

TAURINE SUPPORTS CARDIOVASCULAR RESPONSES TO THERMAL STRESS
IN BROOK TROUT (*Salvelinus fontinalis*)

BY

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List of Abbreviations

\dot{Q}	Cardiac output
$+dP/dT$	Rate of pressure increase
ABT	Arrhenius breakpoint temperature
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
CaMKII	Calmodulin-dependent protein kinases II
CDO	Cysteine dioxygenase
CSAD	Cysteine sulfinatase decarboxylase
CT_{max}	Critical thermal maximum
DO	Dissolved oxygen
ECG	Electrocardiogram
ECM	Extracellular matrix
ETC	Electron transport chain
FDAR	Frequency-dependent acceleration of relaxation
f_h	Heart rate
f_{hmax}	Maximum heart rate
GSH	Glutathione
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSP70	70 kd heat shock proteins
ICM	Intracellular matrix
LDH	Lactate dehydrogenase
LTCC	L-type Ca^{2+} channels
MS-222	Tricaine methanesulfonate
NCX	Na^+/Ca^{2+} exchanger
PCA	Perchloric acid
PLB	Phospholamban
PO	Power output
Q_{10}	Temperature coefficient
RBC	Red blood cells
ROS	Reactive oxygen species
SERCA2a	Sarcoendoplasmic reticulum Ca^{2+} ATPase
SR	Sarcoplasmic reticulum
TauT	Taurine transporter
T_{arr}	Temperature at which arrhythmias appeared
TD	Taurine-deficient
T-tubule	Transverse tubule
V_s	Stroke volume
VSOAC	Volume-sensitive organic osmolyte-anion channels

Abstract

Taurine is involved in a plethora of cellular and physiological processes that are essential for survival during environmental stress – particularly in aquatic ectotherms. The present study aims to characterize the mechanisms for taurine’s contribution to salmonid cardiomyocyte stress tolerance to suggest adaptation processes when facing the osmorepiratory compromise and assist with conservation management. This was done by assessing cardiac performance in taurine-deficient (TD) brook trout (*Salvelinus fontinalis*), which were created with a 5% β -alanine diet. The Arrhenius breakpoint temperature (ABT) test showed that pharmacologically-induced maximum heart rate (f_{hmax}) was significantly lower in TD fish during thermal stress. Heart rate (f_h) sensitivity to heat was assessed by temperature coefficient values (Q_{10}) of 1.80 and 1.61 for control and TD fish, respectively. Cardiac output (\dot{Q}) was also measured *in vivo* during thermal stress and TD animals presented a trend for reduced cardiac scope, as the maximum \dot{Q} was about 10 mL min⁻¹ kg⁻¹ higher in control fish than in TD fish ($Q_{10} = 2.82$ and 1.51, respectively). Control fish increased stroke volume (V_s) with temperature more than TD fish ($Q_{10} = 2.11$ and 1.08, respectively), while both diet treatment groups presented similar f_h sensitivities to high temperature ($Q_{10} = 1.33$ and 1.30, respectively). TD fish survived at the same maximum temperature as control fish and showed evidence of reduced O₂ consumption. Osmotically stressed isolated perfused hearts did not show impaired cardiac performance in TD hearts during acute exposure; thus, suggesting that plasma osmolality changes during thermal stress are not the cause of impaired cardiac performance by TD fish. Different f_h Q_{10} values from the ABT test likely result from the injection of isoproterenol, which indicates that the adrenaline response is blunted in TD fish. Here, excess Ca²⁺ influx by β -adrenergic receptor stimulation may not be adequately regulated within cardiomyocytes to support high cardiac performance. Alternatively, reduced β -adrenergic receptor sensitivity in TD hearts may lower extracellular Ca²⁺ uptake and limit their capacity to increase f_h . An impaired adrenaline response may have also lowered 70 kd heat shock protein (HSP70) expression, which likely amplified any impairments in Ca²⁺ handling. Thus, reduced sensitivity to thermal stress may blunt the heat shock response in TD fish. With this, the present study provides a better understanding of taurine’s role in salmonid cardiomyocytes and organism survival during stress.

Keywords: Arrhenius breakpoint, adrenaline, β -alanine, cardiac scope, heat shock, isolated perfused heart, osmoregulation, osmorepiratory compromise.

Introduction

Taurine Characteristics and Handling

The molecule 2-aminoethanesulfonic acid (taurine) is a non-proteogenic β -amino acid that contains a sulfur group and is most abundant in muscle tissue; particularly in cardiomyocytes, which are the muscle cells of the heart (Schaffer *et al.*, 2010). Taurine is biosynthesized by cysteine dioxygenase (CDO) and cysteine sulfinate decarboxylase (CSAD) from methionine or cysteine (Tappaz, 2004). Taurine is referred to as a conditionally-essential amino acid in fish, as juveniles biosynthesize it and become more dependent on its uptake through their diet in adulthood (Sampath *et al.*, 2020). Despite its short-term biosynthesis, dietary uptake of taurine remains critical for a plethora of cellular functions (Salze & Davis, 2015). This stable free amino acid does not regularly participate in chemical reactions and instead, promotes optimal cardiac function by contributing to osmoregulation, enzymatic activity, Ca^{2+} signalling, antioxidation, and cell membrane stabilization (Salze & Davis, 2015). Taurine is, therefore, highly involved in the kinematic efficiency of the heart during stress responses and recovery (Sampath *et al.*, 2020). As such, a taurine deficiency drastically impacts organism growth, behaviour, and survival (Sampath *et al.*, 2020).

Taurine is transported through cell membranes by transmembrane taurine transporter (TauT) which is comprised of 12 domains (Han *et al.*, 2006). This transporter is a key regulator of cellular processes that rely on taurine and is highly dependent on osmotic factors, such as Na^+ and Cl^- ion concentrations (Tappaz, 2004). More specifically, taurine binds TauT via arginine-324, which can be inhibited with the phosphorylation of serine-322 (Figure 1.1) (Han *et al.*, 2006). Taurine transport by TauT is also inactivated by low pH, hypoosmotic environments, and reactive oxygen species (ROS) (Figure 1.1) (Lambert *et al.*, 2015). Another taurine transporter, PAT1, is dependent on pH and relies on proton coupling for taurine movement through the cell membrane (Figure 1.1) (Baliou *et al.*, 2020). However, this transporter has lower substrate affinity than TauT (K_m of 7.5 mmol L^{-1} and $< 60 \text{ } \mu\text{mol L}^{-1}$, respectively) and is, therefore, less conducive to maintaining

optimal intracellular taurine concentrations (Lambert *et al.*, 2015). This is further supported by Heller-Stilb *et al.* (2002), suggesting that in the absence of TauT activity, PAT1 alone cannot satisfy cellular taurine demands. To facilitate cellular functions, these transporters are regulated by volume-sensitive pathways, which necessitate the influx or efflux of taurine for osmoregulation (Figure 1.1) (Lambert *et al.*, 2015). Furthermore, blocking TauT has been found to cause muscle cell atrophy (De Paepe *et al.*, 2016), likely due to the impaired ability to regulate cell volume. Thus, a taurine deficiency may be detrimental to cardiac function and organism survival in stressful environments.

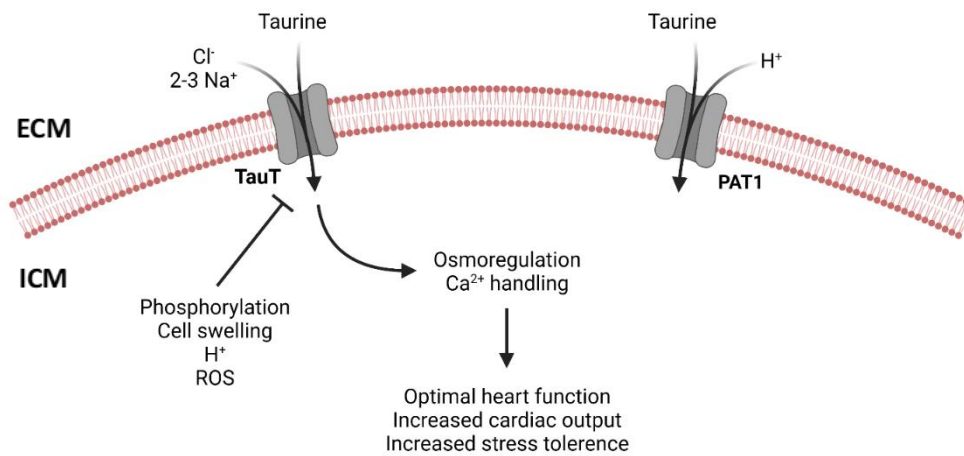


Figure 1.1. Cardiomyocyte containing outer membrane-bound taurine transporters (TauT and PAT1) that facilitate taurine uptake from the extracellular matrix (ECM) to the intracellular matrix (ICM) to promote cellular processes that maintain cardiomyocyte homeostasis. Taurine uptake is inhibited by the phosphorylation of TauT on serine-322, cell swelling in response to hypoosmotic conditions, high intracellular acidity, and ROS. Image was created using BioRender.com.

Cardiovascular Role of Taurine in Non-mammals

The function of taurine in response to cardiac stress is primarily studied in mammalian models, which have relatively stable blood osmolality and body temperatures when compared to other organisms. Therefore, mammals rarely experience thermal and osmotic stress on their cardiovascular system, suggesting that the involvement of taurine in heart function has not been well characterized. In contrast, fish are highly susceptible to thermal and osmotic stress, suggesting that they are more dependent on taurine for maintaining optimal cellular processes in their cardiovascular system than mammals. There

is a clear lack of research describing the role of taurine in the hearts of fish and other ectothermic species. Fish cardiomyocyte taurine concentrations may drop by as much as 60% in response to environmental stressors (Fugelli & Vislie, 1982); hinting towards its importance for stress tolerance and suggesting that taurine may be involved in other mechanisms that have not been identified from mammalian research. This information is critical for understanding the survival mechanisms used by fish during stressful conditions. With rising water temperatures, fish require processes that maintain homeostasis, particularly in their cardiovascular system. As such, evidence for the contributions of taurine to such processes suggests that fish are dependent on this amino acid for their survival – and increasingly so with climate change.

The necessity of mitigating thermal and osmotic stress in fish is highlighted by the osmorepiratory compromise, which describes the trade-off between meeting O₂ demands while minimizing ion loss to the environment from the blood (Wood & Eom, 2021). Here, increased temperatures and stress responses in the organism increase its O₂ demand, which requires increased cardiac output (\dot{Q}) and, therefore, additional blood flow through the gills. However, fish are often limited by the drastic osmolality differences between their blood and the surrounding water. In freshwater fish species, ions are constantly lost to the water from the blood and increasing \dot{Q} amplifies this. As a result, stressed freshwater fish are susceptible to hypoosmotic stress on their cardiovascular system. Under these conditions, fish are challenged with the osmorepiratory compromise and must rely on other mechanisms for maintaining proper heart function.

Taurine in Osmoregulation

While mammals do not typically experience osmotic stress on the heart and are less reliant on taurine for osmoregulation, fish depend on taurine for regulating cardiomyocyte volume and cellular organelle volume when facing the osmorepiratory compromise (Kim *et al.*, 2003). Fishes' dependence on taurine is driven by their susceptibility to varying blood-osmolality when raising \dot{Q} and in many cases, this amino acid is responsible for creating a strong ion gradient in oxidative tissue. As such, taurine greatly contributes to the cardiovascular function of aquatic vertebrates by promoting high \dot{Q} through osmoregulation (Vislie, 1983). In oxidative tissue, such as the heart, taurine facilitates

mitochondrial-driven oxidative phosphorylation. Here, mitochondrial matrix-aurine levels stabilize the proton pumping rate to assist in pH regulation and thus, minimize oxidative damage to cellular components (Hansen *et al.*, 2010). Sarcoplasmic taurine levels in cardiomyocytes also stabilize mitochondrial function by maintaining optimal mitochondrial taurine concentration (Hansen *et al.*, 2010). This ultimately regulates ATP production and thus, the capacity of the organism to fuel intense cardiac performance aerobically.

As a more general function, taurine maintains cardiac function by buffering sarcoplasmic osmolality (Schaffer *et al.*, 2000). For instance, taurine can exit or enter the cell instead of essential ions, which maintains optimal solute concentration for protein function during osmotic stress (Schaffer *et al.*, 2000). Under hypoosmotic conditions, taurine exits cardiomyocytes to lower intracellular osmolality and minimize cell swelling. Whereas an osmotically stressed cell typically relies on K^+ and Cl^- transporters to regulate cell volume, the activation of these transporters can have consequences on cardiomyocytes (Dunham *et al.*, 1993). K^+ and Cl^- transporters are inactivated when phosphorylated and activated when volume-sensitive kinases switch off in response to high cell volume, allowing ions to exit the cell to balance solute concentration (Du & Sorota, 1997). Despite their contribution to osmoregulation, the movement of these ions depolarizes cardiomyocytes by weakening the electrochemical gradient (Du & Sorota, 1997). This effectively reduces the duration and strength of action potentials, leading to weaker heart contractions (Schaffer *et al.*, 2000). As a result, activation of these transporters under osmotic stress decreases \dot{Q} and impedes stress responses by the organism. To better handle stressful conditions, cellular processes need to mitigate the depolarizing effects of volume-sensitive kinases and maintain optimal contractile function by preserving essential ions. Volume-sensitive organic osmolyte-anion channels (VSOAC) and phospholemman channels facilitate the efflux of both taurine and Cl^- in response to volume-induced cellular stress in mammalian cardiomyocytes (Strange & Jackson, 1995; Moorman & Jones, 1998). Taurine efflux from the heart in this manner is a critical component of osmoregulation by stabilizing solute concentration gradients and preserving essential ions in the cell (Schaffer *et al.*, 2000). While such mechanisms are generally conserved among most animals, fish

are expected to undergo amplified taurine handling processes to regulate the relatively frequent and severe osmotic stress that they encounter (Vislie, 1983).

Taurine in Ca²⁺ Signalling

Taurine's osmoregulatory roles in cardiomyocytes also directly impact excitation-contraction coupling for proper heart function. Physiological responses to short-term taurine depletion include impaired diastolic function, as well as abnormal systolic function over extended taurine-deficit periods (Novotny *et al.*, 1991). Here, taurine depletion causes prolonged diastole, which is associated with the interference of Ca²⁺ handling in cardiac muscle tissue (Novotny *et al.*, 1991). In mammalian models, osmotically stressed cardiomyocytes present this trend by undergoing extended relaxation phases in periods of taurine depletion due to irregular Ca²⁺ handling in an attempt to regulate cellular ion concentrations (Schaffer *et al.*, 2000). Specifically, when low taurine concentrations result in slow and insufficient Ca²⁺ transport, imbalanced cell volume can then be accommodated by Ca²⁺ movement across the sarcolemma (Schaffer *et al.*, 2000). This is likely facilitated by reversible Na⁺/Ca²⁺ exchangers (NCX) in the sarcolemma (Schouten *et al.*, 1987; Shiels *et al.*, 2002). As such, current research suggests that taurine-depleted cardiomyocytes have decreased Ca²⁺ sensitivity and are limited in Ca²⁺ uptake capabilities, effectively limiting heart contraction frequency and intensity (Steele *et al.*, 1990). Additionally, increasing extracellular taurine availability increases Ca²⁺ concentrations in the sarcoplasmic reticulum (SR) (Steele *et al.*, 1990), likely due to sufficient Ca²⁺ availability for its primary function in contractile tissue. The mechanism of taurine handling in these processes is of great interest to researchers and may uncover additional roles of taurine in fish.

The development of rhythmic abnormalities and cardiomyopathy in taurine-deficient (TD) hearts result in impaired SR Ca²⁺ transport and decreased \dot{Q} . This is particularly concerning in fish species, as these vertebrates lack transverse tubules (T-tubules) in their cardiomyocytes due to shorter Ca²⁺ diffusion distances and thus, rely largely on extracellular Ca²⁺ supply for cardiac muscle contraction (Vornanen *et al.*, 2002; Shiels, 2017). Taurine's contribution to Ca²⁺ handling is noted to regulate SR Ca²⁺ content (Gates *et al.*, 2022). When phosphorylated, phospholamban (PLB) promotes Ca²⁺ uptake into the SR by increasing the affinity of sarcoendoplasmic reticulum Ca²⁺ ATPase

(SERCA2a) for Ca^{2+} (Figure 1.2) (Huke & Bers, 2007). PLB phosphorylation, however, is hindered by low intracellular taurine levels (Ramila *et al.*, 2015). Here, PLB activity is more directly regulated by modified calmodulin-dependent protein kinases II (CaMKII) and phosphatase 1 activities, which are regulatory proteins for Ca^{2+} uptake by the SR (Figure 1.2) (Ramila *et al.*, 2015). More specifically, intracellular taurine regulates phosphatase 1 activity by minimizing its inhibitory effects on CaMKII (Huke & Bers, 2007). Here, the autophosphorylation of CaMKII at threonine 286/287 enhances the activity of PLB, whereas this autophosphorylation is reversed by phosphatase 1 (Huke & Bers, 2007). As such, taurine deficiency reduces PLB phosphorylation through increased phosphatase 1 activity (Figure 1.2) (Ramila *et al.*, 2015). Phosphatase 1 can also directly dephosphorylate PLB, resulting in faster inhibition of SERCA2a (Berrebi-Bertrand *et al.*, 1998). Impairing this ATPase activity in the SR, therefore, hinders normal heart function by extending the duration of Ca^{2+} uptake from the sarcoplasm during diastole; thus, prolonging the relaxation phases of the heart (Ramila *et al.*, 2015). Inhibition of SERCA2a activity may also result in excess sarcoplasmic Ca^{2+} , which may prevent complete diastole and impair \dot{Q} .

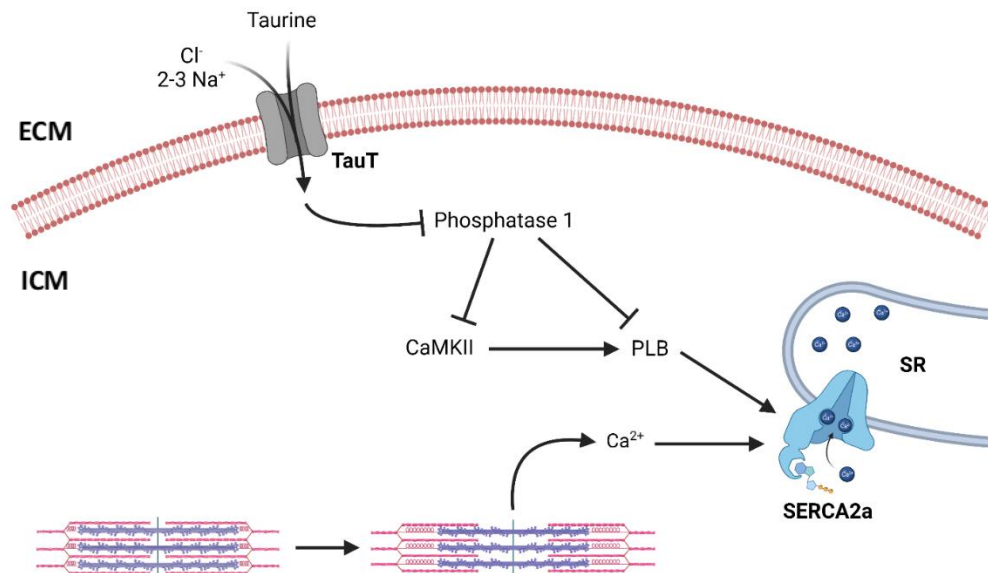


Figure 1.2. Taurine transport into a cardiomyocyte by TauT promotes Ca^{2+} uptake from the sarcoplasm into the SR by SR-membrane bound SERCA2a during diastole. Intracellular taurine upregulates the activity of Ca^{2+} -uptake-promoting factors (PLB and CaMKII) through the inhibition of phosphatase 1. Ca^{2+} is released from troponin after contraction to allow for sarcomere relaxation. Image was created using BioRender.com.

Furthermore, evidence that taurine enhances SR Ca^{2+} handling and contributes to optimal contractile function is supported by Schaffer *et al.* (2010), suggesting that less PLB is found in the TD heart, which, when phosphorylated, modulates Ca^{2+} uptake into the SR. Because of this and intracellular Ca^{2+} efflux for osmoregulation, TD hearts experience prolonged diastole in response to osmotic stress (Schaffer *et al.*, 2010). Better understanding the involvement of taurine in these processes is particularly important to explain frequency-dependent acceleration of relaxation (FDAR) mechanisms. These mechanisms describe the rapid filling of the heart to increase \dot{Q} during high heart rates (f_h), which is essential for stress tolerance (Huke & Bers, 2007). Thus, the direct effects of taurine depletion on Ca^{2+} transport both into and out of the SR and sarcolemma, respectively, are suggested to be critical processes for stress tolerance. However, these may represent a fraction of the complex processes that involve taurine under osmotic stress and contribute to FDAR. With this, long-term taurine depletion during osmotic stress may heavily impact heart function in fish by influencing the expression of Ca^{2+} -handling proteins, such as SERCA2a, in alternate ways that require further research.

Taurine in Thermal Stress Tolerance

Climate change poses survival challenges for all species; perhaps the most notable being aquatic ectotherms. Here, warming waters undoubtedly have negative effects on the metabolic processes of fish by increasing O_2 demand. Increasing water temperatures also results in less dissolved oxygen (DO), which requires fish to increase O_2 uptake through various mechanisms. This can be achieved through an increase in gill perfusion and \dot{Q} . Mechanisms responsible for increasing \dot{Q} , therefore, present promising research opportunities for understanding thermal-stress tolerance in fish as well as contributing to species conservation efforts. Furthermore, the involvement of taurine in cardiovascular processes such as heart contraction strength, duration, and efficiency are not well characterized and may be key to identifying fish's ability to tolerate thermal stress. Fish can acclimate and adapt to temperature changes. Thus, understanding taurine movement and behaviour within cardiomyocytes is critical for predicting how organisms may respond to a changing environment.

Brook trout primarily reside in 15-17°C waters and have an upper incipient lethal temperature (maximum tolerable temperature) of 25°C (Durhack *et al.*, 2021). Under heat stress, these animals will detect decreased DO via peripheral chemoreceptors in the cardiovascular system and increase anaerobic respiration (Milsom & Burtleson, 2007). This signals the heart to increase \dot{Q} until the O₂ demand by the body cannot be met and loss of equilibrium occurs near the incipient lethal temperature. If O₂ demand is not met, animals must rely more on anaerobic metabolism for ATP production. As such, O₂-deprived fish increase lactate synthesis to maintain flux through glycolysis by regenerating anaerobic metabolism substrates.

To mitigate the adverse effects of rising temperatures, taurine's antioxidant properties reduce ROS production and ROS-induced cellular damage, which become more abundant during temperature changes in fish (Cheng *et al.*, 2018). This may be largely due to taurine's role in membrane stability, as the sarcolemma is susceptible to oxidative stress by ROS (Cheng *et al.*, 2018). Additionally, taurine reduces ROS production by supporting mitochondrial protein synthesis (Jong *et al.*, 2012). This increases flux through the electron transport chain (ETC) and reduces the frequency of electron dissociation from ETC complexes (Turrens, 2007). Taurine increases glutathione (GSH) synthesis, which is a key suppressor of ROS (Pushpakiran *et al.*, 2004; Sevin *et al.*, 2013; Cheng *et al.*, 2018). GSH reduces oxidative damage by catalyzing the reduction of ROS to form more stable by-products (Vary *et al.*, 2007). Taurine also increases the expression of CAT, SOD, GR, and GPx genes, all of which play antioxidative roles (Cheng *et al.*, 2018).

Apart from increasing O₂ demand, thermal stress also affects protein stability and enzymatic function. Taurine's involvement in mechanisms that mitigate this is critical for maintaining cardiomyocyte homeostasis. For instance, taurine significantly upregulates 70 kd heat shock proteins (HSP70) (Cheng *et al.* 2018). These proteins are responsible for preserving the proper structure and function of other proteins under environmental stressors and are essential for surviving thermal stress (Currie and Schulte, 2014). This may be a key mechanism describing the function of taurine in thermal stress tolerance and may result in increased capacity for heart function as well as potential adaptations to prolonged high-temperature exposure. In the Ca²⁺ signalling pathway from Figure 1.2, HSP70 may

contribute to protein stabilization. Here, the Ca^{2+} content in the SR of mammalian hearts is significantly higher during thermal stress, likely due to the increased expression of SERCA2a for preserved muscle function as well as the protective effects of HSP70 on this Ca^{2+} ATPase (Figure 1.3) (Chen *et al.*, 2010). These findings are supported by Kim *et al.* (2006), suggesting that HSP70 inhibition interferes with SERCA2a activity. More specifically, HSP70 inhibits the MKK6/p38/MAPK and Raf-1 pathways, which slow the rate of Ca^{2+} uptake into the SR by interfering with SERCA2a (Kim *et al.*, 2006). As such, TD cardiomyocytes are expected to rely more heavily on upregulating SERCA2a and HSP70 during thermal stress due to its increased inhibition by phosphatase 1 (Figure 1.2). Optimal taurine concentrations may, therefore, maintain normal SERCA2a activity at high-temperatures and promote optimal heart function to meet O_2 demands.

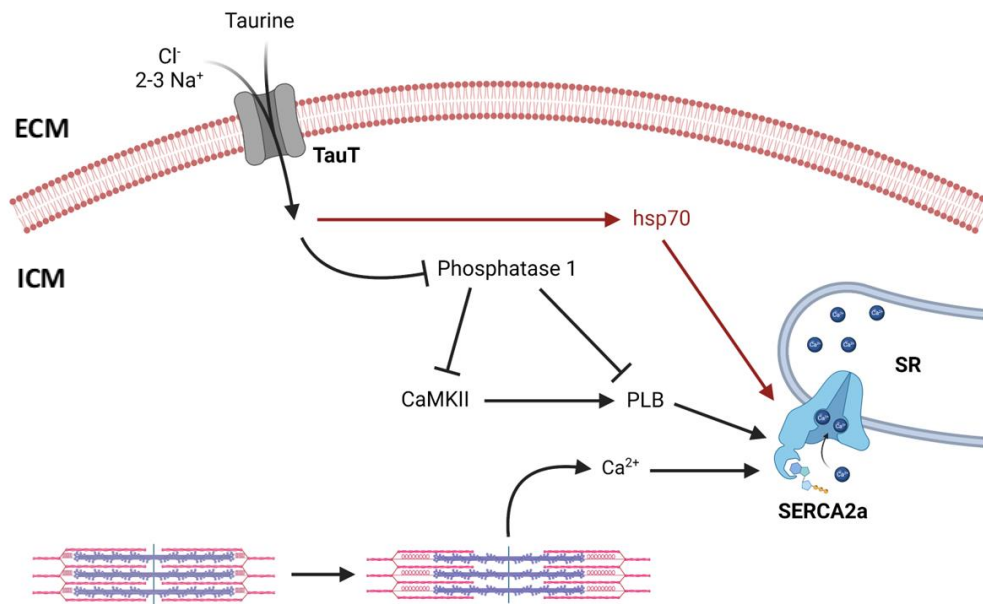


Figure 1.3. Taurine transport into a cardiomyocyte by TauT promotes Ca^{2+} uptake from the sarcoplasm into the SR by SR-membrane bound SERCA2a during diastole. Intracellular taurine increases HSP70 expression, which stabilizes SERCA2a during thermal stress to increase Ca^{2+} uptake following its release from troponin and support high-intensity cardiac performance. Image was created using BioRender.com.

The combination of taurine's influence on HSP70, phosphatase 1, and antioxidation provides promising research opportunities to better understand the limitations that TD cardiomyocytes face under thermal stress. Given this, fish with a taurine deficiency may

have an impaired ability to support proper SERCA2a function and minimize the apoptotic effects of ROS. The enhanced uptake of Ca^{2+} into the SR by SERCA2a upregulation and stability may be a contributing factor to the previously discussed FDAR mechanisms, as rapid relaxation of the heart under thermal stress may contribute to higher \dot{Q} . The following research examines how taurine promotes proper cardiovascular responses to thermal stress. Characterizing such mechanisms that have not been identified in mammals will provide a better understanding of how salmonid species can maintain aerobic metabolism with climate change, as well as how taurine is involved in fish survival during thermal stress.

Taurine in the Adrenaline Response

Typical stress responses in the cardiovascular system of most animals involve an increase in O_2 consumption to support survival processes and behaviour. While taurine directly impacts this within cardiomyocytes by contributing to high \dot{Q} , it is also involved in hormonal signalling and receptor-mediated pathways in the sarcolemma. While the heart can beat independently at its intrinsic f_h (natural frequency), neural and hormonal inputs are required to adjust cardiac function in response to the organism's environment (Keen *et al.*, 1994). Under resting conditions, input from the vagus nerve of the parasympathetic nervous system lowers the f_h below intrinsic by activating muscarinic receptors on cardiomyocytes (Joyce & Wang, 2020). This form of innervation of the heart is released during stress to raise the f_h to intrinsic rate, which can be increased further by input from the sympathetic nervous system (Joyce & Wang, 2020). The sympathetic system relies on adrenergic nerves and circulating adrenaline to stimulate β -adrenergic receptors on the target tissue; thus, control over f_h (chronotropic) and contraction force (inotropic) during stress is largely attributed to this hormone-receptor interaction (Graham & Farrell, 1989; Eliason & Anttila, 2017).

The activation of cardiomyocyte β -adrenergic receptors induces a multitude of cellular processes, one of which involves the influx of extracellular taurine (Figure 1.4) (Huxtable, 1980). Although the purpose of this phenomenon is not well understood, it may be related to taurine's role in Ca^{2+} regulation (Azari & Huxtable, 1980), which is critical for high-intensity cardiac function. Specifically, taurine influx during stress may be critical

to support optimal cardiomyocyte contraction, as activated β -adrenergic receptors also induce L-type Ca^{2+} channel (LTCC) phosphorylation in the sarcolemma, causing Ca^{2+} influx (Figure 1.4) (Shiels *et al.*, 1998; Eliason & Anttila, 2017). Thus, raising intracellular taurine concentration during sympathetic innervation of the heart may enhance the previously discussed mechanisms of Ca^{2+} regulation for FDAR to maximize \dot{Q} (Figure 1.2). Furthermore, β -adrenergic receptor activation influences Ca^{2+} and taurine influx independently (Huxtable, 1980), suggesting that TD animals may maintain Ca^{2+} influx during stress. Consequently, a taurine deficiency in combination with circulating adrenaline may result in insufficient Ca^{2+} regulation to support intense cardiac function during stress.

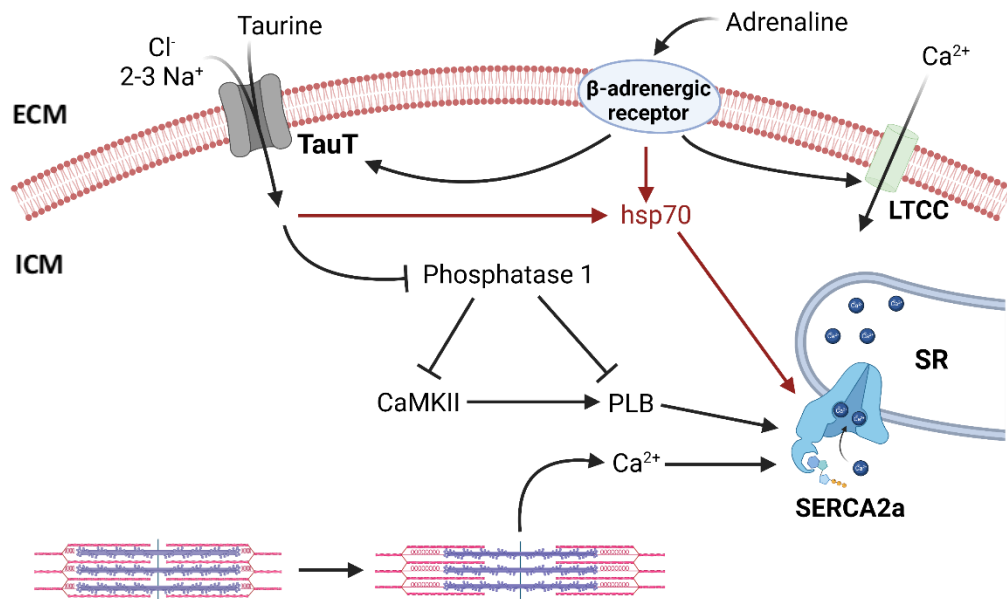


Figure 1.4. Taurine transport into a cardiomyocyte by TauT promotes Ca^{2+} uptake from the sarcoplasm into the SR by SR-membrane bound SERCA2a during diastole. Stimulated β -adrenergic receptors by adrenaline promote Ca^{2+} influx through the phosphorylation of L-type Ca^{2+} channels (LTCC), as well as induce the influx of taurine. Both stimulated β -adrenergic receptors and intracellular taurine increase HSP70 expression, which stabilizes SERCA2a during thermal stress to increase Ca^{2+} uptake following its release from troponin and support high-intensity cardiac performance. Image was created using BioRender.com.

Despite the importance of extracellular Ca^{2+} influx for muscle contraction, cardiomyocytes rely on SR Ca^{2+} stores more with increasing temperature (Keen *et al.*, 1994; Shiels & Farrell, 1997). This is likely due to the enhanced affinity of SR ryanodine

receptors for Ca^{2+} at high temperatures, which are Ca^{2+} release channels that are blocked by ryanodine (Hove-Madsen, 1992; Keen *et al.*, 1994). Additionally, acclimated trout hearts were found to be ten times more sensitive to adrenaline at 8°C than 18°C, which was attributed to higher β -adrenergic receptor density in the sarcolemma of cardiomyocytes (Graham & Farrell, 1989; Keen *et al.*, 1993). Thus, reduced Ca^{2+} influx during prolonged thermal stress may enforce the switch to SR Ca^{2+} supply for heart contraction at high temperatures. This switch may be advantageous at high f_h due to lower Ca^{2+} diffusion distances for muscle contraction and more rapid Ca^{2+} removal from the sarcoplasm during diastole to support FDAR (Henry, 2018). Adrenaline is also involved in supporting optimal cardiovascular responses to thermal stress by increasing HSP70 expression (Figure 1.4) (Currie *et al.*, 2008). Thus, in addition to taurine, stimulated β -adrenergic receptors contribute to SERCA2a stability for Ca^{2+} handling at high temperatures.

Purpose of the Present Study

The present study examines the influence of taurine on the cardiac responses of brook trout (*Salvelinus fontinalis*) to osmotic and thermal stress. Unveiling such mechanisms will improve our understanding of how aquatic species interact with the changing environments and provide insight into adaptation processes that may result from climate change. Thus, taurine's roles in cardiovascular function are of growing interest in conservation research. Additionally, the present study may have clinical applications related to mammalian cardiovascular health by characterizing how taurine contributes to cardiac protection.

To study the effects of taurine, cardiac parameters were assessed between control and TD brook trout. A taurine deficiency was achieved through a 5% β -alanine feed, which has a similar structure to taurine, however, does not contain a sulfonic acid group. β -alanine competes for TauT binding sites to lower intracellular taurine concentration (Ito *et al.*, 2008; Jong *et al.*, 2012). Cardiac performance during thermal stress was assessed by an Arrhenius breakpoint temperature (ABT) test, where f_h was pharmacologically maximized ($f_{h\max}$) and measured by electrocardiography (ECG). Additionally, an ultrasonic flow probe was used to measure ventral aortic flow rate to assess cardiac parameters in response to thermal stress and determine the cardiac scope of TD fish. Osmotic stress tolerance in TD

fish was assessed *in vitro* by measuring the cardiac parameters of isolated brook trout hearts that were perfused with hypoosmotic saline to characterize taurine efflux for osmoregulation. Findings from these experiments suggest mechanisms for taurine's contribution to thermal and osmotic stress tolerance in the cardiovascular system of salmonids and may provide insight into the unique dependence of aquatic vertebrates on taurine for survival.

Materials and Methods

Brook Trout Growth Conditions

Brook trout were acquired from the University of New Brunswick and transported to Mount Allison University's Harold Crabtree Aqualab. Juvenile (body mass 130.7 ± 11.6 g) and mature (body mass 646.6 ± 40.6 g) brook trout were housed in separate aerated freshwater tanks at 16 ± 0.5 °C to maximize aerobic scope (Graham, 1949; Durhack *et al.*, 2021). Brook trout were fed one of two experimental diets once daily to satiation. Control fish were fed 3 mm VITA salmonid chow (EWOS; Surrey, BC, Canada) and TD fish were created by feeding the same chow that was vacuum coated with 5% by mass β -alanine (Allo *et al.*, 1997). All fish were kept on their respective diet for at least 4 weeks prior to 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 No. 101873 and 103144).

Anesthetic Exposure

Brook trout were briefly exposed to 150 mg/L SYNCAINE tricaine methanesulfonate (MS-222) (Syndel Product No. 02168510) in freshwater until respiration ceased. The gills were then perfused with a maintenance dose of MS-222 (83 mg/L) for the duration of the experiments. All water used during stress exposure and experimentation was aerated and maintained at a temperature of 16°C.

Arrhenius Breakpoint Temperature Test

The ABT was determined to assess brook trout performance under heat stress (Casselman *et al.*, 2012; Gilbert and Farrell, 2021; Hardison *et al.*, 2021; Schwieterman *et al.*, 2022). This method was performed on both control ($n = 8$; body mass 147.9 ± 9.9 g) and TD ($n = 8$; body mass 213.9 ± 21.4 g) brook trout (all female).

Anesthetized brook trout were placed ventral side up with aerated 16°C water perfusion through their gills. An injection of isoproterenol (4 µg/kg) and atropine (1.2 mg/kg) in saline (in mmol L⁻¹: 143 NaCl, 2.88 CaCl₂, 0.90 MgSO₄, 3.35 KCl, 2.25 NaH₂PO₄, 5.50 NaHCO₃, and 10 HEPES, pH 7.8) was administered in the ventral region of the fish (1 µg/kg) 30 minutes prior to experimentation and data collection. Isoproterenol and atropine were used to activate and inhibit β-adrenergic and muscarinic acetylcholine receptors, respectively and thus, stimulate f_{hmax} . ECG platinum subdermal needle electrodes (ADInstruments; Product No. MLA1213-DC-19A) were implanted under the skin overlying the heart. Once f_h stabilized, the temperature was increased by 5°C/hour using a DYNEO DD immersion circulator (Julabo) until arrhythmia was detected. The temperature of the water passing the gills was constantly measured using a t-type copper-constantan thermocouple probe (ADInstruments; Colorado Springs, CO, USA; Product No. MLT1400). Both f_h and temperature were simultaneously recorded using PowerLab 8/35 and Bio Amp systems (ADInstruments; Australia), as well as Lab Chart 8 software. Following experimentation, the brook trout were euthanized, weighed, and their ventricles were frozen in liquid N₂ and stored at -80°C for later biochemical analyses.

Thermal Stress Exposure: Ventral Aortic Flow Rate

Cardiovascular adjustments in response to thermal stress were assessed *in vivo* by measuring \dot{Q} in conscious brook trout. This method was performed on both control (n = 8; 4 females and 4 males; body mass 726 ± 143 g; ventricle mass 0.44 ± 0.08 g) and TD (n = 8; 4 females and 4 males; body mass 865 ± 206 g; ventricle mass 0.5 ± 0.1 g) brook trout.

Anesthetized fish were placed on their side with aerated 16°C water perfusion through their gills. A minor incision was made in the skin overlying the ventral aorta. An ultrasonic flow probe (Transonic Systems Inc.; Product No. MC1.5PRB-JS-WC60-CRS10-GX or MC2PSB-SJ-WC90-CRS10-GX) was fit over the ventral aorta to assess \dot{Q} (Figure 1.5). The entire surgical procedure took 35 minutes. The flow probe lead was sutured onto the fish with sufficient slack to minimize probe movement and interference with blood flow. The brook trout were then placed in a perforated tube within a dark recovery tank, which contained aerated water at 16°C and filtered with a Fluval 206 canister filter (Product No. A207). The fish were left to recover for 20 hours, after which the water

temperature was increased by 2°C/hour until the detection of arrhythmia or loss of equilibrium. Cardiac parameters and water temperature (instrument stated above) were recorded using PowerLab 8/35 (ADIInstruments; Australia), T402 flow meter (Transonic Systems Inc.; Ithaca, NY, USA; Product No. T402A91229), and Lab Chart 8 software. Following experimentation, the brook trout were euthanized, weighed, and their ventricle, plasma, and liver were frozen in liquid N₂ and stored at -80°C for later biochemical analyses.

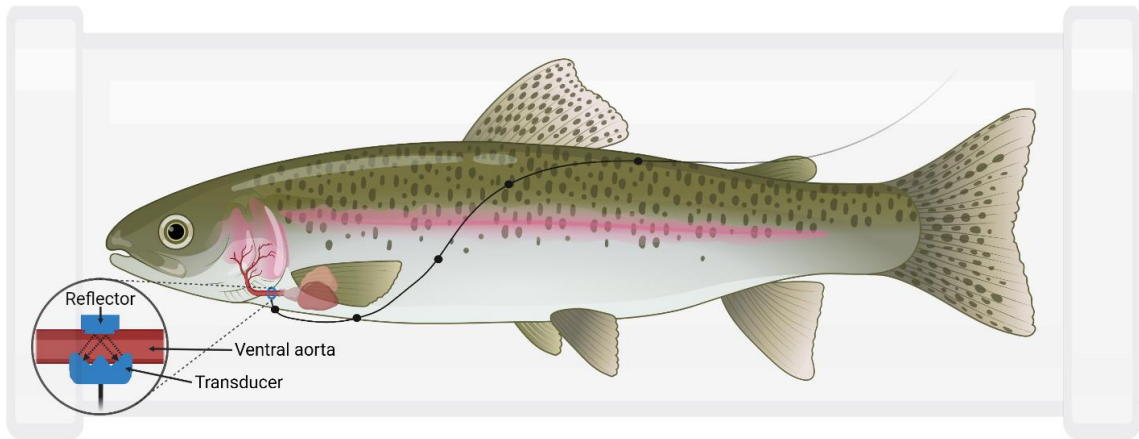


Figure 1.5. Model of the system used to measure ventral aortic flow rate *in vivo* during thermal stress. The ultrasonic flow probe (blue) overlies the ventral aorta and sends an ultrasound via the transducer, which is then reflected off the reflector onto the opposite side of the probe. The probe lead (black line) was sutured (black dots) onto the fish to minimize probe movement. The fish was placed in a perforated tube within a dark recovery tank to minimize lead tangles and probe movement. Image was created using BioRender.com.

Osmotic Stress Exposure: Isolated Perfused Heart

Brook trout hearts were exposed to hypoosmotic stress *in vitro* to mimic the cardiovascular stress that is faced when fish increase \dot{Q} to meet O₂ demands when facing the osmorepiratory compromise. \dot{Q} was measured using an isolated perfused heart preparation. This method was performed on both control (n = 8; 4 females and 4 males; body mass 1226.1 ± 220.1 g; ventricle mass 0.7 ± 0.1 g) and TD (n = 8; 4 females and 4 males; body mass 993.6 ± 169.6 g; ventricle mass 0.6 ± 0.1 g) brook trout.

Anesthetized brook trout were weighed, euthanized, and the heart was removed in a 15-minute surgery. The heart was immediately placed in a saline bath (in mmol L⁻¹: 145 NaCl, 0.90 MgSO₄, 3.35 KCl, 0.4 NaH₂PO₄, 5.50 NaHCO₃, 10 HEPES, 2.93 CaCl₂, 5 glucose, and, pH 7.8; 330 mmol L⁻¹) at 4°C and bubbled with 99.5% O₂/0.5% CO₂. The heart was mounted onto a constant pressure perfusion system via an input cannula at the sinus venosus, an output cannula at the ventral aorta, and supported by a plastic mesh platform (Clow *et al.*, 2004). Saline of optimal osmotic strength (composition stated above) was used as the initial perfusate (minutes 0-20), as well as the final perfusate (minutes 40-60) to assess the heart's ability to recover after being returned to optimal conditions (Figure 1.6). Hypoosmotic stress was induced by switching the perfusate (minutes 20-40) to saline of suboptimal osmotic strength (in mmol L⁻¹: 95 NaCl, 0.90 MgSO₄, 3.35 KCl, 0.4 NaH₂PO₄, 5.50 NaHCO₃, 10 HEPES, 2.93 CaCl₂, 5 glucose, and, pH 7.8; 230 mmol L⁻¹). Minutes 0-20, 20-40, and 40-60 will be referred to as stages 1, 2, and 3, respectively (Figure 1.6). Regardless of the saline concentration used, perfusate contained epinephrine (3 nmol L⁻¹), which was renewed every 30 minutes due to photodegradation (Shiels & Farrell, 1997). The perfusate was maintained at 16 ± 0.1°C using a circulating water bath and a jacket water system in the input and output saline reservoirs, as well as bubbled with 99.5% O₂/0.5% CO₂. The input and output pressures were set to 15 and 25 cm H₂O, respectively, to achieve maximum \dot{Q} and were corrected for tubing resistance. Output pressure was measured at the level of the heart using an MLT844 pressure transducer (MEMSCAP). The output flow was measured using an ultrasonic flow probe (Transonic Systems Inc., Ithaca, NY, USA; Product No. PE2XL) and a T403 flow meter (Transonic Systems Inc.; Ithaca, NY, USA; Product No. T403B41141) (Figure 1.7). Pressure and flow data were acquired using a PowerLab 4/26 system (ADInstruments; Australia) and Lab Chart 8 software. The hearts were stimulated at the input and output cannulas using square wave pulses at 1 Hz (50 V, 10 ms duration) with an SD9 stimulator (Grass Technologies Inc., Warwick, RI, USA). Following experimentation, the ventricle was weighed, frozen in liquid N₂, and stored at -80°C for later biochemical analyses.

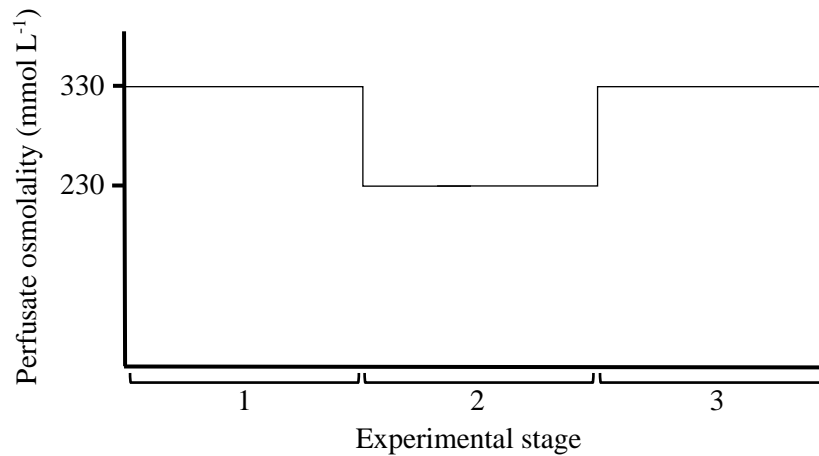


Figure 1.6. The experimental procedure used to expose isolated perfused hearts to hypoosmotic stress. Each stage lasts 20 minutes and exposes the isolated perfused heart to either 330 mmol L⁻¹ or 230 mmol L⁻¹ saline.

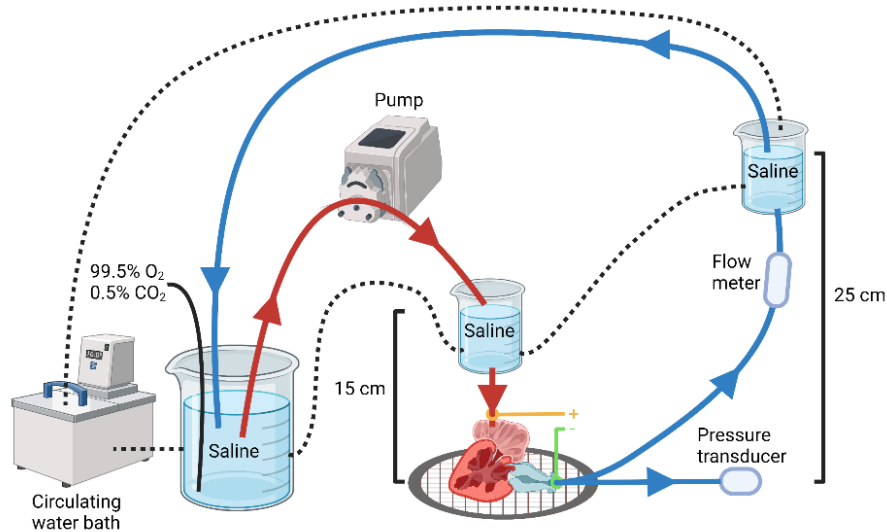


Figure 1.7. Model of the system used to assess cardiac performance in isolated perfused hearts. A jacket water system was used to maintain the perfusate at 16°C (dashed line) and the master saline reservoir was oxygenated (black line). Saline pumped towards and away from the heart is represented by red and blue lines, respectively. Positive (orange) and negative (green) electrodes were placed on the input cannula that was attached to the sinus venosus and the output cannula that was attached to the ventral aorta, respectively. Input and output pressures of the perfusate were 15 and 25 cm H₂O, respectively. A flow meter measured perfusate flow and a pressure transducer measured output pressure at 0 cm H₂O as the heart. Image was created using BioRender.com.

Biochemical Analyses

Tissue Taurine Concentration

Brook trout hearts were sampled following the ABT test and heart taurine concentrations were measured in samples stored at -80°C . The ventricles were homogenized using Kimble Kontes Teflon pestles (DWK Life Sciences, NJ, USA) and a wand-type sonicator (F60 Sonic Dismembrator by ThermoFisher Scientific; Waltham, MA, USA) in 1.5 M perchloric acid (PCA). The homogenate was diluted with 2 PCA volumes of ddH₂O and neutralized with 0.5 PCA volume of 3 M KHCO₃. Prepared samples were vortexed and rested on ice for 5 minutes before being centrifuged at $3000 \times g$ for 5 minutes at 4°C . Taurine concentration in these samples was assessed by high-performance liquid chromatography (HPLC) using a 1200 series HPLC with a diode array detector (Agilent; Santa Clara, CA, USA). This method used a 150 x 4.6 mm ACE Equivalence C18 column (ACE Equivalence, EQV-5C18-1546) mobile phases of 20 mmol L⁻¹ phosphate buffer at pH 7.2 and 45:45:10 methanol:acetonitrile:ddH₂O. Taurine standards (0, 1, 10, and 20 mmol L⁻¹) were used. Samples and standards were mixed with 0.4 mol L⁻¹ borate buffer (pH 10.2) and derivatized in-needle using a mixture comprised of 1 mL phthaldialdehyde reagent and 2-mercaptoethanol before entering the column.

Tissue Lactate Concentration

Brook trout hearts were sampled after the ventral aortic flow rate was measured during thermal stress exposure and heart lactate concentrations were measured in samples stored at -80°C . Samples were thawed on ice and homogenized in 6% PCA using a PowerGen 125 sonicator by Thermo Scientific (Product No. LR60902). The homogenate was centrifuged at $10000 \times g$ at 4°C and the supernatants were assessed for lactate content. A standard curve was made using *L*-lactate (0-2 mmol L⁻¹) to determine sample concentrations. The reduction of NAD⁺ to NADH was catalyzed by lactate dehydrogenase from a bovine heart (CALZYME Laboratories Inc.; San Luis Obispo, CA, USA; Product No. 93401) and measured using a SpectraMax 190 microplate spectrophotometer (Molecular Devices; Sunnyvale, CA, USA; Product No. 94089) at an absorbance of 340 nm. Samples and standards were prepared in 0.2 M glycine buffer by Sigma Life Sciences (Product No. 1003137489). All samples and standards were measured in triplicate.

Proteomics

Quantitative proteomics data were collected from control and TD brook trout hearts ($n = 4$ for both groups) by the biological mass-spectrometry facility at Dalhousie University, Nova Scotia, Canada. Fish were anesthetized, euthanized, and their ventricles were frozen in liquid N_2 and stored at $-80^\circ C$. Protein expression in heart tissue was compared to the present findings (data not shown).

Statistical Analyses

Data were assessed using two-way ANOVAs performed on GraphPad Prism (version 5) to account for diet treatment and exposure to varying osmolality and temperature. Results with significant interactions among variances were analyzed using unpaired T-tests to compare diet treatments alone at each osmolality stage and homogeneity of variance was assessed using F-tests on GraphPad Prism (version 5). Cardiac parameters changing with temperature were plotted and fit with first or second-order polynomial regressions. The cardiac scope was measured by subtracting the highest \dot{Q} value from the \dot{Q} value at rest using the equation of the regression curve. The ABT was determined with a segmented regression performed on RStudio (version 1.3.1073). Thermal sensitivity between $16-22^\circ C$ was assessed by calculating the temperature coefficient (Q_{10}), as all fish reached this level of thermal stress without exhibiting arrhythmias. Box and whiskers plots show the range of values (box) average value (horizontal line), and standard error of the data (error bars). All significant differences were identified using $p < 0.05$ and are indicated by an asterisk.

Results

Arrhenius Breakpoint Temperature Test

Control and TD brook trout differed in mass (148 ± 28 and 214 ± 60 g, respectively), however, no significant effect of mass on cardiac parameters was found (data not shown). The initial f_h of anesthetized brook trout was 84.4 ± 1.4 beats min^{-1} , which increased to 98.5 ± 1.4 beats min^{-1} following the injection of atropine and isoproterenol. Neither metric was significantly different between the diet treatment groups (data not shown). TD brook trout presented more variable temperatures at which f_{hmax} was reached ($p = 0.002$), although this did not differ significantly between the diet treatment groups (p

= 0.328) (Figure 1.8 D). Similarly, the temperature at which TD brook trout became arrhythmic (T_{arr}) was more variable ($p = 0.011$), although this did not differ between the diet treatment groups ($p = 0.204$) (Figure 1.8 E). Control brook trout were found to have significantly higher f_{hmax} , which was about 20 beats min^{-1} higher than TD brook trout ($p = 0.006$) (Figure 1.8 C). Control and TD brook trout presented $f_h Q_{10}$ values of 1.80 and 1.61, respectively. The ABT did not differ significantly with taurine deficiency ($p = 0.138$) (Figure 1.8 F).

Heart taurine concentration was more variable in TD brook trout ($p = 0.045$), although no significant difference was found between the diet treatment groups ($p = 0.505$). Heart taurine concentrations in control brook trout following the ABT test were identical to control brook trout that did not undergo any experimentation (data not shown). In contrast, heart taurine concentrations of TD brook trout following the ABT test were about 10 $\mu\text{mol g}^{-1}$ higher than TD brook trout that did not undergo any experimentation (data not shown); however, this was not significantly different.

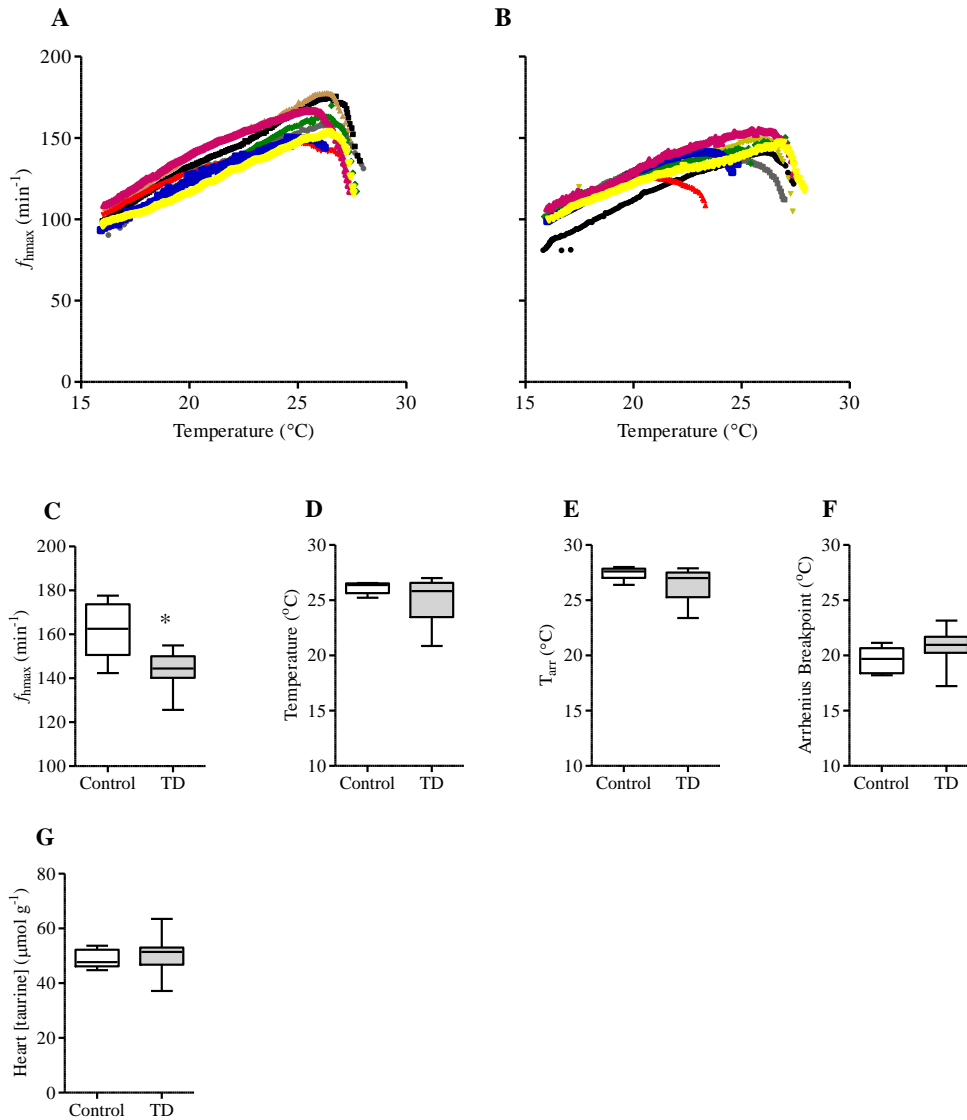


Figure 1.8. Arrhenius breakpoint temperature test for control and TD brook trout ($n = 8$ for both groups). **A)** relationship of maximum heart rate (f_{hmax}) and temperature for control brook trout, **B)** relationship of f_{hmax} and temperature for TD brook trout, **C)** f_{hmax} , **D)** temperature at f_{hmax} , **E)** temperature at which arrhythmias appeared (T_{arr}), **F)** Arrhenius breakpoint temperature, and **G)** heart taurine concentration following the ABT test. Significant differences between diet treatments are indicated by an asterisk ($p < 0.05$).

Thermal Stress Exposure: Ventral Aortic Flow Rate

Cardiac parameters measured *in vivo* during thermal stress were plotted over increasing temperatures and fit with second-order polynomial regressions (Figure 1.9 A-C). Both diet treatment groups showed \dot{Q} of approximately $15 \text{ mL min}^{-1} \text{ kg}^{-1}$ at 16°C ,

however, they diverged considerably with temperature. Here, control brook trout reached a maximum \dot{Q} of about 31 mL min⁻¹ kg⁻¹ at 27°C, while TD brook trout only reached about 21 mL min⁻¹ kg⁻¹ at 26°C (Figure 1.9 A). Control and TD fish presented Q_{10} values for \dot{Q} of 2.82 and 1.51, respectively. Stroke volume (V_s) in the control brook trout was approximately 0.16 mL kg⁻¹ at 16°C and reached a maximum of about 0.26 mL kg⁻¹ at 25°C. A second-order polynomial regression could not represent the V_s for TD brook trout and this data was, therefore, fit with a first-order regression (Figure 1.9 B). V_s in TD brook trout appears to remain at about 0.2-0.22 mL kg⁻¹ until 21°C, after which it increases to 0.24 mL kg⁻¹ until 23°C, then returns to base levels (Figure 1.9 B). The sensitivity of V_s to thermal stress is represented by Q_{10} values of 2.11 and 1.08 for control and TD fish, respectively. The trend for f_h increase in both the control and TD brook trout was relatively linear with temperature, although the control group maintained f_h that was consistently about 10 beats min⁻¹ higher than the TD group (Figure 1.9 C). Control and TD brook trout presented Q_{10} values for f_h of 1.33 and 1.30, respectively (n = 6 for both groups). Gradually increasing error bars across the cardiac parameters is a result of fewer data points at the respective temperature following the loss of equilibrium in some fish. Similarly, a step-like increase in cardiac parameters likely represents the removal of fish from the experiment after displaying arrhythmias. The cardiac scope was measured from data in Figure 1.9 (A) and presented a strong trend for higher cardiac scope in control fish, although not significant (p = 0.0869) (Figure 1.9 D). Neither T_{arr} nor heart lactate differed between the diet treatment groups (p = 0.4179 and 0.9027, respectively) (Figure 1.9 E, F). The relative ventricular masses between control and TD brook trout were 0.061 ± 0.008 and 0.07 ± 0.01 , respectively, and did not differ significantly (p = 0.4488) (data not shown).

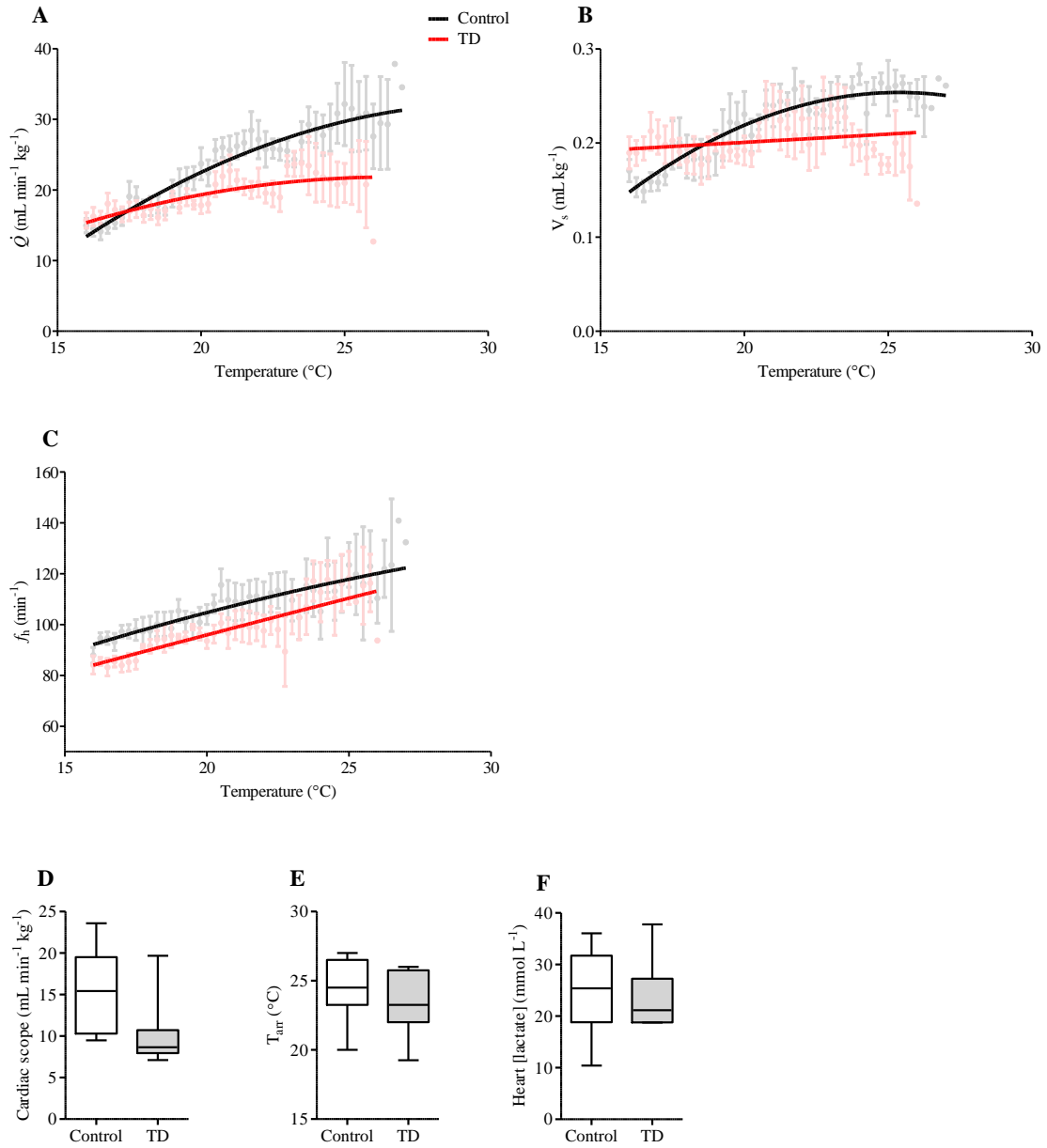


Figure 1.9. Cardiovascular responses in brook trout were assessed by measuring *in vivo* ventral aortic flow during thermal stress. Control and TD brook trout (black and red, respectively; $n = 7$ for both groups) were exposed to rising temperatures ($2^\circ\text{C} \cdot \text{min}^{-1}$) and a second-order polynomial regression was fit to the data. **A)** cardiac output (\dot{Q}), **B)** stroke volume (V_s) (first-order regression fit to the TD data), **C)** heart rate (f_h), **D)** cardiac scope, **E)** temperature at which arrhythmias appeared (T_{arr}), and **F)** heart lactate concentration following thermal stress.

Osmotic Stress Exposure: Isolated Perfused Heart

Statistical analyses done on the data from isolated perfused brook trout hearts exposed to osmotic stress revealed that \dot{Q} significantly differed between the osmolality stages ($p < 0.0001$) but not between diet treatments ($p = 0.2529$) when assessed with a two-way ANOVA (Figure 2.0 A). A significant interaction was found here ($p = 0.0159$), suggesting that the diet treatment groups responded to the second stage of the experiment differently. Subsequent unpaired T-tests were performed on \dot{Q} between the diet treatments at each stage of the experiment (Figure 2.0 B-D). Here, no significant differences were found in stage 1 ($p = 0.1143$), stage 2 ($p = 0.5173$), or stage 3 ($p = 0.2256$). A two-way ANOVA performed on V_s (Figure 2.0 E) also suggested significant differences between the osmolality stages ($p < 0.0001$) but not between the diet treatment groups ($p = 0.2617$). This test presented significant interactions ($p = 0.0079$) and subsequent unpaired T-tests on V_s between diet treatments resulted in no significant differences in stage 1 ($p = 0.1037$), stage 2 ($p = 0.5865$), or stage 3 ($p = 0.2265$) (Figure 2.0 F-H). Power output (PO) (Figure 2.0 I) and the rate of output pressure development ($+dP/dT$) (Figure 2.0 J) were both assessed using two-way ANOVAs and presented significant differences between the osmolality stages of the experiment ($p < 0.0001$ and $p = 0.0005$, respectively) and not between the diet treatments ($p = 0.1433$ and $p = 0.6312$, respectively). Output pressure development was assessed using fewer brook trout ($n = 6$ for both groups) due to undetectable pressure development slopes from incorrect data collection preferences. The time to minimum \dot{Q} following the introduction to stage 2 of the experiment did not differ significantly between the diet treatment groups ($p = 0.2387$) (data not shown). The relative ventricular masses of control and TD brook trout were 0.063 ± 0.008 and 0.06 ± 0.01 , respectively, and did not differ significantly ($p = 0.8875$) (data not shown).

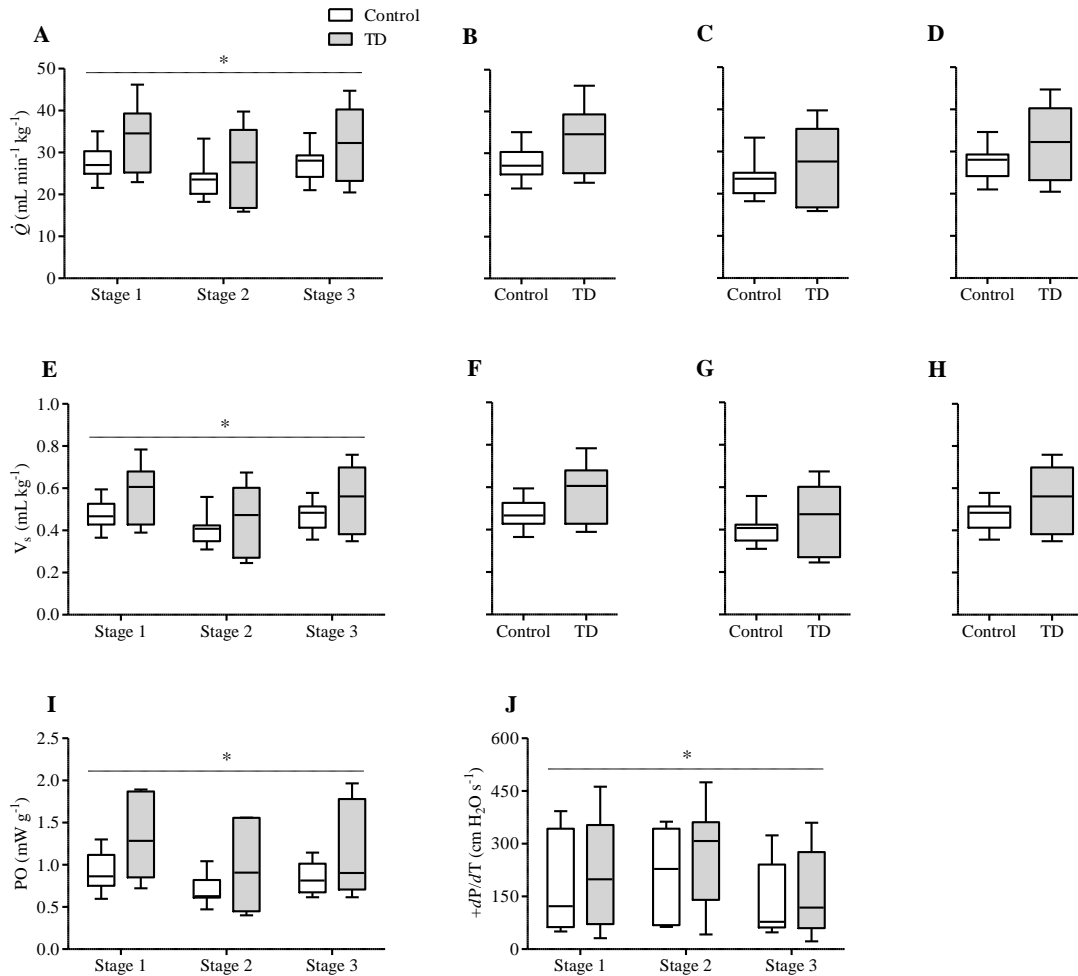


Figure 2.0. Cardiovascular responses in isolated brook trout hearts during osmotic stress. Control and TD (white and grey, respectively; $n = 7$ for both groups) brook trout hearts were exposed to isotonic saline (330 mmol L^{-1}) for 20 minutes (Stage 1), hypoosmotic saline (230 mmol L^{-1}) for 20 minutes (Stage 2), then returned to isotonic saline for 20 minutes (Stage 3). **A)** cardiac output (\dot{Q}) represented by a two-way ANOVA, **B-D)** \dot{Q} represented by unpaired T-tests at the three experimental stages, respectively, **E)** stroke volume (V_s) represented by a two-way ANOVA, **F-H)** V_s represented by unpaired T-tests at the three experimental stages, respectively, **I)** power output (PO) represented by a two-way ANOVA, and **J)** rate of output pressure development ($+dP/dT$) represented by a two-way ANOVA ($n = 6$ for both groups). Significant differences between osmolality stages are indicated by an asterisk ($p < 0.05$).

Discussion

The present study aimed to provide a thorough understanding of the role of taurine in brook trout hearts by characterizing its involvement in cellular processes during cardiovascular stress. Such mechanisms become increasingly relevant with warming

waters that result from climate change and are critical areas of research for predicting animals' responses to changing environments, and for aquatic animal conservation. To assess the effect of taurine on heart function in fish, control and TD brook trout were subject to stress and multiple cardiac parameters were assessed. The animals were exposed to varying conditions of thermal stress to determine their ABT and cardiac scope, and isolated hearts were used to assess cardiac performance during osmotic stress.

Arrhenius Breakpoint Temperature Test

Fish's ability to increase f_h is among the most critical physiological response to meet O_2 demands and tolerate environmental stress (Gilbert & Farrell, 2021). Thus, the ABT test was done using ECG recordings during rising water temperature to assess the effect of taurine on cardiac performance during thermal stress. Findings from the ABT test are presented in Dixon *et al.* (2023). Increasing f_h following the injection of isoproterenol and atropine (increased from 84.4 ± 1.4 to 98.5 ± 1.4 beats min^{-1}) supports the activation of β -adrenergic receptors and the inhibition of muscarinic acetylcholine receptors in anesthetized fish, both of which contribute to $f_{h\text{max}}$. TD brook trout presented more variable $f_{h\text{max}}$ temperature values (Figure 1.8 D), suggesting their dependence on taurine for an optimal stress response. The significantly higher $f_{h\text{max}}$ reached by control fish suggests that their capacity to achieve high f_h may be limited by intracellular taurine availability (Figure 1.8 C). This may be caused by impaired Ca^{2+} handling in cardiomyocytes to limit $f_{h\text{max}}$ (Figure 1.2), as slower Ca^{2+} removal from the sarcoplasm prolongs diastolic phases (Ramila *et al.*, 2015). The importance of taurine for Ca^{2+} handling is amplified during thermal stress due to the increased dependence on SR Ca^{2+} for cardiomyocyte contraction with temperature (Hove-Madsen, 1992; Keen *et al.*, 1994). Ryanodine receptors may be less active in TD SR (Bakker & Berg, 2002), supporting their apparent desensitization to thermal stress, as evident by a lower $f_h Q_{10}$ value in TD fish. With this, it was expected that TD fish would not be able to withstand thermal stress to the same extent as the control fish. However, no significant differences were observed between the temperature at $f_{h\text{max}}$ or T_{arr} (Figure 1.8 D, E); suggesting that both treatment groups can survive at the same water temperatures experienced during the acute exposure. TD fish may have achieved a lower $f_{h\text{max}}$ to support higher V_s , as this provides more time for atrial filling during diastole. A

higher V_s in TD fish may allow them to achieve the same \dot{Q} as control fish at a lower f_h . To test this, subsequent experiments measured \dot{Q} and V_s *in vivo* during thermal stress.

Heart taurine concentrations following the ABT test did not differ significantly between the diet treatment groups (Figure 1.8 G), which was likely attributed to the efflux of taurine for osmoregulation during a thermal stress response by control brook trout hearts. TD fish are not expected to have experienced substantial cellular taurine movement, as findings by Dixon *et al.* (2023) noted 21% less heart taurine in resting TD brook trout than control brook trout and both groups had the same plasma taurine levels. Plasma taurine is, therefore, expected to have been higher in control fish, while TD fish likely experienced minimal changes in this. This finding supports taurine's role in osmoregulation during thermal stress.

Thermal Stress Exposure: Ventral Aortic Flow Rate

The ventral aortic flow rate of brook trout was measured *in vivo* using an ultrasonic flow probe. Flow through this artery was measured during rising water temperature to calculate cardiac scope. Both diet treatment groups achieved the same \dot{Q} under resting conditions ($14 \text{ mL min}^{-1} \text{ kg}^{-1}$ at 16°C), however, control brook trout achieved a maximum \dot{Q} that was approximately $10 \text{ mL min}^{-1} \text{ kg}^{-1}$ higher than TD brook trout. Control fish, therefore, present a trend for a higher cardiac scope, although this was not significant ($p = 0.0869$) (Figure 1.9 D). With a higher maximum \dot{Q} , control fish were expected to outperform TD fish, but both groups became arrhythmic at similar temperatures (27 and 26°C , respectively) (Figure 1.9 A). The lack of change in TD brook trout \dot{Q} suggests that these fish are less sensitive to thermal stress than control fish (Q_{10} values of 1.51 and 2.82, respectively).

TD fish presented higher V_s under optimal conditions by about 0.05 mL kg^{-1} , which may be a result of their lower starting f_h . With thermal stress, TD brook trout increased V_s by only 0.02 mL kg^{-1} , while control brook trout were able to increase their V_s by about 0.11 mL kg^{-1} . The increase in V_s noted here contrasts findings from the literature, as Sandblom & Axelsson (2007) suggest that venous return in trout cannot be increased during acute thermal stress, making them incapable of increasing V_s . However, the

simultaneous increase of V_s and f_h in control fish from this study can likely only be attributed to higher venous return for faster atrial filling. This finding provides novel evidence for cardiovascular adjustments in salmonids during thermal stress and illustrates the importance of taurine in this process. f_h in control fish was maintained at about 10 beats min^{-1} higher than TD fish throughout the entire thermal stress exposure (Figure 1.9 C). Despite this, sensitivity of f_h to heat stress was nearly identical in control and TD fish (Q_{10} values of 1.33 and 1.30, respectively). These findings are consistent with the expected role of taurine in Ca^{2+} regulation to support high-intensity cardiac performance, as slower Ca^{2+} removal from the sarcoplasm into the SR prolongs diastolic phases and lowers f_h (Ramila *et al.*, 2015). Impaired ryanodine receptor activity may also contribute to the lower f_h exhibited by TD fish in this experiment (Bakker & Berg, 2002).

The f_h and V_s relationships with temperature support taurine's involvement in FDAR mechanisms, which promote rapid atrial filling to increase \dot{Q} (Huke & Bers, 2007). Intracellular taurine is expected to accelerate Ca^{2+} uptake into the SR by promoting SERCA2a affinity for Ca^{2+} during diastole (Figure 1.2) (Huke & Bers, 2007; Ramila *et al.*, 2015), thus, allowing for faster heart filling during diastole at high f_h . Control brook trout were able to increase their V_s while increasing f_h , which resulted in a higher \dot{Q} , while TD brook trout achieved little change in V_s and \dot{Q} , despite the increase in f_h . TD fish were, therefore, unable to support a drastic increase in venous return and exhibited an insufficient rate of atrial filling.

Osmotic Stress Exposure: Isolated Perfused Heart

Osmotic stress was induced on isolated brook trout hearts by perfusing them with hypoosmotic saline (230 mmol L^{-1}). All measured cardiac parameters significantly decreased when the hearts were exposed to hypoosmotic stress. Additionally, significant interactions that were observed from \dot{Q} and V_s suggest that the diet treatment groups responded to the stress differently. Despite this, cardiac parameters measured for the diet treatment groups did not differ at any given stage of the experiment. This hints towards the importance of taurine's involvement in neural signalling to the heart, as cardiac responses were measured *in vitro*. These findings also emphasize the complex behaviours of taurine and its involvement in uncharacterized mechanisms throughout the whole organism.

Fish typically face the osmorepiratory compromise during thermal stress to maximize O₂ uptake (Wood & Eom, 2021), but it is unlikely that the acute thermal exposure tested in this study induced substantial osmotic stress on the cardiovascular system. The acute *in vitro* exposure to hypoosmotic conditions in this experiment is an extreme representation of osmotic stress designed to highlight taurine's role in the maintenance of cardiac function. The relatively minor changes observed in cardiac performance by TD hearts under these conditions suggest that any effects of a taurine deficiency on cardiomyocyte osmoregulation *in vivo* are not the only causes of impaired cardiac performance exhibited by TD fish during thermal stress. Chronic exposure to hypoosmotic stress *in vivo* may reveal more significant consequences of taurine deficiency. Regardless, findings from this experiment support the hypothesized mechanisms of taurine that are responsible for the animal's responses to heat stress by suggesting that TD fish can achieve similar stress responses to control fish under acute osmotic stress.

Potential Mechanisms for Impaired Cardiac Performance in TD Brook Trout

Reduced Oxygen Consumption

A blunted cardiac response to thermal stress in TD brook trout may be explained through a combination of several mechanisms that are altered by a taurine deficiency or β -alanine. If all metrics of cardiac performance were shifted down in TD fish, the findings could likely be explained by impaired Ca²⁺ handling alone. However, this was not the case. For instance, it is unclear how TD fish survived the same level of thermal stress as control fish (Figure 1.8, Figure 1.9). Similarly, Dixon *et al.* (2023) observed a significantly higher CT_{max} (critical thermal maximum) by TD brook trout. With reduced blood flow, as evident by reduced \dot{Q} (Figure 1.9 A), delivery of O₂ to the brain of TD fish should be impaired and they should become arrhythmic at lower temperatures. These fish may, therefore, activate processes that reduce O₂ demand to match putative reductions in O₂ uptake and delivery. Others have noted reduced O₂ consumption by TD cardiomyocytes by as much as 30% (Jong *et al.*, 2012). Here, impaired synthesis of specific ETC protein subunits was noted, which reduced flux through the ETC and increased ROS production (Jong *et al.*, 2012). As such, reduced O₂ demand by TD cardiomyocytes may be caused by lower aerobic ATP production (Turrens, 2007; Jong *et al.*, 2012). The blunted capacity for aerobic ATP production may elevate heart lactate concentration if it increases dependence

on anaerobic metabolism, however, this was not found (Figure 1.9 F). The suppression of mitochondrial metabolism in TD hearts is supported by proteomics data, which showed that cytochrome-c, an essential protein for maintaining electron flow through the ETC, was downregulated by 98.9%. Western blots by Dixon *et al.* (2023) showed a 50% reduction in cytochrome-c oxidase subunits in TD hearts. Similarly, Jong *et al.* (2012) noted a 40% reduction in cytochrome-c oxidase subunit expression in β -alanine-treated Wistar rat cardiomyocytes. Furthermore, taurine upregulates GSH for antioxidation (Pushpakiran *et al.*, 2004; Sevin *et al.*, 2013; Cheng *et al.*, 2018). This is supported by the proteomics data, which reveal a 51.3% reduction in GSH S-transferase in TD hearts, thus, suggesting that these fish experience more intense oxidative stress. Apart from changes in metabolic processes, TD fish may initiate other physiological changes to reduce O₂ demand and survive such extreme temperatures; perhaps by redirecting blood flow largely to the brain to maintain consciousness. The involvement of taurine in blood flow regulation, however, is beyond the scope of this study and requires a thorough assessment of whole-animal performance during thermal stress.

Impaired Heat Shock Response

Perhaps the most obvious explanation for the blunted cardiac performance by TD fish involves an impairment in the capacity for muscle contraction by TD cardiomyocytes. It is unlikely that fewer contractile elements are present in the TD fish heart as the PO and $+dP/dT$ measured from the isolated perfused hearts did not differ significantly between the control and TD fish at any stage of the experiment (Figure 2.0 I, J). This contrasts findings from mammalian hearts, where TD animals were found to have less actin and myosin (Eley *et al.*, 1994). If a taurine deficiency does impact contractile material, then perhaps the TD heart performs normally under optimal conditions but cannot sustain prolonged periods of high-intensity activity. Specifically, contractile material performance may decrease with increasing temperature. This phenomenon may be attributed to a blunted heat shock response if a taurine deficiency reduces the expression of HSPs in brook trout hearts, as noted by Cheng *et al.* (2018) (Figure 1.3). This could result in impaired protein stability through the unfolding of contractile proteins and SERCA2a at high temperatures (Dubínska-Magiera *et al.*, 2014). Indeed, the proteomics data suggest that HSP70 content in the unstressed brook trout was 14.8% lower in TD fish. Additionally, the activator

protein of larger HSP, HSP90, was 15.4% lower in these fish. However, these relatively subtle changes in HSP expression are likely insufficient for any significant impairments of cardiac performance during thermal stress. A desensitized heat shock response is expected to limit the maximum tolerable temperature by brook trout, which contrasts with the present findings (Figure 1.9 E) and those of Dixon *et al.* (2023), but this may be more evident during prolonged exposures to thermal stress. Additionally, a reduced heat shock response by TD fish hearts may impact countless other survival processes that only become apparent beyond the timeframe of the thermal exposure used in this study. Given the subtle changes in HSP70 expression in resting fish, future research should assess HSP70 levels after thermal stress (Currie *et al.*, 2008).

Impaired Adrenaline Response

Comparing the relationships of f_h with temperature between the ABT test and data collected by measuring ventral aortic flow rate suggests different rates of f_h increase. Specifically, $f_h Q_{10}$ values calculated from the ventral aortic flow rate data are more similar than the Q_{10} values from the ABT test; comparable by the ratios of Q_{10} from TD fish to Q_{10} from control fish of 0.98 and 0.89 for the two experiments, respectively. Thus, anesthetized TD fish from the ABT test did not raise f_h as rapidly as the control fish (Figure 1.8 A, B), whereas both diet treatment groups increased f_h at the same rate when measuring ventral aortic flow in conscious fish (Figure 1.9 C). It is important to note that these Q_{10} values were calculated for the increasing f_h from 16-22°C, as some fish became arrhythmic after this point. Assessing Q_{10} for higher temperatures may present more drastic differences in these findings. f_h in TD fish from the ABT test, therefore, appears to be suppressed. The major difference in these two experiments is the atropine and isoproterenol injection that was administered to the anesthetized fish in the ABT test, suggesting that the adrenaline response may be impacted by either a taurine deficiency or β -alanine. Several potential explanations for this phenomenon are presented below.

Adrenaline Response: Hypothesis 1

The present findings may be explained if neither treatment groups experience any impairments in neural innervation of the heart or hormonal signalling. Here, thermal stress is expected to induce a typical adrenaline response by the cardiomyocytes of both diet

treatment groups, which involves an enhanced influx of Ca^{2+} (Shiels *et al.*, 1998; Eliason & Anttila, 2017). Although stimulated β -adrenergic receptors simultaneously promote taurine influx (Huxtable, 1980), TD animals may not have the capacity to support this process or the blockade of TauT transporters by β -alanine may inhibit taurine movement. The independent influx of Ca^{2+} without additional taurine (Huxtable, 1980) may impair Ca^{2+} flux in TD brook trout cardiomyocytes. This was likely amplified by the injection of isoproterenol in the ABT test, as this enhances Ca^{2+} influx (Huxtable, 1980). The combination of excess Ca^{2+} and taurine deficiency may, therefore, result in the inability to regulate sarcoplasmic Ca^{2+} movement between contraction phases. Excess free- Ca^{2+} may prevent complete diastole, restricting atrial filling and limiting V_s and \dot{Q} . This hypothesized Ca^{2+} handling impairment in TD brook trout is supported by taurine's role in downregulating phosphatase 1 (Figure 1.2). Specifically, Ramila *et al.* (2015) noted significantly less phosphatase 1 in TD mice hearts, which prevents CaMKII and PLB activation. Thus, TD fish may experience impaired SERCA2a affinity for Ca^{2+} , limiting Ca^{2+} removal from the sarcoplasm during diastole (Figure 1.2). To test this hypothesis, future studies can inject anesthetized fish with atropine, but not isoproterenol, to assess cardiac responses to thermal stress. If isoproterenol enhances Ca^{2+} influx, TD brook trout would be expected to raise their f_h at the same rate as control brook trout.

Importantly, other factors also appear to impair the ability of TD fish to maintain Ca^{2+} homeostasis, as proteomics data suggest a 43.3% reduction in S100 Ca^{2+} -binding protein expression. This is a family of proteins that contribute to Ca^{2+} movement for cardiomyocyte contraction by promoting SERCA2a and ryanodine receptor activity in the SR (Donato *et al.*, 2013). It is unclear whether taurine directly affects S100 protein expression, however, this phenomenon may have amplified the adverse effects of a taurine deficiency on Ca^{2+} handling.

Adrenaline Response: Hypothesis 2

The present findings may also be explained by the impairment of processes in the sympathetic nervous system during thermal stress. For instance, TD fish may have impaired adrenaline release or reduced β -adrenergic receptor sensitivity. Reduced expression of these receptors at high temperatures may also be responsible, as have been

noted in acclimated trout hearts (Graham & Farrell, 1989; Keen *et al.*, 1993). With blunted sympathetic innervation of the heart, organisms would not achieve an adequate stress response to initiate crucial survival processes. Specifically, reduced sensitivity to adrenaline may impair Ca^{2+} influx for muscle contraction (Shiels *et al.*, 1998; Eliason & Anttila, 2017). In contrast to hypothesis 1, reduced sympathetic activity may cause insufficient Ca^{2+} supply to facilitate forceful contractions during systolic phases, limiting FDAR processes intended to increase \dot{Q} . The dependency of cardiomyocytes on trans-sarcolemmal Ca^{2+} influx for contraction at low temperatures, as opposed to SR Ca^{2+} contribution, may explain why both diet treatment groups achieved the same starting \dot{Q} at 16°C (Figure 1.9 A) (Keen *et al.*, 1994; Shiels & Farrell, 1997). SR Ca^{2+} stores are increasingly critical for cardiomyocyte contraction at high temperatures, as the higher affinity of ryanodine receptors for Ca^{2+} enables high f_h for FDAR (Hove-Madsen, 1992; Keen *et al.*, 1994). This hypothesis is supported by the inability of TD brook trout to increase their f_h or V_s to the same extent as the control fish (Figure 1.9 B). As a result, these fish achieved a minimal increase in \dot{Q} during thermal stress. The enhancement of ryanodine receptor activity by taurine may also contribute to Ca^{2+} deprivation in the sarcoplasm of TD hearts, as SR Ca^{2+} stores become less accessible (Bakker & Berg, 2002).

Currie *et al.* (2008) noted the association between adrenergic and heat shock responses, suggesting that the stimulation of β -adrenergic receptors significantly increases HSP70 expression in trout red blood cells (RBC) during thermal stress (Figure 1.4). If taurine impairs adrenaline release or reduces β -adrenergic receptor sensitivity, TD fish may experience a blunted heat shock response in combination with impaired extracellular Ca^{2+} influx. As a result, TD cardiomyocytes may not be able to adequately protect SERCA2a (Figure 1.4) and other proteins involved in muscle contraction or regulate other cellular processes.

To test these explanations for taurine's involvement in the adrenaline response, plasma adrenaline levels must be determined following thermal stress to assess the fish's ability to release this hormone into circulation. Resting adrenaline is expected to be approximately 5 nmol L⁻¹ (Milligan *et al.*, 1989), and has been noted to increase up to 1 $\mu\text{mol L}^{-1}$ in stressed trout (Mc Donald & Milligan, 1992). Additionally, β -adrenergic

receptor sensitivity to adrenaline may be tested by measuring the force of contraction of ventricular muscle strips in the presence of gradually increasing adrenaline concentrations (Shiels *et al.*, 1998). β -adrenergic receptor density on cardiomyocyte sarcolemma may also provide insight into impaired sympathetic control of heart function in TD brook trout. HSP70 mRNA and protein expression should also be measured to support both an impaired adrenaline response, as well as to assess the sensitivity of TD fish to thermal stress (Currie *et al.*, 2008). Lastly, characterizing intracellular Ca^{2+} flux will provide insight into Ca^{2+} movement during thermal stress, as this hypothesis suggests that a higher concentration will be found in control hearts.

Adrenaline Response: Hypothesis 3

The impaired cardiac thermal sensitivity in TD fish observed in the ABT test (Figure 1.8 A, B) may be explained if either taurine deficiency or β -alanine exerts some consistent inhibition on the pacemaker during stress, despite the inhibitory effect of atropine on muscarinic receptors. Blunted inhibition of parasympathetic innervation of the heart may explain the minor changes in \dot{Q} and V_s in TD brook trout under thermal stress (Figure 1.9 A, B). If this is the only neural signalling pathway being altered, then TD fish would present the same adrenaline function as the control fish. To test this hypothesis, the capacity for adrenaline release and β -adrenergic receptor sensitivity must be measured as previously described.

It is also possible that TD brook trout both promote parasympathetic and inhibit sympathetic innervation of the heart through a combination of the previously discussed mechanisms. This would counteract the effects of both isoproterenol and atropine in the ABT test. It is unlikely, however, that both neural processes are influenced to the same extent, as this would not explain the impaired cardiac thermal sensitivity of TD fish from the ABT test. If this was the case, it would be expected that both diet treatment groups would increase f_h at the same rate with rising temperature, as is seen in Figure 1.9 (C). Thus, impaired thermal sensitivity of f_h by TD fish following drug administration in the ABT test suggests that there is a disproportionate effect of a taurine deficiency on parasympathetic and sympathetic innervations of the heart.

Conclusions

Taurine is evidently involved in a plethora of cellular processes in vertebrates, many of which likely remain unknown. The major findings from the present study are that TD fish appear desensitized to thermal stress and that taurine potentially impacts the adrenaline response in brook trout hearts in some way. The mechanisms by which this takes place likely involve a combination of Ca^{2+} handling, ROS mitigation, and maintaining protein stability via the heat shock response. Given this, impaired cardiac performance in TD fish was likely attributed to alterations in Ca^{2+} -handling processes at either the sarcolemma or in the SR. Alternatively, these fish may have experienced cardiac Ca^{2+} -deprivation if the capacity for sympathetic innervation of the heart was reduced or a blunted heat shock response destabilized SERCA2a and contractile proteins. The data provided strong evidence for the involvement of taurine in neural and hormonal regulation of cardiac performance, which is critical for enhancing FDAR to support O_2 uptake and delivery during stress. As such, future research may uncover a whole host of impairments in performance during stress by TD animals.

This study demonstrates that taurine plays a critical role in cardiac responses to thermal stress in salmonids. Heart taurine levels drastically vary between species, so this research may help predict how different organisms will respond to environmental stress. Climate change is increasing the frequency and severity of thermal stressors in freshwater habitats, so understanding how fish tolerate these stressors is critical for informing best conservation practices. Additionally, this research may present clinical applications related to mammalian cardiovascular health; taurine's effects appear to be greatly amplified in fish, making it easier to determine mechanisms of action that may be too subtle to detect in mammalian models.

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