The effect of exhaustive exercise on the thermal tolerance of brook charr (*Salvelinus fontinalis*)

by

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ABSTRACT

This study examined whether exercise influences acute thermal tolerance in brook charr (*Salvelinus fontinalis*). To address this, fish were assigned to one of 4 groups (i) control; (ii) 5-minute exhaustive exercise; (iii) thermal stress only; and (iv) 5-minute exhaustive exercise prior to thermal stress. Blood samples were also taken from the fish to measured stress parameters (plasma lactate and glucose) between the groups. Acute thermal tolerance, measured as the temperature where the fish lost equilibrium, were similar between fish exposed to thermal stress only and fish exposed to exercise prior to the thermal stress (30.2 ±0.5°C and 29.6±0.8°C, respectively). However, blood lactate and glucose were about 25% higher in charr exercised prior to the thermal stress (lactate 8.2 mmol/L and glucose 9.5mmol/L). These findings show that there are no differences in the thermal tolerance between groups, but there are additional physiological effects in fish exercised prior to thermal stress.

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STATEMENT OF RESEARCH CONTRIBUTION

All of the work was done by Emma Traynor, with guidance from Dr. Kieffer. The experiments were conducted from September 2019 to January 2020.

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List of Symbols, Nomenclature or Abbreviations

CT_{Max}, Critical thermal Maximum

CT, Critical thermal

LOE, Loss of equilibrium

OCLTT, Oxygen-and- capacity limiting thermal tolerance

hr, Hour

°C, Degrees Celsius

W, Watt

L, Litre

mg, Milligrams

g, Grams

cm, Centimeters

nm, Nanometer

μL, Microlitre

mmol, Millimole

S.E, Standard Error

P, Probability value

ANOVA, Analysis of variance

Introduction

It is well known that temperature influences the distribution, physiology and ecology of ectothermic animals (Beitinger and Fitzpatrick, 1979; Pörtner and Farrell, 2008; Sunday et al., 2012). Understanding the thermal tolerance of fish helps researchers define an organism's acute thermal limits (reviewed in Lutterschmidt and Hutchison, 1997); this continues to be a topic of recent research in relation to climate change (Pörter and Knust, 2007; Eliason et al., 2011; Roessig et al., 2014). Thermal tolerance can be measured using lethal (i.e., incipient lethal temperature) and non-lethal (i.e. critical thermal-CT-methodology) methods (Terblanche et al., 2007). The critical thermal methodology (maximum CT and minimum CT) is the most used method to test acute thermal tolerance in ectotherms (Lutterschmidt and Hutchison, 1997) because: (1) it is more ecologically relevant, (2) it is non-lethal (unless tissue samples are collected), (3) it is a relatively fast test (e.g., can be completed within an hour) and (4) fewer animals are required for statistical power (reviewed in Beitinger and Lutterschmidt, 2011). Over the last few decades, a large database on CT_{Max} in fish has been collected; various reviews reveal that numerous factors, such as heating rates, body size and acclimation temperature, significantly influence the thermal tolerance of various fish species (reviewed in Becker and Genoway, 1979; Beitinger and Bennett, 2000; Lutterschmidt and Hutchison, 1997).

Despite its popularity and practicality, the data collected using the CT methodology can be limited in its scope. Most studies using CT_{Max} methods only examine one stress (i.e., the thermal stress itself) and modify abiotic factors, such as acclimation temperature or heating rate (Beitinger and Bennett, 2000). It has been suggested that the acute thermal tolerance of an organism might be affected differently if an additional stressor is provided either before or

during the CT_{Max} test. For example, Ellis et al. (2013) found that the CT_{Max} of brook charr (*Salvelinus fontinalis*) was reduced when the fish were exposed to a hypoxia stress during the thermal tolerance testing, compared to fish that did not undergo the hypoxic stress during the thermal stress. Thus incorporating multiple stressors into the thermal tolerance protocol may provide more ecologically relevant information (Crain et al., 2008; Darling and Côté, 2008; Todgham and Stillman, 2013; Heye et al., 2019). Equally as in important, adding additional stressors into thermal tolerance experiments may also allow further insight into the potential mechanism(s) behind the loss of equilibrium (LOE) endpoint that is often used to define acute thermal tolerance temperature. It has been hypothesized that the LOE associated with CT testing is the result of motor function loss linked with the nervous system (Friedlander et al., 1976; Jutfelt et al., 2019) and/or oxygen- and- capacity- limited thermal tolerance (OCLTT) (cardiovascular limitations; Ekström et al., 2016; Pörtner, 2010). The OCLTT hypothesis suggests that the loss of equilibrium associated with a CT_{Max} could be related to the organism's tissues not receiving sufficient oxygen at higher temperatures via the circulatory system. This hypothesis has been partially tested by Ellis et al. (2013) where brook charr exposed to a hypoxic environment during a CT_{Max} thermal stress test had a lower acute thermal tolerance-CTmax- and shorter time to loss of equilibrium than under standard conditions. Other researchers have shown that CT_{Max} was independent of oxygen availability over a wide range of oxygen levels (Clark et al., 2013; Ern et al., 2014; Norin et al., 2014; Wang et al., 2014). One limitation within the Ern et al. (2014) study was the thermal tolerance test was conducted at 2°C/hr. This is a much slower heating rate than the 18°C/hr rate recommended for CT_{Max} studies (Lutterschmidt and Hutchison, 1997). Thus, it is possible that the thermal stress wasn't delivered at a rate sufficient enough to stress the animal over a short-term basis.

The focus of the current study is three fold: (i) to examine how an exhaustive exercise stress prior to a thermal stress impacts critical thermal tolerance; (ii) to examine the hematology/stress response (measured as hematocrit, lactate and glucose) in fish that have been stressed prior to and during the CT_{Max} test, as most thermal tolerance studies don't include tissue level responses and measurements (Carline and Machung, 2001; Galbreath et al., 2004; Galbreath et al., 2006; Zhang and Kieffer, 2014; Bard and Kieffer, 2019) and (iii) through extrapolation, the new information from (i) and (ii), above, could provide further evidence against the OCLTT hypothesis which is currently under criticism (Ern, 2019; Ern et al., 2014; Norin et al., 2014; Jutfelt et al., 2018). Exhaustive exercise has been shown to challenge many physiological systems in fish, (Milligan, 1996; Wood, 1991; Kieffer, 2000; Kieffer 2010), including the cardiovascular system (Pörtner, 2010; Ekström et al., 2016). Exhaustive exercise results in the production of high levels of lactate and metabolic protons in brook charr (Hyndman et al., 2003; Kieffer et al., 1994) and a long recovery process, greater than two hours (Hyndman et. al., 2003). A plausible prediction is that the stress of exhaustive exercise could reduce the thermal tolerance of the fish because there would be a cardiovascular imbalance with providing oxygen for recovery from exercise with the energy requirements to support oxygen delivery during the acute thermal tolerance test.

Material and Methods

2.1 Animal Care

Brook charr (*Salvelinus fontinalis*) originated from brood stock used at the University of New Brunswick, Fredericton New Brunswick. Charr were transferred to the University of New Brunswick, Saint John and held indoors in large flow-through tank continuously supplied with

aerated, dechlorinated ambient Saint John water. Fish were acclimated to 18±1°C and fed a commercial salmonid pellet (EWOS 4 mm salmonid pellet). A 12 hr light: 12 hr dark photoperiod was used throughout the study. Twenty-four hours prior to experimentation, feeding was suspended to reduce possible dietary influences on metabolism (Kieffer et al., 1994). The University of New Brunswick Animal Care Committee approved the following experimental protocol, meeting guidelines of the Canadian Council of Animal Care.

2.2 Experimental setup

Acute thermal tolerance was determined using a modified critical tolerance (CT_{Max}) methodology as described in Bard and Kieffer (2019). The CT_{Max} was done in an insulated testing arena (41.9 x 24.8 x 29.8 cm), filled with 18°C de-chlorinated Saint John municipal water. A heating tank (45 x 56 cm; ≅30L) was placed elevated and adjacent to the testing arena and equipped with a 1800 W heater (Pentair Aquatic Ecosystems, Apopka, Florida, USA) and air diffusers. The heater was set to a heating rate of ≅14°C/hour throughout the study, within the recommended heating rate for CT_{Max} studies (Becker and Genoway, 1979). The water from the heating tank flowed to the test tank by gravity and was then pumped back to the heating tank via a submersible pump (Loligo Systems, Viborg, Denmark) to maintain temperature within the setup. The submersible pump was isolated from the test chamber by a perforated black Plexiglass shield. Electronic temperature probes (Loligo systems, Viborg, Denmark) were also placed at both ends of the test tank to record temperature within the testing arena.

2.3 Experimental Groups and Experimental Procedure.

Individual charr (≅130g, see Table 1) were netted and removed from their holding tank and assigned haphazardly to one of the following four experimental groups: (control, neither thermally stressed nor exhaustively exercised fish; n=4), group 2 (5-minute exhaustive exercise stress only, n=8), group 3 (thermal stress only, n=8), or group 4 (thermal stress following a 5-minute exhaustive exercise stress, n=8).

Fish in group 1 (no thermal stress or exhaustive exercise stress) were netted from the holding tank and placed in the testing tank overnight (~12hr). The following morning, these fish were anaesthetized (a pH7, buffered solution of MS-222[l g/L of MS-222 with 2 g/L sodium bicarbonate]) within the test arena. Fish began to lose equilibrium after a few minutes and were fully anaesthetized after 5 minutes. At this point, the fish were no longer responsive to touch they were removed from the test tank, blotted dry and a 1mL sample of blood was removed from the caudal vasculature using a needle and syringe. Following this, the blood was placed in a centrifuge for further processing (see below).

Fish in group 2 (exhaustive exercise only) were netted from the holding tank and placed in the test tank over-night (~12hr); the next morning, individuals were removed via net from testing arena and quickly transferred to a small circular bucket filled with 18±1°C water and exercised by manual chasing for 5 minutes. Manual chasing is a standard technique used in many fish exercise studies (reviewed in Wood, 1991; Milligan, 1996; Kieffer, 2000). Fish were considered to be physically exhausted when they lost equilibrium and were no longer responsive to manual chasing (Kieffer et al., 1994). It should be noted that the work done during exercise was not quantified (e.g. exact time to fatigue). Instead, the fish were exercised to a behavioral

state of exhaustion (Kieffer et al., 1994). Exhausted fish were then placed into an anesthetic solution prior to removal of a blood sample (as noted above for control fish).

Fish in group 3 (thermal stress testing only) were netted from their holding tank and placed in the test tank over-night (~12hr); following the 12 hr holding period, the fish were exposed to an increasing temperature of ≅14°C/hr at a constant rate until the loss of equilibrium (LOE) occurred (following the method described in Bard and Kieffer 2018). LOE as an end-point is indicated when the fish rolled ventral side up and is unable to right itself (modified Lutterschmidt and Hutchison, 1997) within a 10 second time frame; the temperature in which the LOE occurs is the acute maximum thermal tolerance (i.e., CT_{Max}). At CT_{Max}, the fish was removed from the test arena, anaesthetized and measured for length, mass and a blood sample taken. The test arena was drained, wiped down and rinsed, and filled with new de-chlorinated water at 18°C following each trail.

Fish in group 4 (thermal stress following exercise) were netted from the holding tank and placed in the test tank overnight (~12hr). The next morning, the fish was removed via a net from the testing arena and transferred to a small circular bucket filled with 18±1°C water and exercised to exhaustion by manual chasing for 5 minutes as outlined above for fish in group 2 (see above). After the 5-minute exercise period, fish were then placed back into the testing arena. Fish were then exposed to an increasing temperature at a constant rate of ≅14°C/hr until the loss of equilibrium (LOE) occurred (see above for fish in group 3). Once the fish reached its CT_{Max}, it was removed from the test tank, anaesthetized, a blood sample taken, and the mass and length recorded.

2.4 Blood sampling and analysis

The blood sample removed from the fish was analyzed for various physiological endpoints. Approximately 70μL of the whole blood was used for duplicate hematocrit determination (Zhang et al., 2017). Hematocrit provides an estimate of the oxygen carrying capacity of the blood. The remainder of the whole blood was then centrifuged for 2 minutes at 6700g and the resulting plasma was pipetted into smaller tubes and frozen at −20°C freezer for future glucose and lactate analyses (Bard and Kieffer, 2019). Glucose and lactate are measures of the secondary stress response in fish, and lactate also provides evidence for the activation of anaerobic processes in fish (Kieffer 2000).

2.5 Lactate and glucose analysis

Once the plasma samples were removed from the −20°C freezer and thawed, plasma glucose levels were determined using an OneTouch Ultra glucose meter (OneTouch Ultra 2; code 25 test strips; www.onetouch.ca; Penny and Kieffer, 2014). Plasma lactate concentration were measured by using a lactate meter (Stat Strip; Nova Biomedical; www.novabiomedical.com; Waltham, USA). The relationship between lactate values determined by assay (Bard and Kieffer, 2019) and those determined using the meter is described by the following equation: $lactate_{assay} = 1.13*lactate_{meter} = 0.54$ ($R_2 = 0.96$).

2.6 Statistical analysis

All data was analyzed using SIGMASTAT version 4.0 software (https://systatsoftware.com). An unpaired t-tests was used to compare the CT_{Max} values of group 3 (thermal stress only), and group 4 (exercise + thermal stress). A one-way analyses of variance

(ANOVA) was used to assess whether differences existed in the blood variables between the four fish groups. When the ANOVA indicated a significant difference ($P<0.05$), a Holm-Sidak post-hoc test was used to determine significant differences between groups.

Results

Mass and length

Mean (±S.E) mass and length were 135.1± 21.74 g and the length 24.07±1.0 cm, respectively, and these values were not significantly different between the four fish groups (Table 1).

Table 1: Mean mass (±S.E) and length (±S.E) of brook charr (*Salvelinus fontinalis*) used in the study. No significant differences existed between groups ($P> 0.05$, One-way ANOVA).

Group	N	Weight (g)	Length (cm)
Control	4	121.0±0.4	23.5±1.1
Exercise only	8	129.0±21.2	23.9±1.2
Thermal Stress only	8	136.4±20.7	24.2±0.7
Exercise + Thermal Stress	8	145.4±23.9	24.4±1.0

Thermal tolerance

There was no significant difference in acute thermal tolerance between fish in the thermal stress only group and exercise plus thermal stress group (t-test, t=-1.679, P=0.115). The median (±S.E) acute thermal tolerance was 30.2 ± 0.5°C (n=8) in the thermal stress only group and 29.6 ± 0.8°C (n=8) in the exercise + thermal stress group (Figure 1A). The time it took to reach the endpoint of loss of equilibrium (LOE) was about 62 minutes, and was not significantly different between thermal stress group and exercise plus thermal stress groups (t-test, t= -0.492, P=0.315; Figure 1B).

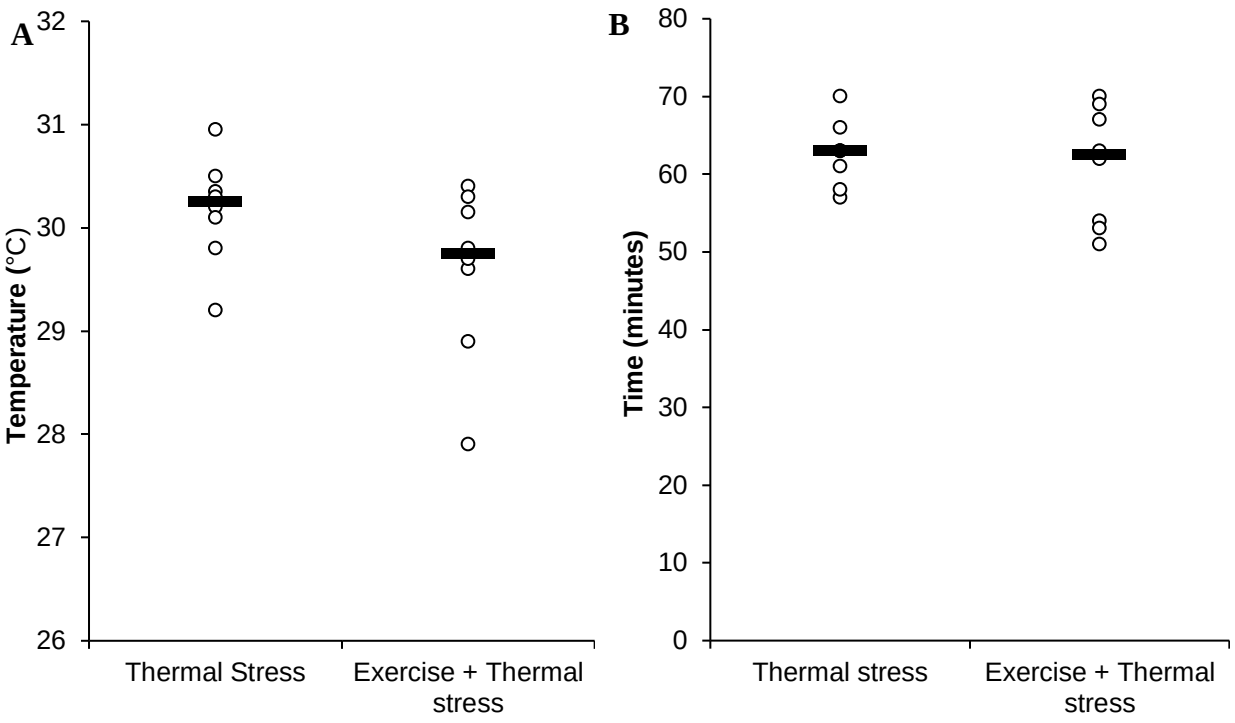

Figure 1. Median temperature (°C) **(A)** and time (minutes) **(B)** for the point where brook charr lost equilibrium (LOE) when exposed to an acute temperature stress only (n=8) or exposed to an exercise stress prior to the thermal stress (exercise + thermal stress; n=8). Open circles represent data from individual charr fish and black horizontal bars represents the group median.

Hematological responses

Hematocrit, and plasma lactate and glucose concentrations were used to measure the physiological status, pre- and post-stress. Hematocrit showed no significant difference between any of the groups (ANOVA, $P>0.05$)(Figure 2A), and the overall values averaged 28%. Under control conditions, lactate levels were low, and these levels increased significantly following exhaustive exercise (Figure 2B). The post-exercise lactate levels were similar (~6mmol/L) to those noted for fish exposed to a thermal stress only ($P>0.05$; Figure 2B). Plasma lactate levels were highest in the charr exposed to exhaustive exercise prior to thermal stress and these levels were significantly higher compared with the exercise only and thermal stress only fish ($P< 0.05$) (Figure 2B). Glucose levels were low under control conditions then rose with the highest values seen in the thermal stress following exercise group (Figure 2C). The post-exercise glucose levels were similar to that of the thermally stressed only fish ($P>0.05$; Figure 2C). There was a significant difference in the glucose levels between thermal stress only and thermal stress following exercise ($P< 0.02$) and between exercise stress only and thermal stress following exercise ($P<0.02$; Figure 2C).

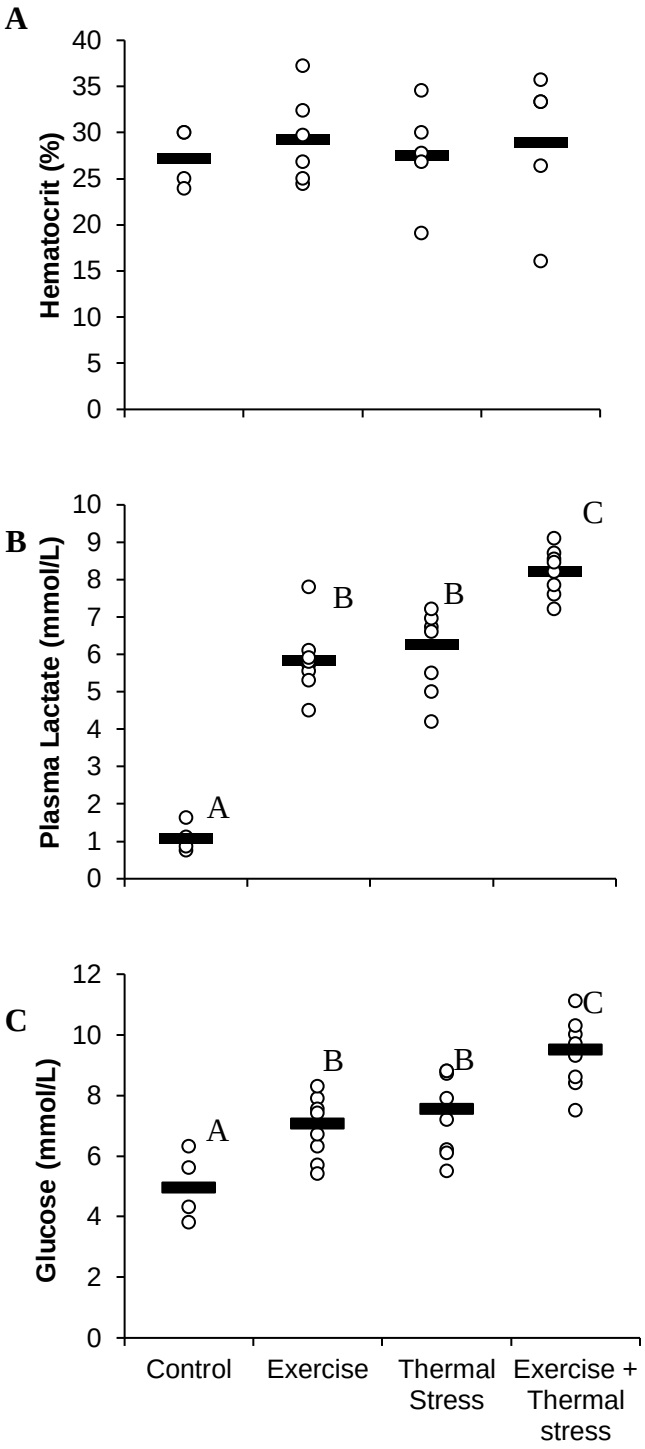

Figure 2. Median hematocrit (%)(A), plasma lactate (mmol/L)(B), and plasma glucose (mmol/L)(C) of brook charr that was a part of an control group(no stress)(n=4), an exhaustive exercise stress only (n=8), an thermal stress(n=8), and an exercise + thermal stress (thermal stress following exercise)(n=8) group of fish. The open circles represent the data for individual charr,

and the black horizontal bar represents group medians. Different capital letters indicate a significant difference ($P<0.05$) from each other.

Discussion

The purpose of this experiment was to: (i) examine how exhaustive exercise stress prior to a thermal stress impacts acute critical thermal tolerance (measured as loss of equilibrium; LOE) in brook charr, and (ii) examine the hematology/secondary stress response in brook charr following thermal stress. My results show that the thermal tolerance in charr that were thermally stressed following exhaustive exercised were similar to those fish that had just undergone thermal stress. However, when examining the hematological responses between groups of fish, there were differences in the lactate and glucose levels in fish challenged to a combined stress (exercise followed by thermal stress) compared with those only thermally stressed or exercise stressed only. The stress response associated with thermal stress and exercise stress are similar to previous studies conducted in the MADSAM lab (Hyndman et al., 2003; Ellis et al., 2013). Despite the differences in the physiological responses between groups of the fish, the LOE associated with thermal stress was not different. Thus, LOE in the present study is likely not the result of a limitation in the ability of the fish to deliver oxygenated blood to the tissues at high temperatures. This is partially supported by the lack of differences in the hematocrit values (a measure of the oxygen carrying capacity of blood) between the 4 groups (Figure 2A). The data from this thesis is useful in helping to identify some of the factors that influences the acute thermal tolerance of fish. However, it is limited (e.g. no measure of tissue hypoxia, exercise is the only stressors presented to the fish) in the value in terms of providing evidence for or against the OCLTT hypothesis as the cause of LOE in fish at high temperatures. To better test the OCLTT hypothesis, additional experiments should be conducted using a broader range of

hypoxic conditions during the thermal stress, as has been done in previous studies (e.g. Ern et al., 2014).

Overall, acute thermal tolerance values for brook charr in the current study were similar to those in a previous study (Benfey at al., 1997). However, when brook charr were exposed to decreasing levels of oxygen (15, 9, 6 and 3 mg/L dissolved oxygen) during a thermal stress trial, the temperature at loss of equilibrium decreased significantly from 29.2°C (@ 15 mg/L dissolved oxygen) to 27.7°C (@ 3 mg/L dissolved oxygen) (Ellis et al., 2013). The difference in the two studies (present and Ellis et al., 2013) may reflect the means in which hypoxia was delivered to the fish prior to thermal stress. Water hypoxia is known to significantly reduce blood oxygen levels (Smith and Jones, 1982; Furimsky et al., 2003; Timmerman and Chapman, 2004). Exercise can modify blood oxygen levels, but likely not to the same extent as that of hypoxic stress (Randall, 1982; Farrell, 2002). Also, in the present study, fish underwent an exhaustive exercise stress before a thermal stress, whereas Ellis et al. (2013) applied the hypoxic stress simultaneously with the thermal stress. Despite this lack of change in acute thermal tolerance in the present study, levels of lactate and glucose were significantly higher in the fish exposed to exercise prior to thermal stress compared with thermally stressed only fish. Specifically, fish in the thermal stress following exercise group had lactate and glucose levels 25% higher than in thermal stress only fish (Figures 2 B, C). This suggests that thermal stress following exhaustive exercise is more physiologically demanding for the fish compared with thermal stress alone.

Exhaustive exercise is normally associated with an increase in the utilization in anaerobic metabolism in fish thus creating higher levels of lactate both in the muscle and blood compartments (Boutilier et al., 1993; Ferguson et al., 1993; Kieffer, 2000; Milligan and Wood, 1986; Rees et al., 2009). High intensity exercise can only be maintained for several minutes

(Wood, 1991; Kieffer, 2000), but the recovery time post-exercise can last for 6-12 hours, with peaks in blood lactate occurring between 1-4 hr (Milligan, 1996; Wood, 1991). Hyndman et al. (2003) noted that post-exercise blood lactate levels in brook charr (at 19C) were ~7mmol/L immediately following exercise and increased to ~17 mmol/L by 2hr into recovery. In the current study, fish that had been exercised would already have higher levels of lactate in their blood—levels were ~ 6 mmol/L, compared to fish that had not undergone exercise. It is highly possible that the increased lactate and glucose levels that appear in the blood of fish that have been exercised and then thermally stressed could be related to the thermal conditions and/or time-course used in the current study.

Figures 3 highlights the potential alternate explanations for the higher lactate levels in the exercise + thermal stress group of fish. It is well known that temperature influences the movement of lactate from the muscle to the blood in fish. Kieffer et al. (1994) inferred that environmental temperatures influence lactate dynamics between muscle and blood in fish because: (i) rate of diffusion of the metabolic end-product out of the muscle is enhanced at warmer temperatures, and/or (ii) the increase in blood perfusion to the muscle at warmer temperatures (Figure 3). Kieffer and Tufts (1996) then showed that warmer temperatures during recovery influences the increased movement of protons (and likely lactate) from the muscle to the blood. Furthermore, Galloway and Kieffer (2003) found that plasma lactate concentration in fish recovering acutely at 18°C were slightly higher at 1 hr and 2 hr post-exercise compared with fish recovering at 12°C. Thus, at warmer temperatures, more lactate appears in the blood of salmonids (Kieffer et al. 1994; Kieffer and Tufts 1996; Galloway and Kieffer 2003). Wang et al. (1997) provide further evidence that the movement of lactate from the muscle to the blood in rainbow trout (*Oncorhynchus mykiss*) operates through either a lactate/proton symport or free

diffusion of lactic acid. These diffusion-related processes will be affected by temperature (Sidell and Hazel, 1987). Coupled with this, it is known that blood perfusion to the muscle increases at warmer temperatures (Barron et al., 1987; Farrell, 1991) likely because of the higher cardiac output at higher temperatures in fish (Farrell, 1991; Farrell, 1984; Kolok and Farrell, 1994). Taken together, the potential increase in perfusion of blood to the white muscle which could affect the amount of lactate diffusing from the muscle to the blood at the warmer temperatures, such as those that occur during the thermal tolerance test (Figure 3).

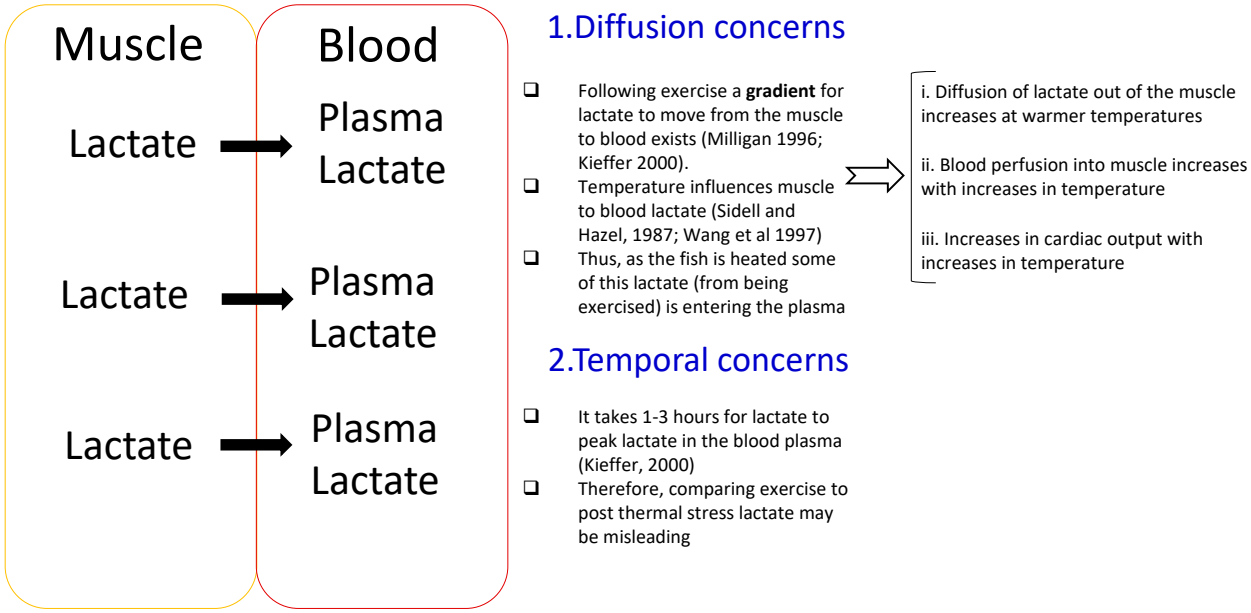

Figure 3. Visual representations of the possible mechanism of why blood lactate appears in higher concentrations in fish exercised to exhaustion prior to the thermal tolerance test.

One of the methodological, and perhaps confounding, issues with the present experimental design is also related to the time-course used for blood sampling. For example, fish from the exercise only group were sampled for blood immediately following the exhaustive exercise (i.e., at ~5 minutes). Fish exposed to thermal stress only and exercise prior to a thermal

stress were sampled between 51-70 minutes after the test began. These sampling times were potentially problematic because it is well known that there is a temporal relationship between post stress recovery times and peak lactate and glucose levels in fish (see above, reviews in Milligan, 1996; Kieffer, 2000). As noted above, in active fish, such as rainbow trout and brook charr, plasma lactate typically peaks within 1-4 hrs following exercise stress (Kieffer, 2000; Milligan, 1996). Ellis et al. (2013) also noted that post-thermal stress values of glucose peaked about 2 hrs in brook charr. Therefore, the patterns of the physiological response between exercise only and thermal stress only may reflect the discrepancies in the time course for blood sampling (Figure 3). Had a 1 hr, post-exercise recovery sampling period been included in the present study, there likely would be differences in lactate and glucose levels between the exercise only and thermal stress only groups. Future studies could address these temporal concerns.

In conclusion, these results showed no effect of exhaustive exercise on the thermal tolerance of brook charr (Figure 1A). Hematocrit also showed not to be affected by thermal stress, exhaustive exercise stress or a combination of both (Figure 2A). Blood lactate and glucose levels showed differing responses depending on the stressors the fish was exposed to (Figure 2B, C). Thus, my results do not directly support nor refute the OCLTT hypothesis as the cause of the LOE in fish at high temperatures. However, the results show the importance of multiple stress testing as well as the value of taking blood samples when conducting acute thermal tolerance tests. Consequently, an extension of this project could be to record the hematological changes in brook charr, following a thermal stress test over a longer time course.

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