C., T.J.M.; Project administration: T.-A.M.D., C.M.P., A.J.M., S.G.L., T.J.M.; Funding acquisition: T.J.M.

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Data availability

Data are available from the Dryad digital repository (Dixon et al., 2023): doi:10.5061/dryad.w9ghx3fsd

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