

Cardiac Taurine Deficiency Impairs Physiological Performance and Mitochondrial Function
in Brook Trout (*Salvelinus fontinalis*)

By

Emma-Lee M. Rhyno

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Abstract

Taurine is a nonessential amino acid abundant in skeletal and cardiac muscle tissues. Taurine plays a critical role in many physiological processes, such as osmoregulation, cardioprotection, and oxidative stress mediation. Studies in other animal models have shown that taurine deficiency is associated with the progression of cardiovascular and mitochondrial diseases, as well as interruptions in energy metabolism. This study evaluated the effects of taurine on the physiological performance and mitochondrial function in brook trout, *Salvelinus fontinalis*. Dietary supplementation of β -alanine, a competitive inhibitor of the taurine transporter, was administered over a minimum time frame of four weeks to decrease intracellular taurine concentrations. Two treatment groups were used in this study, a group of fish fed a control diet and a taurine-deficient (TD) group fed the same diet infused with 5% β -alanine. The physiological impacts of taurine deficiency were assessed through evaluating the critical thermal maximum (CT_{max}) and tolerance to acute hypoxia through time-to-loss of equilibrium (LOE) tests. CT_{max} was evaluated by increasing tank water temperature at a set ramp rate until fish could no longer maintain equilibrium. CT_{max} was found to be significantly higher in ($p=0.017$), while tolerance to acute hypoxia was observed to be significantly lower ($p=0.015$) in TD trout than in the control group. FluoRespirometry evaluated mitochondrial efficiency at different points along the electron transport chain. Increased mitochondrial leak rates were observed in TD trout, indicating a greater degree of proton leak into the intermembrane space ($p=0.0056$). Activities of mitochondrial complexes I and II were not affected by TD. The average respiratory control ratio (RCR) of the TD trout was significantly decreased in comparison to the control group, indicating that a greater portion of oxygen consumption in TD mitochondria is not coupled with ADP phosphorylation and is instead dissipated as heat energy ($p=0.0051$). Respirometry also revealed that maximum oxygen consumption of complex IV of the electron transport chain was significantly decreased by TD ($p=0.0216$). Expression of complex IV protein COX3 was significantly downregulated in TD cardiac muscle tissue, indicating potential defects in protein synthesis correlated with TD ($p=0.007$). Our findings provide further insight into the roles of taurine, and pathology of taurine deficiency in the cardiovascular system of fish. This understanding can enable us to further gauge the effects of environmental fluctuations on this species.

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List of Abbreviations

tRNA	Transfer RNA
TauT	Taurine Transporter
CDO	Cysteine dioxygenase
CSD	Cysteinesulfinate decarboxylase
HTDH	Hypotaurine dehydrogenase
PKC	Protein kinase C
TD	Taurine deficient
ECC	Excitation-contraction coupling
SR	Sarcoplasmic Reticulum
LTCC	L-Type calcium channels
SERCA	Sarcoplasmic reticulum Ca ²⁺ ATPase
ATP	Adenosine triphosphate
ETC	Electron transport chain
IMM	Inner mitochondrial membrane
OXPHOS	Oxidative phosphorylation
FMN	Flavin mononucleotide
nDNA	Nuclear DNA
mtDNA	Mitochondrial DNA
ROS	Reactive oxygen species
CoQ	Coenzyme Q
MnSOD	Mitochondrial superoxide dismutase
CT _{max}	Critical thermal maximum
LOE	Loss of equilibrium
OXPI	State III Respiration (Complex I)
OXPI-II	State III Respiration (Complex I-II)
ET	Electron transport capacity
OXPII	Complex II OXPHOS
RCR	Respiratory control ratio
CCO	Cytochrome-c oxidase (Complex IV)

Introduction

Taurine

Taurine (2-aminoethansulfonic acid) is the most abundant free amino acid found in cardiac and skeletal muscle. As one of the most prominent organic acids across mammalian species, taurine exhibits a range of biological functions. Taurine is primarily known for its essential role as an osmolyte in the maintenance of cell volume but has been found to assist the body in cardioprotection, calcium transport, and oxidative stress mediation (Vislie, 1983; Sturman, 1993; Wang *et al.*, 2016).

Taurine was originally isolated from ox bile by Tiedemann and Gmelin (1827). Taurine, unlike other amino acids, contains an acidic sulfonate group in place of a carboxylic acid and an amino group bound to the β -carbon (Figure 1). Without the carboxyl group, taurine is unable to form peptide bonds and is therefore non-proteogenic. The absence of peptide bonds means that taurine is not included in the genetic code as it is not coded by tRNA (transfer RNA) (Huxtable, 1992; Salze and Davis, 2015).

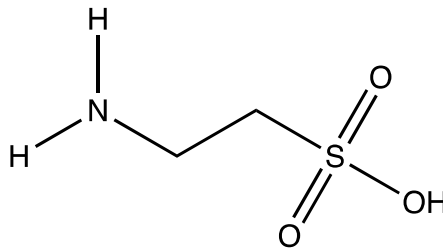


Figure 1: Chemical composition of taurine (2-aminoethansulfonic acid, C₂H₇NO₃S). Created using ChemDraw software.

Growing interest in the physiological significance of taurine stems from the steep gradients it establishes across cell membranes. As a zwitterion and a polar amino acid, taurine is highly water-soluble and resists diffusion through non-polar, lipophilic substances (Brosnan & Brosnan, 2006; Huxtable, 1992). This results in intracellular taurine concentrations that far exceed the concentrations in blood plasma. In rainbow trout (*Oncorhynchus mykiss*,) taurine concentrations in cardiac muscle compared to plasma were 48.7 $\mu\text{mol/g}$ and 0.73 $\mu\text{mol/g}$, respectively (Huxtable, 1992). To overcome its high gradient, taurine is actively transported into the cell through the Na/Cl⁻-dependent taurine cotransporter (TauT) (Huxtable, 1992, Pinto *et al.*, 2012).

Taurine Biosynthesis

Jacobsen and Smith (1968) first described five different biosynthetic pathways of taurine. Although the pathways are mechanistically different across organisms, taurine synthesis begins with sulfur-containing amino acids like methionine and cysteine (Brosnan & Brosnan, 2006; Wang *et al.*, 2016). The most extensively studied mammalian synthetic route is pathway 1, the cysteinesulfinate-dependent pathway (Salze and Davis, 2015). Pathway 1 converts cysteine to taurine, a process catalyzed by three enzymes; cysteine dioxygenase (CDO), cysteinesulfinate decarboxylase (CSD) and hypotaurine dehydrogenase (HTDH). These three sequentially convert cysteine into cysteine sulfinic acid, then to hypotaurine, and finally to taurine. Literature suggests that the cysteine metabolism of *O. mykiss* is similar to the mammalian pathway 1. Studies involving intraperitoneal injections of cysteine into rainbow trout observed increases in CDO activity, a result also observed in mammalian studies (Yokoyama *et al.*, 1997).

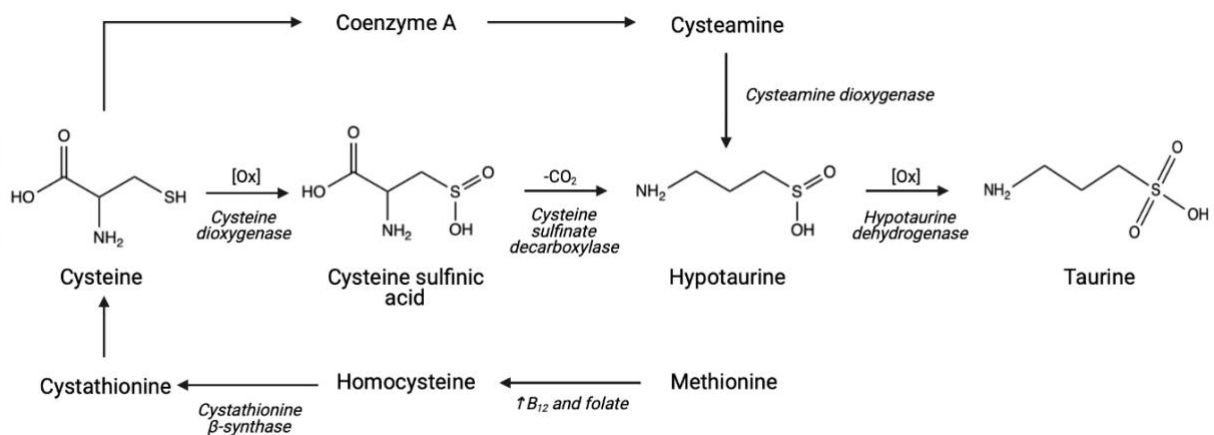


Figure 2: Biosynthetic pathway of taurine from cysteine and methionine. Pathway 1 involves the conversion of cysteine to taurine through a hypotaurine intermediate. Image adapted from De Luca *et al.*, (2015).

The capabilities of organisms to synthesize taurine varies between species and is tissue dependent. Rainbow trout and Japanese flounder are both species of teleost fish that have high and low abilities to synthesize taurine, respectively (Wang *et al.*, 2016). Marine fish species, such as the Japanese flounder, have a low taurine synthesis capacity due to low activity of CSD in methionine metabolism (El Sayed, 2014). Conversely, freshwater teleosts, such as trout and salmon, can synthesize taurine in their livers due to high CSD activity. Therefore, they do not require exogenous taurine supplementation. Freshwater fish utilize a

transsulphuration pathway to generate taurine from dietary sulphur-containing amino acids, such as methionine (Sampath *et al.*, 2020; El Sayed, 2014).

Taurine Transport

Unlike most amino acids, taurine is not metabolized or used in the formation of proteins. Taurine remains free and inert within cells; with reabsorption rates in the range of 40-99.5%, while other amino acids have reabsorption rates of 98-99% (Han *et al.*, 2006; Patters *et al.*, 2006). Transcriptional factors, like protein kinase C (PKC) influence taurine accumulation within the cell. PKC phosphorylation of the serine residue in the fourth segment of TauT is responsible for the transfer of taurine across the membrane (Patters *et al.*, 2006).

Taurine transport via TauT is dependent on cotransport with Na^+ and Cl^- . TauT has twelve hydrophobic transmembrane domains and both the N- and C-terminals are exposed to the cytosol. The extracellular loop at the N-terminal is the site of Na^+ and Cl^- binding, while taurine forms ionic bonds with arginine on the fourth helix (Lambert *et al.*, 2015).

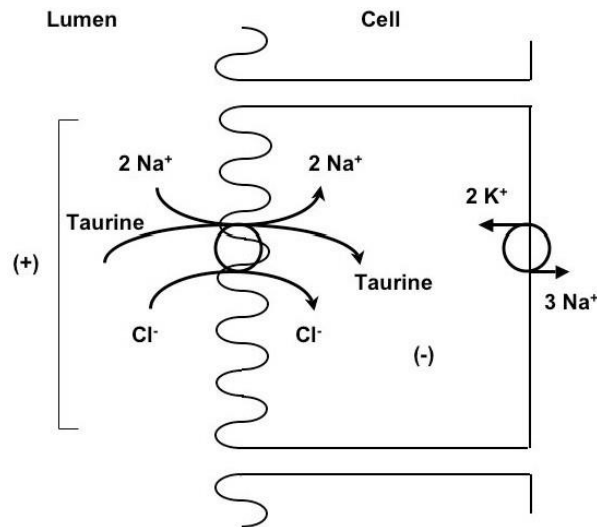


Figure 3: Ion movement through the sodium/chloride-dependent taurine cotransporter, (TauT) in a mammalian renal cell. (Chesney *et al.*, 2010).

The energy required for the active transport of taurine into the cell is generated from the movement of sodium along its gradient. Sodium concentration gradients are maintained by the enzyme $\text{Na}^+\text{K}^+\text{ATPase}$. In the absence of Na^+ , taurine is not imported into the cell (Han *et al.*, 2006; Chesney *et al.*, 2010). Cl^- is also required for full taurine uptake; unlike

Na⁺, it can be partially substituted with bromide (Patters *et al.*, 2006). TauT stoichiometry requires two Na⁺ and one Cl⁻ for the transport of one unit of taurine (Figure 3).

β-Alanine

β-Alanine (Figure 4) is a non-essential β-amino acid that is naturally produced within the body. β-alanine is commercially available as a dietary athletic supplement due to its ability to increase muscle carnosine levels (Liu *et al.*, 1993). As a structural analogue of taurine, β-alanine can access TauT and competitively inhibit taurine uptake, effectively reducing taurine levels when administered (Schaffer, 2015; Shaffer & Kocsis, 1981). Unlike taurine, β-alanine is not inert and can be metabolized to malonate semialdehyde and carnosine (Mendelson, 2008).

Research conducted by Shaffer and Kocsis (1981) observed increased urinary taurine excretion and decreased tissue taurine content in rats following oral supplementation of 3% β-alanine. Increased taurine excretion indicates that taurine uptake into cells was compromised. Other inhibitors of taurine, such as its guanidino analogue taurocyamine, were compared with β-alanine to assess their ability to deplete intracellular taurine. Neither substitute showed taurine decreases comparable to levels observed with β-alanine, indicating that β-alanine may be the most competitive inhibitor of taurine uptake (Shaffer & Kocsis, 1981; Liu *et al.*, 1993).

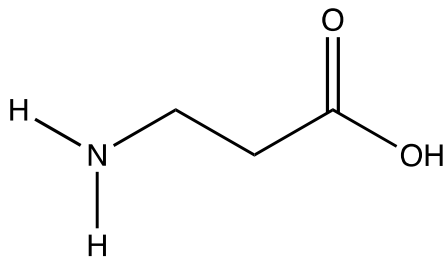


Figure 4: Chemical composition of β-alanine (3-aminopropanoic acid, C₃H₇NO₂).
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Taurine and the Heart

Particularly high concentrations of taurine are found in excitable cardiac and skeletal muscle tissues. Within the heart, higher levels of taurine are found in the ventricle. Based on this observation, taurine content is hypothesized to be linked with cardiac workload (Eley *et al.*, 1994; Schaffer *et al.*, 2010). Taurine is required for maintenance of normal heart function, as depletion had been observed to lead to cardiomyopathy in studies involving

taurine deficient (TD) cats (Pion *et al.*, 1987). Though the mechanism of TD-induced heart failure is not yet clear, decreased Ca^{2+} handling and reduced sensitivity of contractile proteins to Ca^{2+} has been associated with decreased taurine and heart failure (Schaffer *et al.*, 2010). Skeletal muscle function is also impaired in TauT knockout mice (Schaffer *et al.*, 2010).

One of taurine's most prominent physiological roles is controlling Ca^{2+} levels in the heart. Excitation-contraction coupling (ECC) is the process by which electrical impulses are converted into cardiac muscle contractions (Figure 5). This process is crucial in maintaining normal contractile rhythms and preserving heart health.

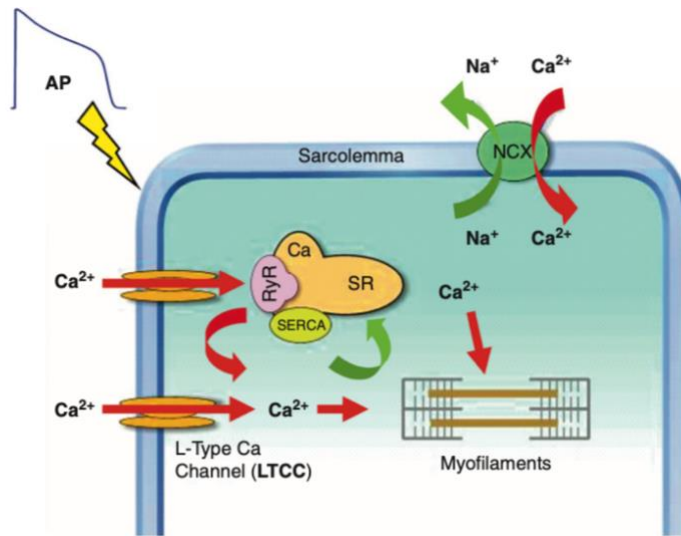


Figure 5: Calcium movement during excitation-contraction coupling in a fish cardiomyocyte. The sarcolemma (SL) is excited by an action potential which opens L-type calcium channels (LTCC) allowing Ca^{2+} influx (red arrows). Ca^{2+} can also enter the cell through the Na-Ca exchange system. Ca^{2+} influx triggers calcium release from the SR through ryanodine receptors (RyR). This influx causes a rise in Ca^{2+} that initiates contraction at the myofilaments. When calcium is removed from the cytosol (green arrows), relaxation occurs. Image taken from Shiels, (2011).

Fish cardiomyocytes have a large surface area-to-volume ratio that increases the effectiveness of ion flux pathways (Shiels, 2011). Ca^{2+} influx into the cytosol initiates ECC which, in most fish is acquired from extracellular Ca^{2+} . However, in rainbow trout and tuna, calcium release from the sarcoplasmic reticulum (SR) plays a greater role in Ca^{2+} influx (Shiels, 2011). Depolarization of the membrane causes the L-type calcium channels (LTCC) to open. These channels are the main pathway for calcium influx. At this point, extracellular Ca^{2+} concentrations are at least 100-fold greater than within the cell, so ions flow readily into the cell down their gradient (Shiels, 2014; Huxtable, 1992). Intracellular Ca^{2+} can also be

increased by release from the SR through ryanodine receptors (RyR) (Schaffer *et al.*, 2010). High intracellular Ca^{2+} promotes Ca^{2+} binding to troponin C, that removes tropomyosin from myosin to enable binding of actin, resulting in tension that drives a muscle contraction. The “coupling” process of ECC is initiated during muscle contraction as calcium is returned to the SR via the SR Ca^{2+} ATPase (SERCA) or out of the cell through the Na-Ca exchanger (D’Oria *et al.*, 2020; Huxtable, 1992). This decrease in intracellular calcium weakens the actin-myosin bonds and enables muscular relaxation.

In the heart, taurine exerts cardioprotective properties to maintain optimal organ function. Taurine increases sensitivity of contractile proteins to Ca^{2+} , and the activity of SERCA is inhibited by oxidative stress. Taurine deficiencies contribute to systolic and diastolic dysfunction as calcium binding to troponin is impaired, altering the degree of muscle contraction (Schaffer *et al.*, 2010).

Mitochondrial Respiratory Chain Complexes

Mitochondrial respiration is the most vital feature of living organisms as it supplies most of the ATP necessary to support physiological function. In animals, including teleost fish, respiration occurs by the electron transport chain, also known as the respiratory chain (Hansen *et al.*, 2010). The electron transport chain (ETC) is a complex carrier mechanism

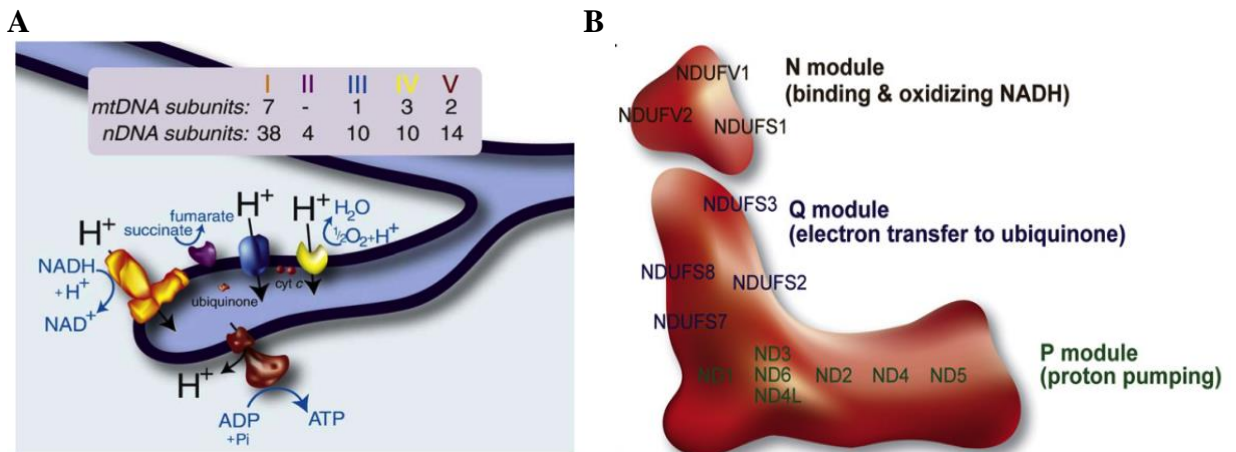


Figure 6: Mitochondrial electron transport chain complexes. A) Visual description of OXPHOS through the ETC on the IMM. The chain begins as electrons enter via CI or CII, coloured orange and purple, respectively. Electrons exit the chain at complex IV (yellow) causing the reduction of O_2 to $2\text{H}_2\text{O}$. The number of nuclear (nDNA) and mitochondrial (mtDNA) subunits are illustrated above. (Lazarou *et al.*, 2009). **B)** Schematic assembly of mammalian CI. The matrix branch and membrane arm join to form an L-shaped structure. Relative locations of core subunits are indicated and are highly conserved between prokaryotes and eukaryotes. (Mimaki *et al.*, 2012).

located on the inside of the inner mitochondrial membrane (IMM). The ETC is made up of four transmembrane protein complexes, I-IV that function as electron carriers in oxidative phosphorylation (OXPHOS) (Figure 6.A). In this study, complex I is of interest due to the documented effects of taurine deficiency on its activity by Jong *et al.* (2012).

Mitochondrial ATP is produced through the coupling of ADP phosphorylation and electron transfer through oxidoreductase reactions (Zhao *et al.*, 2019). OXPHOS begins when electrons enter the transport chain through complex I (CI, NADH ubiquinone oxidoreductase) or complex II (CII, succinate ubiquinone oxidoreductase) (Picard *et al.*, 2011). CI transfers electrons from NADH to ubiquinone via flavin mononucleotide (FMN) and iron-sulfur complexes, and transfers protons and electrons out of the matrix in a ratio of 4:2. Electrons are then transferred to complex III (CIII, cytochrome c oxidoreductase), to cytochrome c, and then to complex IV (CIV, cytochrome c oxidase). CIV terminates the ETC and reduces oxygen to two water molecules using the generated electrons (Picard *et al.*, 2011). The CI-CIII-CIV pathway pumps five protons per electron, generating the membrane potential (Lazarou *et al.*, 2009). This generated potential then induces a conformational change in ATP synthase, producing ATP.

Complex I

CI (NADH ubiquinone oxidoreductase) is the largest multi-subunit enzyme complex of the mitochondrial electron transport chain, weighing ~980 kDa in mammalian cells (Guo *et al.*, 2018, Figure 6.B). In general, CI binds to and oxidizes NADH to generate two electrons which are then transferred to ubiquinone. CI is located on the IMM, protruding into the matrix in an L-shaped structure. CI is composed of 45 different subunits, 14 of which are conserved core subunits and 31 are accessory subunits (Zhao *et al.*, 2019; Lazarou *et al.*, 2009).

The peripheral branch of CI extends into the mitochondrial matrix and is composed of two divisions; the electron input and output modules, designated by 'N' and 'Q', respectively (Mimaki *et al.*, 2012). The N-module is the site of NADH oxidation and utilizes FMN as the primary electron acceptor. The Q-module is where the electron transfer to ubiquinone takes place, occurring via FMN and iron-sulfur clusters (Zickermann *et al.*, 2015). Both modules of the peripheral branch contain nDNA encoded subunits of the NDUFV and NDUFS families, however not all subunits are required to be present for complex function (Cardol *et al.*, 2008).

The membrane arm of CI is perpendicular to the peripheral branch and is located on the IMM. The membrane arm is also the site of proton translocation, termed the P-module. The P-module contains three hydrophobic protein subunits, ND2, ND4 and ND5 which act as antiporters in proton movement, similar to Na⁺ or K⁺ antiport systems (Lazarou *et al.*, 2009; Mimaki *et al.*, 2012). Due to similar function in their bacterial homologs, it is hypothesized that mitochondrial-encoded subunits (ND1-ND5) play a role in proton movement and ubiquinone binding (Cardol *et al.*, 2008; Zickermann *et al.*, 2015).

Taurine and Complex Assembly

Complex subunits must assemble in a specific configuration to ensure proper function, and taurine plays an important role in this assembly as its conjugation with the leucine codon (UUG) in the wobble position of tRNA^{Leu(UUR)} is required for normal decoding and post-transcriptional modification (Shaffer & Kim, 2018). Taurine deficiency interferes with this conjugation and contributes to the malformation of complexes as mitochondrial protein synthesis is interrupted (Rikimaru *et al.*, 2012). The degree to which complex assembly is impaired depends on the frequency of UUG leucine codons within the amino acid sequence. The assembly of CI is poorly understood due to its large size, lack of image resolution, and control by both nuclear and mitochondrial DNA (Zhao *et al.*, 2019; Cardol *et al.*, 2008). Protein ND5 of CI has been of specific interest in TD animal studies. ND5 has two UUG leucine codons, and impairments in CI function and ND5 protein expression have been observed in TD rat cardiomyocytes (Jong *et al.*, 2012). Another protein of CI that has been found to be significantly impacted by taurine deficiency in rats is ND6, due to its eight UUG codons. However, ND6 expression was not targeted in this study as protein sequence conservation was not strong enough between organisms to obtain a commercial antibody that would be reactive in our trout species.

Deficiencies in CI due to impaired assembly is one of the most common pathological ETC defects, as all 14 core subunits have documented genetic defects to date (Mimaki *et al.*, 2012). MELAS (mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes) and MERRF (myoclonic epilepsy and ragged-red fiber syndrome) are branches of mitochondrial disease caused by mutations and malformations of the tRNA^{Leu(UUR)} wobble position (Jong *et al.*, 2011; Tsutomu *et al.*, 2011). Symptoms of these mitochondrial diseases

include cardiomyopathy and muscle weakness, which are also associated with TD (Schaffer *et al.*, 2013; Tsutomu *et al.*, 2011).

Taurine and Oxidative Stress

Along with decreased ATP generation, deficient complex formation also contributes to increased oxidative stress (Christen *et al.*, 2018). When complexes are not properly formed, the movement of electrons along the electron transport chain is slowed, creating a bottleneck in transport (Jong *et al.*, 2011; Schaffer *et al.*, 2012). Due to this, electrons are diverted from the chain and bind with environmental oxygen to produce superoxide anions, a type of reactive oxygen species (ROS) (Lazarou *et al.*, 2009).

Oxidative stress is characterized by the excess production of ROS, which can lead to mitochondrial damage, mutations, interruptions in calcium homeostasis and impairment mitochondrial defense mechanisms, all of which are related to various diseases (Guo *et al.*, 2013). Under normal physiological conditions, ROS production is stable and an essential part of aging and necessary cell proliferation. Mitochondria directly produce ROS such as free-radical superoxide (O_2^-) and non-free radical hydrogen peroxide (H_2O_2). Exogenous sources of free radicals include pollution and radiation, and endogenous sources arise within the cell from the mitochondria (Phaniendra *et al.*, 2015). Free radicals alter normal redox states, which causes oxidative stress. The majority of intracellular ROS come from the mitochondrial electron transport chain as electrons leak out into the matrix. CI and CIII are the major sites of superoxide radical production due to their electron transferring capabilities (Jong *et al.*, 2012). Liu *et al.* (2002) demonstrated that ROS production occurs at the FMN of CI through reverse electron transfer and leakage to couple with molecular oxygen.

Electron transfer from CI or CII to coenzyme Q (CoQ) produces a reduced form of CoQ, which regenerates itself via an unstable semiquinone anion (Picard *et al.*, 2011; Guo *et al.*, 2013). This anion transfers its electrons to molecular oxygen in the mitochondrial matrix, forming the ROS superoxide, O_2^- . The superoxide is converted to hydrogen peroxide, another ROS, by mitochondrial superoxide dismutase (MnSOD) (Guo *et al.*, 2013). Free radicals can cause oxidative damage to nucleic acids, specifically mitochondrial DNA (mtDNA) due to their proximity to the release site. Mutations in mtDNA can lead to further impairment of ETC function and pathology (Zhao *et al.*, 2019). ROS also contribute to protein denaturation by oxidizing certain amino acids and forming cross-links. Sulphur-containing amino acids,

like methionine and cysteine, are especially susceptible to damage via oxidation from ROS (Zhao *et al.*, 2019; Hansen *et al.*, 2010).

Taurine is critically involved in the regulation of oxidative stress. However, the antioxidant mechanisms of taurine are poorly understood. Jong *et al.*, (2012) postulated that taurine's antioxidant activity is correlated with improved mitochondrial function by reducing the generation of ROS. Their hypothesis was tested by inducing taurine deficiency in rat cardiomyocytes using β -alanine. Aconitase activity and the glutathione redox state are two markers of oxidative stress. In TD cardiomyocytes, aconitase activity and the redox capacity of glutathione were decreased by 45% and 43%, respectively (Jong *et al.*, 2012). When β -alanine and taurine were administered in equal quantities, aconitase and glutathione redox activities were unaffected when compared with control cells containing only taurine. Their results indicate that β -alanine mediated taurine deficiencies cause oxidative stress in mammalian mitochondria.

Decreases in complex activity from TD-induced oxidative stress occurs due to reduced levels of core complex subunits. Specific to CI, downregulation of core subunits ND5 and ND6 have been observed in TD rat cardiac muscle cells (Jong *et al.*, 2012; Schaffer *et al.*, 2014).

Oxidative Stress and the Cardiovascular System

Typically, oxidative balance within cardiomyocytes is tightly regulated by antioxidant systems, which modulate the creation and removal of ROS (Münzel *et al.*, 2017). At low levels, ROS are required for cell differentiation, proliferation, and ECC. Increased ROS and oxidative stress within the myocardium have been observed to cause left ventricular dysfunction in murine models (D'Oria *et al.*, 2020).

In rat cardiomyocytes, ROS have been observed to impact contractile function in ECC by inhibiting calcium uptake at the SERCA (Takimoto and Kass, 2006). The SERCA enables cardiac muscle relaxation during diastole which inhibits calcium uptake and prevents adequate repolarization of the ventricle before the next contraction occurs. This leads to a rapid succession of ventricular contractions, eventually weakening the heart muscle and leading to the development of cardiomyopathy (Köhler *et al.*, 2014). Although the precise mechanism of the development of cardiomyopathy in fish is unknown, it is predicted that oxidative stress impairs ventricular diastole such that the heart cannot properly relax before

the next contraction. Due to this, the diastolic blood pressure begins to rise to levels near the systolic pressure, hereby decreasing pulse pressure (Schaffer *et al.*, 2010; D’Oria *et al.*, 2020). Decreased pulse pressure in TD animals can therefore be a biomarker of the development of cardiovascular diseases (Bkaily *et al.*, 2019).

Experimental Objectives

The objective of this study was to evaluate the impacts of taurine deficiency in brook trout, *Salvelinus fontinalis*, on the cellular and whole-animal level. Intracellular taurine levels in trout were depleted following a four-week diet of 5% β -alanine infused feed. Prior studies conducted by researchers in the MacCormack Lab indicated that taurine levels are depleted to a minimal level after this feeding regime. In rat studies conducted by Jong *et al.* (2012), taurine deficiencies increased mitochondrial oxidative stress by increased superoxide generation, and decreased levels of CI and CIII.

The implications of taurine deficiencies have been frequently observed in mammals, however a gap in knowledge surrounds the impacts on teleost fish. By initiating taurine deficiencies in *S. fontinalis* using β -alanine, we expected to observe decreases in ETC activity at complex I and decreased expression of proteins encoding core subunits ND1 and ND5. Western blot analyses using antibodies specific to the ND1 and ND5 proteins were used to identify their abundance in trout ventricle tissue. The protein encoding complex IV, COX3, was used as a control in this study as it was not reported to be decreased under conditions of TD (Jong *et al.*, 2012). Measurement of mitochondrial O₂ consumption will be used to determine complex efficiency along the ETC.

Experiments conducted by Gates (2019) and Otley (2020) in the MacCormack Lab showed that taurine deficiencies in *S. fontinalis* did not impact critical swimming speed or physical fitness in chase-to-exhaustion tests but did cause significant decreases in pulse pressure. By depleting intracellular taurine and compromising cardiac function, we expected to observe differences in the tolerance to thermal stress and hypoxia between control trout (normal diet) and our β -alanine diet test group.

Acclimation to differing environmental temperatures involves cardiorespiratory adaptations to facilitate metabolism in sub-optimal conditions. Thermal tolerance is quantified as the critical thermal maximum (CT_{max}), which is the water temperature (°C) at which loss of equilibrium (LOE) occurs (Baker *et al.*, 2020). LOE indicates the maximal

level of cardiac output that the organism can sustain and assessing the CT_{max} of TD *S. fontinalis* will indicate whether oxidative stress impacts their cardiac function. Hypoxia tolerance is also assessed by loss of equilibrium. With altered respiratory chain complexes and impaired cardiac function, we hypothesized that TD trout will be less tolerant to oxygen depletion.

Methods

Animal Care

Fifty-eight brook trout (*Salvelinus fontinalis*) were transported from the Bailey Hall Fish Lab at the University of New Brunswick, Fredericton, to Mount Allison University's Harold Crabtree Aqualab facility in spring of 2020. Fish were housed in two 750 L partially recirculating tanks of freshwater at 16°C. The total volume of the tank system was 2400 L, freshwater was circulated through a biofilter and UV light. Twenty-nine trout were kept in one tank as a control group and twenty-nine were transferred to the second tank as the experimental group. The control fish were fed a diet of 4.0 mm Optimum sinking pellets (Corey Nutrition, Fredericton, NB, Canada) every other day until satiation. The experimental trout were fed 3mm EWOS Vita sinking pellets that had been vacuum coated with 5% β -alanine by mass (EWOS, Surrey, BC, Canada). The experimental fish were fed for a minimum of four weeks before conducting any experiments. Prior studies conducted by Gates (2019) revealed that this time frame is required to significantly decrease intracellular taurine concentrations. Sex distribution of fish used in experiments was divided evenly between males and females. All experiments were conducted in accordance with the Mount Allison Animal Care Committee (Protocol #101873).

Thermal Tolerance

The thermal tolerance of seven control (391.09 ± 109.06 g) and seven β -alanine fed-trout (446.46 ± 189.02 g) was assessed through determining their voluntary thermal maximum (VTM) and critical thermal maximum (CT_{max}) in degrees Celsius following established protocols (Gallent *et al.*, 2017, Becker & Genoway, 1979). Fish were removed from their housing tanks and tested individually in a 15-gallon glass aquarium filled with tank water at 16°C. Following a five-minute acclimation period, the water temperature was increased at a rate of $0.3^{\circ}\text{C min}^{-1}$ using a DYNEO DD heating immersion circulator (Julabo; Seelbach, Germany). The temperature at which the fish began to show signs of physical distress was recorded as the VTM. Behaviour traits used to determine VTM were congruent with the physical distress responses observed by Nilsson *et al.* (2019). Indicators used to mark physical distress were emergence attempts, increased burst of swimming speed followed by rest and erratic swimming patterns that included collisions with the tank walls, rapid direction changes, and sideways bending of the body. CT_{max} was recorded as the

temperature when loss of equilibrium occurred. Immediately upon reaching CT_{max} , fish were removed from the heated water, weighed, and transferred to a recovery tank at 16°C. Once they regained the ability to maintain equilibrium, they were returned to their 750L housing tanks.

Hypoxia Tolerance

The hypoxia tolerance of eight control (502.02 ± 127.71 g) and eight β -alanine fed-trout (456.28 ± 163.31 g) was assessed by measuring the time-to-loss of equilibrium when exposed to reduced oxygen saturation (%O₂). Fish were removed from their housing tanks and tested four-at-a-time in 100L tanks at 16°C. Following a 24-hour acclimation period, the O₂ saturation was decreased at a rate of 1.5% per minute to a minimum saturation of 10% by pumping nitrogen gas into the tanks using a WitroxCTRL oxygen control system (Loligo Systems; Copenhagen, Denmark). A timer was started when the O₂ levels reached 10% and the time at which the fish reached LOE was recorded. Fish were immediately removed from the tanks, weighed (g) and transferred to a fully oxygenated recovery tank at 16°C. Once fish regained equilibrium and resumed normal swimming behaviours, they were returned to their housing tanks.

Mitochondrial Respirometry

All chemicals used in the following protocol were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

Muscle Fibre Permeabilization

Tissue samples were obtained from the apex of the ventricles in eight control (504.96 ± 103.61 g) and eight β -alanine-fed (475.45 ± 116.06 g) trout. Duplicate samples from each heart were assessed. Once dissected, samples were immersed in 2 mL cold BIOPS buffer (2.77 mM CaK₂EGTA, 7.23 mM K₂EGTA, 5.77 mM Na₂ATP, 6.56 mM MgCl₂·6 H₂O, 20 mM taurine, 20 mM imidazole, 0.5 mM dithiothreitol, 50 mM MES hydrate, 15 mM Na₂Phosphocreatine and 50 mM sucrose, pH 7.1 at 0°C). Muscle fibre bundles were pulled from the tissue using fine forceps and were immediately transferred to fresh BIOPS with 50 µg/mL saponin in a 6-well culture plate and shaken on ice for 30 min to permeabilize the tissue. Fibres were then washed in 2 mL of mitochondrial respiration media, MiRO5 (0.5 mM EGTA, 3 mM MgCl₂, 60 mM lactobionic acid, 20 mM taurine, 10 mM KH₂PO₄, 20 mM HEPES, 110 mM D-sucrose and 1 g/L fatty acid-free BSA, pH 7.1 at 30°C), for 15 min.

Fibres were blotted dry and weighed before use in respiration assays. Fibre bundle weights were between 1-3 mg.

Respiration Assay

Respirometry followed protocol described by Iftikar & Hickey, 2013. Duplicate ventricle fibre samples were added to two chambers filled with 2 mL of MiRO5 media in an Oroboros O2k Fluorescence Respirometer (Oroboros Instruments, Innsbruck, Austria, CAT No. 10002-03). Mitochondrial function of the ETC throughout OXPHOS was assessed using a substrate-uncoupler-inhibitor titration (SUIT) protocol. SUIT protocol chemicals were prepared in advance according to (Oroboros O2k-Procedures, 2016) and added to the chambers in the following order (Figure 7).

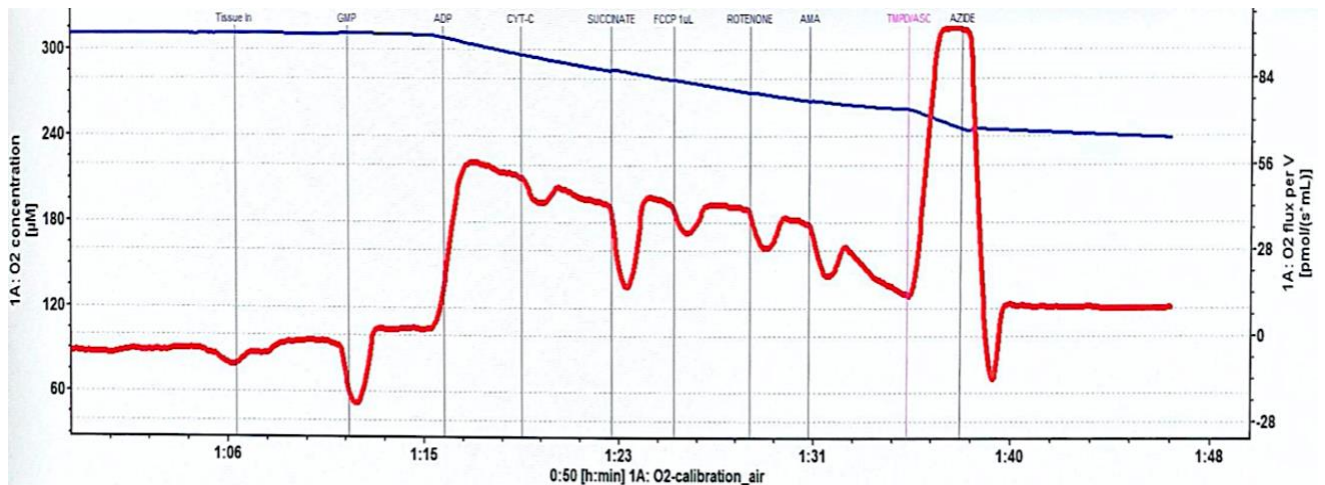


Figure 7: Mitochondrial respiration assay trace from permeabilized muscle fibres of *S. fontinalis* ventricle tissue measuring mitochondrial O₂ flux (pmol s⁻¹mL⁻¹, red line, right y-axis) and oxygen concentration (µM, blue line, left y-axis). Additions of SUIT protocol substrates are indicated with vertical lines

CI substrates malate (1 mM), glutamate (10 mM) and pyruvate (5 mM) were first added to initiate the Krebs's cycle and measure the baseline "Leak" state II respiration through CI when phosphorylation of ADP is not occurring. ADP (5 mM) was then added to initiate OXPHOS to measure state III respiration, "OXPHOS I." Cytochrome-C (10 µM) was added to evaluate the integrity of the outer mitochondrial membrane. Increases in O₂ flux greater than ~15% indicate significant membrane damage and will nullify the data collected from the sample in question. Succinate (10 mM) was then added to stimulate FADH₂ production and enable electrons to enter the ETC via CII as well as CI, "OXPHOS I-II." The OXPHOS uncoupler

carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP, 0.5 μM) was added to uncouple the mitochondria. FCCP was added in 1 μL increments to a maximum of 5 μL until O_2 flux stopped increasing to give the electron transport capacity “ET”. Rotenone (0.5 μM) was added to inhibit CI function and reveal the O_2 consumption rate of CII-fueled OXPHOS “OXF II.” Antimycin A (2.5 μM) was added to inhibit electron flow to CIII and decrease O_2 consumption. *N,N,N',N'*-tetramethyl-p-phenylenediamine dihydrochloride (TMPD, 0.5 M) and ascorbate sodium salt (2 mM) were added simultaneously to measure the steady-state activity of cytochrome-c-oxidase. Ascorbate functioned to keep TMPD in its reduced state. Finally, sodium azide (0.1 M) was added to cease all complex function to measure the residual background O_2 consumption.

Throughout the titration protocol, O_2 flux ($\text{pmol O}_2 \text{ s}^{-1} \text{ mL}^{-1}$) at the different respiratory stages was calculated using Oroboros O2k DatLab data acquisition software (Oroboros Instruments, Innsbruck, Austria, CAT No. 20700).

The respiratory control ratio (RCR) was calculated as the ratio of OXF I to Leak respiration rates (Equation 1). The RCR is indicative of the coupling capacity of the mitochondrial electron transport system.

$$RCR = \frac{OXF I}{Leak} \quad \text{Equation 1}$$

The maximal rate of CIV O_2 consumption “CCO” was calculated as the steady-state rate following the addition of TMPD and ascorbate, subtract the background rate of consumption after sodium azide was added (Equation 2).

$$CCO = TMPD - Azd \quad \text{Equation 2}$$

Protein Quantification

Antibody Selection

This project aimed to quantify the abundance of three protein that encoded core subunits of different electron transport chain complexes. Two of the target proteins in this study encode two core subunits of CI, NADH-ubiquinone oxidoreductase subunit 5 (MT-ND5, UniProt ID: Q9XN31) and NADH-ubiquinone oxidoreductase subunit 1 (MT-ND1, (UniProt ID: Q9XN37). The third target protein encoded a core subunit of CIV, cytochrome-c oxidase subunit 3 (MT-COX3, UniProt ID: Q9XN34).

To detect the presence of protein bands through Western blotting, appropriate primary antibodies were selected for each target. Antibodies reactive in *S. fontinalis* and specific to

the target proteins were not found across antibody vendors. Commercial unconjugated polyclonal antibodies for MT-ND5, MT-ND1 and MT-COX3, were found with reactivity in *H. sapiens*, *M. musculus*, *R. norvegicus* and *D. rerio*. Bioinformatic analyses of protein sequence conservation revealed high levels of similarity between key active binding sites across each organism. Protein sequences were obtained from UniProt (The UniProt Consortium, 2021) and compared using Jalview Version 2 (Waterhouse *et al.*, 2009).

All antibodies were ordered from MyBioSource (San Diego, CA, USA). Antibodies targeting MT-ND5 and MT-ND1 required blocking peptides, which were also purchased from MyBioSource.

Tissue Sampling

Five control (537.05 ± 125.76 g) and five β -alanine fed-trout (433.63 ± 149.74 g) were removed from their housing tanks and were given a lethal dose of tricaine methanesulfonate anaesthetic (TMS, 300 mg/L; Aqualife) buffered with sodium bicarbonate (NaHCO_3 , 600 mg/L) in a darkened chamber filled with tank water. Loss of consciousness was marked by the cessation of ventilation across the gills, and at this point the fish were removed from the chamber and transferred to a surgical table. To ensure death, spinal cords were severed above the operculum. Immediately after severing the spinal cord, two samples of each fish's ventricle were taken and frozen in liquid nitrogen. Tissue samples were stored at -80°C until required for further analysis.

Protein Extraction and Sample Preparation

Frozen tissue samples were thawed, and 20-30 mg were weighed into bead-filled tubes. Each pellet received 300 μL 1X protein extraction buffer composed of 4X protein solubilization buffer (Agrisera, CAT No. AS08 300), ddH₂O and 50X Pefabloc (Bioshop, CAT NO. AEB602). Samples were lysed thrice for 60 s at 6.5 m/s in a FastPrep-24 5G benchtop homogenizer (MP Biomedicals, CAT No. 116004500) with 60 s of cooling on ice between lysing. Samples were then centrifuged at 16 162 g for 5 min and their supernatants were removed and re-centrifuged at 16 162 g for 2 min. The homogenized supernatants were then separated into aliquots for use in protein quantification assays and western blots. Total protein content of the ten ventricle tissue samples was measured using a BCA Protein Assay Kit (ThermoFischer Scientific, CAT No. 23225) compared to standards of bovine gamma globulin (BGG, Bio-Rad) following assay protocols performed by Bonisteel *et al.* (2018).

Absorbances were measured at 562 nm using a SpectraMax microplate reader. Sample concentrations were assessed in triplicate, and averaged to determine the

MT-COX3 samples were prepared at 3 ug total protein content with dithiothreitol (DTT) and Novex Bolt LDS sample buffer (4X, 1X, Life Technologies, CAT No. B0008). Optimal sample loads for MT-ND5 and MT-ND1 proteins are still being determined. Prepared samples were heated at 70°C for 5 min and spun in a benchtop centrifuge for a few seconds before loading into Bolt 4-12% Bis-Tris Plus 15-well acrylamide gels (Invitrogen, CAT No. NW04125). Protein migration was compared to a standard ladder of visible NovexSharp Pre-Stained standard (Invitrogen, CAT No. 57318) and MagicMark XP western standard (Invitrogen, CAT No. LC5603).

Gel Electrophoresis and Transfer

Blots were electrophoresed at 200V for 30 min in 1X MES SDS running buffer (Life Technologies, CAT No. B0002). The gels were then transferred to PVDF membranes at 20V for 60 min in 1X Bolt transfer buffer (Life Technologies, CAT No. BT00061). Following transfer, PVDF membranes were blocked in 2% Amersham ECL blocking solution (Cytiva, CAT No. RPN2125) for one hour at room temperature with gentle agitation.

Antibody Incubation

After blocking, blots were incubated with primary antibodies for one hour with gentle agitation (MyBioSource, CAT No. MT-ND5: MBS8242338, MT-ND1: MBS129270, MT-COX3: MBS9708822). Antibodies were prepared at 1:1000 dilutions in ECL blocking solution. After the primary incubation, membranes were washed with 1X TBS-T twice briefly, once for 15 min and thrice for five min. Membranes were then incubated with 1:1500 dilutions of Goat Anti-Rabbit IgG HRP secondary antibody (Bio-Rad, CAT No. 6423791) for one hour with gentle agitation. The same wash protocol was repeated following this incubation period.

Blot Imaging and Analyses

Following incubation, the PVDF membranes were wet with a 1:1 chemiluminescent solution of Luminol (Amersham ECL Select, CAT No. 2235V1) and peroxide and visualized with a VersaDoc Molecular MP 5000 Imager (Bio-Rad, CA, USA). Quantity One 1-D analysis software (Bio-Rad, CA, USA) was used to process the images and Bio-Rad 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). Unpaired t-tests were used to compare control and β -alanine-fed trout data, along with F-tests to determine homogeneity of variances and Shapiro-Wilk's normality tests; all of which used an α of 0.05. If data failed assumptions, nonparametric Mann-Whitney rank tests were used to compare between the two groups.

Results

The group of experimental trout fed β -alanine-infused feed appeared to be healthy and were visibly and behaviourally indistinguishable from the control group throughout the course of the following experiments. Additionally, there were no significant effects of body mass or sex on the data collected in this study, nor were there significant differences in fish size between treatment groups.

Previous work by Gates (2019) observed significant decreases of 16.8% in *O. mykiss* heart taurine concentrations following four weeks of 5% β -alanine-infused feeding. Prior studies have found that the critical swimming speed (U_{crit}) and time-to-exhaustion in chased trout are not affected by taurine depletion (Gates, 2019; Otley, 2020). However, fluctuations between systolic and diastolic blood pressure in TD *S. fontinalis* were significantly decreased compared to control fed trout (Otley, 2020). This cardiac impairment suggests that physiological performance may be affected, which prompted our investigation into the impacts of depleted taurine on the tolerance of *S. fontinalis* to thermal and hypoxic stress.

CT_{max} & VTM

The average VTM of β -alanine fed fish was increased, but not found to be significantly different than that of the control fish ($p=0.077$, Figure 8). CT_{max} was significantly increased in the β -alanine fed trout, at an average temperature of $30.88 \pm 0.23^{\circ}\text{C}$, while the CT_{max} of the control fish was $29.84 \pm 0.96^{\circ}\text{C}$ ($p=0.017$).

Carline & Machung (2011) performed thermal tolerance experiments on different species of wild trout acclimated at varying temperatures. The CT_{max} of brown trout acclimated at 12.0°C was observed at 28.2°C , and the CT_{max} of brook trout acclimated at 10°C was observed at 28.5°C . Although higher than the average observed in those studies, our data are realistic as CT_{max} has been observed to increase with acclimation temperature (Currie

et al., 1998). Additionally, studies involving rainbow trout acclimated at 18°C showed an average CT_{max} of 31.2°C (Recsetar *et al.*, 2011).

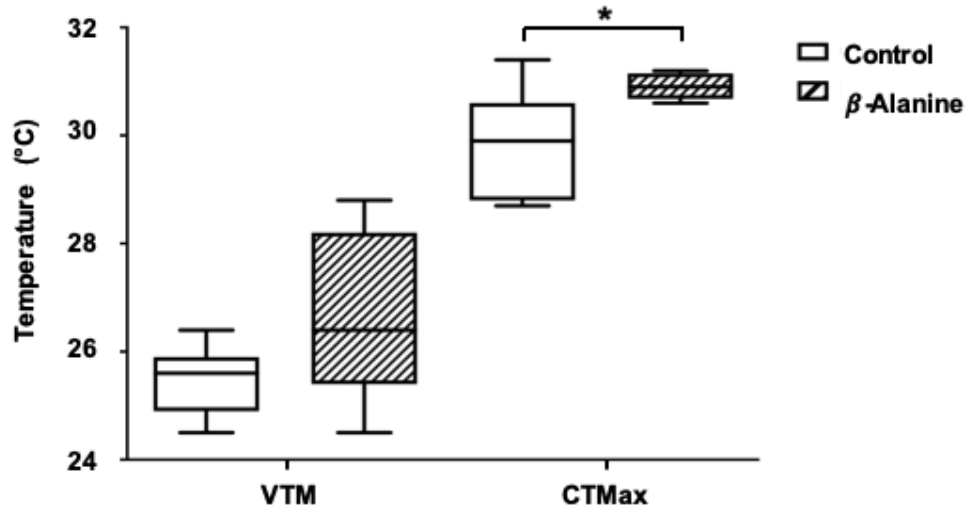


Figure 8: Average voluntary thermal maximum and critical thermal maximum (°C) of *S. fontinalis* fed a control diet (n=7) compared with *S. fontinalis* fed a diet infused with 5% beta-alanine (n=7). Asterisks denote significant differences (p<0.05).

Acute Hypoxia

The mean time-to-LOE was significantly decreased in the beta-alanine fed trout when oxygen saturation was depleted to 10% (p=0.015, Figure 9). Loss-of-equilibrium experiments revealed higher resistance to acute hypoxia in control trout, with greater variability but an average time-to-LOE of 59.63 ± 40.52 min vs. 13.5 ± 6.35 min for the beta-alanine group.

A study conducted by Antila *et al.* (2011) on juvenile Atlantic salmon assessed their tolerance to acute hypoxia following the same protocol used in this study. The time-to-LOE of their salmon ranged from 22.9 to 120 min at 10% O₂ saturation, similar to the LOE of the control trout in our study that ranged from 17 to 135 min.

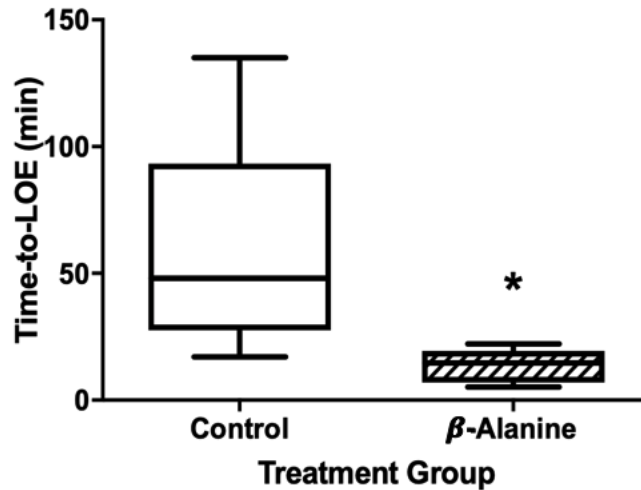


Figure 9: Time-to-Loss of Equilibrium (min) of *S. fontinalis* fed a control diet (n=8) compared to *S. fontinalis* fed a diet infused with 5% β -alanine (n=8). Oxygen saturation was decreased at 1.5% per minute to a minimum of 10% with a WitroxCTRL oxygen control system. Asterisks denote significant differences ($p < 0.05$).

Mitochondrial Function

Permeabilized ventricle fibers of fish fed the β -alanine-infused diet showed significantly higher Leak O₂ flux rates than the control fish ($p = 0.0056$, Figure 10). Leak O₂ rates of β -alanine mitochondria were on average $8.2 \pm 1.94 \text{ pmol s}^{-1}\text{mL}^{-1}$, compared to $5.10 \pm 3.07 \text{ pmol s}^{-1}\text{mL}^{-1}$ in control mitochondria. However, β -alanine treatment did not exert any significant differences on OXP I and OXP I-II fueled oxygen flux states ($p = 0.6838$ and $p = 0.6496$, respectively). Mitochondrial respirometry experiments conducted by Chung *et al.*, (2017) identified leak rates observed in heart tissue of Atlantic killfish that averaged between 4 and 6 $\text{pmol s}^{-1}\text{mL}^{-1}$ when acclimated at 15°C. These results are not unlike the leak rates observed in our control group, that were acclimated at 16°C.

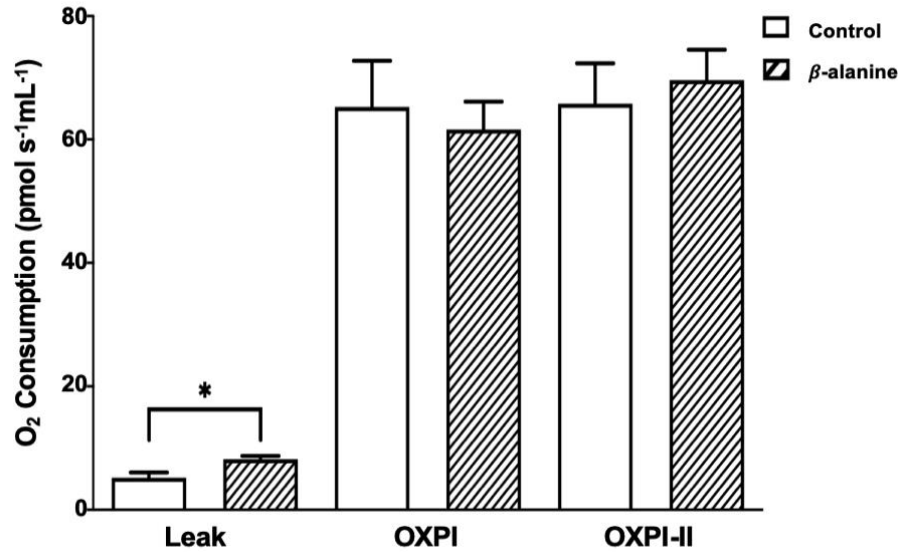


Figure 10: Mean O₂ consumption (pmol s⁻¹mL⁻¹) during mitochondrial respiration in permeabilized ventricle fibers from *S. fontinalis* fed a control diet (n=8) compared to *S. fontinalis* fed a diet infused with 5% β-alanine (n=8). Asterisks denote significant differences (p<0.05).

Lower OXP-I and higher Leak rates in the β-alanine mitochondria resulted in a 36% significantly decrease in RCR rates when compared with the control RCR of 12.32 ± 4.67 pmol s⁻¹mL⁻¹ (p=0.0051, Figure 11). Similar to results obtained from control trout in our experiments, RCR measured from Atlantic killfish, heart mitochondria approximated 12 pmol s⁻¹mL⁻¹ (Chung *et al.*, 2017). In a study conducted by Salin *et al.* (2018), RCR of liver mitochondria from brown trout, *S. trutta*, averaged 10.34 ± 0.05 pmol s⁻¹mL⁻¹, which resembles data obtained in this study.

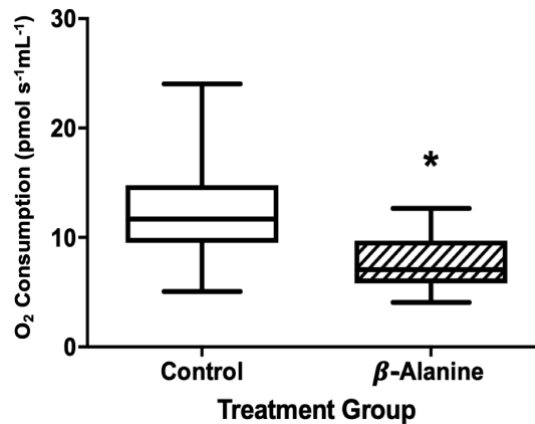


Figure 11: Mean mitochondrial respiratory control ratios (RCR) in permeabilized ventricle fibers from *S. fontinalis* fed a control diet (n=8) compared to *S. fontinalis* fed a diet infused with 5% β-alanine (n=8). Asterisks denote significant differences (p<0.05).

β -alanine-fed fish exhibited significantly decreased levels of CIV activity, marked by lower mean O₂ consumption rates of $61.80 \pm 12.66 \text{ pmol s}^{-1}\text{mL}^{-1}$ ($p=0.0216$, Figure 12.A). The CIV activity in control mitochondria averaged an O₂ consumption rate of $75.89 \pm 17.81 \text{ pmol s}^{-1}\text{mL}^{-1}$. Western blots of ventricle tissue revealed a significant $\sim 39\%$ downregulation in expression of mitochondrial protein COX3 in β -alanine samples ($p=0.007$, Figure 12.B, C).

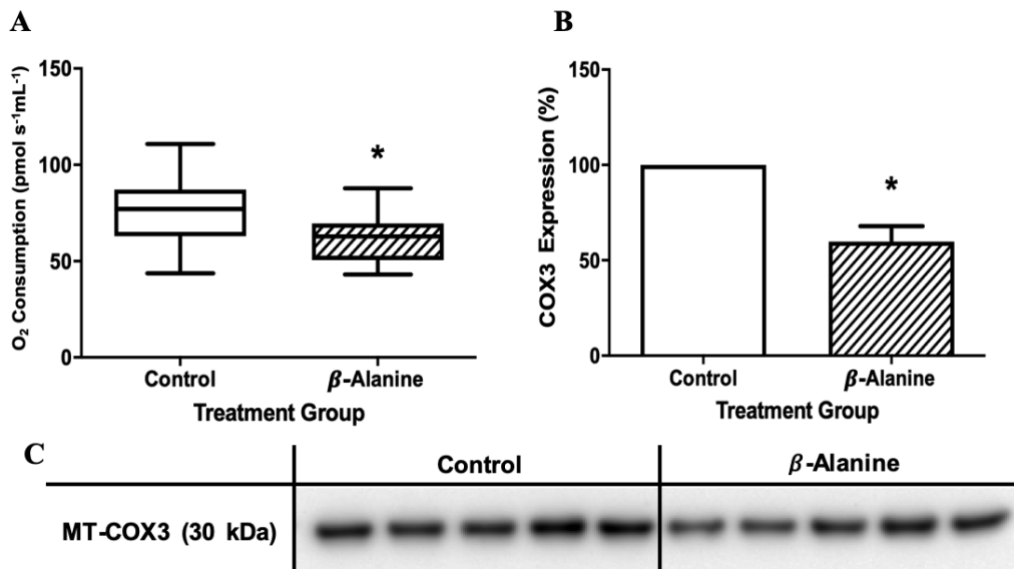


Figure 12: Impacts of taurine deficiency on CIV in *S. fontinalis* mitochondria. **A)** Mean steady-state O₂ consumption of CIV following the addition of TMPD and ascorbate in control ($n=8$) and β -alanine-fed ($n=8$) trout ventricle fibers. Measured with OROBROS DatLab software. **B)** Percent protein expression of MT-COX3 in β -alanine-fed ($n=5$) ventricle samples relative to control ($n=5$) as determined from band intensity analyses from western blots. **C)** Western blot analysis of mitochondrial protein COX3 of CIV in control and β -alanine ventricle tissues. Asterisks denote significant differences ($p<0.05$).

Discussion

This study aimed to evaluate the impacts of taurine deficiency on the physiological performance and mitochondrial function in brook trout. Experiments assessing tolerance to thermal and hypoxic stress were used to gauge the *in vivo* impacts of TD on the whole animal. High-resolution FluoRespirometry was used to measure the activity of the complexes of the electron transport chain to determine the effects of TD on mitochondrial function. Finally, western blots were conducted to detect the expression of mitochondrial proteins ND5, ND1 and COX3 of complexes I and IV. We found that TD improved thermal tolerance but decreased acute hypoxia tolerance following 4 weeks of β -alanine feed, indicating that thermal and hypoxic stress responses could be handled differently in this species.

Additionally, TD cardiac mitochondrial efficiency was impaired through observations of increased proton leak and decreased CIV activity. Expression of CIV protein COX3 was also downregulated in TD trout ventricles, a novel finding in this species that indicates potential damage on the transcriptional level.

Taurine Deficiency Increases Apparent Thermal Tolerance

Our CT_{max} experiments yielded interesting results that contradicted our initial predictions. In our thermal trials, all fish remained relatively stationary until the water temperature reached approximately 24°C, where they began swimming around the holding tank. However, this behaviour was not considered as a state of distress, which is similar to observations of salmon swimming behaviours at 26°C (Nilsson *et al.*, 2019). Water temperatures of 28°C elicited distressed behavioural responses in *S. salar*, resembling the patterns observed and recorded as the VTM in our experimental groups (Nilsson *et al.*, 2019). Stress-responses included collisions with the tank walls, emergence attempts breaking the water surface, rapid changes in swimming direction, sideways body contortions and rapid shaking of the head and body. Bodily contortions and shaking were observed closer to the recorded CT_{max} of our trout, similar to Nilsson *et al.* (2019). These responses are believed to be elicited in response to pain. Mechanothermal nociceptors in the skin of *O. mykiss* detect instantaneous thermal stimuli and fish react accordingly with a flight response before critical thermal endpoints are reached (Ashley *et al.*, 2007). The response threshold of these particular nociceptors is between 28-33°C, which is within, or near, the range of both VTM and CT_{max} observed in this study and several others (Ashley *et al.*, 2007; Nilsson *et al.*, 2019; Carline & Machung, 2011; Recsetar *et al.*, 2011). While TD did not exert any significant effects on the VTM of our brook trout, CT_{max} was significantly increased, with less fish-to-fish variation than observed in the control group. The increase in CT_{max} of TD trout raises speculation that the standard stress response has been altered and differs from the response employed in hypoxia.

Loss-of-equilibrium at high temperatures is thought to be caused by limited oxygen delivery within body tissues, which would suggest that thermal tolerance and hypoxia tolerance may be positively correlated in teleost fish (Anttila *et al.*, 2013). Decreased thermal tolerance is characterized by a decrease in an organism's aerobic scope (Pörtner & Knunst, 2007). Aerobic scope, or aerobic metabolic rate, describes a fish's ability to take in oxygen

and distribute it appropriately to bodily tissues (Pörtner, 2010). Insufficient oxygen supply limits aerobic scope and requires fish to resort to anaerobic metabolism, which is significantly less efficient than mitochondrial aerobic respiration (Pörtner, 2010; Pörtner & Knunst, 2007; Anttila *et al.*, 2013). In past studies using *O. mykiss*, aerobic scope and critical swimming speed were not significantly affected by TD (Gates, 2019). Additionally, Otley (2020), observed that TD did not impact chase-to-exhaustion in *S. fontinalis*. These observations allow us to deduce that any differences in responses of our treatment groups to thermal stress are not due to decreased aerobic scope or physical fitness. However, pulse pressure, the difference between systolic and diastolic blood pressure, was found to be significantly decreased in TD trout (Otley, 2020). This evidence of cardiac impairment indicates that there may be impacts on the physiological responses of TD trout to thermal stress that may not be associated with decreased oxygen delivery.

It is possible that our increased CT_{max} results are due to the fact that taurine can act as a neurodepressant. In the brain of the anoxia-tolerant western painted turtle, extracellular taurine activates inhibitory gamma aminobutyric acid (GABA) receptors to prolong survival when O₂ supply is low (Miles *et al.*, 2018). By inhibiting cellular taurine uptake with β -alanine and therefore increasing extracellular taurine concentrations, the TD trout could make behavioural and physiological modifications that render them less likely to succumb to thermal stress. At present, this is a speculative assumption that requires further research.

Taurine Deficiency Decreases Acute Hypoxia Tolerance

Out TD trout were significantly less tolerant to acute hypoxia at 10% O₂ saturation. Time-to-loss of equilibrium in our TD trout was recorded at a minimum of 17 minutes and a maximum of 2 hours and 15 minutes, supported by findings from acute hypoxia studies in Atlantic salmon and threespine stickleback (Anttila *et al.*, 2013; Regan *et al.*, 2017).

Physiological responses to hypoxia are similar to those employed in thermal stress; as environmental oxygen availability declines, impaired oxygen delivery to the brain causes shifts in metabolism to favor anaerobic respiration (MacCormack *et al.*, 2016; Marcel *et al.*, 1996; Anttila *et al.*, 2013). In this case, glycolysis is heavily relied on as the primary source of ATP. In anoxia-intolerant organisms, the metabolic demands of the body cannot be met by anaerobic ATP (Hosseini-Javaheri & Buck, 2021). Interestingly, anoxia-tolerant organisms such as painted turtles, crucian carp and goldfish reduce the metabolic demands of the brain

to conserve ATP in anoxia. During anoxia, gamma aminobutyric acid (GABA) is released which activates inhibitory GABA receptors, resulting in a phenomenon known as “spike arrest” that reduces ATP expenditure by downregulating the synaptic transmission in the brain (Hochachka *et al.*, 1996; Miles *et al.*, 2018). Studies in western painted turtles have shown that taurine, which is structurally similar to GABA, also activates inhibitory GABA receptors in anoxia tolerance (Miles *et al.*, 2018). By depleting physiological taurine concentrations with β -alanine, it is probable that the neuroprotective mechanisms employed in GABA receptor inhibition are interrupted, which would decrease hypoxia tolerance (Wu *et al.*, 2009). Compounded with mitochondrial damage from TD that impacts ATP production, ATP would be rapidly depleted, causing the fish to instead burn through metabolic reserves, resulting in earlier loss of equilibrium.

Taurine Deficiency Increases Mitochondrial Leak

Taurine contributes to the attenuation of oxidative stress by minimizing the production of superoxide anions from the electron transport chain (Jong *et al.*, 2012; Schaffer *et al.*, 2014). As electrons are shuttled along the chain, energy is produced and released, or “leaked”, into the intermembrane space as protons, establishing a proton gradient (Salin *et al.*, 2018). Taurine deficiency increases the production of ROS as electrons are diverted from the electron transport chain (Cardol *et al.*, 2008).

Mitochondrial leak, or state II respiration, was determined by measuring O₂ consumption after the addition of GMP which donates electrons to CI. Leak O₂ consumption rates were significantly increased in TD trout mitochondria, indicating an increase in proton leak into the intermembrane space which consumes excess O₂ when not coupled with ADP phosphorylation. Proton leak is the primary contributor to incomplete OXPHOS coupling and is indicative of mitochondrial integrity. Mitochondrial respirometry experiments conducted by Chung *et al.*, (2017) identified leak rates observed in heart tissue of Atlantic killfish that were similar to values obtained in the control group of this study.

CI and CI-CII fueled OXPHOS were measured after the addition of saturating ADP and succinate, respectively. Insignificant changes in O₂ consumption were observed between treatment groups, indicating that electron flux through CI and II was not altered by TD. CI-II state III respiration of human cardiac muscle has been previously observed at approximately 54 pmol s⁻¹mL⁻¹ (Park *et al.*, 2014). State III respiration rates in our study were found to be

greater than this literature, but differences can be attributed to respiration efficiency differences across species. Our findings contradicted those found in rat model studies conducted by Jong *et al.* (2012). Similar to our study, intracellular taurine was depleted using β -alanine, but there was a decrease in CI activity by 45% (Jong *et al.*, 2012). However, their study assessed complex activity spectrophotometrically with isolated cells treated with β -alanine-containing media over 48 hours and may not be as representative of whole-animal responses. The impacts of TD on the decrease in CI activity in other studies is linked with the dependence of protein synthesis on leucine (UUG) decoding (Tsutomy *et al.*, 2011; Schaffer *et al.*, 2014). Without taurine, decoding of the UUG codon is impaired which further impacts complex assembly and activity in rat mitochondria (Jong *et al.*, 2012). It is possible that, in *S. fontinalis*, protein expression is not impacted to the same degree as in rats. Determining the abundance of CI proteins ND1 and ND5 through western blots will help to indicate the impacts of TD on the protein level and could provide insight into the possibility of differentially affected decoding.

Since the activities of CI and CII were not impacted by TD, the mitochondrial RCR depended solely on the degree of proton leak. RCR provides a quantitative indication of the coupling of the mitochondrial system. With higher leak rates, the β -alanine-fed trout had significantly lower RCRs. Therefore, a greater proportion of their oxygen consumption is not coupled to ADP phosphorylation, and is dissipated as excess heat energy. Several studies looking into mitochondrial respiration in aquatic species found that the average RCR of Atlantic killfish and brown trout were approximately 12 and 10.34 $\text{pmol s}^{-1}\text{mL}^{-1}$, respectively (Chung *et al.*, 2017; Salin *et al.*, 2018). These observations were similar to the RCR observed in control heart mitochondria in our experiments. To support our findings, respiratory control ratios were found to be decreased in liver mitochondria of TD TauT knockout mice (Warskulat *et al.*, 2006).

Mitochondrial defects translated to impaired physiological performance in our TD trout. It is conceivable that inadequate OXPHOS coupling negatively impacts the inner physiology of brook trout. Leaky mitochondrial and uncoupled OXPHOS lead to increased O_2 consumption and decreased ATP production throughout the mitochondrial electron transport chain. ATP is required for adequate muscle contractions, and ROS have been observed to exert negative effects on ECC through inhibiting calcium uptake at the SERCA

(Takimoto & Kass, 2006). When functioning normally, the SERCA ensures that cardiac muscle relax adequately prior to the subsequent contractions. If the SERCA cannot access sufficient calcium levels, ventricular contractions become rapid and eventually weakening the heart muscle, leading to cardiomyopathy (Köhler *et al.*, 2014). Compounded with uncoupled oxygen consumption, the development of cardiomyopathy in fish may contribute to significant intolerances to environmental fluctuations.

Taurine Deficiency Impacts Complex IV Function

Following the addition of the direct electron donor TMPD, maximal O₂ consumption of cytochrome c oxidase, CIV, was decreased in the β -alanine-fed trout. Supporting the decreased complex activity, significant downregulation of protein MT-COX3 expression of CIV was observed in TD trout. Impaired CIV expression and activity in brook trout is a novel finding, contrary to results obtained in other rat model studies, which only observe defects in CII and CIII. The study conducted by Jong *et al.*, (2012) remarked that CIV activity was not impacted by TD as it does not contain leucine residues that are translated from UUG codons. Observing the decrease in CIV activity and protein expression in this study raises speculation that either a) at least one leucine residue in the *S. fontinalis* COX3 protein sequence is translated from UUG or b) if there aren't any leucine residues within the protein sequence that are translated from UUG, TD may impair the decoding of leucine from the other five codons in this trout species. Further analysis into the codon translation of MT-COX3 would provide insight into the presence of UUG leucine or the potential of alternative transcriptional defects caused by TD.

Mutations proteins contributing to CIV formation are linked with the pathology of mitochondrial diseases and cardiomyopathy (Kirino & Suzuki, 2005; Suzuki *et al.*, 2002; Abdulhag *et al.*, 2015). Deficiencies in CIV formation are currently linked with genetic mutations and the direct role of taurine deficiency in CIV malformation has yet to be fully explored. In Mto1 taurine-modification knockout murine cell models, CIV activity has been observed to decrease, although only by 8% (Fakruddin *et al.*, 2018). In these knockout cells, translation of all mtDNA-encoded genes was also decreased. Applying the context of their results to the observations in our experiments, we can predict that the decrease in CIV activity and protein expression could be a result of taurine-dependent translational errors.

Conclusions and Future Directions

The objective of this study was to determine the physiological and mitochondrial effects of taurine deficiency in brook trout, *S. fontinalis*. To support our hypothesis, we observed significant differences with respect to both physiological performance and mitochondrial function in the β -alanine fed trout. However, while taurine deficiency significantly decreased their tolerance to hypoxic stress, it significantly increased their apparent tolerance to thermal stress. This study also discovered that mitochondrial function was impacted by TD through increased mitochondrial leak and impaired O₂ consumption rates at CIV. Additionally, expression of protein MT-COX3 of CIV was downregulated in TD, indicating potential defects in post-transcriptional modifications. Taurine-deficient CIV defects are a novel finding in *S. fontinalis* and could indicate potential differences in mitochondrial protein transcription that have not been characterized in teleost fish. Our findings indicate that taurine plays an essential role in maintaining the normal physiological stress response in this species. By preserving complex integrity, taurine facilitates normal mitochondrial function that assists in the construction of whole-animal responses to stress.

Future investigation into the behavioural differences between control and TD brook trout would be interesting to pursue. This could provide insight into the increased CT_{max} in TD trout to understand if there are any behavioural modifications made that may render the affected fish less likely to succumb to thermal stress. Understanding the role of taurine in teleost fish from the inside-out can help to conceptualize the relationships between their physiological and behavioural responses to stress and how they may be repressed or elevated. Environmental variations brought on by global warming could cause fluctuations in water temperatures and oxygen content. Under stressful conditions, the standard response to such changes are the suppression of normal behaviours, reduced metabolism and decreases in aerobic scope (Killen *et al.*, 2013). This study concluded that taurine plays a protective role in hypoxia tolerance, but a depressive role in thermal tolerance. This provokes future discussion of a presently unknown response that could have broad applications in describing trout responses to environmental flux. It is predicted that, in the next 100 years, global water temperatures will increase by approximately 1-4°C (Alfonso *et al.*, 2020). If teleost fish are more tolerant to warmer temperatures when intracellular taurine is depleted, they may be one of the best suited aquatic species to withstand global warming.

A limitation presented in this study was the extensive non-specific antibody binding of complex I proteins, resulting in inconclusive western blots. Further optimization using blocking peptides will hopefully yield accurate blots and enable the quantification of ND5 and ND1 protein expression. To further analyse the role of taurine on cardiac function in brook trout, it would be interesting to include a test group that receives dietary taurine supplementation. Doing so would provide insight as to whether taurine has further cardioprotective properties when found beyond normal physiological levels. Expanding our present study to also include juvenile life stages of trout would help to determine if taurine deficiency has detrimental effects on growth and development.

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