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The Contribution of Skeletal Muscle Metabolism to Aerobic Scope of Rainbow Trout (Oncorhynchus mykiss)

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The Contribution of Skeletal Muscle Metabolism to Aerobic Scope of Rainbow Trout (*Oncorhynchus mykiss*)

by

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Abstract

Beyond cellular maintenance, fish require additional energy to accomplish activities such as swimming, digestion, growth, and reproduction. Aerobic scope is the capacity to increase metabolic rate above the minimum (i.e. standard vs maximal metabolism) to meet the demands required for these other activities. Increasing temperatures often increase the standard metabolic rate, reducing and limiting aerobic scope, and it has been suggested this may ultimately limit heat tolerance in fishes. Many different tissues collectively contribute to metabolic rate, but we do not know if specific tissues dominate energy use in fishes, nor how they are impacted by temperature. This thesis investigated the hypothesis that red, aerobic muscle is the primary contributor to the aerobic metabolism, and thus aerobic scope, of a swimming fish. Swim tunnel respirometry using rainbow trout (*Oncorhynchus mykiss*) and measures of oxygen consumption on isolated, working muscle were used to determine their respective aerobic scopes, using oxygen as a proxy for aerobic metabolism across a range of temperatures, up to those approaching the critical thermal maximum of rainbow trout. Further, the mass of red muscle in fish, along with measures of metabolic rate, were used to assess the contribution of red muscle metabolism to that of the whole fish. It was found that red muscle was not a major contributor of aerobic metabolism compared to the whole fish, and thus it was not a major component of aerobic scope. However, red muscle showed similar effects of temperature on both resting and maximum metabolism as that seen in whole fish, increasing the standard metabolic rate at high temperatures but not affecting the maximum metabolic rate. Further, red muscle power output was significantly reduced at high temperatures while metabolic rate was not, potentially implicating the cost of maintenance of muscle as one contributing factor to the high mortality rate of fish at high temperatures.

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Dedication

This thesis is dedicated to Barbara Ann Fowlow

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
$\dot{V}O_2$	The rate of oxygen consumption
$\dot{V}O_{2min}$	Minimum rate of oxygen consumption ($mgO_2\ kg^{-1}\ hr^{-1}$)
$\dot{V}O_{2max}$	Maximum rate of oxygen consumption ($mgO_2\ kg^{-1}\ hr^{-1}$)
SMR	Standard aerobic metabolic rate
MMR	Maximum aerobic metabolic rate
AAS	Absolute aerobic scope (MMR – SMR)
FAS	Factorial aerobic scope (MMR/SMR)
CT_{max}	Critical thermal maximum
U_{crit}	Critical swimming speed
$U_{\dot{V}O2max}$	Swim speed at $\dot{V}O_{2max}$
Q_{10}	Temperature coefficient
PO_2	Partial pressure of oxygen

Chapter One: **Introduction**

1.1 Background

1.1.1 Metabolism and aerobic scope in fish

Fish have been of interest for some time now in the context of metabolism, in part due to their ectothermic nature and the ability to study the effects of environmental temperature on their metabolism (Fry and Hart, 1948; Priede, 1985; reviewed by Schulte, 2015). How energy is allocated within the various processes that constitute metabolism can vary with species and individuals, and is partitioned between requirements for processes of cellular maintenance (e.g. maintaining and renewing proteins, etc.) and other metabolic demands including reproduction, growth, locomotion, and digestion/feeding (Calow, 1985). Aerobic metabolism requires the use of oxygen to sustain metabolism, whereas anaerobic metabolism does not require oxygen. Aerobic metabolism is the primary interest of this study, intended to examine the aerobic, metabolic allocation of energy of fish and the impact of temperature on metabolism. Since aerobic metabolism is often measured as a rate of oxygen consumption, it is referred to as aerobic metabolic rate and any mention of metabolism here forth will be in reference to the aerobic metabolism, or aerobic metabolic rate.

As mentioned previously, aerobic metabolism involves the allocation of energy to different processes within the organism, based on the requirements of the organism at that time. However, some energy must always be available to maintain the minimum metabolic rate. The lowest metabolic rate an organism can have that will still sustain life without causing cellular damage (reviewed by Chabot et al., 2016). The conditions of reaching this minimum would require no activity (i.e. locomotion), growth, digestion or reproductive costs. The terminology for this concept varies in the literature, represented as either basal metabolic rate (BMR) or standard

metabolic rate (SMR), and may vary depending on the animal in question. BMR typically refers to the absolute minimum rate of oxygen consumption at complete rest by any individual organ or living tissue, whereas SMR is often used for a living animal that is at rest (i.e. no voluntary movement). BMR is rarely used because it has been said to be impossible to measure in a living fish; some fish can never be truly at rest, some tend to exhibit indeterminate growth, and for some, resting rates of metabolism are temperature dependent (Brett, 1971; reviewed by Chabot et al., 2016; Ege and Krogh, 1914; Priede, 1985). In fish, SMR refers to the minimum aerobic metabolism in a post absorptive, stationary state at a given temperature, and will be used from now on in reference to a fish's minimum metabolic rate.

In contrast to SMR, there is also a maximum aerobic metabolic rate, which is the greatest rate of aerobic metabolism that animals can achieve. It is possible for some animals to reach a higher total metabolic rate when also considering anaerobic processes, as some fish that enter into an anaerobic state during intensive swimming can incur an oxygen debt (reviewed by Norin and Clark, 2016). However, if considering only sustained, aerobic activity, as is the case in my study, the maximum aerobic metabolic rate (MMR) reflects only aerobic activity. Demonstrating that an animal has attained MMR is difficult due to variation between individuals and because it often relies on a physical effort being exerted, which can vary depending on the motivation of each individual (Brett, 1964; Fry and Hart, 1948; Jutfelt et al., 2018). One approach for studies involving fish is to measure the maximum rate of oxygen consumption ($\dot{V}O_{2max}$) while pushing the animal to swim at their highest speed during steady-state locomotion to determine their maximum aerobic capacity. To do so, swim tunnel respirometers can be used to enclose the fish and record the change in oxygen within the chamber.

SMR and MMR define the lower and upper bounds, respectively, of aerobic metabolic rate. Aerobic scope is a metric used to assess an organism's capacity to increase their aerobic metabolic rate, above that required for basic maintenance, to support other metabolic processes such as locomotion, reproduction, etc. (Fry, 1947; Norin and Malte, 2011; Weibel et al., 2004). Aerobic scope is often presented as absolute aerobic scope (AAS), the difference between an animal's MMR and SMR (Fry and Hart, 1948), or as factorial aerobic scope (FAS), the ratio of MMR to SMR (MMR/SMR) (reviewed by Halsey et al., 2018). Both are useful to assess the difference between the two measures of metabolic rate, but each is uniquely helpful in identifying the effects of other factors (e.g. temperature, growth, hypoxia) on metabolic rate. Aerobic scope is discussed in more detail below, in the context of effects of temperature on metabolism.

1.1.2 Effect of temperature on metabolism

Changes in the rate of oxygen consumption (and often aerobic scope of a fish) are known to occur with variation in size of fish and as a result of abiotic factors such as temperature and hypoxia (Clarke and Johnston, 1999; Norin and Clark, 2016; Pörtner and Peck, 2010). Temperature is of particular interest for poikilothermic ectotherms (e.g. most fish) because the various components of their metabolism are directly affected by their environmental temperature. SMR tends to increase with temperature increases, although not necessarily at the same rate across the spectrum of temperature, such that SMR can increase exponentially as environmental temperatures are raised (reviewed by Chabot et al., 2016). This increase in SMR could be associated with heat shock proteins, increased protein production or other physiological responses to acute temperature increases (Iwama et al., 1998; Fowler et al., 2009). MMR tends to

increase with increasing temperatures as well, at times increasing equally to SMR (Norin et al., 2014), but more often plateauing or declining at high temperatures, thus narrowing the aerobic scope at higher temperatures (Chen et al., 2015; Farrell, 2009). These changes in metabolic rate with changes in temperature can impact aerobic scope to a very large extent, most often narrowing the amount of energy available for other activities beyond resting metabolism at higher temperatures (Eliason et al., 2011; Pörtner and Peck, 2010). MMR appears to show greater variability in response to temperature than SMR, as the relationship with temperature can be dependent on the species, ontogeny, or acclimated temperature of the fish.

Results of some studies suggest that MMR and SMR approach one another at warmer temperatures, such that the aerobic scope narrows, decreasing the scope for activity beyond what is required for resting state (Ferreira et al., 2014; Fry and Hart, 1948; Weibel et al., 2004). At some specific temperature, the fish reaches the maximum temperature at which they can survive, which denotes their critical thermal maximum (CT_{max}). CT_{max} has been proposed to be limited, in part, by the increase in standard metabolic rate, and thus decreasing aerobic scope, that occurs with increased temperature (Fry and Hart, 1948; Hinds et al., 1993). Therefore, the implications of aerobic scope and survival are frequently associated with temperature (Beitinger et al., 2000; Clark et al., 2013; Farrell, 2016; Farrell et al., 2008; Fry and Hart, 1948; Norin and Clark, 2016). For example, it is suggested that aerobic scope could be a determinant of CT_{max}, which assumes that decreasing aerobic scope is a key limiting factor in fish survival at high temperatures, although some suggest that this limitation may be of only minor importance in this regard, if at all (Clark et al., 2013; Norin et al., 2014). Others suggest that aerobic scope can at least act as a good indicator for the thermal niche of an organism, as there is substantial interspecific variation of AAS that appears to follow the thermal habitats of different animals (Farrell, 2009; Raby et

al., 2016). Further, there can be intraspecific variation of AAS as well, depending on the acclimation state of the fish, and so it is important to consider their habitat temperature when examining the association between aerobic scope and CT_{max}; adding to the idea of how important temperature can be to an ectothermic organism's metabolism (Sandblom et al., 2014).

Based on a number of studies, it is now well established that different species of fish have different CT_{max} and relationships with aerobic scope, and this is likely related to the variety of thermal environments that different species can inhabit and the thermal tolerance within each species (Anttila et al., 2013; Beitinger et al., 2000; Farrell, 2009; Farrell et al., 2008; Johnston and Walesby, 1977). It remains unclear, however, what physiological factors are contributing to determining CT_{max}, and thus limiting the survival of fish at extreme temperatures. Further, it is not clear how various aspects of metabolism contribute to total metabolism and aerobic scope, nor the effects of temperature on these relationships. There have been some attempts to identify the physiological contributions to aerobic scope, such as comparing aerobic scope to cardiovascular performance, gill size (for oxygen uptake), blood flow rate, and distribution throughout the body (Barron et al., 1987; Ferreira et al., 2014; Gerry and Ellerby, 2014; Neumann et al., 1983). From studies such as these, muscle has been identified as a large oxygen consumer during swimming, which can be related to their importance in powering locomotion (Gerry and Ellerby, 2014). Since swimming appears to be the most energetically costly activity of the organism beyond basic costs of SMR (Gerry and Ellerby, 2014; Johnson and Johnston, 1991), it is expected that there will be implications for the effects of increasing temperature on muscle metabolism and hence aerobic scope. However, the extent to which temperature affects fish muscle metabolism, its relation to whole-body metabolism, and the aerobic scope itself, has yet to be determined. Establishing a better understanding of temperature effects on all aspects of

fish metabolism could lead to a better understanding of why temperature changes can be so detrimental to the survival of fishes (De Staso and Rahel, 1994; Farrell, 2009; Farrell et al., 2008; McMahon et al., 2008; Sandblom et al., 2014).

1.1.3 Fish muscle: function and oxidative capacity

Fish that swim using body-caudal fin undulations undergo lateral bending of the body and tail to drive forward locomotion and overcome drag (Johnsrude and Webb, 1985; reviewed by Sfakiotakis et al., 1999). This form of locomotion relies on alternating contractions of the axial myotomal musculature. These muscles in fish are typically categorized into two main types that differ in their oxidative and contractile properties. Red, oxidative (i.e. aerobic) muscle is recruited for steady, sustained swimming, while white, glycolytic (i.e. anaerobic) muscle powers rapid, powerful bursts of swimming that cannot be sustained for long periods (Altringham and Ellerby, 1999; Hammond et al., 1998; Jayne and Lauder, 1994; Johnston and Moon, 1980; Johnston et al., 1993; Rome et al., 1984). These muscle fibre types vary in size, protein isoforms, contractile characteristics, mitochondrial content, myoglobin content, lipid content and capillarization (Johnston et al., 1977; Sänger and Stoiber, 2001; reviewed by Syme, 2005). Red muscle fiber types are typically located superficially (i.e. under the skin) along the lateral line of most fishes and have a high mitochondrial content and a greater oxidative capacity compared to white muscle, making red muscle the primary aerobic muscle in fish (Johnston et al., 1977). This, along with electromyogram (EMG) studies, aid in our understanding of fibre type recruitment during different swimming conditions (Gerry and Ellerby, 2014; Jayne and Lauder, 1993; Jayne and Lauder, 1994; Rome et al., 1984; Rome et al., 1993; Shadwick and Syme, 2008). Highly aerobic red muscle is recruited for slow, steady and sustained swimming, whereas white muscle,

with less aerobic capacity but higher glycolytic capacity, is well-suited for fast, short-term swimming. However, the idea of separating the muscles into two distinct categories for muscle function (i.e. red used for sustained swimming and white used for burst) has received some criticism, particularly when considering certain species of fish (Johnston and Moon, 1980). Some have suggested that white muscle also contributes to thrust during sustained swimming, but whether this is via exclusively glycolytic or partially oxidative metabolism is not well studied (Sänger and Stoiber, 2001). That being said, red muscle is the primary aerobic muscle type for fish when swimming at slower speeds (i.e. below the critical swim speed, U_{crit}, the fastest sustainable swim speed prior to exhaustion) (Farrell, 2008; Gerry and Ellerby, 2014).

When examining the aerobic capacity of fishes, therefore, red muscle is clearly an important and perhaps primary contributor. Based on Gerry and Ellerby's study (2014), it does appear that approximately 60% of oxygenated blood is carried to the red muscle while the fish is exercising in a sustained fashion, contributing to our expectation that aerobic red muscle may dominate in terms of a contribution to aerobic capacity and scope of the whole fish. Additionally, EMG studies (e.g. Hammond et al., 1998; Rome et al., 2000; Syme et al., 2008) confirm that red skeletal muscle is being activated to perform greater work with increasing swim speeds and thus demanding more energy. However, white muscle should not be ignored as a potential contributor to the aerobic cost of locomotion. White muscles in fish contain 1-4% mitochondria, indicating that there is some aerobic capacity for these muscles (reviewed in Sänger and Stoiber, 2001 and Syme, 2005). Additionally, red muscle contributes only 1-3% of the total body mass in rainbow trout, sometimes more in other species of fish (Sänger and Stoiber, 2001), whereas white muscle is approximately 50% (Goolish, 1989). Thus, although red muscle might be more oxygen demanding on a mass-specific basis, the relative proportion of each muscle type, and the very

large mass of white muscle in particular, will affect their overall contribution to aerobic metabolism of the fish.

In summary, the impact of temperature on the activity and oxidative metabolic rate of muscle tissue is not well understood. Muscle is a large contributor to the total fish mass and is expected to be the primary driver in metabolic rate during exercise, particularly aerobic muscle during sustained swimming. Therefore, it is useful to understand the contribution of muscle metabolism to aerobic scope, as well as the effect of temperature on muscle metabolism at temperatures approaching CT_{max}, to better understand if and how muscle contributes to aerobic metabolic rate and aerobic scope of the whole fish, and if the metabolic demands of muscle might contribute to determining CT_{max}.

1.2 Hypotheses

Forced swimming is commonly used as an experimental means to attain maximum aerobic metabolism in fish, which can then be measured via oxygen consumption. Using this approach, the effects of temperature, particularly warm temperatures, on SMR, MMR and aerobic scope of fish can be measured. Creating a similar circumstance with muscle tissue, making it work as it would in a swimming fish while measuring the rate of oxygen consumption, can be used to determine the muscle's resting and maximum aerobic metabolism, and hence aerobic scope. For example, fish exhibit increased tail beat frequencies at higher swim speeds, where the muscles produce greater power and thrust to overcome increased drag (Altringham and Ellerby, 1999; Bernal et al., 2005; Coughlin, 2000; Syme et al., 2008; Webb, 1971). This might indicate that muscle should be the primary consumer of oxygen and dominate aerobic metabolism while the fish is swimming, particularly at high speeds. Therefore, it was hypothesized that muscle would be the primary contributor to the aerobic metabolism, and thus aerobic scope, of a swimming fish. Additionally, if muscle dominates aerobic metabolism of fish, particularly MMR, it is expected that temperature will affect metabolism and thus aerobic scope of muscle tissue similar to that seen in intact fish, implicating muscle as a primary contributor to the reduced aerobic scope of ectothermic fish in warmer water. Further, if CT_{max} is a reflection of reduced aerobic scope, I hypothesize that a decline in aerobic scope at warm temperatures seen in intact fish should correspond to the CT_{max} of the fish, and if muscle dominates aerobic metabolism, then a similar relationship should exist between aerobic scope and temperature in muscle. In order to test these hypotheses, I determined the aerobic scope of intact fish across a range of temperatures up to those approaching CT_{max} and compared it with the aerobic scope

measured from fish aerobic skeletal muscle (i.e. red muscle). If my hypotheses are supported, I expect to see that metabolic rate and aerobic scope of muscle will exhibit a similar relationship with temperature as they do in the whole fish, with aerobic scope of both muscle and fish declining at temperatures approaching CT_{max}, which is 27-29^{o}C in rainbow trout based on other studies (e.g. Beitinger et al., 2000; Scott et al., 2014). I also expect that the decline in aerobic scope of whole fish will be largely due to an exponential increase in SMR with increasing temperature, and that the MMR of red muscle will constitute most of the aerobic scope of whole fish.

1.3 Experimental Approaches

1.3.1 Swim tunnel respirometry as a measure of aerobic metabolism in fish

Measures of aerobic metabolism often use rates of oxygen consumption as estimates of aerobic metabolic rate (i.e. indirect calorimetry), because oxygen is vital to the creation of adenosine triphosphate (ATP), the currency of energy used by cellular processes in animals (Priede, 1985), and so the rate of oxygen use is a good, although not necessarily perfect, reflection of the rate of ATP production and use. Many studies have employed methods of measuring oxygen consumption as an indicator of aerobic metabolism, as well as aerobic scope, in fish (e.g. Clark et al., 2013; Gräns et al., 2014; Guderley, 2004; Norin and Malte, 2011; Norin et al., 2014; Soofiani and Priede, 1985; Zhang and Kieffer, 2017). Such studies have examined the resting and maximum oxygen uptake for a fish as indicators of their standard and maximum aerobic metabolic rate, where maximum rates are measured during rapid but sustained swimming which relies predominantly on aerobic metabolism (i.e. not during fast escape responses that invoke anaerobic metabolism) (Coughlin, 2002). Respirometry is a type of indirect calorimetry that attempts to measure the aerobic metabolism of an organism *in vivo*, using the release (i.e. CO_2) or use (i.e. O_2) of metabolic gases as an indicator of metabolic rate (Farrell, 2009; Fry, 1947; Priede, 1985; Tierney, 2011). This is opposed to direct calorimetry, which uses the release of heat as a measure of metabolism and is a much more difficult measurement to make on a living organism.

A common method of indirect calorimetry in fish is to use swim tunnel respirometers to measure metabolic rate via the rate of oxygen uptake from the environment (reviewed by Chabot et al., 2016; Nelson, 2016; Norin and Malte, 2011; Svendsen et al., 2016). This approach can also

be used as an indicator of an organism's metabolic rate at different temperatures. The technique involves enclosing a fish in an air-tight compartment filled with water, and then measuring the decline in oxygen in the water within the compartment as a measure of metabolic rate. The fish's swim speed can be controlled by the rate of water flow within the chamber, allowing measurements of metabolic rate at different intensities of exercise, until the maximum rate of oxygen consumption is determined (Tierney, 2011). A common method of swim tunnel respirometry uses intermittent recordings of oxygen, in which the rate of oxygen consumption ($\dot{V}O_2$) is recorded for a set amount of time, and then the water in the chamber is flushed out and replaced with oxygenated water, at which point there is a stabilization period before $\dot{V}O_2$ is recorded again. This ensures the fish is continuously in normoxic conditions.

There are various techniques used in swim tunnel studies, including swim tunnel respirometers in the lab and chase protocols, in which the fish are manually chased in a container to induce maximal swimming. These variations can also yield different results and makes comparisons between studies difficult to interpret (Nelson, 2016; Rummer et al., 2016). Additionally, some argue that measuring metabolic capacity in a controlled lab setting may be an inaccurate or incomparable measure for wild organisms (Treberg et al., 2016). Although these are legitimate concerns, measuring aerobic scope in a controlled manner can provide useful information that likely approximates metabolic capacity of an organism, helping us to understand the impact that changes (environmental, anthropogenic, etc.) may have on metabolism.

Recent literature has also addressed the notion that certain techniques used to measure oxygen consumption may not reflect aerobic metabolism exclusively, when oxygen consumption post-activity may be associated with anaerobic compensation (Norin and Clark, 2016). This is due to costs of anaerobic metabolism during activity that need to be repaid later when the fish is

less active, and suggest the activity in question may not be exclusively steady state, aerobic, and sustainable (Brett, 1971; Priede, 1985). Thus, it is important to consider the techniques used for measuring aerobic capacity, as well as for comparison between studies.

It is also important to consider factors such as allometric scaling with body mass, acclimation conditions, and the effect of feeding when undergoing measures of metabolic rate, including swim tunnel studies, because such factors can contribute to variation in aerobic scope (Clarke and Johnston, 1999; Glazier, 2014; Sandblom et al., 2014). Many have shown that the acclimation temperature can alter aerobic scope, often showing that fish acclimated to higher temperatures have a slightly shifted aerobic scope and a higher CT_{max} (e.g. Chen et al., 2015). Additionally, the size of the fish is a critical determinant for the aerobic metabolic rate, as total rate of oxygen consumption seems to scale logarithmically with mass (Clarke and Johnston, 1999; Goolish, 1991; Killen et al., 2007). These scaling relations can also have inter- and intra-specific variation, however, growth and metabolism do appear to have allometric scaling effects, in both whole organism and in muscle growth as well (Clarke and Johnston, 1999; Goolish, 1989; Killen et al., 2007).

1.3.2 Work-loop technique to assess aerobic metabolism of muscle

As with whole fish, the energy required for muscle to undergo aerobic working contractions can be measured as a function of oxygen consumption by the muscle, as has been done in other studies (e.g. Harwood et al., 2002; Josephson and Stevenson, 1991; Stainsby and Otis, 1964; Syme, 1994; Trinh and Syme, 2007). The more work done, the more oxygen will be used, up to the limits of maximal aerobic metabolism. Work is a measure of mechanical energy, and the work done on an object is the product of the force exerted on an object and displacement

of the object. For muscle in a living fish, this would be the force exerted by the muscle to move the load (e.g. the myotome). Power is the rate of doing work, which in turn should impact the rate of oxygen consumption, again up to the limits of aerobic metabolism. Power will be a function of the work done during a single contraction and how many contraction/relaxation (i.e. work) cycles are completed in a given period of time (i.e. contraction frequency), which in a swimming fish is the number of tail beats per second (Johnson and Johnston, 1991; reviewed by Syme, 2005; Syme et al., 2008). Thus, by manipulating the power output of a muscle, the aerobic metabolic rate can be altered, and measured.

The work-loop method (Josephson, 1985) can be used to manipulate and examine the mechanical power output of muscle tissue. In this method, the muscle is phasically activated (i.e. stimulated to contract) while its length is cycled through shortening and lengthening motions. This mimics the movements the muscle experiences in a swimming fish, and the maximum power a muscle is capable of producing in a fish while swimming can be approximated (Coughlin, 2000; Hammond et al., 1998; Listrat et al., 2016; Lou et al., 2002). The amount of energy used by the working muscle can simultaneously be assessed via the change in oxygen levels recorded within the chamber that contains the working muscle, and so we can assess the aerobic metabolic rate of a contracting muscle, at rest through maximal.

The work-loop technique has been used in parallel with recordings of oxygen previously (Harwood et al., 2002; Syme, 1994; Trinh and Syme, 2007), but to our knowledge, it has never been examined for fish skeletal muscle and certainly not in the context of the effects of temperature on muscle performance. The work-loop technique in conjunction with oxygen measurements is thus a valuable approach for this study because it allows the muscle to perform

work at its maximum capacity, affording the ability to measure the maximal metabolic rate of oxygen uptake ($\dot{V}O_{2max}$).

1.3.3 Study species

Juvenile rainbow trout were selected as the model organism to study the comparison of aerobic scope in whole organisms with that of excised muscle tissue. Working with juveniles avoided the complications of interpretation that would be introduced through energetic costs of gonadal development and investment, which are relevant when considering aerobic scope of the entire fish (Nelson, 2016). The energy requirements for gonad development can be extensive in adult fish, and often is more costly for females than males (Hayward and Gillooly, 2011; Hendry and Berg, 1999; Jonsson and Jonsson, 2003). Rainbow trout are also an ideal model organism as they are relatively large; with large red muscles allowing for accurate isolation during dissections. They are also a well-studied organism regarding their physiology and thermal effects on their physiology and are readily available with established husbandry practices. In an ecological context, rainbow trout are locally, nationally and internationally important as native and imported species, components of the ecosystem, and as sportfish.

Chapter Two: **Aerobic Metabolism of Swimming Rainbow Trout**

2.1 Introduction

Metabolic rates normally range from minimal while resting, to relatively large during exhaustive swimming (Priede and Young, 1977). The lowest metabolic rate of a fish is their standard metabolic rate (SMR) and is measured under non-digesting conditions in resting animals, whereas the maximum aerobic metabolic rate (MMR) is typically measured while swimming at some high, but sustainable, speed. Aerobic scope, the difference between SMR and MMR, is the available energy for activities above SMR, such as locomotion, feeding, digestion and reproduction (Eliason and Farrell, 2016; Farrell, 2016; Farrell et al., 2001; Fry, 1947).

Environmental temperature changes are known to have profound effects on the physiology of fishes, including metabolic rate, and will impact the allocation of energy to the various processes that consume it (Brett, 1971; Claireaux and Lagardère, 1999; Fry, 1947; Lefrançois and Claireaux, 2003; Pörtner and Peck, 2010). The concerns of environmental warming are of interest regarding ectothermic organisms, like most fish, because they are not able to regulate their internal body temperature and are reliant on the temperature of their environment, which will in turn impact aerobic scope and the ability to survive in particular habitats. Ectotherms in particular often have an aerobic scope that is typically maximal at or near the thermal preference, and a reduced aerobic scope when in temperatures outside their thermal preference (Brett, 1964; Farrell, 2009; Fry, 1947; Pörtner and Knust, 2007; Wieser, 1985). Many fish species have shown signs of reduced aerobic scope at extreme temperatures relative to their native habitat (e.g. Farrell, 2009; Ferreira et al., 2014), however, it is important to note that this is not necessarily the case with all fish (Farrell, 2009; Norin et al., 2014). A reduced aerobic scope at extreme temperatures suggests that there is less energy available for growth, reproduction and

locomotion. Thus, very high temperatures could result in death (e.g. Carline and Machung, 2001; Pörtner and Knust, 2007).

Many have attempted to explain why temperature limits performance in aquatic ectotherms through studies of whole animal aerobic scope, cardiac pumping performance and thermal tolerance limits (Clark et al., 2011; Clark et al., 2013; Cooke et al., 2000; Eliason et al., 2013; Neumann, 1983; Overgaard et al., 2012; Pörtner and Knust, 2007; Priede and Young, 1977). However, fish seem to show significant interspecific differences in their thermal tolerances and aerobic scope, making it difficult to identify what is limiting their survival at increasing temperatures (Clark et al., 2011; Ferreira et al., 2014; Norin and Malte, 2011; Norin et al., 2014; Zhang and Kieffer, 2017). A common proposal is that fish, like other animals, are dependent on oxygen to perform many essential activities (i.e. swimming, reproduction). Therefore, if aerobic energy is limited by high temperatures, fish will not be able to survive in those conditions for long (reviewed by Clark et al., 2013; Farrell, 2016; Halsey et al., 2018; Norin and Clark, 2016). Oxygen availability at high temperatures could be limited by hypoxic environmental conditions, by the cardiac pumping capacity, by the rates that certain tissues use oxygen, and/or by elevated basic metabolic costs to maintain cellular function (Barron et al., 1987; Brett, 1971; Gamperl et al., 2002). Therefore, the focus of this study will be to examine the effects of temperature on aerobic metabolic rate in fish, as a prelude to understanding how the metabolism of muscle contributes to whole fish metabolism and aerobic scope.

Rainbow trout are a good model organism to study the effect of temperature on fish because they often live in regions with high seasonal and daily thermal fluctuations (Committee on the Status of Endangered Wildlife in Canada (COSEWIC), 2014; Gamperl et al., 2002). Additionally, rainbow trout have been used to study metabolism under a variety of environmental

and physiological conditions (Farrell, 2008; Myrick and Cech, 2000; Steffensen and Lomholt, 1991; Wieser, 1985; Zhang et al., 2018). Rainbow trout also inhabit lakes and rivers across most of the world, and there is evidence of variation among the different strains that show adaptations to temperature within their current habitat (Carline and Machung, 2001; Chen et al., 2015; Myrick and Cech, 2000; Scott et al., 2014; Zhang et al., 2018).

I sought to determine the effects of temperature on aerobic scope of rainbow trout under laboratory conditions to identify how they compared to other studies and other strains of rainbow trout, and to then use this data to understand how muscle might contribute to whole-body aerobic scope. For the latter, I compared the whole organism aerobic scope to muscle aerobic scope of trout that were reared under the same conditions, to identify if similar patterns and limits exist in the aerobic scope of muscle compared with intact fish that might suggest if muscle is a primary determinate of aerobic scope in fish (see Chapter 4). Gaining a better understanding of the thermal tolerance and its mechanistic basis for specific strains used to stock lakes throughout Alberta also helps to improve our understanding of how local temperature changes may influence the energy allocations of these trout.

2.2 Methods

2.2.1 Animal care and handling

All procurement, care and handling of animals was approved by the University of Calgary animal care committee following guidelines of the Canadian Council on Animal Care. Rainbow trout (BEBE 2n strain) were obtained from Allison Creek Hatchery, Crowsnest Pass, Alberta, Canada, May 2018, at an average mass of approximately 10 g. All fish were initially housed in a circular holding tank, 180 cm diameter x 70 cm height, approximately 1400 L, flow-through water supply, at a water temperature of 12 ± 1ºC, for several months. Fish were fed to satiation daily on commercial trout pellets (EWOS Food Supply, micro 1.2mm and 2mm, Bergen, Hordaland, Norway) and kept on a 14:10h light:dark photoperiod. As the fish approached the size required for experiments (approx. 40 g), they were moved into 12 L flow-through holding tanks (~40 mL s^{-1} water recirculation rate) and held at 12-13ºC (Ranco Electronic Temperature Control Pump, ETC Supply, Delphos, OH, USA) for 1-3 months prior to experimentation. The 12L tanks were located in the room housing the swim tunnels, with 1-3 fish per tank depending on individual aggression and dominance behaviour. The rainbow trout used for the swim tunnel experiments averaged 89.8 ± 4.8 g (47 – 165 g, n = 36) and were fasted for a minimum of 48 hours (maximum of 54 hours) prior to measurements of metabolic rate (Clark et al., 2013).

2.2.2 Swim tunnel respirometer

Two, 4L swim tunnel respirometers were used simultaneously (model SW10050, Loligo Systems, Viborg, Denmark) (Figure 2.1). The water from both swim tunnel systems was drained and replenished prior to each new experiment. An external water compartment (i.e. water jacket)

surrounded the swim tunnel chamber and was used as a source for replenishment of water in the swim tunnel chamber and as temperature control for the swim tunnel. The swim tunnel chamber housed a propeller connected to a motor that circulated water throughout the oval-shaped chamber, with a rectangular holding compartment (7.5 cm height x 7.5 cm width x 30 cm length) for the fish, and a honey-comb block placed in front of the holding compartment to induce laminar flow. The holding compartment had a wire mesh backing at the rear end to constrain the fish to that region of the tunnel.

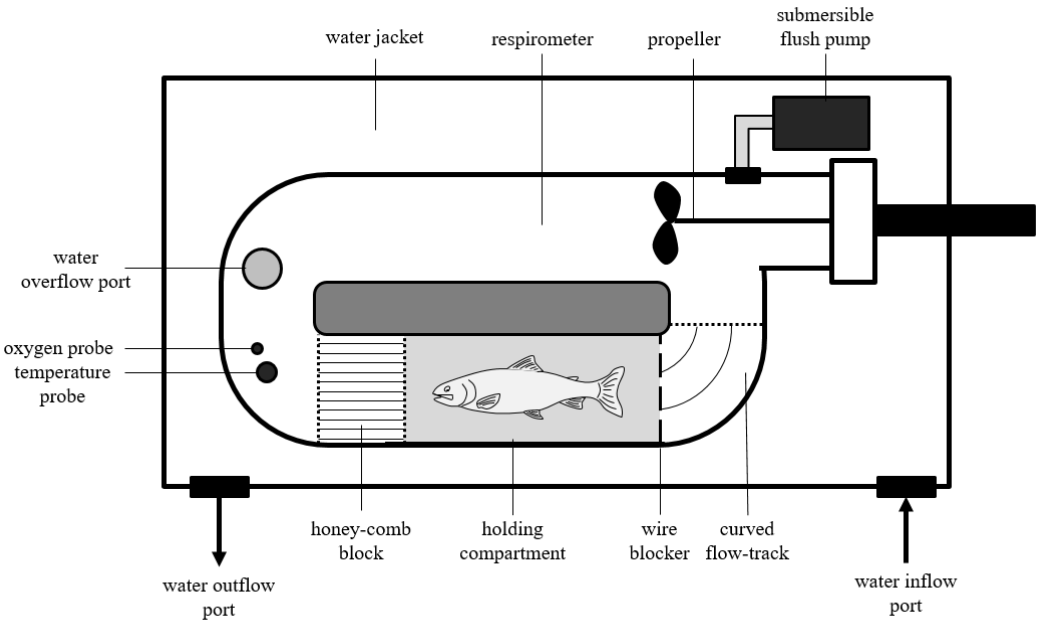

Figure 2.1 Schematic (top view) of swim tunnel respirometer used to measure metabolic rate of fish. Water inflow and outflow ports were connected to a temperature-controlled water bath that circulated freshly aerated water into the water jacket surrounding the respirometer chamber. The submersible flush pump moved water from the water jacket into the respirometer during the flush period. Excess water from the respirometer moved out via the water overflow port. The propeller induced water flow within the respirometer, which moved through the honey-comb block to promote laminar flow as the water entered the holding compartment (i.e. where the fish was held). The wire blocker prevented the fish from leaving the holding compartment. The curved flow-track promoted circulation of water and limited turbulence within the respirometer. The oxygen and temperature probes were held in place by the respirometer lid and used to measure changes in oxygen content and temperature within the respirometer during experimentation. See methods for further detail.

A temperature probe, and fiber-optic dipping probe used to measure oxygen (Witrox 1, Loligo Systems Viborg, Denmark), were held in place within the swim tunnel chamber via adjustable openings on the swim tunnel lid. The temperature probes in the respirometers were checked for accuracy using a digital thermometer (Traceable Digital Thermometer, VWR International, Radnor, PA, USA). Oxygen dipping probes were calibrated in water bubbled with atmospheric air for 100% air saturation, and 1% sodium sulphite was mixed into a separate water container to determine 0% air saturation. Oxygen recordings were measured under intermittent flow-through respirometry conditions: 100 seconds of fresh water flushing the swimming chamber, a 40 second waiting period to allow for oxygen levels to stabilize, and a 160 second closed-system measuring period (AutoResp 2.2.0 Software, Loligo Systems, Viborg, Denmark). The flush-wait-measure cycles are referred to as loops, and a single loop lasted 5 minutes.

Flow velocity in the holding compartment was calibrated with a digital anemometer (Hontzsch AC10000, Handheld Flowtherm NT, Hontzsch Flow Measuring Technology, Waiblingen, Germany). The set flow velocity was controlled using the AutoResp 2.2.0 Software package (Loligo Systems, Viborg, Denmark) which controlled the speed of the motor and propeller and was used to record and store the calibrated data from the oxygen and temperature probes. A linear relationship was established between the output (RPM) from the motor controller and the water flow velocity recorded by the digital anemometer for each swim tunnel at multiple speeds, and the corrected velocity was adjusted post-experiment. Blocking effects in the holding compartment (i.e. effects of the fish itself influencing the flow and speed of water around it) were not considered for this experiment, as an accurate measure of fish swimming speed was not critical for assessing maximal rates of oxygen consumption. The swim tunnels were cleaned weekly with 5% bleach (sodium hypochlorite) to limit bacterial growth and reduce

background oxygen consumption. Water quality was checked with AquaChek chlorine strips post-bleaching and prior to new experiments (AquaChek, Loveland Colorado, USA).

Before measures of respirometry commenced, each swim tunnel respirometer and temperature jacket were filled with fresh water from the holding tank system. Once filled, water was drained by gravity from the water jacket region of the respirometers into a circulating chiller (Isotemp model 3006, Fisher Scientific, Waltham, MA, USA) initially set to 12ºC and aerated with an air stone. This aerated water was then pumped from the chiller back into the water jackets surrounding the swim tunnel respirometers (20 mL/s circulation rate), continuously recirculating throughout the experiment. A submersible flush pump (Eheim 1048 Universal Pump, Deizisau, Germany) moved water from the water jacket into the swim tunnel respirometer during the flushing periods to allow fresh water to replenish the testing chamber. The swim tunnel lid also had an overflow valve to allow for the release of excess water. The lids of the swim tunnels were tightly fastened and any bubbles that had formed were removed prior to experimental measurements. The swim tunnel respirometers were initially set to 12ºC, the same temperature as the holding tanks.

Fish were netted from their holding tank, transferred to a transport container and weighed immediately prior to being introduced to one of two swim tunnel respirometers. Air exposure was never longer than 5 seconds and the time from removal from holding tank to being placed in the swim tunnel was less than 1 minute.

The fish were then left to rest in the swim tunnel chamber for 1 hour with continuous flow of water, at which point the temperature was increased by 2ºC hr^{-1} until reaching the randomly assigned testing temperature. The length of the fish was determined at this point using

pre-measured orientation lines within the holding compartment (i.e. vertical reflective lines on the compartment wall to help fish with orientation).

Testing temperatures ranged from 12ºC to 24ºC, and six fish were tested at each temperature, each fish being tested at only one temperature. A maximum test temperature of 26ºC was also attempted on six fish, which approaches the CT_{max} for rainbow trout (Beitinger et al., 2000); however, at this temperature the fish did not maintain orientation during overnight acclimation and so a maximum test temperature of 24ºC was used for the remainder of experiments. All trout recovered successfully post experimentation upon return to their respective holding tanks, with the exception of the trout that were exposed to 26ºC, of which four of the six tested fish died overnight. Some fish did not swim (one at 12ºC and one at 18ºC), despite increasing flow velocity, and remained pressed against the wire blocker. Data from these fish were removed from the experiment.

Standard metabolic rate (SMR) was recorded as the rate of oxygen decline ($\dot{V}O_{2\ min}$) using the intermittent respirometry technique previously described. SMR measurements commenced between 16.00 and 18.00 h and were recorded overnight for the following 14 to 17.5 hours, such that the room was dark and quiet with no other activity. The variation in time over which these measures were made was due to differences in time required to attain the test temperature. Water flow through the holding compartment was 0.20 ± 0.03 L s^{-1} (3.60 ± 0.30 cm s^{-1}) during resting measurements, to help the fish maintain orientation but the flow was not fast enough to induce swimming (Chabot et al., 2016; Clark et al., 2013). This flow was also important to ensure mixing within the respirometer for accurate oxygen content readings. There was an exponential decline in the rate of oxygen consumption over the ensuing several hours after the fish was placed in the respirometer, as the fish became accustomed to the conditions

post-experiment. SMR was determined as the average of the 50 lowest values of metabolic rate recorded throughout the period of rest. This represents 30% of the approximately 180 recordings of SMR made during the experiments on each fish.

Maximum metabolic rate (MMR) was determined using an adapted ramp-U_{crit} protocol (adapted from Farrell, 2008) (Figure 2.2). In this approach, the fish swam at increasing increments of water velocity of ~5 cm s^{-1} every 5 minutes up to 30 - 50 cm s^{-1}, depending on fish size, which was estimated to be 50% of maximum swim speed (i.e. U_{crit}) based on preliminary tests. Once ~50% maximum speed was attained, the time between increases in flow speed was increased to 15 minutes (i.e. every 3 loops of a flush, wait, measure cycle) until the fish reached a final swim speed at the point of exhaustion. This ramp-U_{crit} protocol was used to rapidly approach the maximum rate of oxygen consumption ($\dot{V}O_{2max}$) in a steady swimming state without exhausting the fish or inducing a burst-and-glide swimming gait that may be utilizing anaerobic metabolism (reviewed by Zhang et al., 2019). MMR was recorded as the average of the three highest $\dot{V}O_2$ recordings and occurred when increasing swim speed no longer resulted in increased $\dot{V}O_2$. The swim speeds of those three $\dot{V}O_{2max}$ trials that represented MMR were averaged and used to represent the swim speed at $\dot{V}O_{2max}$ (i.e. $U_{\dot{V}O2max}$; Table 2.1). Fish were continually observed during measures of MMR, and any $\dot{V}O_2$ recordings during non-steady swimming behaviours (i.e. sudden bursts) were excluded, although this did not occur often.

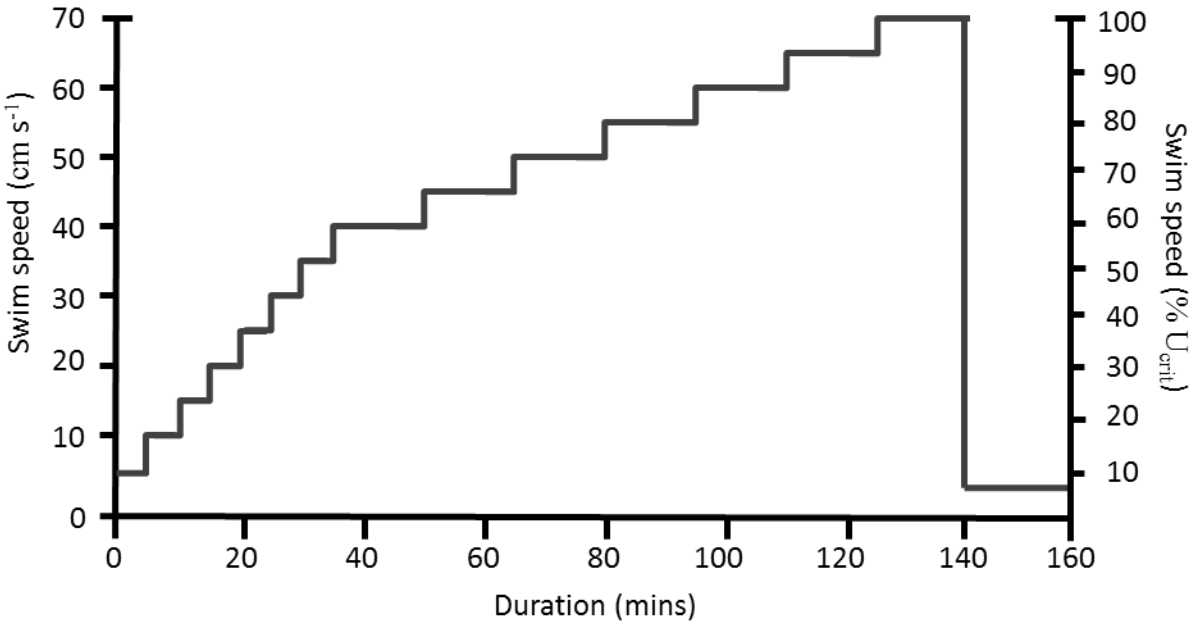

Figure 2.2 Example of the swim speed increments used throughout the adapted ramp-U_{crit} protocol to determine MMR. 5 cms^{-1} speed increases occurred every 5 minutes until approximately 50% of the maximum swim speed (U_{crit}) (40 cm s^{-1} in this example), at which 5 cm s^{-1} speed increases occurred every 15 minutes until U_{crit} was reached, and swim speed was then dropped back to resting flow levels (~3.5 cm s^{-1}) for recovery.

MMR was often reached before the swim speed at which the fish became exhausted (the fish would continually fall back against the wire grid at the back of the holding compartment), and so the speed at exhaustion (i.e. final swim speed) was also recorded. At this point, swim speed was reduced to 3.60 ± 0.30 cm s^{-1} to allow recovery. The final swim speed was recorded, but the exact time of exhaustion was not (i.e. how long at that speed the fish was able to swim prior to exhaustion). Therefore, U_{crit} could not be calculated as per Brett (1964), however, the final swim speed approaches U_{crit}, and estimating U_{crit} was not a necessary component of this study, as only attaining MMR was necessary.

Post experimentation, fish recovered at a flow rate of about 3.5 cm s^{-1} for one hour before the water temperature was reduced back to 12ºC at a rate of 3ºC hr^{-1}. During the one hour recovery period, the fish were moved into the external water jacket of the swim tunnel and the chamber was resealed without a fish in the measuring compartment to record background oxygen consumption rates within the swim tunnel at the testing temperature, and this value was subtracted from the measures obtained with a fish present.

2.2.3 Rate of oxygen consumption in whole fish

$\dot{V}O_2$ (mgO_2 kg^{-1} hr^{-1}) for both SMR and MMR was calculated via the respirometer's AutoResp program using the following equation:

$$VO_2 = \frac{\Delta[O_2] * v}{m * t},$$

Change in oxygen content (mgO_2 L^{-1}) is calculated from the slope of the linear regression of oxygen decline for each measuring period, *m* is the mass of the fish, *v* is the volume of the closed respirometer (L) from which oxygen was recorded, and *t is* the duration of each recording.

The effect of temperature on $\dot{V}O_2$ was calculated as Q_{10}:

$$Q_{10} = \left(\frac{K_1}{K_2}\right)^{\left(\frac{10}{t_1 - t_2}\right)}$$

K_1 and K_2 represent the rate of oxygen consumption at their corresponding temperatures (t_1 and t_2, respectively) (Table 2.2). Q_{10} is a temperature coefficient measuring the change in metabolic rate over 10°C.

2.2.4 Tail beat frequency of swimming fish

Videos were taken of 7 fish from a dorsal view while swimming at the higher swim speeds (>50% final swim speed) (Nikon Coolpix 8700 camera, Nikon, China) at 30 frames per second (fps). Tail beat frequency (TBF, in beats per second) was measured using ImageJ software, Fiji (ImageJ 1.52p, http://imagej.nih.gov/ij/). Tail beat frequencies were recorded for each swim speed. These frequencies were used for comparison to the optimal cycle frequency for maximal power output from the red skeletal muscles (Chapter 3). The higher TBF's were too fast to accurately assess with a 30 fps frame rate of the recording device, and so only approximate values of TBF's could be estimated at high speeds, however, approximations are adequate as these values of TBF were only used as additional information about muscle function in swimming fishes (Chapter 4).

2.2.5 Analysis

Linear regressions of oxygen content over time were derived from the AutoResp 2.2.0 Software package (Loligo Systems, Viborg, Denmark) and used to calculate $\dot{V}O_2$ as described above. The effect of temperature on SMR, MMR, absolute aerobic scope (AAS=MMR – SMR) and factorial aerobic scope (FAS=MMR/SMR) were analyzed using a one-way ANOVA and residuals were examined for normality and equal variance using the Shapiro-Wilk test and Levenes test, respectively, in RStudio (R version 3.6.1, car package add-on). Where ANOVA indicated statistical significance, Tukey's honest significant difference (HSD) post-hoc tests were conducted to identify which specific temperatures were significantly different from one another. The statistical significance was determined with α of 5% ($P < 0.05$) and variation indicated as SEM.

2.3 Results

The range in fish sizes used in this study was fairly broad (47 – 165 g, 89.7 ± 20 g average across all fish, n = 34), therefore, linear regressions for both SMR and MMR fitted against fish body mass was used to determined if there was an effect of size on metabolic rate; there was no significant relationship between body mass and the temperature groups tested (Figure 2.3). There were also no significant differences in body mass of the fish and metabolic rates (Figure 2.4).

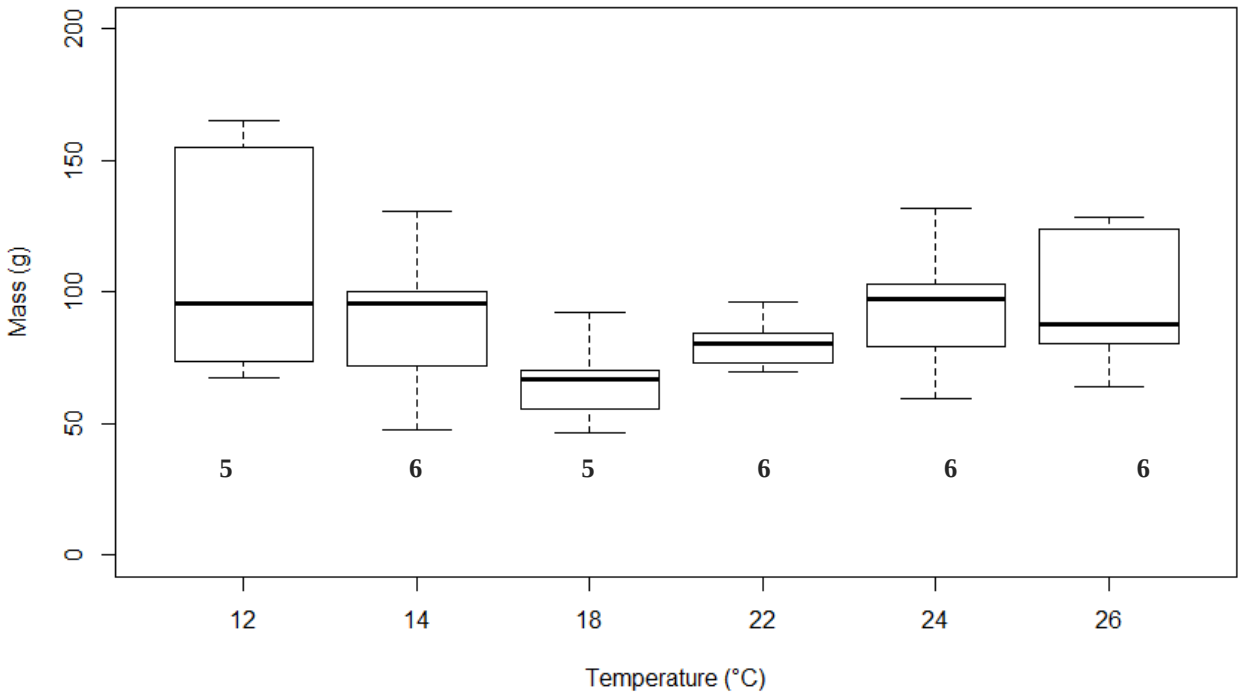

Figure 2.3 Boxplot of mass (g) of the groups of fish tested at each temperature. The dark horizontal line represents the median of the data (50th percentile), the upper and lower margins of the box indicated the interquartile range (25th and 75th percentile), and vertical dashes and whiskers are the maximum or minimum values (i.e. 1st and 3rd quartile ± 1.5 x interquartile range). Numbers below the boxes represent the n-value. There was no significant difference between each temperature (P = 0.181).

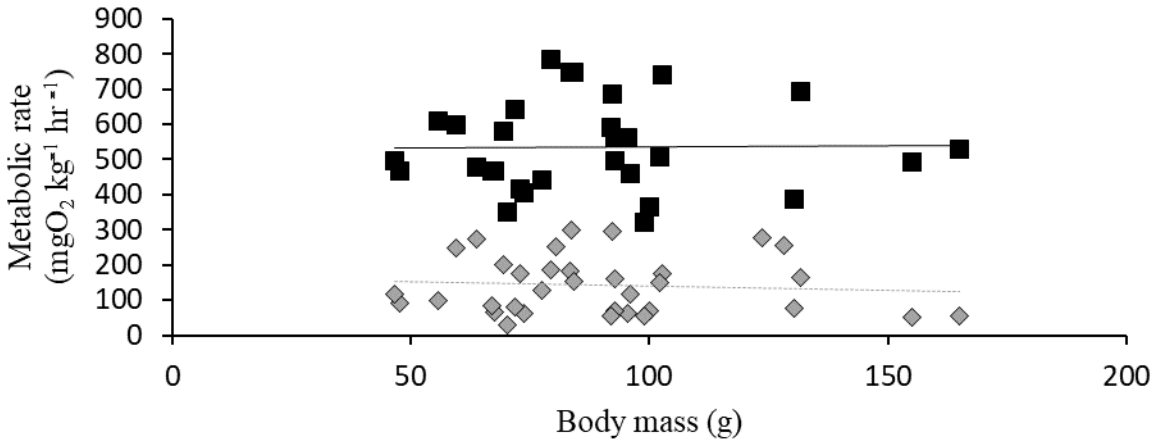

Figure 2.4 The relationship of standard metabolic rate (SMR) and maximum metabolic rate (MMR) to body mass of rainbow trout (*O. mykiss*) acclimated to 12ºC and tested at different temperatures. SMR (grey, diamond) linear regression y = -0.2263x + 162.12, R^2 = 0.0061 (P = 0.661). MMR (black, square) linear regression y = 0.00677x + 530.2, R^2 = 0.0002 (P = 0.937).

Condition factor was recorded as a reference for the state of health of the trout and was calculated as: $factor = 100 * m/L^3$, where m is body mass (g) and *L* is total body length (cm) (Ricker, 1975). The condition of the fish were similar across all temperatures, and all greater than 1, indicating healthy fish ratios between length and mass (Table 2.1).

Table 2.1 Physical measures and respirometry data from rainbow trout (*O. mykiss)* acclimated to 12ºC and tested at different temperatures. Values are means ± S.E.M. Each fish was tested at a single temperature. Condition factor (CF) is a reference of health of a fish (100 x mass/length^3), such that values over 1 indicate healthy mass to length ratios. N is sample size. SMR is standard metabolic rate ($mgO_2\ kg^{-1}\ hr^{-1}$), MMR is maximum metabolic rate ($mgO_2\ kg^{-1}\ hr^{-1}$), AAS is absolute aerobic scope which is the difference between MMR and SMR ($mgO_2\ kg^{-1}\ hr^{-1}$), and FAS is factorial aerobic scope which is MMR/SMR. * n = 6, ** n=2.

Temp. (ºC)	N	CF	Mass (g)	Length (cm)	SMR	MMR	AAS	FAS
12.2 ± 0.0244	5	1.25 ± 0.0521	111 ± 20.4	20.4 ± 10.5	58.3 ± 2.87	491 ± 26.9	433 ± 27.9	8.53 ± 0.708
14.1 ± 0.0310	6	1.18 ± 0.0706	90.3 ± 11.5	19.7 ± 1.24	75.7 ± 5.10	446 ± 47.0	371 ± 44.5	5.98 ± 0.501
18.1 ± 0.0183	5	1.3 ± 0.125	66.3 ± 7.69	17.3 ± 1.07	77.4 ± 15.7	503 ± 46.7	426 ± 41.1	7.68 ± 1.52
22.0 ± 0.0244	6	1.17 ± 0.135	80.6 ± 3.89	19.3 ± 0.761	159 ± 13.5	566 ± 62.2	407 ± 58.0	3.64 ± 0.367
24.1 ± 0.0449	6	1.24 ± 0.0598	94.7 ± 9.96	19.7 ± 0.885	182 ± 14.5	647 ± 44.5	466 ± 45.2	3.64 ± 0.295
25.8 ± 0.0386	2** - 6*	1.03 ± 0.0603	95.4 ± 10.4	21.0 ± 1.03	275 ± 7.74*	581 ± 104**	298 ± 93.3**	2.04 ± 0.291**

2.3.1 Effects of temperature on aerobic metabolism

SMR significantly increased with warming temperature (Figure 2.5), whereby fish at higher temperatures had notably higher SMR values compared to lower temperatures (Table 2.3). The temperature coefficient (Q_{10}) determine the effect of temperature on reaction rates (Alsop and Wood, 1997). SMR ranged from 1.09 to 6.30, the largest being between 18ºC and 22ºC (Table 2.2). When including the limited data from 26ºC, there was an even greater Q_{10} value of 10.0 between 24ºC and 26ºC (Table 2.2).

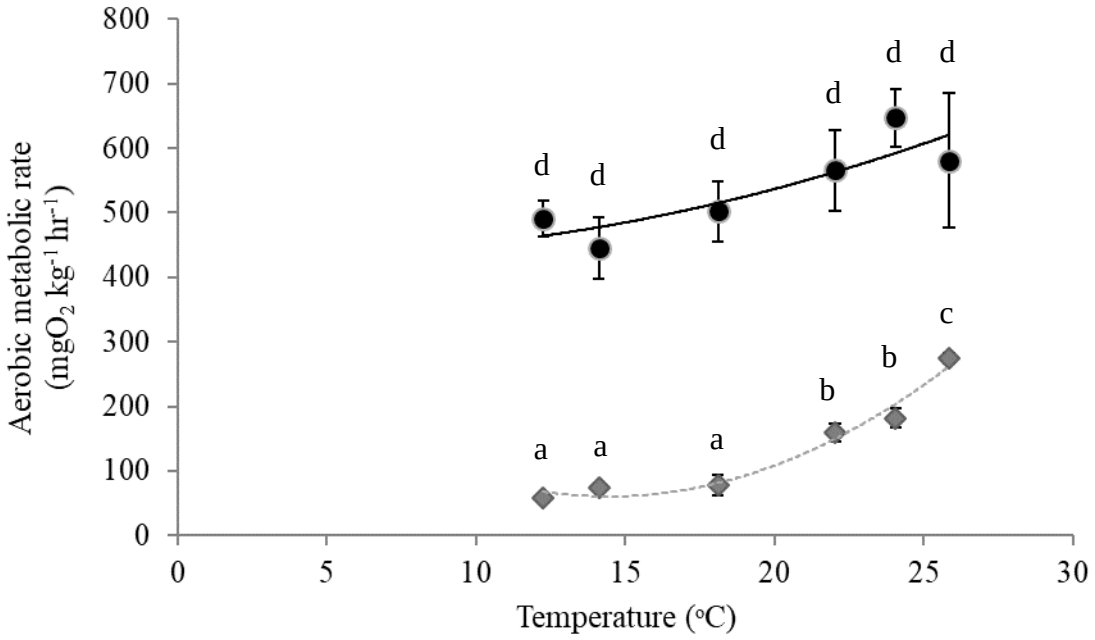

Figure 2.5 SMR (grey, triangle) and MMR (black, circle) of rainbow trout (*O. mykiss*) acclimated to 12ºC at different temperatures. Sample size at 14ºC, 22ºC and 24ºC n = 6; 12ºC and 18ºC n = 5; 26ºC SMR n = 6, MMR n = 2. SMR fitted regression $y = 1.55x^2 - 44.7x + 383$, $R^2 = 0.97$; MMR fitted regression $y = 0.370x^2 - 2.57x + 440$, $R^2 = 0.76$. Values are mean ± SEM. Different letters indicate values that are significantly different from one another ($P<0.05$).

Table 2.2 Temperature coefficients (Q_{10}) of SMR and MMR from rainbow trout (*O. mykiss*) acclimated to 12ºC, shown for each temperature interval.

	Temperature Interval						
	12 - 14ºC	14 - 18ºC	18 - 22ºC	22 - 24ºC	24 - 26ºC	12 - 24ºC	12 - 26ºC
SMR	3.76	1.09	6.30	1.93	10.00	2.62	3.13
MMR	0.60	1.35	1.35	1.95	0.55	1.26	1.13

The positive relationship between SMR and temperature was not seen in MMR (Figure 2.5), and none of the temperature groups differed significantly for MMR (one-way ANOVA, $F = 2.22$, d.f. = 5, $P = 0.0856$; Table 2.4, Figure 2.5). The Q_{10} value for MMR ranged between 0.6 and 1.95, with the greatest value between 22 and 24ºC (Table 2.2).

Table 2.3 Statistical analysis of respirometry data from rainbow trout *(O. mykiss*) acclimated to 12ºC, comparing groups across all test temperatures (12ºC to 26ºC). Groups were compared using a one-way ANOVA, Shapiro-Wilk test on residuals and Levene's test on variance. ANOVA revealed statistically significant differences ($P<0.05$), Tukey's post-hoc analysis was performed and reported in the graphs showing differences between tests with letters. Final swim speed is the speed at exhaustion. U_{VO2max} is the speed at which MMR was reached.

	ANOVA			Shapiro-Wilk Test		Levene's Test	
	F	d.f.	P	W	P	F	P
Body Mass	1.94	5	0.138	0.965	0.451	1.68	0.171
SMR	57.8	5	$6.92x10^{-14}$	0.975	0.611	1.69	0.169
MMR	2.22	5	0.0856	0.951	0.177	1.17	0.353
AAS	0.939	5	0.473	0.940	0.0929	0.614	0.690
FAS	9.38	5	$4.67\ x10^{-5}$	0.974	0.653	2.04	0.108
Final Swim Speed (cm/s)	0.343	5	0.881	0.955	0.228	2.05	0.107
U_{VO2max} (cm/s)	1.11	5	0.381	0.988	0.975	1.11	0.381

AAS did not differ between temperatures (ANOVA, F = 0.939, d.f. = 5, P = 0.473, Table 2.1; Figure 2.6). The maximum mean AAS was 466 ± 45.2 $mgO_2\ kg^{-1}\ hr^{-1}$ and was observed at 24ºC, which dropped a notable 36% between 24ºC and 26ºC, although the difference was not statistically significant. Conversely, FAS declined significantly with increasing temperature (ANOVA, F = 9.384, d.f. = 5, P = $4.67\ x10^{-5}$; Table 2.1), particularly between 12ºC compared to 22ºC and 24ºC, and between 18ºC compared to 22ºC and 24ºC (Figure 2.7). The maximum FAS was 8.53 ± 0.708 and was observed at 12ºC, declining significantly to 3.64 ± 0.295 at 24ºC, and even further to 2.04 ± 0.291 when including 26ºC.

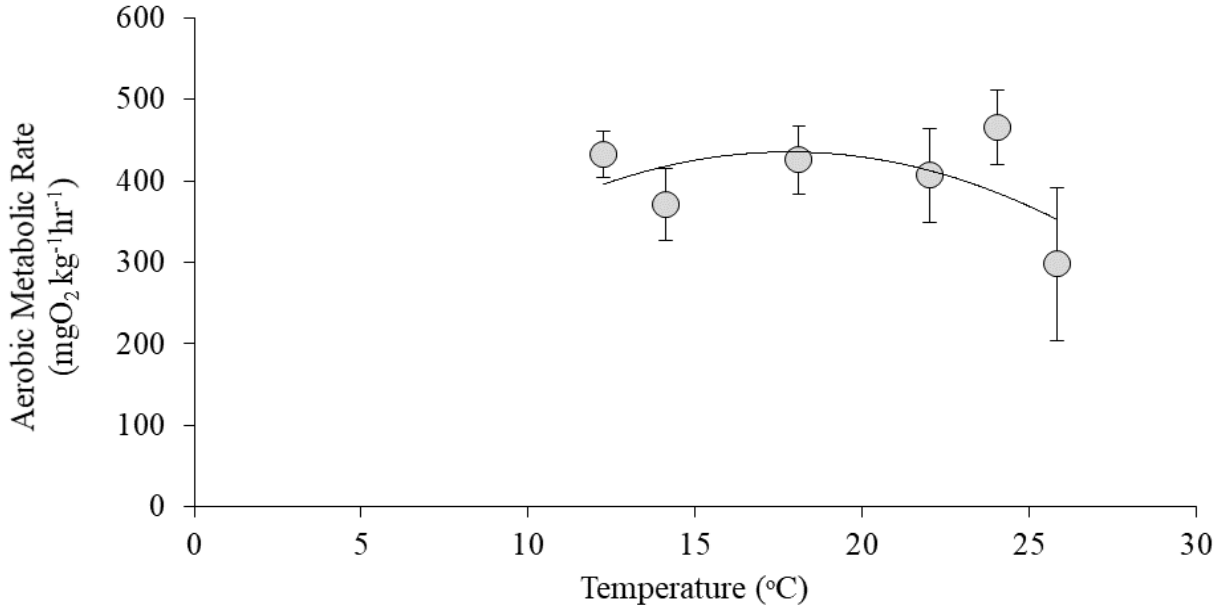

Figure 2.6 Absolute aerobic scope (MMR-SMR) of rainbow trout (*O. mykiss*) acclimated to 12ºC at different temperatures. Sample size at 14ºC, 22ºC and 24ºC n = 6; 12ºC and 18ºC n = 5; 26ºC n = 2. Fitted regression $y = -1.29x^2 + 45.9x + 26.8$, $R^2 = 0.24$. Values are mean ± SEM. Aerobic scope was not different between any of the temperatures tested (P = 0.473).

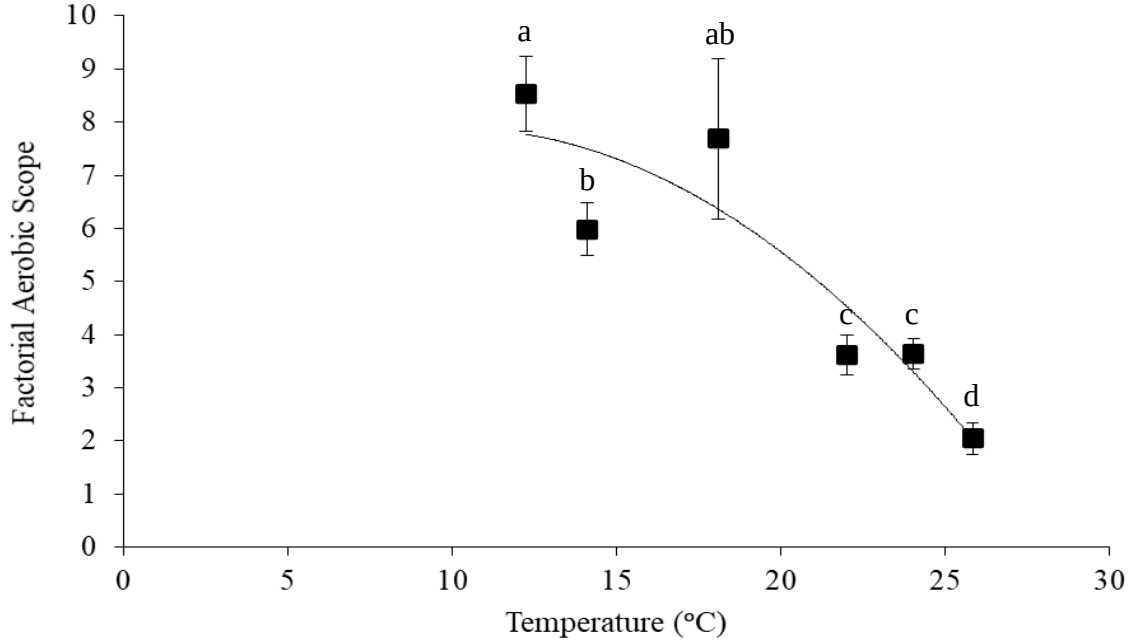

Figure 2.7 Factorial aerobic scope (MMR/SMR) of rainbow trout (*O. mykiss*) acclimated to 12ºC at different temperatures. Sample size at 14ºC, 22ºC and 24ºC n = 6; 12ºC and 18ºC n=5; 26ºC n=2. Fitted regression $y = -0.0234x^2 + 0.4694x + 5.5287$, $R^2 = 0.83$. Values are mean ± SEM. Different letters indicate values that are significantly different from one another (P<0.05).

2.3.2 Swim speed and tail beat frequency

In most of the fish, the point of exhaustion (i.e. final swim speed, approximate-U_{crit}) was reached after the speed at which $\dot{V}O_{2max}$ was obtained ($U_{\dot{V}O2max}$). $U_{\dot{V}O2max}$ was reached at approximately 75% of the final swim speed. There was no significant effect of temperature on swim speed (final swim speed, P = 0.881; $U_{\dot{V}O2max}$, P = 0.381; Table 2.4). Tail beat frequency at $\dot{V}O_{2max}$ averaged at 5.4 ± 0.5 Hz across all temperatures. There was insufficient data to assess an effect of temperature on TBF.

Table 2.4 Effect of temperature on swim speed at the point of exhaustion (Final swim speed, approximate-U_{crit}), and at the speed where MMR was measured (U_{VO2max}) which is given as the average of the three recorded maximum metabolic rates. BL is total length of the fish. Values are means ± S.E.M.

Temperature (°C)	Sample Size	U_{VO2max} (cm s^{-1})	U_{VO2max} (BL s^{-1})	Final swim speed (cm s^{-1})	Final swim speed (BL s^{-1})
12.2 ± 0.0244	5	44.1 ± 2.98	2.21 ± 0.261	67.8 ± 2.57	3.35 ± 0.184
14.1 ± 0.0310	6	57.0 ± 3.62	2.93 ± 0.204	70.0 ± 5.33	3.63 ± 0.338
18.1 ± 0.0183	5	54.8 ± 6.30	3.27 ± 0.494	72.2 ± 4.22	4.22 ± 0.319
22.0 ± 0.0439	6	48.0 ± 2.82	2.49 ± 0.131	65.2 ± 5.07	3.29 ± 0.248
24.1 ± 0.0449	6	48.7 ± 5.19	2.45 ± 0.170	69.3 ± 6.04	3.50 ± 0.195
25.8 ± 0.0386	2	53.1 ± 10.9	2.59 ± 0.277	60.7 ± 18.5	2.93 ± 0.617

2.4 Discussion

2.4.1 Effects of temperature on standard metabolic rate

There was a significant increase in SMR with increasing temperature (Figure 2.5) adding to the existing literature on SMR of salmonid species (e.g. Raby et al., 2016; Voutilainen et al., 2011; Chen et al., 2015; Verhille et al., 2016). Scott et al. (2014) studied 4 strains of 12ºC acclimated rainbow trout (3g body mass, much smaller than in the present study), and reported an average SMR of ~160 $mgO_2\ kg^{-1}\ hr^{-1}$, ~2.7 times the SMR from the present study at 12°C. Similarly, Gamperl et al. (2002) recorded higher values of SMR ($121 \pm 8\ mgO_2\ kg^{-1}\ hr^{-1}$), by about 2 fold, in wild populations of redband trout (*O. mykiss* spp.) at 12ºC and of similar mass to the fish used in the present study. These differences could be a factor of the life history of hatchery-reared rainbow trout compared to wild populations of trout, differences in fish size, feeding history, individual variability, possibly a factor of differences in strains bred in different geographical locations and climates, or simply variability due to experimental conditions and approaches to analysis (Gamperl et al., 2002; Killen et al., 2007; Norin and Malte, 2011; Sandblom et al., 2014; Farrell, 2016; Zhang et al., 2018; Zhang et al., 2019). For example, in the present study SMR was calculated as the lowest 50 values recorded (~20-25% of all values measured), whereas some have reported the average of the lowest 10%, and using the lowest 10% vs 20-25% would tend to yield a lower average value (Chabot et al., 2016). The method of reporting SMR has yet to be standardized within the literature (reviewed by Chabot et al., 2016).

2.4.2 Effects of temperature on maximum metabolic rate

The maximum recorded $\dot{V}O_2$ in other studies of rainbow trout ranged from 350 to 730 $mgO_2\ kg^{-1}\ hr^{-1}$ (Gamperl et al., 2002; Kieffer et al., 1998), which encompasses the range of MMR

measured in this study (491 to 647 $mgO_2\ kg^{-1}\ hr^{-1}$). While not statistically significant, there was a trend of MMR to increase with increasing temperatures between 14ºC and 24ºC, and perhaps decline at 26ºC, although there were only 2 MMR values measured at 26ºC. A decline in MMR at 26ºC might be anticipated given that this temperature is nearing the CT_{max} of rainbow trout (Beitinger et al., 2000; Chen et al., 2015; Scott et al., 2014). Further, a trend of increasing MMR with increasing temperatures and an eventual plateau or decline approaching CT_{max} has been seen in other species of fish (Ferreira et al., 2014; Tirsgaard et al., 2015; Zhang and Kieffer, 2017). For example, MMR of Pacific pink salmon (*Oncorhynchus gorbuscha*) increased gradually until a plateau at ~21ºC - 28ºC, the maximum tested temperature (Clark et al., 2011). However, it is becoming clear that not all fish show the same MMR response with temperature (reviewed by Norin and Clark, 2016). Verhille et al. (2016) reported a linear increase from 13ºC to 25ºC in a wild population of *O. mykiss*, and Norin et al. (2014) saw that in tropical, eurythermal fish (i.e. juvenile barramundi, *Lates calcarifer*), MMR continued to increase as temperatures approached their thermal limit, and to a greater extent than the increase in SMR, which resulted in a continuously increasing aerobic scope despite death at high temperatures. Also, MMR's relationship with temperature can vary between similar species with different habitats (Seth et al., 2013) or within a species based on size (Tirsgaard et al., 2015). Therefore, the effects of temperature on MMR appear highly variable between individuals and species.

Differences in experimental protocols used in different studies to measure aerobic scope could have a distinct impact on the results, and likely explains some of the variation between studies, even on the same species. For example, oxygen consumption is the sum of all internal metabolic demands supported by aerobic metabolism, and certain organs that have priority for oxygen supply and thus consumption while swimming (i.e. heart and red muscle) may consume

oxygen at the expense of other organs, delaying their aerobic costs with immediate anaerobic compensation (reviewed by Chabot et al., 2016). Therefore, studies employing shorter durations of swimming might not fully account for the whole aerobic cost of swimming for all tissues. Also, the variation seen in MMR within, and possibly between, species could also be attributed to differences among individuals and their motivation to swim under the experimental conditions. It is important to understand this variation, because regulatory policy regarding local habitat can be informed by the results of studies of aerobic scope, but such studies need to reliably capture accurate measures that consider reliable accounts of natural variation (discussed by Eliason and Farrell, 2016; Verhille et al., 2016), and a broad and thorough understanding of variation among strains, populations, species, individuals, life-stages, and thermal plasticity is important in order to cover all potential variables affecting survival of wild populations.

2.4.3 Effects of temperature on aerobic scope

Absolute aerobic scope, the difference between SMR and MMR, is the amount of aerobic energy available for processes beyond cellular maintenance, and has been used to make predictions about the animal's ability to perform in certain temperatures (Fry, 1947; reviewed by Farrell, 2016). It was expected that AAS would increase with temperature until an optimal temperature, and then decrease with further increase in temperature, particularly as temperature approached CT_{max}, as has been seen in some other studies (Chen et al., 2015; Farrell, 2009). However, this trend was not clearly identified from my results (Figure 2.6), instead showing no significant effect of temperature on AAS. The lack of an effect of temperature on AAS has been observed in some other studies of salmonids (Poletto et al., 2017; Verhille et al., 2016), potentially suggesting the increased mortality rate at high temperatures is likely not a result of

reduced aerobic scope, at least for rainbow trout. Heat shock proteins, oxidative stress, reactive oxygen species and/or other protective responses to heat could be attributing to death of an organism at high temperatures, however this needs further investigation.

AAS is dependent on the combined effects of temperature on both SMR and MMR. SMR increased by 3.1-fold from 12ºC to 24ºC in the present study, and by 2.5-fold from 13ºC to 25ºC in a study on rainbow trout by Verhille et al. (2016). Therefore, an increase in SMR with temperature appears consistent. But MMR appears to be more variable in response to temperature increases, resulting in differences in the effects of temperature on AAS between studies of the same species, making the effects of temperature on the availability of aerobic energy difficult to interpret (e.g. Verhille et al., 2016). For example, at 26ºC, only ~2ºC lower than the predicted CT_{max} of rainbow trout (~28ºC; Scott et al., 2014), MMR was still 2.1 times greater than SMR, resulting in a substantial AAS of ~300 $mgO_2\ kg^{-1}\ hr^{-1}$, still about 2/3 of the maximum AAS observed (Table 2.1). And a long-term acclimation study of thermal changes on Atlantic halibut showed that AAS continually increased with increasing temperature, never reaching an optimal temperature or declining prior to the fish reaching their thermal limit (Gräns et al., 2014). This again suggests that aerobic scope is not the primary determinant of thermal tolerance, but rather thermal tolerance is potentially limited by the increase in SMR to some unsustainably high rate. While the present study does not strongly support the notion that CT_{max} is reached at the point where aerobic scope approaches zero, it is still possible that at temperatures beyond 24ºC MMR would rapidly decline (as discussed by Chen et al., 2015), and the present study may not have encapsulated that effect, in part due to the limited data at 26ºC. Halsey et al. (2018) note this common theme in respirometry studies, where fish do not tolerate

warm temperatures making measures of MMR difficult and therefore data is limited at warm temperatures.

There is mounting evidence that aerobic scope is highly variable depending on the species, acclimation temperature of the individuals tested, wild vs. hatchery-reared populations, and age of the fish, rarely ever exhibiting the bell curve shape that would suggest aerobic scope limits CT_{max} (Eliason and Farrell, 2016; Farrell, 2016; Farrell et al., 2008). Current theories are trying to identify other physiological causes of death at high temperatures, if not due to a reduced AAS or other limits to oxygen availability (Brijs et al., 2015; Ekström et al., 2016). Reduced cardiac performance has been proposed as a contender for the cause of death at CT_{max}, resulting in a limited heart rate and cardiac pumping capacity and appears to be reduced at high temperatures for many fish (Farrell et al., 2008; Eliason et al., 2013). Cardiac arrhythmia, an irregularity in heart rate which ensues only a few degrees earlier (~2ºC) than thermal limits, has been observed in several species of salmonids (Anttila et al., 2013; Chen et al., 2015; Clark et al., 2008; Farrell, 2009; Ferreira et al., 2014; Steinhausen et al., 2008; Verhille et al., 2013), and has been seen to occur in *O. mykiss* that were incrementally warmed to ~25ºC (Chen et al., 2015). That being said, cardiac failure is not necessarily a result of limited oxygen supply either, or there may be some other physiological effect of temperature on cardiac performance (Ekström et al., 2016). There is also some evidence that temperature negatively effects mitochondrial metabolism in Atlantic cod at temperatures approaching their CT_{max} (Rodnick et al., 2014). Therefore, the effect of temperature on ectotherm survival may not be clearly described by limited AAS.

Factorial aerobic scope is a metric used to identify how much an animal can increase their metabolic activity relative to their SMR (reviewed by Halsey et al., 2018). The maximum FAS

recorded in the present study was 8.5 (at 12ºC) (Figure 2.7). This value is about 2.5 - fold higher than the FAS at 13ºC (3.32 ± 0.41) of a California strain of *O. mykiss* (10.5 - 79.6 g body mass) (Verhille et al., 2016), and 1.5-fold higher than the predicted FAS at 12ºC (~5.4) for southern Australian *O. mykiss* (33.6 ± 0.4 g body mass) (Chen et al., 2015), but similar to the maximum FAS, 8.5, of adult male pink salmon (*O. gorbuscha*) at 11ºC (Clark et al., 2011).

In the present study, FAS declined with increasing temperature and was 3.6 at 24ºC and 2.0 at 26ºC. A reduced FAS at higher temperatures suggests that there is a reduced capacity for metabolic work, which has been seen in many species, including Pacific salmon and shortnose sturgeon (reviewed by Deslauriers and Kieffer, 2012; Eliason and Farrell, 2016; Killen et al., 2007; Zhang and Kieffer, 2017). FAS also decreased with increase temperature in the study by Chen et al. (2015), remaining at 1.4 – 1.8 at the highest temperature tested (25ºC), which is slightly lower than in the present study. These relatively higher FAS values, compared to other studies, are potential indicators that this strain of rainbow trout was either less aerobically demanding for SMR at high temperatures, or had higher metabolic capacities in an active state. In support of the former, the predicted SMR for the southern Australia population of *O. mykiss* at 24ºC is 2.2 times greater than the recorded SMR from the present study (Chen et al., 2015). It is noteworthy that fish living in different thermal habitats can share similar FAS values, making FAS appear largely independent of acclimation temperature and not supporting the notion that colder habitats might result in greater scope of activity (Johnston et al., 1991; Lowe and Davison, 2006). However, the implications are difficult to interpret, as they often involve comparisons between-species, with the potential caveat of notable differences in performance (Pörtner, 2002) and other differences between species.

There are some criticisms of presenting aerobic scope as FAS, including that FAS can be very sensitive to changes in SMR, where FAS can appear to decline linearly with temperature but AAS remains stable or bell-curved (reviewed by Halsey et al., 2018; Pörtner, 2002). Also, a large FAS as a result of a very small SMR does not necessarily imply a large amount of energy is available above SMR. Further, the observed FAS in the present study was ~2 at high temperatures (26ºC), which indicates that MMR is still double SMR at temperatures approaching CT_{max}. MMR being double that of SMR might imply that there is still significant aerobic scope available for activity beyond SMR, but fish mortality still occurred at this temperature without the fish even being pushed to swim. Thus, a high FAS does not necessarily indicate the fish is in a state of good health. This again suggests that CT_{max} can occur despite available aerobic scope, whether measured as FAS or AAS, and thus aerobic scope approaching zero is not limiting survival at high temperatures.

2.4.4 Thermal preference and CT_{max}

Optimal growth temperature for rainbow trout is 13.1ºC (McMahon et al., 2008) so there should not have been any growth limitations on these trout as a result of environmental constraints. This was confirmed by the condition factor of these fish (Table 2.3), which were considered healthy and comparable to other studies on rainbow trout (Gamperl et al., 2002; Kieffer et al., 1998).

The acclimation temperature of 12ºC used in the current study is at the lower end of the preferred temperature range of rainbow trout (Calow, 1985; Garside and Tait, 1959; McCauley et al., 1977; McMahon et al., 2008; Pörtner, 2002; Pörtner and Knust, 2007; Schurmann et al., 1991). If the thermal preference is associated with maximal aerobic scope, this might explain

why we did not see much change in AAS over at least the lower range of temperatures tested (Figure 2.6). Likewise, Verhille et al. (2016) showed that rainbow trout, along with several other teleosts, have AAS that are within 95% of their maximum even at temperatures approaching CT_{max}, suggesting that the thermal optimum can be relatively broad for certain species (Lefevre, 2016). However, a direct comparison of thermal preference and the effects of temperature on aerobic scope from multiple populations of *O. mykiss* has yet to be examined. In a geographical context, some rainbow trout that occupy cooler or warmer climates vary slightly in the relationship between temperature and aerobic scope, as well as their thermal preference ranges (Scott et al., 2015; Chen et al., 2015; reviewed by Lefevre, 2016). It is possible that a shift in acclimation temperature can change AAS presumably because some fish, given enough time, will shift their basal metabolic demands to minimize the energy costs when exposed to a new but constant temperature (reviewed by Lefevre, 2016). However, this is not the case in all fish, as Trinidadian guppies have shown limited capacity for acclimation, suggesting that certain fish may be more physiologically capable of acclimating to temperature changes than others (Muñoz et al., 2012). Therefore, differences in habituated temperatures between strains of the same species might have an effect on fitness in extreme temperatures (Chen et al., 2015), and this may result in variability between populations in aerobic scope and the response of aerobic scope to temperature.

Q_{10} values are used to determine the effect of temperature on reaction rates (Alsop and Wood, 1997), and in this case, Q_{10} values were used to determine the effect of acute thermal changes on the rate of aerobic metabolism. A Q_{10} value of 1 would indicate that there is perfect thermal compensation of metabolic rate between the temperatures tested (i.e. no effect of temperature). The Q_{10} values of SMR were about double those for MMR between 12ºC and 24ºC

(Table 2.2). Similarly, the highest Q_{10} in groups of *O. mykiss* studied by Chen et al. (2015) was 3.1 between 8ºC and 25ºC, very similar to the present study. This suggests that the effect of temperature is primarily on SMR of *O. mykiss*, as previously discussed. The Q_{10} of SMR is generally between 1 and 3 in fish species, with no significant difference between acute and acclimated temperature changes (reviewed by Lefevre, 2016). However, acclimation was seen to change the Q_{10} coefficient of SMR in shorthorn sculpin (*M. scorpius*) (Sandblom et al., 2014). Of note, considerably different values of Q_{10} were obtained between each pair of testing temperatures, which also supports observations that aerobic scope is not constant across temperature (Farrell, 2016) (Table 2.2). In fact, a substantial increase in Q_{10} between 24ºC and 26ºC was also observed by Rodnick et al. (2004), showing that there can be greater effects of temperature between some temperature ranges over others.

2.4.5 Swimming velocity at MMR and tail beat frequency

The final swim speed attained by the fish in this study, about 2.7 BLs^{-1} (Table 2.4), which is likely slightly higher than U_{crit}, but remains consistent with existing literature of similarly sized fish (e.g. Gamperl et al., 2002). The final swim speed was more than twice the U_{crit} of larger trout under similar experimental conditions, but less than half of smaller strains of trout (Table 2.5). There was no significant effect of temperature on final swim speed, which is consistent with Gamperl et al. (2002), however, some other studies on salmonid species have noted that U_{crit} was maximized at temperatures below 18ºC and declined above 23ºC (Brett, 1964; Gamperl et al., 2002; Taylor et al., 1996). It is somewhat difficult to compare these results between studies due to differences in protocols and swim speed calculations, and the final swim speed attained by the trout in the present study is not necessarily equal to U_{crit}, but the present study and existing

literature implicate a potential effect of size on swim speed, whereby larger trout exhaust at relatively slower swim speeds than juveniles (Bainbridge, 1958; Brett, 1964b; Webb et al., 1984; Table 2.5).

Table 2.5 Results from literature that examines swim speed and metabolic rates for rainbow trout (*O. mykiss*). Data presented as ranges and as means ± SEM, as available. CF is condition factor. T_{accl} is acclimation temperature and in parenthesis are the test temperature, no parenthesis indicate test temperature is equal to acclimation temperature. * is final swim speed.

N	T_{accl} (°C)	Mass (g)	Length (cm)	CF	SMR	MMR	U_{crit} (BLs^{-1})	Location	Reference
9	12 (13.9)	4.16 ± 0.16	7.38 ± 0.10	1.0	-	-	7.5 – 10.5	Raven Brood Trout Station, Caroline, AB, Canada	Ralph et al. 2012
14	12 (13.9)	10.4 ± 0.43	9.97 ± 0.16	1.1	-	-	5 – 6.5	Allison Creek Brood Trout Hatchery, Crowsnest Pass, AB, Canada	Ralph et al. 2012
6-7	15 ± 1	10 – 20	9 – 12	-	~64	~320	4.52 ± 0.18	Rainbow Springs Hatchery, Thamesford, ON, Canada	Alsop and Wood 1997
8-10	12	-	10 – 14.7	-	-	136 – 200	4.5–7	Fraser Valley Hatchery, Abbotsford, Canada	Scott et al., 2014
15	8–10	13.2 ± 1.17	10.9 ± 0.35	1.18 ± 0.02	-	-	6.05 ± 0.2	-	Jones 1971
14	21-23	21 ± 1.6	12.5 ± 0.35	1.08 ± 0.02	-	-	6.34 ± 0.22	-	Jones 1971
44	12.5–13.6	10.5 – 79.6	12.6 ± 2.9	-	130.8 ± 27	397.2 ± 61.8	-	Tuolumne River, California, USA	Verhille et al. 2016
44	12.5–13.6	10.5 – 79.6	12.6 ± 2.9	-	322.2 ± 24.6	673.2 ± 51.6	-	Tuolumne River, California, USA	Verhille et al. 2016
8	24	50.4 ± 2.9	16.7 ± 0.4	-	197.6	756.8	3.55 ± 0.19	Rock Creek, Oregon, USA	Rodnick et al., 2004
9	24	56.5 ± 3.8	17.9 ± 0.4	-	201.2	775.6	3.41 ± 0.17	12-mile Creek, Oregon, USA	Rodnick et al., 2004
9	12–14	58 ± 6	17.6 ± 0.5	1.03 ± 0.02	121 ± 8	572 ± 45	3.5 ± 0.1	Little Blitzen River, Oregon, USA	Gamperl et al., 2002
7	24	62.9 ± 5.6	18.5 ± 0.5	-	200.5	6.72	3.88 ± 0.23	Bridge Creek, Oregon, USA	Rodnick et al., 2004
9	12–14 (24)	71 ± 5	18.8 ± 0.4	1.05 ± 0.02	304 ± 28	937 ± 62	3.2 ± 0.2	Little Blitzen River, Oregon, USA	Gamperl et al., 2002
8	12–14	92 ± 11	20.3 ± 0.9	1.07 ± 0.02	165 ± 12	827 ± 52	3.1 ± 0.2	Bridge Creek, Oregon, USA	Gamperl et al., 2002
7	12–14	108 ± 12	21.4 ± 0.9	1.07 ± 0.02	383 ± 38	960 ± 42	3.5 ± 0.2	Bridge Creek, Oregon, USA	Gamperl et al., 2002
5	**12–13**	**111 ± 20.4**	**20.4 ± 1.05**	**1.25 ± 0.05**	**58.3 ± 2.87**	**491 ± 26.9**	***3.35 ± 0.184**	**Allison Creek Brood Trout Hatchery, Crowsnest Pass, Canada**	**Present Study**
8	7	493 ± 17	76.7 ± 1.8	-	-	210.3	2.13 ± 0.05	West Creek Trout Farm, Abbotsford, B.C., Canada	Burgetz et al. 1998
27	5.5–7.0	437 ± 12	-	-	-	-	1.05 ± 0.03	Simon Fraser University, BC, Canada	Farrell, 2008
11	11–13	446 ± 12	-	-	-	-	1.05 ± 0.02	Simon Fraser University, BC, Canada	Farrell, 2008
5	5.5–8	650 ± 6	38.9 ± 0.5	-	-	-	1.64 ± 0.07	-	Jain et al., 1997
7	24	662.6 ± 74.7	39.6 ± 1.6	-	212.2	689.4	3.21 ± 0.23	Bridge Creek, Oregon, USA	Rodnick et al., 2004
4-9	9–10.5	900 – 1500	40 – 53	-	~48	~370	~2	-	Kiceniuk and Jones, 1977

Notably, a different strain of *O. mykiss* ssp. (redband trout), of similar size to the fish used in the present study, had nearly the exact same U_{crit} as the final swim speed of the fish in the present study, at both 12–14ºC and 24ºC, despite having nearly double the SMR and MMR as the fish used in the present study at both temperatures (Gamperl et al., 2002). It is possible that there is some effect of wild vs. hatchery-reared habitats on metabolism (Gamperl et al., 2002); potentially even a genetic shift in response to thermal adaptations that shifts the metabolic rate, such that warmer geographically sourced trout could have a higher CT_{max} and greater metabolic rates, but reduced aerobic scope (Gamperl et al., 2002; Rodnick et al., 2014; Chen et al., 2015). The advantage or disadvantages of these trade-offs need to be examined further.

We also examined the tail-beat frequency (TBF) of several of the fish to determine the TBF in relation to the swim speed at $\dot{V}O_{2max}$. From the limited available data, we determined a TBF of 5.4 Hz when the fish were swimming at a rate that invoked $\dot{V}O_{2max}$ (2.7 BLs^{-1}). This is similar to the value estimated from other studies on rainbow trout (about 200 g body mass), which would predict a TBF of 4.7 Hz at 2.7 BLs^{-1} (Webb, 1971). TBF will impact the power available from the muscle for swimming, which will become important when examining the effects of temperature on muscle power and metabolic rate (Chapter 4).

2.5 Conclusion

Many studies have explored the effects of temperature on metabolic rate, including aerobic scope, in rainbow trout, as well as the effects of other abiotic factors on metabolism (Carline and Machung, 2001; Chen et al., 2015; Gamperl et al., 2002; Kieffer et al., 1998; Myrick and Cech, 2000; Scarabello et al., 1992; Scott et al., 2014; Verhille et al., 2016; Wieser, 1985). These studies collectively provide evidence that temperature will affect the metabolic rates of fish, but that temperature most significantly alters SMR, as compared with effects on MMR and aerobic scope. In regards to the relationship between metabolic rate and CT_{max}, there was a marked elevation in SMR as temperature approached CT_{max}, some evidence of a fall in MMR, little evidence of changes in AAS, but a considerable fall in FAS, although the limited data at temperatures close to CT_{max} makes this difficult to assess. The present study suggests that rainbow trout might be reaching CT_{max} with still ~200 $mgO_2\ kg^{-1}\ hr^{-1}$ AAS, supporting the idea that there is likely some other physiological effects of temperature beyond the aerobic scope limiting survival at warm temperatures. Thus, it remains equivocal if aerobic scope is a predictor of CT_{max}, whereas the large increase in SMR may be more predictive of an energetic challenge at warm temperatures.

Additionally, swim speed at $\dot{V}O_{2max}$ was ~75% of approximated-U_{crit} in this study. If U_{crit} represents the maximal aerobic swim speed, but $\dot{V}O_{2max}$ is attained at substantially slower swim speeds, this possibly reveals that either aerobic red muscle relies on low but sustainable levels of anaerobic metabolism at high sustained swim speeds, or that white muscle may be recruited in a sustainable manner along with red muscle during swim speeds approaching U_{crit}. Further studies on the aerobic capacity of both muscle types might help shed light on the aerobic requirements of swimming fish.

The results of this study also add to the collective understanding that increased temperatures likely result in a markedly enhanced metabolic rate at a resting/maintenance level, impressing the importance of environmental temperature on the physiology of trout. Yet how this elevated metabolism contributes to metabolic budgets is not clear. There is continued and important debate within the literature regarding the value of using aerobic scope as a predictor of the impact of temperature changes on ectotherm metabolism (Farrell, 2016; Norin and Clark, 2016; Norin et al., 2014; Overgaard et al., 2012). While this study will not resolve this debate, it is the intention of this study to try adding to the existing physiological examinations attempting to identify if and why aerobic scope narrows as temperature approaches thermal limits, to distinguish, for the first time, the aerobic scope of skeletal muscles, and how they might contribute to the aerobic scope and energy budgets of fish as temperatures increase. This will inform future discussions surrounding the effects of temperature on metabolic rate and how it may limit energy availability in living organisms.

Chapter Three: **Aerobic Metabolism of Skeletal Muscle**

3.1 Introduction

Body caudal-fin (BCF) locomotion in fish describes the use of lateral undulations of the body to power forward locomotion. The primary contributor of power comes from skeletal muscle, either red, aerobic muscle, or white, anaerobic muscle, depending on the speed and power required for a given task (e.g. Altringham et al., 1993; Burgetz et al., 1998; Coughlin, 2000). In most ectothermic BCF fish, there is a superficial layer of aerobic red skeletal muscle along the lateral line of the body, and the remainder of the muscle tissue that exists more medially is anaerobic white muscle. White muscle in fish is used for quick bursts of swimming, while red muscle is used for routine, sustained swimming at speeds up to ~70-80% of the maximal sustained swimming speeds, and therefore is suspected to be the most metabolically demanding muscle type in salmonids (West et al., 1993; Coughlin and Rome, 1996). During routine swimming, the majority of blood is directed to the red muscle (Gerry and Ellerby, 2014), despite red muscle being only a small portion of the whole fish (reviewed by Bernal et al., 2001; Ellerby et al., 2000). White muscle appears to make only a limited contribution at sustained swim speeds, likely only being employed at speeds approaching U_{crit} and during burst swimming in rainbow trout (Jayne and Lauder, 1994). Therefore, if red muscle were the primary muscle used for sustained locomotion then it would be expected that red muscle is also a major consumer of aerobically produced energy, however, direct measures of aerobic metabolic rate of skeletal muscle in fish have not been made.

As was seen in the previous chapter, temperature can have a substantial impact on the metabolic rate of a swimming fish, increasing the resting metabolism significantly with increasing temperature. Due to the poikilothermic nature of rainbow trout, it is likely that muscle

metabolism would also be affected by temperature. In fact, temperature is known to play a key role in impacting the metabolic rate of muscle mitochondria, which supports the notion that the metabolic rate of aerobic muscle would be affected by temperature (Guderley, 2004). Muscle power output drives locomotion and also appears to be affected by temperature (reviewed by Syme, 2005; e.g. Rome et al., 2000; Seebacher and James, 2008). Therefore, understanding the effects of temperature on muscle power and aerobic metabolic rate will help understand the effects of these parameters on fish.

In order to measure the contractile abilities of isolated segments of muscle, mimicking how they would function in a moving animal, such as a swimming fish, Josephson (1985) devised the work-loop technique. This technique provides the opportunity to study the muscles in vitro, isolated from the animal, and determine the maximum work and power they are capable of producing (e.g. Barclay, 1994; Coughlin, 2000; Harwood et al., 2002; Syme, 1994). In teleosts, red, white and cardiac muscle have all been examined to determine their work output (e.g. (Coughlin and Rome, 1996; Hammond et al., 1998; Harwood et al., 1998; Rome et al., 1999). Many studies of muscle performance in fish examine the effect of temperature on skeletal muscles as well (see Syme, 2005 for review), and some have even examined the effects of temperature on power output of red skeletal muscle in rainbow trout specifically (e.g. Shuman and Coughlin, 2018). However, none of these studies have measured the aerobic capacity of fish skeletal muscle. Thus, it is not currently known what effect temperature has on the aerobic scope of skeletal muscle, as only estimates of muscle metabolic costs have been made. As a result, the work-loop technique could be applied in conjunction with measures of oxygen consumption to elicit maximal oxygen consumption (i.e. MMR) of working muscle tissue, as well as SMR in resting tissue. These measures, made at different temperatures, can then be compared with

measures of aerobic metabolism and aerobic scope in fish, to better understand how muscle contributes to metabolism of the whole fish.

As previously mentioned, oxygen consumption and thus aerobic metabolic rate can be used as a proxy for ATP utilization and thus aerobic metabolism. Measures of the resting rate of oxygen consumption in fish skeletal muscle has been conducted before, both as a resting rate of muscle tissue in tuna (Gordon, 1968) and using the oxidative capacity of muscle mitochondria (e.g. Bouchard and Guderley, 2003). Rates of oxygen consumption have also been recorded while muscles are working, to identify the oxidative demand of working muscle, which would reflect maximal aerobic rates under appropriate circumstances. However, such measures have primarily been conducted on species other than fishes, or in cardiac muscle (e.g. Harwood et al., 2002; Josephson and Stevenson, 1991; Seebacher et al., 2014; Syme, 1994), and the effects of temperature on the maximal metabolic rate of skeletal muscle in fishes has not been investigated. Simultaneous measures of oxygen consumption and work output also allows us to estimate the mechanical efficiency of the muscle. Mechanical efficiency is a measure of the mechanical power output ($\dot{E}p$; the rate of mechanical work done by the muscle) relative to the rate of metabolic energy utilization ($\dot{E}O_2$; the rate of energy used by the muscle, estimated from oxygen consumption). By this definition, the mechanical efficiency of teleost red skeletal muscle has been estimated to be ~20%, calculated based on measures of work and heat output from other studies of fish (calculated by Syme, 1994, from Curtin and Woledge, 1993; Smit et al., 1971), but has not previously been measured directly from oxygen consumption in fish muscle.

A similar approach to measure oxygen consumption of working muscle as used by Syme (1994) and Trinh and Syme (2007) was used in the present study to measure, we believe for the first time, the metabolic energy expenditure of resting and working red skeletal muscle in

juvenile rainbow trout. Muscle aerobic scope at various temperatures was then calculated using the resting rates of oxygen consumption and the maximal rates of oxygen consumed by the muscle while performing work. White, anaerobic skeletal muscle was also measured under resting conditions to identify the potential oxidative contribution of white muscle to resting metabolic rates in fishes. Based on my hypothesis that red skeletal muscle will dominate energy use in fish and thus aerobic scope, it was expected that temperature changes would impact the resting and maximal rates of oxygen consumption in the muscle similar to the effects seen in whole fish aerobic scope tests.

3.2 Methods

3.2.1 Animal care

All procurement, care and handling of animals was approved by the University of Calgary animal care committee following guidelines of the Canadian Council on Animal Care. Rainbow trout (BEBE 2n strain) were obtained from the Raven Station Hatchery, Clearwater County, Alberta, Canada, in June 2019 (~ 2 g body mass), and were kept at temperatures of 12 ± 1°C for several months prior to experiments until grown to testing size (range 61.4 - 107.8 g body mass, average 78.1 ± 1.7 g). All fish were fed to satiation daily on commercial trout pellets (EWOS Food Supply, micro 1.2mm and 2mm, Bergen, Hordaland, Norway) and kept on a 14:10h light:dark photoperiod.

3.2.2 Muscle preparation

Fish were euthanized by a blow to the head and pithing of the brain and spinal cord rather than chemical euthanasia to prevent any effects of chemical euthanasia on muscle excitability (Roberts and Syme, 2016). Immediately following euthanasia, a rectangular section of muscle approximately 2 cm in length and 0.5 cm in depth was removed from the region of the lateral line at ~45% of the fish body length (proximal to lateral line from the anterior edge of the dorsal fin) and placed into a dissection petri dish maintained at 4ºC, with the skin side down. The preparation was pinned in place in all four corners to maintain tension on the muscle. The petri dish was filled with fresh saline solution (in mM: NaCl 132; KCl 2.6; $CaCl_2$ 2.7; $MgSO_4$ 1.0; NaH_2PO_4 1.0; glucose 10; HEPES buffer 10, salinity: 12.874 g/L: pH adjusted to 7.8 with 6 M NaOH) (adapted from Altringham and Johnston, 1990). Prior to use, saline solution was filtered through a Nalgene Rapid Flow Sterile Disposable Bottle with PES Membrane (0.2 micron pore

size; Thermo Fisher Scientific, Waltham, Massachusetts, USA) to reduce the possibility of bacteria being an alternate source of oxygen consumption, and bubbled with pure oxygen.

Red muscle preparations were carefully cut away from white muscle and then separated from the skin. The preparation was then trimmed to a length of 2 – 4 myomeres, averaging 7.28 ± 0.14 mm in length (range 5.5 – 9.5 mm) and 29.3 ±1.88 mg mass. A 60V stimulus pulse, 1ms duration (Grass S44 Stimulator, Grass Instruments, West Warwick, Rhode Island, USA) was occasionally applied to the muscle during dissection to help maintain excitability/viability of the preparation.

Silk sutures (5–0 gauge) were tied around short segments of myosepta that extended from either end of the muscle sample. The pins holding the muscle in place were removed and the muscle preparation was then placed into a glass chamber used for measurements of work and oxygen consumption, described below. The sutures affixed to each end of the preparation were tied onto the hooked ends of silver wires that passed through glass capillary tubes extending through the ends of the glass chamber, and the opposite ends of these wires were attached to the arm of a servomotor (model 300C-LR Dual-Mode Lever System, Aurora Scientific, Aurora, ON, Canada) that controlled muscle length, and a force transducer (5g model 400a, Aurora Scientific, Aurora, ON, Canada) that measured muscle force, used for measures of power output discussed later.

In addition to red muscle, white, anaerobic muscle was removed ventral to the location of red muscle excision, in the hypaxial region. White muscle (68.9 ± 1.62 mg mass; 10.4 ±0.139mm muscle length) was prepared in the same way as described for red muscles and used to measure the resting aerobic metabolism of white muscle. White muscle in rainbow trout is not active

during sustained swimming, therefore the working measures of white muscle were not relevant to this study and only SMR of white muscle was necessary to consider.

3.2.3 The experimental chamber

The chamber used for measuring work and oxygen consumption in the muscle was laser milled into a 4 x 7.6 x 1.5 cm glass block by the University of Calgary Science Glass Workshop (Figure 3.1). The muscle preparations were placed into a 4.7 x 32.1 x 5 mm well cut into the bottom/centre of the glass chamber. This well was filled with filtered, oxygenated saline as described above. Three circular depressions were cut into the bottom of the well (~1 mm depth and 2.2 mm diameter) to house three glass-encapsulated magnetic stir bars (2 mm length). The stir bars mixed the saline in the chamber and were powered by a magnetic stir plate (Cimarec model S130815, Barnstead International, Dubuque, IA, USA) that sat beneath the glass chamber. Each ends of the well that contained the muscle preparation had 1.07 mm diameter holes milled into them, just large enough for smooth passage of the silver wires and glass capillary tubes (1.05 mm diameter) connecting the muscle within the chamber to the servomotor and force transducer outside of the chamber, while also limiting the possibility of atmospheric air moving into the chamber during the experiment. The end of the glass capillary tubes inside of the chamber were enclosed with sealant to prevent movement of air or saline into the capillary tubes. The volume of the well that housed the muscle preparation was 0.797 mL, accounting for the volume displaced by the glass capillary tubes within the chamber, the wells that housed the magnetic stir bars, and the stir bars themselves.

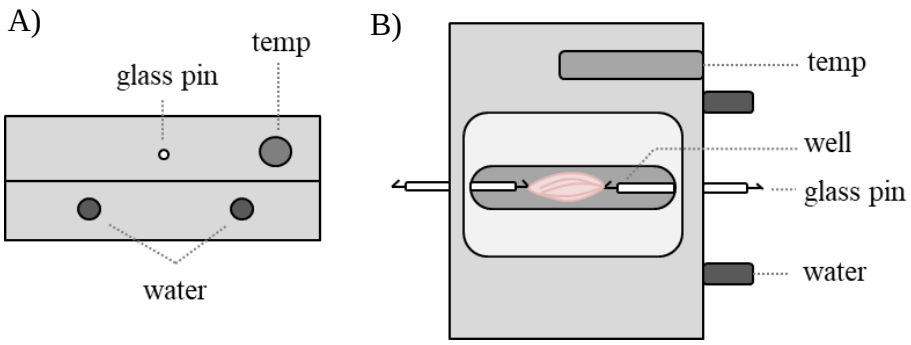

Figure 3.1 Schematic of the glass chamber used for measuring muscle power and oxygen consumption. A) End view of chamber, showing one of the pins that was attached to the muscle, the location of the temperature probe, and the location of channels through which water circulated through the chamber to control temperature. B) Top view of the chamber showing the well in which the muscle was located and the two silver pins to which it was attached that were in turn surrounded by a glass capillary tube. The well holds the muscle (depicted in pink) between the pins. Three magnetic stir bars (not shown) were located on the bottom of the well, below the muscle.

Pieces of fine copper magnet wire were soldered to the silver hooks that the muscle was tied to, and were attached to a stimulator circuit outside of the glass chamber to stimulate the muscle directly through the silver hooks attached to each end of the muscle. The silver hooks attached to the muscle preparation were coated with chloride via bleach (sodium hypochlorite) dipping for 5 minutes, creating a silver chloride electrode which effectively eliminated electrolysis and any production of oxygen and hydrogen gas bubbles in the saline when the muscle was stimulated. Additionally, a circuit was custom designed to convert the DC voltage pulses from the stimulator (SD9 Stimulator, Grass Product Group, West Warwick, RI, USA) into pairs of alternating pulses, each pulse with equal voltage-time area to the other, to help avoid electrolysis and ensure the pins remained coated with chloride throughout the experiment (Figure 3.2). The stimulus pulse duration from the stimulator was 2 ms delivered at pulse interval of 5 ms for all tests, resulting in pairs of ~1 ms pulses alternating in polarity from the custom circuit, delivered at a frequency of 200 Hz. The accuracy of the pulse amplitude and voltage-time area

was verified by viewing with a digital oscilloscope (model DS2202A, Rigol Technologies, Beaverton, OR, USA). These durations and frequencies were manipulated in preliminary studies to determine values that resulted in consistent and maximal activation of the muscle with no electrolysis within the saline, and the same values were then used throughout the remaining experiments.

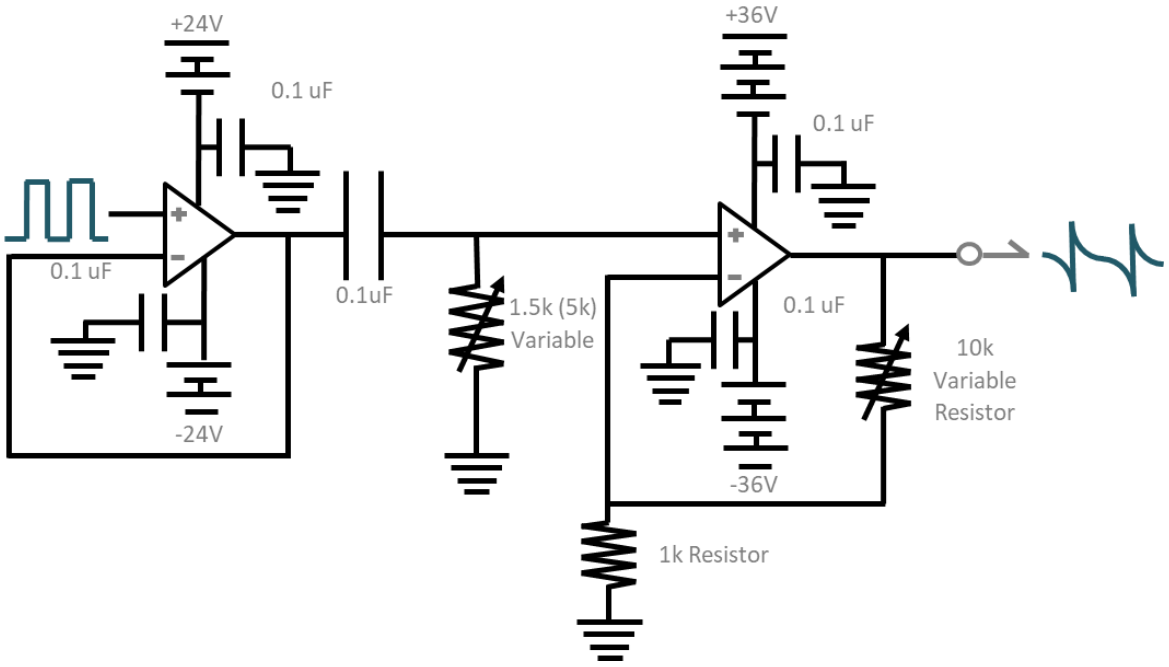

Figure 3.2 Circuit diagram of the amplifier used to convert the DC pulses from the stimulator into biphasic voltage pulses used to stimulate the muscles. The input amplifier (left) and associated capacitor/resistor converted the DC pulse into a biphasic pulse with equal area (voltage/time integral) of each pulse, and the output amplifier (right) and variable resistor was used to adjust the gain and provide a current source to stimulate the muscle. The square wave DC input pulses are shown on the left, and the biphasic AC output pulses are shown on the right. uF is microfarads. Resistances are in ohms.

The milled glass chamber also had a series of tubes running beneath the well that contained the muscle, through which water for temperature regulation flowed from a temperature-controlled circulator (Isotemp 3016D, Fisher Scientific, Waltham, MA, USA). Another hole (4.5 mm diameter, 25.7 mm depth) was milled into the glass chamber parallel to the muscle well, and housed a temperature probe (Fibox 3, PreSens Precision Sensing, Regensburg, Germany). Actual temperature within the chamber was calibrated using the known set temperature of the temperature-controlled circulator and the temperature recorded within the glass chamber using a digital thermometer (Traceable Digital Thermometer, VWR International, Radnor, PA, USA).

3.2.4 Measuring mechanical power from isolated skeletal muscle

Data were collected and experimental parameters (i.e. stimulus frequency, duration, and phase) were controlled with custom-written LabView software (ver 6.1, National Instruments, Austin, TX). The length of the muscle was initially adjusted to obtain maximal force output. This optimal resting length was found by increasing the muscle length by increments of 0.5 mm between isometric contractions, until the force output no longer increased with increased length. This muscle resting length was used for the remainder of the experiments.

The conditions that resulted in maximal, or near maximal power output from the muscle were then determined, and subsequently used to elicit maximal rates of oxygen consumption from the muscle. The work-loop technique was used to assess the effect of cycle frequency on power output, where cycle frequency (Hz) is the rate of cycling of muscle length, equal to the number of cycles of lengthening and shortening in one second, analogous to the tail-beat frequency of a swimming fish. For example, a cycle frequency of 3 Hz indicates 3 cycles per

second, with each cycle having a duration of 333.3 ms. In the work-loop technique, the muscle length is cycled in a sinusoidal pattern of lengthening and shortening (i.e. muscle strain) (see Syme 2005 for review), and the muscle is stimulated to contract during this cycle. The work recorded during the lengthening of the muscle is termed negative or lengthening work, while the work recorded during shortening is termed positive or shortening work. Together, the difference between shortening and lengthening work is the net work done during a complete cycle of shortening and lengthening (Figure 3.3). The amount of work produced is influenced by the cycle frequency, the stimulus duration during each cycle, the timing of the onset of stimulation during the strain cycle (i.e. stimulus phase), and the relative change in length of the muscle during the cycle (i.e. strain amplitude). All of these parameters were manipulated to determine the combination that maximized work output at each combination cycle frequency and temperature.

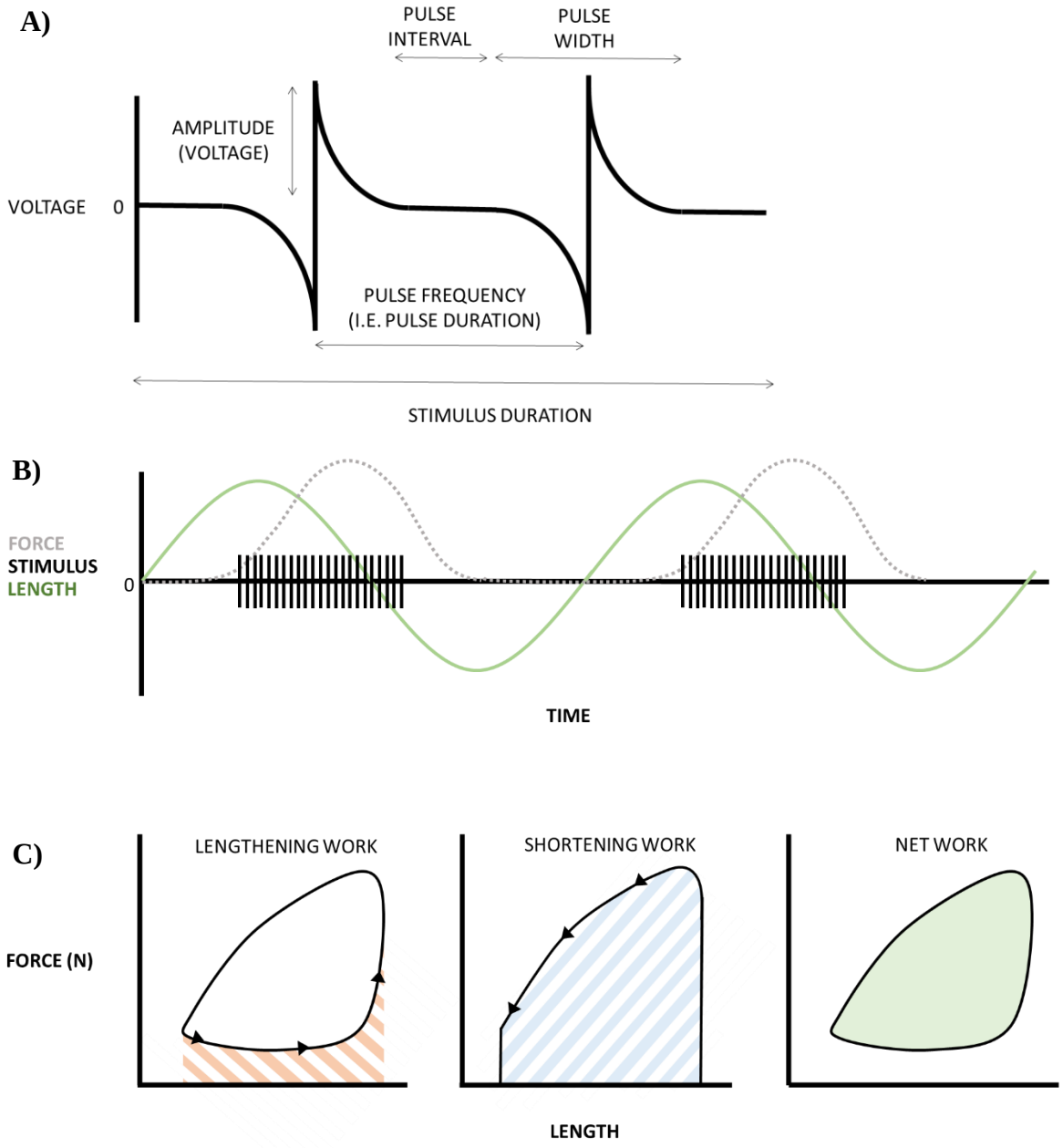

Figure 3.3 A) Schematic of the alternating voltage pulses used to stimulate the muscle. B) A schematic exampe of the relationships between the stimulus (series of voltage pulses), muscle length cycling (green sinusoidal trace), and force production (black dotted line). The onset of the stimulus pulses occur near the onse of muscle shortening. The amplitude of the length cycle is the strain amplitude. Force is produced when the muscle is stimulated to contract. C) Work loops formed by plotting force vs. length of the muscle. Net work done during a complete cycle (right) is the area inside the loop, and is the difference between shortening work (area beneath the upper/shortening arm of the loop, middle) and lengthening work (area beneath the lower/lengthening arm of the loop, left).

Power output was then determined by multiplying the net work done during a single cycle by the cycle frequency, to obtain a measure of power in watts (joules of work per second; W). These values of power were then used to assess at what cycle frequency power output was maximal, and how temperature affected this relationship, so that the conditions that resulted in near maximal power output during measures of oxygen consumption could be used to maximize aerobic metabolism. This was done using a preliminary set of measures on a sample of muscles ($n = 3 – 9$, depending on muscle temperature), used to determine the stimulus duration, phase, strain amplitude and cycle frequency that yielded the maximum power output at each experimental temperature (12ºC, 14ºC, 18ºC, 22ºC, 24ºC, and 26ºC). The relationship between power output and cycle frequency was determined for each muscle at each temperature, fitted with a second order polynomial, and the cycle frequency that maximized power output was determined by taking the first derivative of the polynomial to determine the peak of the curve.

At each temperature, the average of the cycle frequencies that yielded maximal power was then used to approximate (rounding to the nearest whole number) the cycle frequency that would maximize power output. The approximated parameters allowed for quicker determination of the maximum power output of each muscle at each temperature, which was determined prior to each oxygen experiment (between 3 and 6 Hz). This was important because fewer number of stimuli were applied to the muscle in order to find maximum power output and thus limit muscle fatigue and potential deterioration of the preparations. Further, it was not critical that each muscle be working at precisely its maximal capacity, only near maximal power as a means to elicit maximal aerobic metabolism, but these tests prior to oxygen tests ensured the muscle was always working at near maximum capacity.

3.2.5 Oxygen measurements from isolated skeletal muscle

The partial pressure of oxygen (PO_2) in the saline surrounding the muscle was measured using a fibre-optic oxygen meter (Fibox 3, PreSens Precision Sensing, Regensburg, Germany). An oxygen sensitive 'spot' was attached to a glass plate that was placed over the well containing the muscle, creating a closed system for experimentation, with the spot in contact with the saline. A fibre-optic probe, connected to the oxygen meter, was placed over the spot during experiments, and was used to measure the PO_2 of the saline surrounding the muscle during experiments, while maintain a sealed system that was not exposed to atmospheric air.

The rate of oxygen decline in the saline was also determined without a muscle present to verify that the chamber was closed, and oxygen was not leaking out of the chamber. This was tested with saline that had a PO_2 at 100% air saturation, 3x air saturation, pure oxygen (about 5x air saturation), and with pure nitrogen (0% oxygen), and no significant leak was observed. The chamber was routinely rinsed with bleach prior to experiments to kill bacteria growth as well as to re-chlorinate the hooks in the chamber. Further, a baseline rate of oxygen consumption was recorded at each temperature on 5 separate occasions to determine the rate of oxygen depletion within the chamber in the absence of a muscle (due to bacteria, minor leak, etc.), and the average value at each temperature was subtracted from the experimental rate of oxygen consumption recorded with muscles present.

Saline was bubbled with pure oxygen prior to being placed in the well with the muscle for all experiments. Initial tests showed that if atmospheric levels of oxygen were used, it was depleted rapidly from the saline during the lengthy experiments, leading to the concern that insufficient oxygen levels were available to the muscle. Thus, starting with a higher PO_2 preserved high levels of oxygen in the saline throughout the experiment (average PO_2 at start was

~50 kPa). Also, by increasing the PO_2 of the saline, there is improved diffusion of oxygen into the tissue mass, which no longer has a functional circulation. Preliminary tests showed no significant difference between the amount of work produced by a muscle when in either air saturation or pure oxygen conditions, therefore, while air saturation appears adequate to support high rates of work, pure oxygen was used in all subsequent oxygen measurement experiments to ensure maximal and adequate levels of oxygen were present throughout the measurements.

Standard metabolic rate was determined as the rate of decline of oxygen content in the chamber while the muscle was at rest (i.e. not being stimulated). Fresh, oxygenated saline was introduced to the well containing the muscle, approximately 10 minutes was given to allow the temperatures to equilibrate, and then the rate of decline of PO_2 in the saline was measured for approximately 15-25 minutes. For measures of maximal metabolic rate, fresh saline was introduced, the muscle was left in a resting state to obtain a pre-stimulus baseline rate of oxygen consumption, and the muscle was then stimulated using the conditions determined to produce near maximal power output in 5 series of 20 cycles each, with a 30 second rest between each series, to reduce fatigue in the muscle. Post stimulus, the muscle was again left at a resting state and oxygen consumption was recorded for an additional ~20 minutes (total of 35 to 45 minutes). The temperature of the chamber was then set to the next value to be tested and the muscle given ~ 15 minutes to equilibrate before fresh saline was rinsed through the bath and the next set of measures of resting and maximal metabolic rate were performed. The sequence of test temperatures was randomly assigned for each muscle so that muscles were not tested in the same sequence of temperatures. However, muscles exposed to temperatures greater than 24ºC did not appear to recover well, and thus 26ºC temperatures were normally tested last. The experimental

duration for a single muscle never lasted longer than 8 hours, while once stimulated on a regular bases during experiments muscles were able to perform work for more than 24 hours, suggesting the results reflect metabolism of a healthy muscle and are comparable across the duration of the experiment (i.e. initial power output was within 80% of initial values when tested at the same temperature).

Post experimentation, the sutures and dead tissue were cut away from the preparation and the remaining muscle tissue was placed on filter paper to remove excess water before the muscle mass was recorded (MT5 microbalance, Mettler-Toledo, Switzerland).

3.2.6 Calculating SMR and MMR of skeletal muscle

Oxygen data was analyzed using custom software written in LabView (ver 6.1, National Instruments, Austin, TX). Standard metabolic rate was measured as the rate of oxygen decline in the chamber while the muscle was at rest (i.e. not induced to undergo contractions). Maximal metabolic rate was measured as the change in PO_2 in the saline as a result of the muscle doing work. This was calculated by first fitting linear regressions to the pre- and post-stimulus (i.e. resting) portions of the PO_2 trace, and then the vertical difference between the two regressions (change in PO_2) was measured at the time point approximately midway through the period of stimulation to calculate the change in PO_2 as a result of the muscle doing work (Figure 3.4). This value was divided by the period that the muscle was made to work to attain MMR. Using these values of change in PO_2, along with the oxygen compliance of the saline (dependant on temperature), atmospheric pressure at the time of the experiment, and the volume of the chamber, the molar amount of oxygen used by the muscle could be calculated. Further, established relationships between ATP production per mole of oxygen used (assumed as 450kJ $mole^{-1}$) were

used to estimate the metabolic rate of the muscle in units of energy. The molar amounts of oxygen consumption were converted into mass of oxygen (32g mol^{-1} O_2) and the Joule equivalent (Gnaiger, 1983) assuming oxidation of glucose as the substrate. The rate of oxygen consumption was standardized using both mass of muscle and relative mass of whole fish (both expressed as mg O_2 kg^{-1} hr^{-1}).

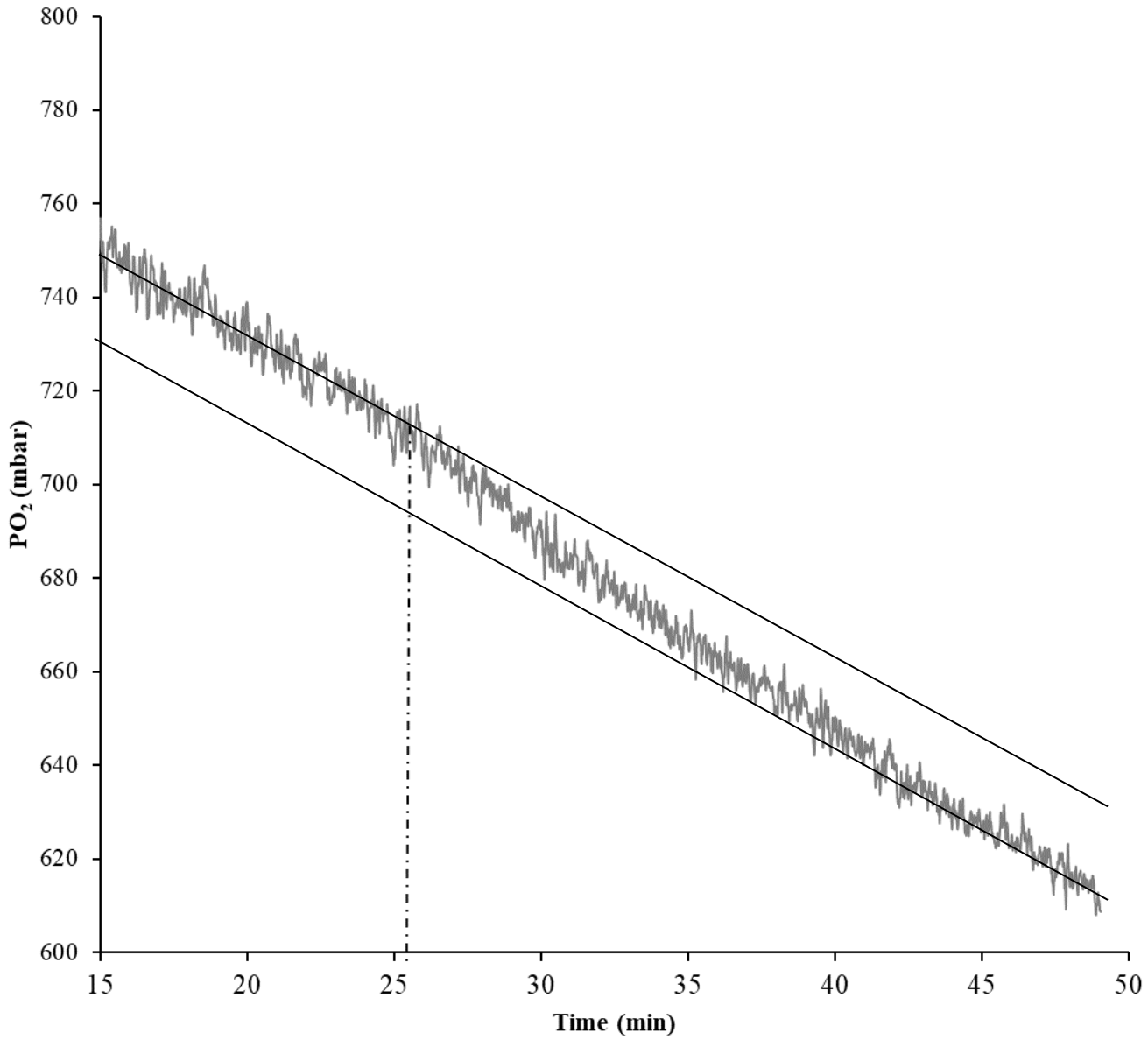

Figure 3.4 Example of oxygen recording from red skeletal muscle of rainbow trout, showing PO_2 of the saline over time. Grey line is raw trace of PO_2 (mbar) of the chamber. Dashed line is time stimulus and work loops started. PO_2 declines slowly as the muscle is resting before stimulation (15-25 minutes), then declines more rapidly as the muscle consumes more oxygen while contracting and doing work (about 25-30 min), and then returns to the resting rate of oxygen consumption after stimulation ends (about 30 minutes onwards). The amount of oxygen used by the muscle, as a result of doing work, was calculated by fitting linear regressions to the pre- and post-stimulus resting portions of the data, and measuring the vertical separation between these lines at approximately the 30 minute mark, which is about half way between the onset and end of the more rapid rates of oxygen consumption.

Q_{10}, the relative change in metabolic rate for a 10ºC increase in temperature, was calculated for SMR and MMR between each pair of tested temperatures, and across the full range of temperatures tested (12 – 26ºC).

3.2.7 Effects of epinephrine, oligomycin and ethanol on red muscle

The effects of epinephrine were tested on a sample group of red muscle to determine if circulating catecholamines, as are known to exist in rainbow trout (Perry and Reid, 1992), affect work and oxygen consumption. The effects of epinephrine on SMR and work output of the first 10 cycles were tested at concentrations: control (0 nM), 10 nM, 100 nM, 500 nM and 1000 nM. These values were chosen based on measured circulating plasma concentration of ~1 nM in resting and unstressed rainbow trout, and stress induced levels of nearly 300 nM (Perry and Reid, 1992). Work and oxygen consumption were measured in the same way as previously described for each concentration (n = 3 – 5), and the difference relative to control was determined.

Three muscles were also acutely exposed to oligomycin, a blocker of mitochondrial oxidative phosphorylation, specifically ATP synthase, to provide further evidence that the oxygen decline observed during these experiments could be attributed to muscle mitochondrial utilization. For three muscles, 1 mg ml^{-1} oligomycin was added to the saline bathing the muscle preparation and left for ~2 hours (Bouchard and Guderley, 2003). Oxygen consumption of the muscle in this state was recorded to observe the effect of oligomycin on aerobic capacity. Additionally, 50% ethanol was added to the saline to ensure complete cell death, and the effects of these different treatments on oxygen consumption were compared.

3.2.8 Muscle volume distribution in trout

Measures of muscle metabolic rate were used to determine the contribution of muscle metabolism to that of the whole fish, which required a measure of the total mass of muscle in the fish. Muscle volumes in the whole fish were estimated from 25 rainbow trout (fish mass 40.3 – 234.8 g). Fish were euthanized and cut transversely into 6 segments at the following locations: immediately caudal to the gills, anterior to dorsal fin, posterior to dorsal fin, anterior to pelvic fins, anterior to adipose fin, and at the caudal peduncle (Figure 3.5). These segmental cuts were made consistently for all fish. Photographs were taken of each section from both the caudal and anterior aspect of the segment, with a ruler placed in the field of view. Photographs were taken with a digital camera (Nikon Coolpix 8700, Nikon, China). Areas of red muscle, white muscle, and total area were calculated for each surface of each segment using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA; Schneider et al., 2012), and along with the segment lengths were used to determine total red muscle and white muscle volume for each section (Figure 3.6). Area was measured using the area tool and length measures using the measure tool in ImageJ. The length was converted from pixels to a known metric using a ruler that was present in each image to indicate a relative length to pixel metric for each photo. Estimates of volume were calculated using the equation;

$$Volume = \frac{[(area)_{anterior} + (area)_{posterior}]}{2} * length\ of\ segment;$$

A linear change in area of each muscle type was assumed from the anterior to posterior surface of each segment. Volumes were also standardized to total fish mass to determine if there was a scaling effect of muscle volume with mass.

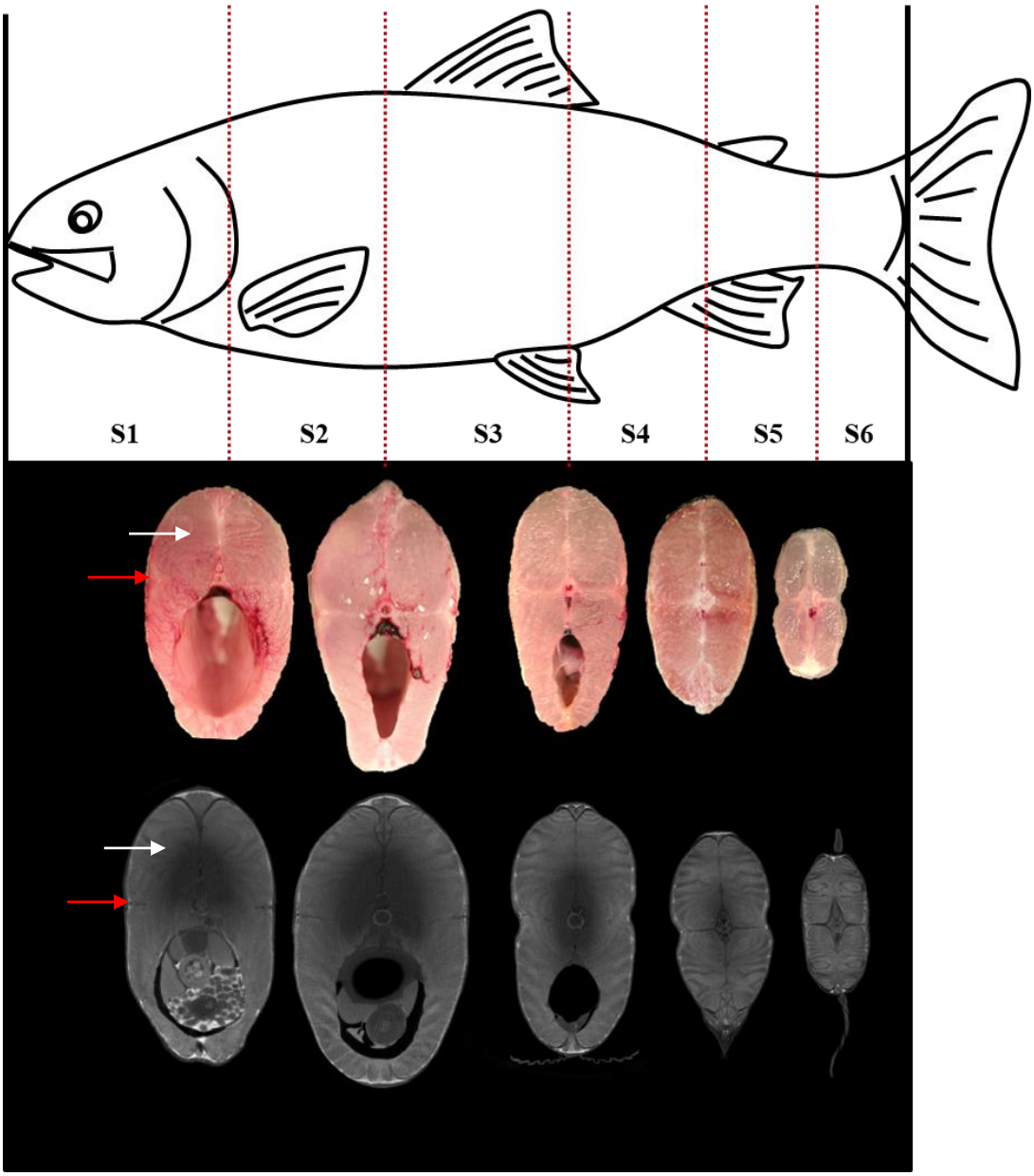

Figure 3.5 Schematic of locations from which the transverse sections were cut to calculate volumes of red and white muscle. Dotted lines indicate where the transverse cuts were made, and the corresponding anterior view from each muscle section is shown. Muscle volume for each section was then calculated using the segment length (S1-S6) and area of red and white muscle in each section, assuming a linear change in area from the anterior to posterior surface of each section, and a linear decline to zero muscle volume at the head and the caudal peduncle (i.e. segment 1 and segment 6). First images show transverse view analyzed in ImageJ software, second represents the 3D image from Amira software. Red arrows indicate location of red muscle for each image type, whereas white arrows indicate white muscle.

These muscle volumes calculated from the digital images were then compared to volume measurements from a single fish calculated using a Skyscanner 1173 μCT scanner (Bruker, Kontich, Belgium). The fish head was removed at the gill arch to allow perfusion of solutions into the body. The fish was fixed in 10% neutral buffered formalin (NBF) for 88 hours and then held in 5% Lugol's iodine solution to stain the muscle preparation for 68 hours. 3D image stacks were reconstructed in NREcon v1.6.9 (Bruker, Kontich, Belgium) for transverse analysis. Image stacks were then imported into Amira v5.3.3 (Visage Imaging, Berlin, Germany) for volume measurements (Figure 3.5). Manual differentiation of white and red muscle areas were determined, and stack interpolation was used to quantify muscle area and volume along the body axis. Only the right side of the fish was measured for red and white muscle volume from the gill arch to the caudal peduncle; volumes were multiplied by 2 and standardized to the total volume of the fish, also determined using the Amira software.

3.2.9 Muscle efficiency

Mechanical efficiency ($\dot{E}p/\dot{E}O_2$) is a measure of the mechanical power output (i.e. rate of doing work, $\dot{E}p$) of a muscle relative to the rate of metabolic energy utilization ($\dot{E}O_2$). $\dot{E}O_2$ is the rate of energy utilization of the muscle, measured from $\dot{V}O_2$, and was calculated using the assumption that glucose was the only exogenous fuel source (J min^{-1} kg^{-1}) (Syme, 1994). $\dot{E}p$ is the rate at which the muscle does work, standardized by muscle mass (J min^{-1} kg^{-1}). While it is acknowledged that the fuel substrates used by the muscle likely consisted of a mixed source of carbohydrates, lipids and perhaps proteins, it was not possible to assess this or the relative contributions of each substrate, and thus it was assumed that glucose was the sole substrate. This

assumption will cause a small error in estimates of metabolic rate, but this error would be systematic across all measures and thus should not impact interpretation (West et al., 1993).

While the force produced by the muscle tended to be relatively stable across the experiments, the work output from the muscle tended to fall with successive contractions in each series of contractions, recovering between series, perhaps reflecting fatigue in the isolated preparations that would likely not be present in muscle working in an intact fish, or at least to the same extent (Figure 3.6). Therefore, $\dot{E}p$ was calculated in two different ways. First, work from only cycles 3 through 12 (10 cycles) of the first set of 20 contractions was used to determine average work output before the onset of fatigue, and this average was multiplied by 100 (the total number of cycles tested) to estimate net power from the muscle if fatigue did not occur. Second, work from all 100 cycles was added to represent the actual work and power output of the isolated muscle over the period of measurements. Efficiencies were then calculated using each measure of power separately.

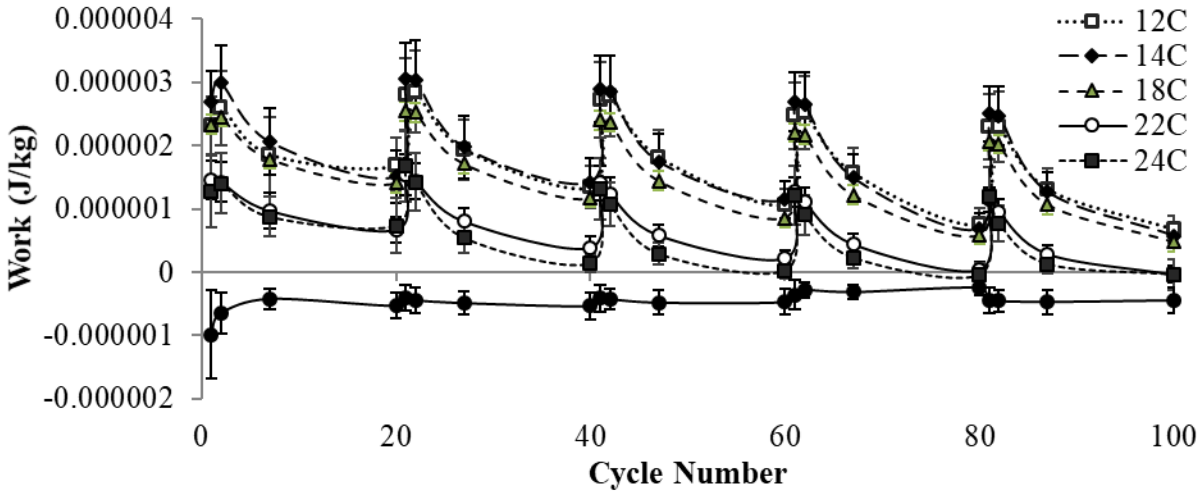

Figure 3.6 The work done by red muscle (J/kg) over the course of 100 muscle contractions, given as 5 sets of 20 contractions separated by 30 second periods of rest, for each temperature. Values are mean ± SEM.

3.2.10 Analysis

A one-way repeated measures ANOVA was run on SMR, MMR, AAS and FAS to assess the effects of temperature. Red muscle MMR, AAS and FAS, as well as white muscle SMR were not normally distributed, therefore logarithmic transformations were applied to satisfy the assumptions of the ANOVA test. A one-way repeated measures ANOVA was also run on red muscle *Ep*, EO_2 and red muscle mechanical efficiency to test the effects of temperature. Normality was tested using a Shapiro-Wilk test on residuals and equal variance was determined using a Levene's test. Statistical significance of differences between temperatures were then determined post-hoc using a Tukey's test.

A one-way ANOVA was conducted to determine the effects of different concentrations of epinephrine, and of oligomycin and ethanol on rates of oxygen consumption of red muscle. A pair-wise T-test was conducted to determine the statistical difference between white muscle SMR initially and post ethanol exposure.

3.3 Results

3.3.1 The effect of temperature on red muscle metabolic rate and efficiency

SMR of red skeletal muscle significantly increased with warming temperature (one way repeated measures ANOVA, $F = 20.6$, d.f. = 5, $p < 0.001$; Table 3.1; Figure 3.7) such that there was significantly higher SMR with warmer temperatures, increasing 3.4 fold from 12ºC to 26ºC (Table 3.1; Figure 3.7). The Q_{10} from 12ºC to 26ºC, calculated from the mean SMR at each temperature, was 2.4 (Table 3.2). Temperature had no significant effect on MMR (one-way repeated measures ANOVA, $F = 0.706$, d.f. = 5, $p = 0.623$; Table 3.3). The Q_{10} from 12ºC to 26ºC, calculated from the mean MMR at each temperature, was 0.955. AAS also did not differ between temperatures (one-way repeated measures ANOVA, $F= 1.17$, d.f. = 5, $p = 0.346$; Figure 3.8). However, FAS significantly declined with increasing temperatures (one-way repeated measures ANOVA, $P = 0.012$, $F = 3.48$, d.f. = 5, $p = 0.012$; Figure 3.9), particularly between 12ºC compared to 24ºC and 26ºC (Table 3.3, Figure 3.9).

$\dot{E}O_2$ at maximal metabolic rate was not significantly affected by temperature (one-way repeated measures ANOVA on LOG transformation, $F = 0.529$, d.f. = 5, $P = 0.753$; Table 3.4). In contrast, $\dot{E}p$ declined significantly with increased temperature (one way repeated measures ANOVA, $F = 9.281$, d.f. = 5, $P < 0.001$; Table 3.5), therefore mechanical efficiency, both measures, declined significantly with increased temperature ($F = 9.101$, d.f. = 5, $P < 0.001$; Figure 3.10). The parameters of obtaining maximum power output were also reported (Table 3.5).

Table 3.1 Red and white muscle aerobic metabolic rate tested at each temperature, expressed per mass of muscle tissue ($mgO_2\ kg^{-1}_{muscle}\ hr^{-1}$) and per mass of fish ($mgO_2\ kg^{-1}_{fish}\ hr^{-1}$). Values expressed per mass of fish are standardized by the percentage of red muscle relative to whole fish (red: 2.73%, white: 70.8%). SMR (standard metabolic rate), MMR (maximum metabolic rate), AAS (absolute aerobic scope MMR - SMR), FAS (factorial aerobic scope; MMR/SMR). Baseline is the mean change in oxygen in the absence of muscle (i.e. resting rate of chamber). Data are mean ± S.E.M.

Temperature		12ºC	14ºC	18ºC	22ºC	24ºC	26ºC
Baseline ($x\ 10^{-8}\ mgO_2\ s^{-1}$)	N	6	6	6	6	5	6
	Resting rate	14.7	8.76	7.81	21.5	33.8	49.8
Red muscle	N	10	9	13	11	7	7
per mass of tissue ($mgO_2\ kg^{-1}_{muscle}\ hr^{-1}$)	SMR	115 ± 9.90	156 ± 10.8	242 ± 26.3	266 ± 23.4	268 ± 38.5	389 ± 43.2
	MMR	882 ± 177	770 ± 141	948 ± 155	816 ± 161	606 ± 76.1	827 ± 79.4
	AAS	767 ± 172	613 ± 144	706 ± 158	550 ± 156	338 ± 87.0	438 ± 113
	FAS	7.62 ± 1.24	5.30 ± 1.14	4.41 ± 0.833	3.08 ± 0.547	2.84 ± 0.884	2.51 ± 0.639
per mass of fish ($mgO_2\ kg^{-1}_{fish}\ hr^{-1}$)	SMR	3.14 ± 0.27	4.27 ± 0.30	6.60 ± 0.72	7.27 ± 0.64	7.33 ± 1.05	10.6 ± 1.18
	MMR	24.1 ± 4.82	21.0 ± 3.94	25.9 ± 4.25	22.3 ± 4.40	16.5 ± 2.08	22.6 ± 2.17
	AAS	21.0 ± 4.70	16.7 ± 3.94	19.3 ± 4.32	15.0 ± 4.25	9.22 ± 2.38	12.0 ± 3.07
	FAS	7.62 ± 1.24	5.30 ± 1.14	4.41 ± 0.833	3.08 ± 0.547	2.85 ± 0.884	2.51 ± 0.639
White muscle	N	11	14	15	16	14	11
per mass of tissue ($mgO_2\ kg^{-1}_{muscle}\ hr^{-1}$)	SMR	59.7 ± 16.0	54.2 ± 11.8	60.0 ± 9.80	88.2 ± 15.2	104 ± 20.3	130 ± 29.7
per mass of fish ($mgO_2\ kg^{-1}_{fish}\ hr^{-1}$)	SMR	49.1 ± 13.9	46.4 ± 11.4	47.7 ± 8.19	67.3 ± 11.7	75.6 ± 15.4	91.8 ± 21.0

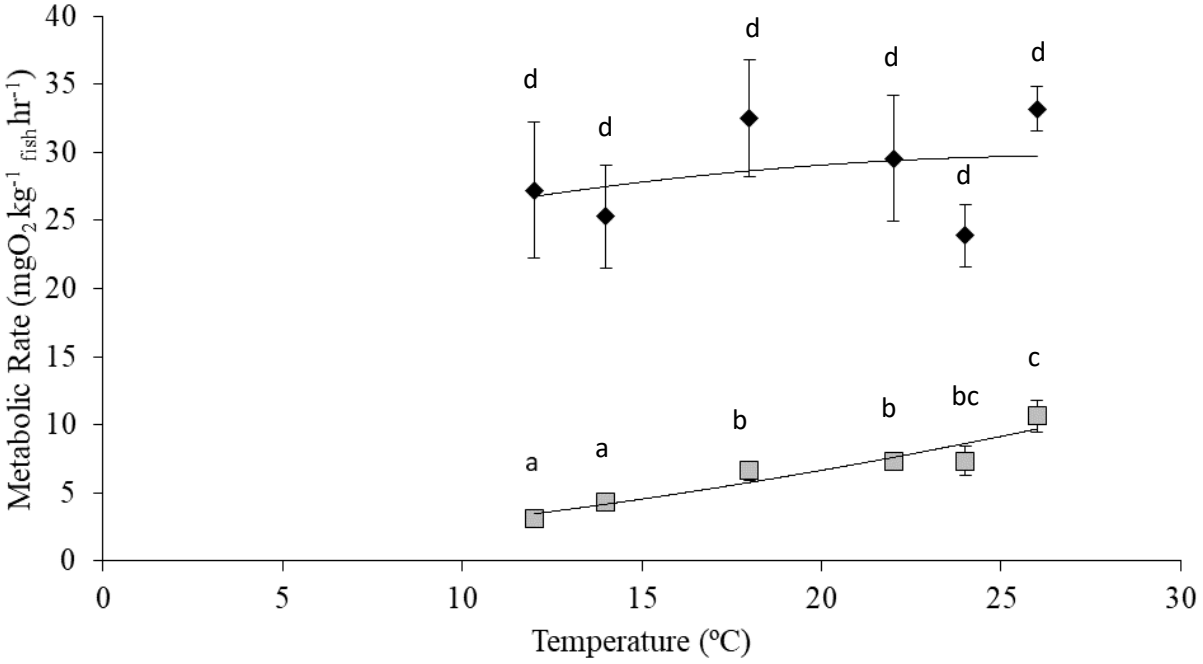

Figure 3.7 SMR (grey, square) and MMR (black, diamond) of rainbow trout (*O. mykiss*) red skeletal muscle, from fish acclimated to 12ºC and tested at various temperatures. Sample size at 12ºC n=10, 14ºC n=9, 18ºC n=13, 22ºC n = 11, and 24ºC and 26ºC n = 7. MMR fitted polynomial line (black, solid) ($y = -0.0127x^2 + 0.645x + 20.3$; $R^2 = 0.0488$). SMR fitted polynomial line (grey, dotted) ($y = 0.0076x^2 + 0.155x + 0.4878$; $R^2 = 0.8997$). Values are mean ± SEM. Different letters indicate significantly different values ($P<0.05$).

Table 3.2 Q_{10} values for SMR and MMR of red and white muscle across a range of temperatures, determined from the means of the metabolic rates for each temperature.

T_{Q10} (ºC)	Red SMR	Red MMR	White SMR
12ºC to 26ºC	2.39	0.955	1.76
12ºC to 14ºC	4.68	0.506	1.44
14ºC to 18ºC	3.00	1.55	1.19
18ºC to 22ºC	1.26	0.748	2.12
22ºC to 24ºC	0.939	0.333	1.96
24ºC to 26ºC	7.11	3.21	3.01

Table 3.3 Statistical effects of temperature on aerobic metabolic measurements of muscle tissue. Data was standardized to muscle mass and logarithmically transformed before one-way repeated-measures ANOVA. All red muscle values were log-transformed to fit the assumptions of the statistical tests. SMR (standard metabolic rate: $mgO_2\ kg^{-1}\ hr^{-1}$), MMR (maximum metabolic rate: $mgO_2\ kg^{-1}\ hr^{-1}$), AAS (absolute aerobic scope: MMR-SMR), FAS (factorial aerobic scope: MMR/SMR).

Muscle Type	Metabolic Measure	P value	F value	DF	Normality Test (Shapiro-Wilk)	Equal Variance Test
Red	SMR	<0.001	23.3	5	0.598	0.678
	MMR	0.753	0.529	5	0.259	0.524
	AAS	0.493	0.899	5	0.689	0.970
	FAS	0.012	3.07	5	0.406	0.748
White	SMR	0.005	3.809	5	0.092	0.417

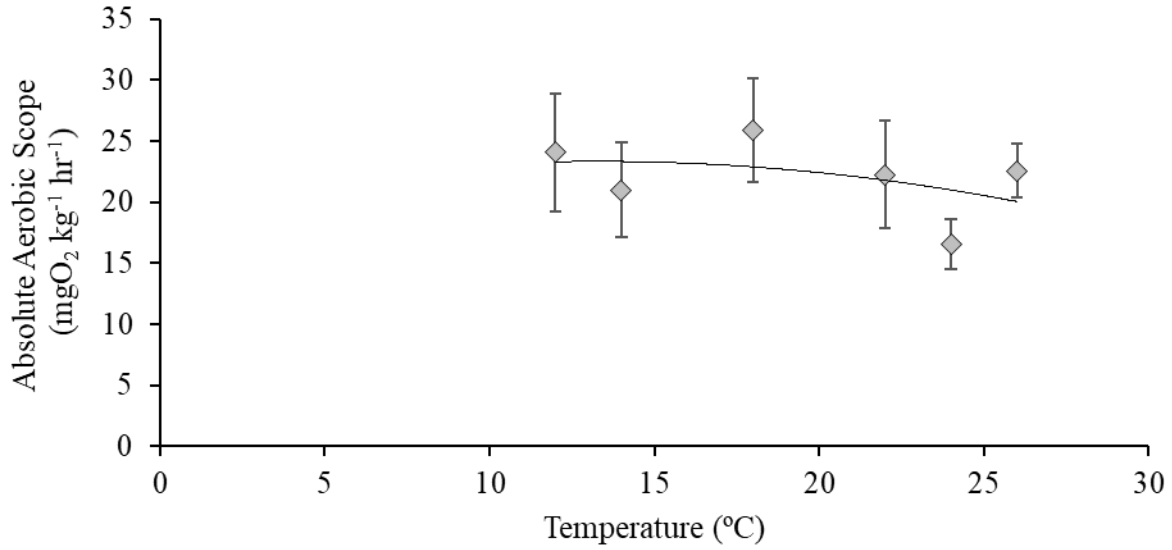

Figure 3.8 Absolute aerobic scope (AAS = MMR – SMR) of rainbow trout (*O. mykiss*) red skeletal muscle, from fish acclimated to 12ºC and tested at various temperatures. AAS fitted polynomial line $y = -0.028x^2 + 0.3845x + 19.277$; $R^2 = 0.745$). Values are mean ± SEM. Aerobic scope did not significantly differ between temperatures (P = 0.493).

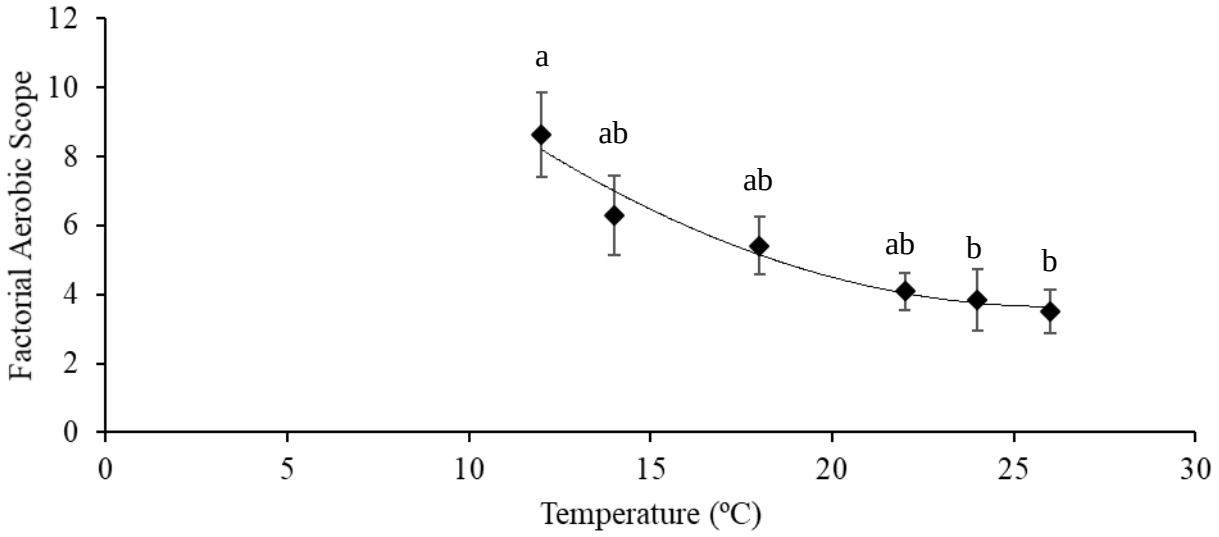

Figure 3.9 Factorial aerobic scope (FAS=MMR/SMR) of rainbow trout (*O. mykiss*) red skeletal muscle, from fish acclimated to 12ºC and tested at various temperatures. FAS fitted polynomial line $y = 0.0229x^2 - 1.195x + 18.245$; $R^2 = 0.959$. Values are mean ± SEM. Different letters indicate significantly different values ($P<0.05$).

Table 3.4 Effects of temperature on $\dot{E}O_2$ (rate of energy utilization estimated from oxygen uptake), $\dot{E}p$ (muscle power output calculated using the average of all cycles in the entire series of 100 contractions) and $\dot{E}p$ corrected (muscle power output calculated using the average of cycles 2- 13 in each series of 100 contractions, before the onset of fatigue). Efficiency calculated using $\dot{E}p$ or $\dot{E}p$ corrected, calculated as ($\dot{E}p/\dot{E}O_2$ x100).

Temp (ºC)	N	$\dot{E}O_2$ (J kg^{-1} min^{-1})	$\dot{E}p$ (J kg^{-1} min^{-1})	Efficiency (%)	*$\dot{E}p$ corrected* (J kg^{-1} min^{-1})	Efficiency corrected (%)
12	10	206.9 ± 41.4	7.17 ± 2.27	4.91 ± 0.937	10.1 ± 3.03	6.46 ± 1.61
14	9	180.3 ± 33.0	8.99 ± 3.00	8.69 ± 2.02	16.0 ± 4.77	11.5 ± 2.97
18	13	222.2 ± 36.5	6.47 ± 1.80	6.13 ± 1.24	14.2 ± 2.54	8.52 ± 2.04
22	11	191.2 ± 37.7	7.56 ± 2.28	2.85 ± 1.13	8.53 ± 3.08	4.88 ± 1.57
24	7	142.0 ± 17.8	3.41 ± 1.29	1.82 ± 0.986	6.13 ± 2.09	3.93 ± 1.58
26	7	193.7 ± 18.6	4.11 ± 1.56	-2.77 ± 0.753	-4.97 ± 1.44	-2.46 ± 0.66

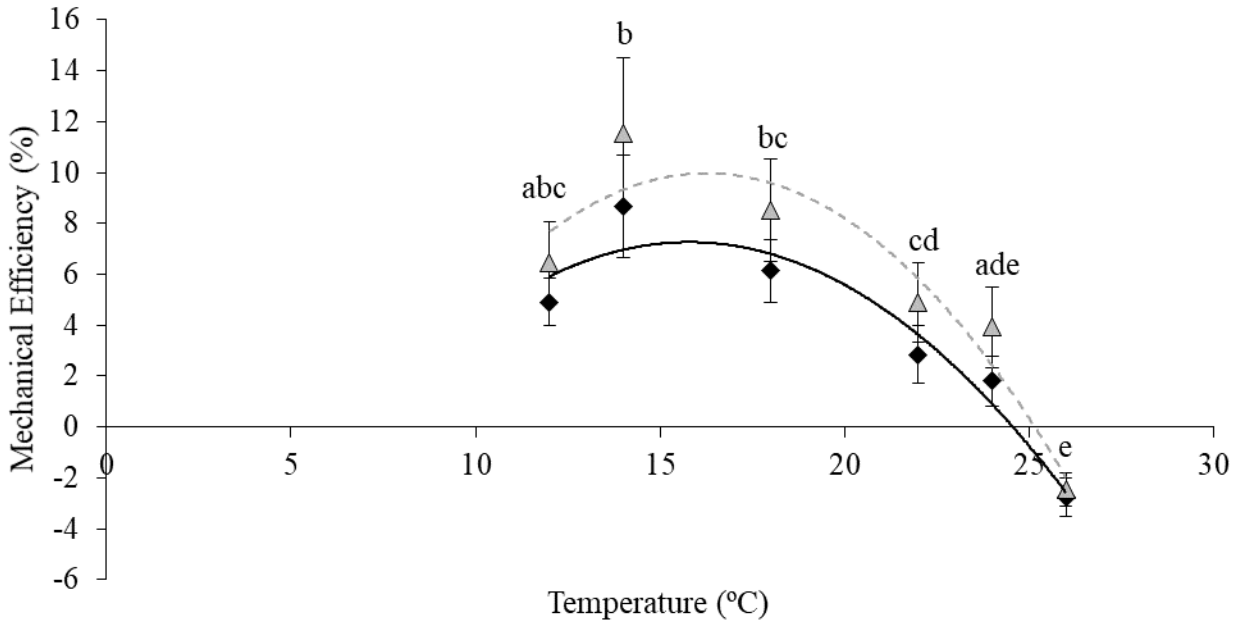

Figure 3.10. Mechanical efficiency ($\dot{E}p/\dot{E}O_2$) of red skeletal muscle of rainbow trout (*O. mykiss*) from fish acclimated to 12ºC and tested at various temperatures. Net mechanical efficiency using all values recorded during the 100 contractions (black, diamond) fitted regression $y = -0.0943x^2 + 2.98x - 16.2$; $R^2 = 0.925$; net mechanical efficiency using only the average power output of first 10 contractions, before the onset of significant fatigue (i.e. average of first 10 loops x 100) (grey, triangle), fitted regression $y = -0.126x^2 + 4.10x - 23.4$; $R^2 = 0.904$. Values are mean ± SEM. Different letters indicate values that are significantly different from one another ($P < 0.05$).

Table 3.5 Parameters used to elicit maximal power from red skeletal muscle at each temperature, used to determine the cycle frequency and stimulus that produced maximal power from each muscle during measures of muscle MMR.

Temp. (ºC)	N	Cycle Frequency (Hz)	Stimulus Duration (ms)	Muscle Strain Amplitude (%)	Stimulus Phase (%)
12	10	3.25 ± 0.112	134 ± 8.49	4.20 ± 0.690	6.4 ± 0.859
14	9	3.28 ± 0.121	146 ± 8.89	4.56 ± 0.444	5.33 ± 0.764
18	13	3.69 ± 0.223	138 ± 9.36	4.31 ± 0.429	6.77 ± 0.411
22	11	4.04 ± 0.305	126 ± 8.99	4.36 ± 0.577	7.45 ± 0.623
24	7	3.64 ± 0.237	149 ± 9.97	5.71 ± 1.25	6.14 ± 0.634
26	7	4.56 ± 0.414	121 ± 15.6	5.14 ± 1.42	7.71 ± 0.714

3.3.2 The effect of temperature on white muscle metabolic rate

SMR of white muscle also significantly increased with warming temperature (one-way repeated measures ANOVA, F =3.81, d.f. = 5, p = 0.005; Table 3.3; Figure 3.11), particularly between the coolest and the warmest tested temperatures. Q_{10} of $\dot{V}O_{2min}$ was 1.76 between 12ºC and 26ºC, with the greatest increase being between 24ºC and 26ºC with a Q_{10} of 3.01 (Table 3.3).

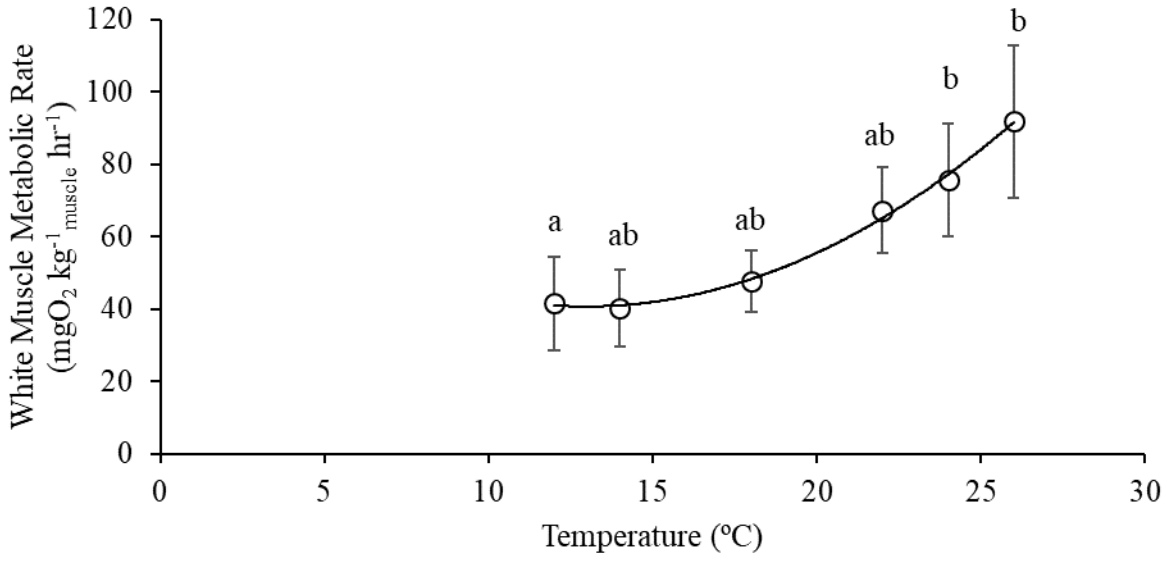

Figure 3.11 Standard metabolic rate (SMR) of rainbow trout (*O. mykiss*) white skeletal muscle, from acclimated to 12ºC and tested at various temperatures. Sample size: 12ºC n = 15, 14ºC n = 12, 18ºC n = 15, 22ºC n = 14, 24ºC n = 12, and 26ºC n = 11. Polynomial fitted line $y = 0.2997x^2 - 7.7763x + 91.165$; $R^2 = 0.9962$. Values are mean ± SEM. Different letters indicate significantly different values (P>0.05).

3.3.3 Muscle volume distribution

Two groups of fish were examined for muscle volume distribution. The initial group (n = 20) were of from the population of fish used to examine aerobic scope in whole fish (see Chapter 2), whereas the second group (n = 5) was from the population of the fish used to measure metabolic rate of muscle. The two groups did not significantly differ in muscle volumes relative to total fish volume (T-test; T = -1.80, d.f. = 8.75, P = 0.107), and fish mass of the second test group (47 – 84 g) was within the range of masses examined in group 1 (40 – 235 g), therefore, the two groups (n = 25) were combined to calculate the mean percentage of each muscle type in a whole fish (average 109.3 ± 8.6 g body mass).

The total volume of red skeletal muscle was 2.73 ± 0.115% of the whole fish volume, whereas the amount of white skeletal muscle was 70.8 ± 0.85% of the whole fish volume (Table 3.1). This was consistent with the 3D scan of a single fish of similar mass (97.3 g) that revealed 2.4% red muscle and 68.5% white muscle relative to with the whole measured fish volume (Figure 3.5).

There was no significant relationship between body mass and relative percentage of muscle volume for either tissue type (Table 3.1; Figure 3.13). The maximum cross-sectional area of red muscle occurred between 62% and 78% of the fish standard length (Figure 3.12). Estimated muscle mass was also determined assuming a density of 1.05 g cm^{-3} (Goolish, 1989).

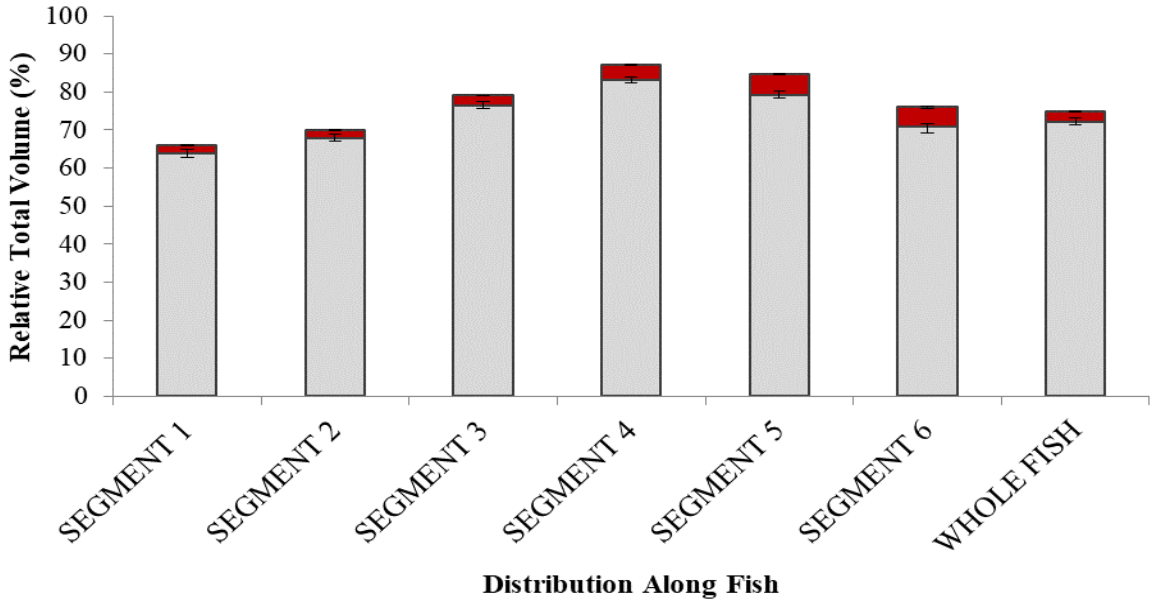

Figure 3.12 Red and white muscle volume relative to total volume within each segment (n = 25). The estimated percentage of the whole fish that is red muscle is 2.73%, whereas the contribution of white muscle to whole body volume is 70.8%. Red (top bar) is red muscle; grey (bottom bar) is white muscle. Values are means ± SEM.

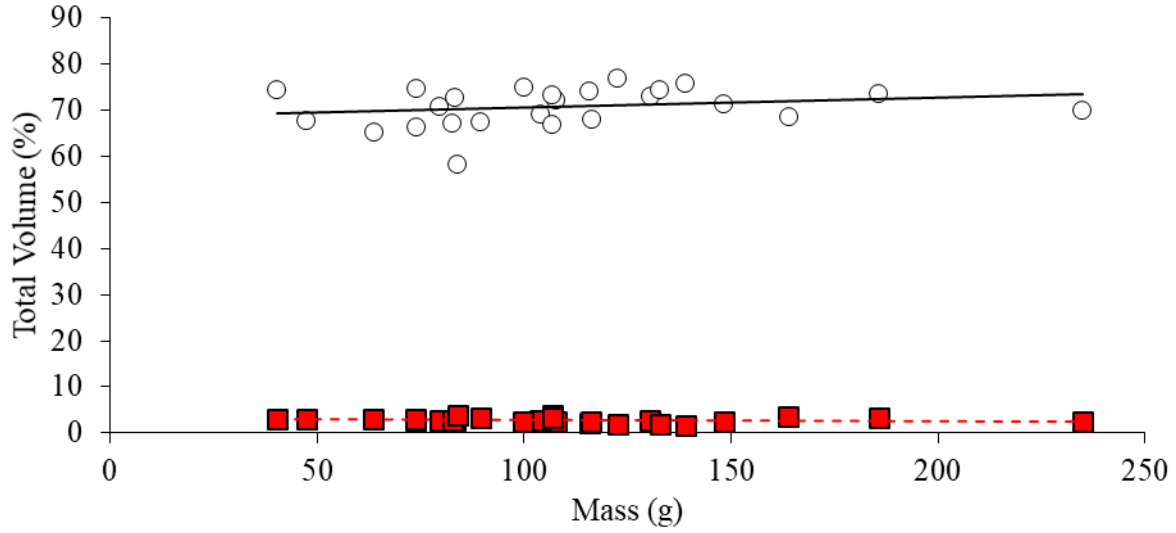

Figure 3.13 Total volume of red and white muscle tissue as a percentage of the total fish volume related to fish mass of rainbow trout (*O. mykiss*) acclimated to 12ºC. Sample size of red and white muscle is n = 25. Red muscle (square, red) linear regression y = -0.0029x + 3.05, R^2 = 0.0479; white muscle (circle, open) linear regression y = -0.0221x + 68.4, R^2 = 0.0507. The slopes of each muscle type showed no significant relationship between muscle mass and volume (P>0.05).

3.3.4 Effects of epinephrine, oligomycin and ethanol on muscle performance

Epinephrine was added in various concentrations to determine if increased circulating catecholamines, as would be expected to increase in stressed situations (Perry and Reid 1992), would result in increased SMR and power output. There was no significant effect of any concentration of epinephrine on SMR at 22ºC (one way repeated measures ANOVA, $F = 10.939$, d.f. = 8, $p < 0.001$; Figure 3.14), which may suggest that in these isolated preparations the muscle contractile response was already maximized. Epinephrine also had no effect on muscle power output (one-way ANOVA, $F = 0.345$, d.f. = 4, $P = 0.841$; Figure 3.14).

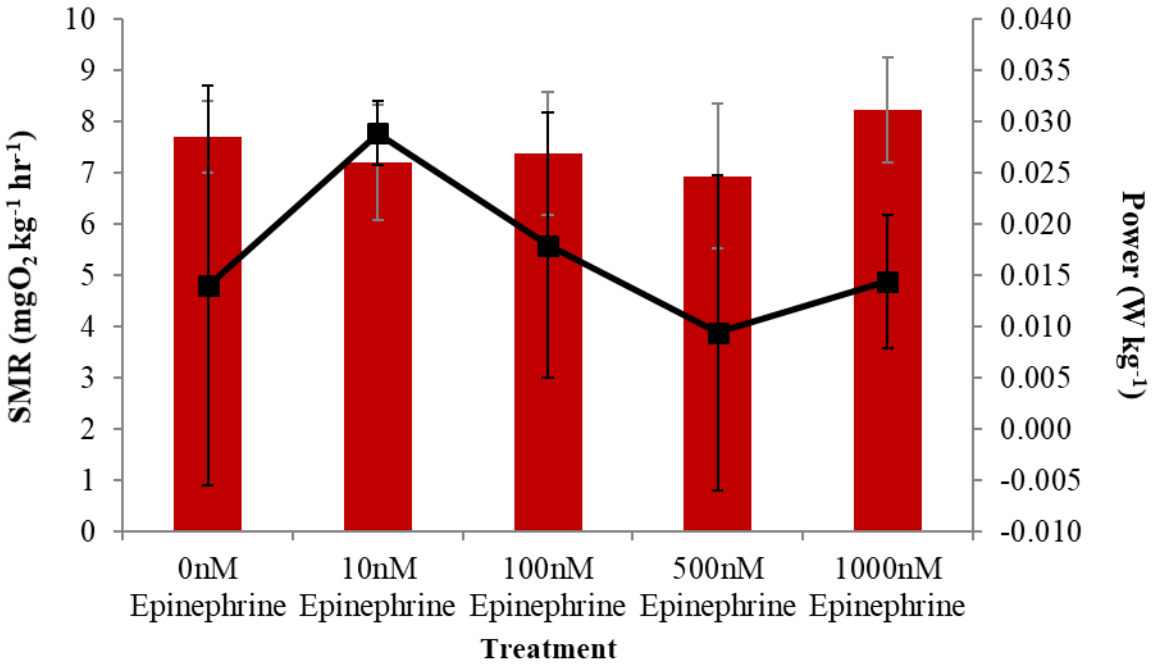

Figure 3.14 Effects of epinephrine on SMR and red muscle power output at 22ºC. Bars indicate standard metabolic rate (SMR), and line indicates the average power (W kg^{-1}) output at the start of each series of contractions (i.e. cycles 1, 21, 41, 61 and 81). Values are mean ± SEM. There was no significant difference between any of the concentrations of epinephrine for either SMR or power ($P = 0.841$). SMR Control (0nM epinephrine) n = 7, 10nM n = 4, 100nM n = 4, 500nM n = 3, 1000nM n = 3. Power at each concentration n = 3.

Oligomycin (n = 3) and ethanol (n = 5) were used to confirm that the decline in oxygen in the saline was attributable to muscle metabolism. Red muscle SMR significantly decreased when oligomycin and ethanol were added to the saline (Figure 3.15) (Control: 7.70 ± 0.69 $mgO_2\ kg^{-1}\ hr^{-1}$; Oligomycin: 4.30 ± 0.15 $mgO_2\ kg^{-1}\ hr^{-1}$; Ethanol: 2.71 ± 1.01 $mgO_2\ kg^{-1}\ hr^{-1}$). This result is similar to results from rat skeletal muscle, where oligomycin resulted in a 51% decline in SMR, also measured as rate of oxygen consumption (Rolfe et al., 1999), adding support to the notion that the observed rate of oxygen consumption can be attributed to SMR of fish skeletal muscle. Oligomycin inhibits oxidative ATP synthase, but we would not expect $\dot{V}O_2$ to reach zero because of proton leaking (Guderley, 2004). Ethanol was added in an attempt to completely reduce all oxygen consumption and cause cell death such that no O_2 would be required, although it appears this was not completely successful. A paired t-test was conducted on white muscle SMR compared to white muscle in 50% ethanol, and there was also significant decline in SMR (Paired T-test, T =67.725, d.f. = 1, P = 0.0094, Figure 3.16).

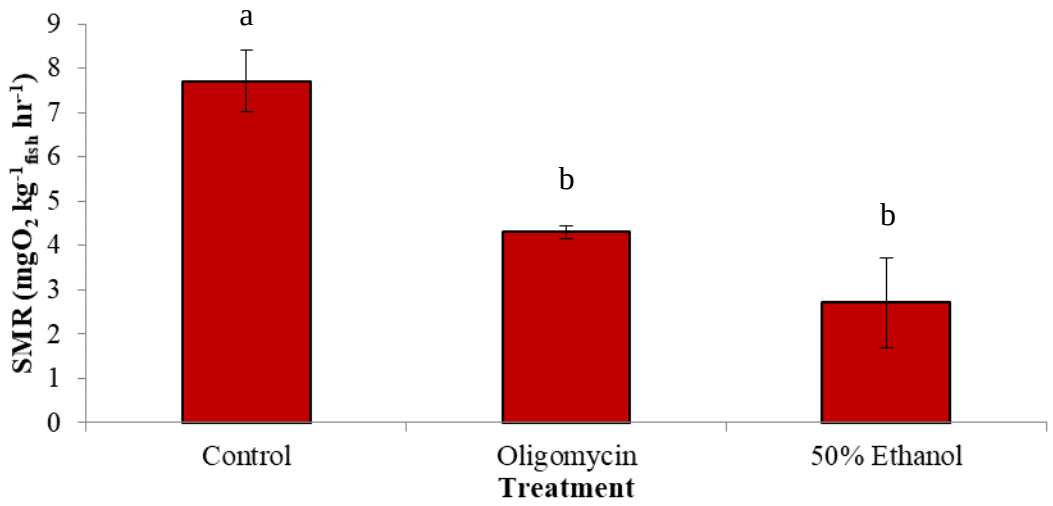

Figure 3.15 Standard metabolic rate (SMR) of red skeletal muscle of rainbow trout (*O. mykiss*) initially, with the addition of oligomycin (1mg/mL) and 50% ethanol. Values are mean ± SEM. Different letters indicate significantly different values ($P < 0.05$). Control n = 7, oligomycin n = 3, ethanol n =5.

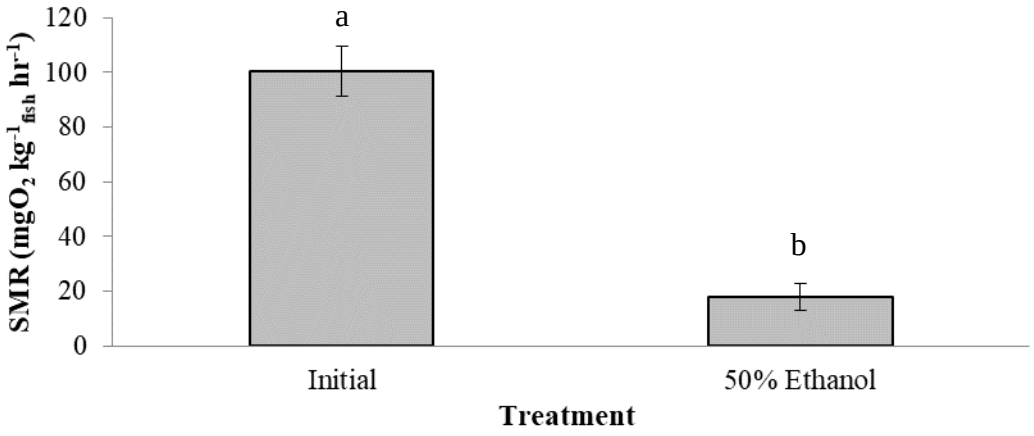

Figure 3.16 Standard metabolic rate (SMR) of white skeletal muscle of rainbow trout (*O. mykiss)* initially and with the addition of 50% ethanol. Values are mean ± SEM. Different letters indicate significantly different values ($P < 0.05$).

3.4 Discussion

3.4.1 The effect of temperature on aerobic metabolism of red muscle

Oxygen consumption of red muscle mitochondria in rainbow trout has been previously examined, demonstrating that mitochondrial oxygen consumption increases with increasing temperature (e.g. Bouchard and Guderley, 2003; St-Pierre et al., 1998). This agrees with the measured SMR of red skeletal muscle, which increased from 115 at 12ºC to 389 $mgO_2\ kg^{-1}\ hr^{-1}$ at 26˚C, a Q_{10} of 2.4 (Table 3.1; Table 3.2). A similar Q_{10} has been observed in other studies of whole animal metabolic rates in teleosts (reviewed by Clarke and Johnston, 1999; Rome and Swank, 1992), which suggests a similar pattern of change in metabolic rate of muscle as in the intact animal.

Temperature appears to have a similar effect on resting metabolic rates in rainbow trout at the cellular, tissue and whole organism level. Oxygen consumption of red muscle mitochondria in rainbow trout increases with increasing temperature (e.g. Bouchard and Guderley, 2003; St-Pierre et al., 1998). This agrees with the SMR of red skeletal muscle, which also increased with increased temperature (Table 3.2; Table 3.3), and a similar Q_{10} has been observed in other studies of whole animal metabolic rates in teleosts (reviewed by Clarke and Johnston, 1999; Rome and Swank, 1992), and tissue oxygen uptake increased by 3-fold from 15ºC to 35ºC in rat hindlimb skeletal muscle (Seiyama et al., 1990). These similar values of Q_{10} suggest a similar pattern of change in metabolic rate with temperature across hierarchies of organization, which likely reflects the reliance at all levels on oxidative metabolism in mitochondria.

There also appear to be similarities across taxa and tissue types regarding the increase in metabolic rate from rest to the working state in muscle, and the effects of temperature on this relationship. For example, the ratio of MMR to SMR (i.e. FAS) decreased from 4.2 at 15ºC to 2.6

at 25ºC in frog *(Xenopus laevis*) skeletal muscle (Seebacher et al., 2014), and from 5.3 at 14ºC to 2.8 at 24ºC in rainbow trout skeletal muscle (Table 3.2). Similarly, the ratio of MMR to SMR of rainbow trout cardiac muscle is ~4 at 15ºC (Harwood et al., 2002), although the SMR and MMR of cardiac muscle are 1.7 and 1.4 times greater than skeletal muscle, respectively (Gibbs et al., 1967; Harwood et al., 2002). Further, FAS in dog *(Canus familiaris*) skeletal muscle is 5.2 at 37ºC (Duran and and Renkin, 1976). Therefore, the relative increase in metabolic rate of muscle from a resting to active state, which should also reflect the cost of locomotion, seems similar for dog, frog and trout muscles within their thermal optima, and the rate of oxygen uptake of excised muscle tissue from several species seems to be similarly affected by changes in temperature.

Unlike SMR, MMR did not change as temperature was increased from 12ºC to 26ºC, with a mean Q_{10} of 0.96 (Table 3.4). This consistency of MMR, despite temperature change, was also observed by Seebacher et al. (2014) using frog muscle. Although not statistically significant, MMR appeared to decline as temperature increased from 18ºC to 24ºC, but then increased as temperature increase to 26ºC. A fall in MMR with increased temperature would be consistent with the fall in maximal power produced by the muscle (Figure 3.8), and would suggest that MMR in muscle is closely associated with the rate of energy used to produce power. However, an increase in MMR from 24ºC at 26ºC would be unexpected based on the continued fall in power output with increased temperature. This dissociation between power and metabolic rate at very warm temperatures may reflect failure of some aspects of homeostasis at very warm temperatures, such as membrane permeability and ion regulation (Fangue et al., 2009; Kraffe et al., 2007; Sappal et al., 2014). Further, this increase in muscle MMR from 24ºC to 26ºC occurred at temperatures approaching CT_{max} (see Chapter 2), supporting the idea that the muscle is also failing at these temperatures, and that temperatures approaching CT_{max} have a negative impact

(i.e. a substantial increase) on the aerobic metabolic cost of muscle contraction despite failure of the capacity to do work by the muscle. This was further supported by the significant decline in mechanical efficiency, as efficiency transitioned from positive to negative values between 24ºC and 26ºC (Table 3.4).

AAS did not decline significantly with increased temperature (Figure 3.8), which would suggest that failure of scope for sustaining aerobic metabolism does not occur in muscle at warmer temperatures. This may reflect the failure of muscle to produce power when very warm, which in itself should lead to reduced metabolic demands. Conversely, FAS showed a significant decrease with increasing temperature, declining from about 8.6 at 12ºC to 3.5 at 26ºC (Figure 3.9). The decline was due predominantly to a rise in SMR with increased temperature, rather than a fall in MMR. This decline is consistent with other studies demonstrating a greater effect of temperature on SMR than on MMR, and that muscle is negatively affected at extreme temperatures (reviewed by Farrell, 2008; Sänger and Stoiber, 2001). However, MMR of red muscle was still more than 3-fold greater than SMR at temperatures approaching CT_{max} of rainbow trout, and AAS was reduced but still substantial at 26ºC (repeated measures ANOVA, F=3.074, d.f. = 5, P = 0.406; Figure 3.8). If muscle aerobic scope was limiting CT_{max}, then it would be expected that FAS would be near 1, and that AAS would be near zero at temperatures approaching CT_{max}, which they were not.

3.4.2 The effect of temperature on the resting aerobic metabolism of white muscle

SMR of white muscle increased with temperature as well, and had a Q_{10} of 1.8 from 12ºC to 26ºC (Table 3.2), compared to 2.4 in red muscle. Aerobic metabolic rate of sculpin white muscle has a similar Q_{10} of 1 – 2 in swimming fish, which is tail-beat frequency dependent

(Johnson and Johnston, 1991), although this would reflect active metabolism and not just SMR. These data suggest that temperature effects on white muscle metabolic rate are slightly less than red muscle. When standardized to muscle mass, SMR of red muscle was 2 to 3 times greater than white muscle at all temperatures. This is likely due to the anaerobic nature of white muscle, which only has half the mitochondrial volume per mass of tissue seen in red muscle (reviewed by Sanger 1993).

Despite the mass-specific metabolic rate of red muscle being greater than white muscle, there is far more white muscle in the fish than red muscle. Red muscle is usually less than 10% of the whole fish, but can range from 0% to nearly 30% (Bernal et al., 2003; Greer-Walker and Pull, 1975). When the metabolic rates of red and white muscle were expressed relative to the amount of red and white muscle in these rainbow trout (2.73% and 70.8%, respectively), the rate of resting oxygen consumption was significantly greater in white muscle than in red muscle, making the SMR of total white muscle 7.2 to 13.2 times greater than red muscle, depending on temperature (Table 3.1). While white muscle is normally considered anaerobic and red muscle aerobic, it might not be surprising that white muscle contributes so significantly to SMR. SMR is mostly attributed to cellular maintenance, which would be supported aerobically in both red and white muscle, and white muscle volume was nearly 24 times greater than red muscle (Figure 3.15). However, while not measured in the present study, the oxidative contribution of working anaerobic (white) muscle is expected to be relatively small (Arthur et al., 1992; Driedzic et al., 1983).

3.5 Conclusion

In summary, it was observed that SMR for both red and white muscle increased with increasing temperature, implying a metabolic effect of temperature on cellular maintenance processes directly in muscle tissue. The lack of effect of increasing temperature on MMR, but significant decline in power output at higher temperatures, suggests that muscle contractile properties appear to be inhibited at high temperature, despite no change in aerobic energy use. The decline in muscle efficiency with increased temperature supports this notion, such that effective energy conversion into mechanical work is diminished at extreme temperatures. The significant failure of power output at 26ºC, despite increased or maintained oxygen consumption, could point to some hindrance of cellular performance as temperature approaches the CT_{max}. Therefore, it is possible that these increased metabolic costs but reduced muscle contractile capacity at high temperatures could be an important contributor to fish death. Further, while AAS did fall at warmer temperatures, it was still substantial and did not appear to be collapsing toward zero as temperature approached CT_{max}, suggesting that collapse of aerobic scope is not the cause of muscle failure or death at CT_{max}. However, high aerobic costs of skeletal muscle at rest might contribute to a reduced aerobic scope in the whole fish.

Chapter Four: **Synthesis of Muscle and Whole Fish Studies**

4.1 Discussion

In order to overcome the drag forces acting against fish when they swim, a certain amount of power is required by the muscle, and the faster a fish swims the greater this power requirement will be. Assuming that metabolic rate of muscle will be closely related to its mechanical power output, I maximized power output in the muscle to determine the maximum metabolic rate of the muscle. While this may be an over representation of red muscle power in a swimming fish (reviewed by Syme 2005), my objective was to maximize metabolic rate of the muscle and so this is not a significant concern. Maximal power output in red muscle occurred at cyclic frequencies nearly identical to the maximum TBF of a fish swimming at V_{O2max} as recorded in the swim tunnel experiments. Tail beat frequencies for my fish at V_{O2max} were about 5.4Hz, and the greatest power output from red muscle occurred at frequencies between 3 – 5 Hz (Table 3.7). Further, these frequencies are consistent with EMG studies done by Coughlin (2000), who saw the highest aerobic swim speeds were achieved with tail beat frequencies of ~3.5 – 4 Hz, and is similar to other studies that have examined TBF of rainbow trout of similar size (e.g. Webb, 1971; Webb et al., 1984). Thus, the muscle contraction conditions used to measure maximal metabolic rate from the muscle were similar to those used by swimming fish.

4.1.1 Comparing whole fish metabolic rates to muscle metabolic rates

The mean metabolic rates of muscle relative to the whole fish were compared at each temperature (Table 4.1; Figure 4.1). These values were not statistically comparable because individual measurements of muscle metabolic rate and whole fish metabolic rate were not taken from each fish, and thus the ratios are simply the average of two means. As discussed in the

previous chapter, red and white muscle metabolic rates were standardized to the whole fish by multiplying the rate of oxygen consumption per mass of tissue by the relative proportion of muscle in the whole fish. SMR for both muscle types increased with temperature, similar to the increase in SMR seen by the whole fish, such that red muscle SMR accounted for 3.9 – 8.5% of whole fish SMR across temperatures and red muscle MMR contributed between 3.7% and 6.5% of the whole fish MMR across all temperatures (Table 4.1, Figure 4.2). White muscle SMR contributed much more significantly to whole fish SMR, being 71.4% of total SMR at 12ºC, and declining to 33.4% at 26ºC (Figure 4.1).

Table 4.1 The percent contribution of the metabolic rate of muscle to the corresponding metabolic rate of the whole fish, at different test temperatures (e.g. red muscle MMR/whole fish MMR*100). Muscle metabolic rate was calculated by multiplying the mass-specific metabolic rate of muscle tissue by the total mass of muscle in a fish, as measured from the cross-section images (see Chapter 3). SMR is standard metabolic rate, MMR is maximum metabolic rate, and AAS is absolute aerobic scope. Values were calculated using the mean measured from muscles and the mean from whole fish.

Contribution to Whole fish (%)	12ºC	14ºC	18ºC	22ºC	24ºC	26ºC
Red SMR	5.38	5.72	8.54	4.57	4.03	3.87
Red MMR	5.55	5.67	6.46	5.22	3.69	5.71
Red AAS	5.57	5.66	6.08	5.48	3.55	7.58
White SMR	71.4	53.9	61.7	42.3	41.6	33.4

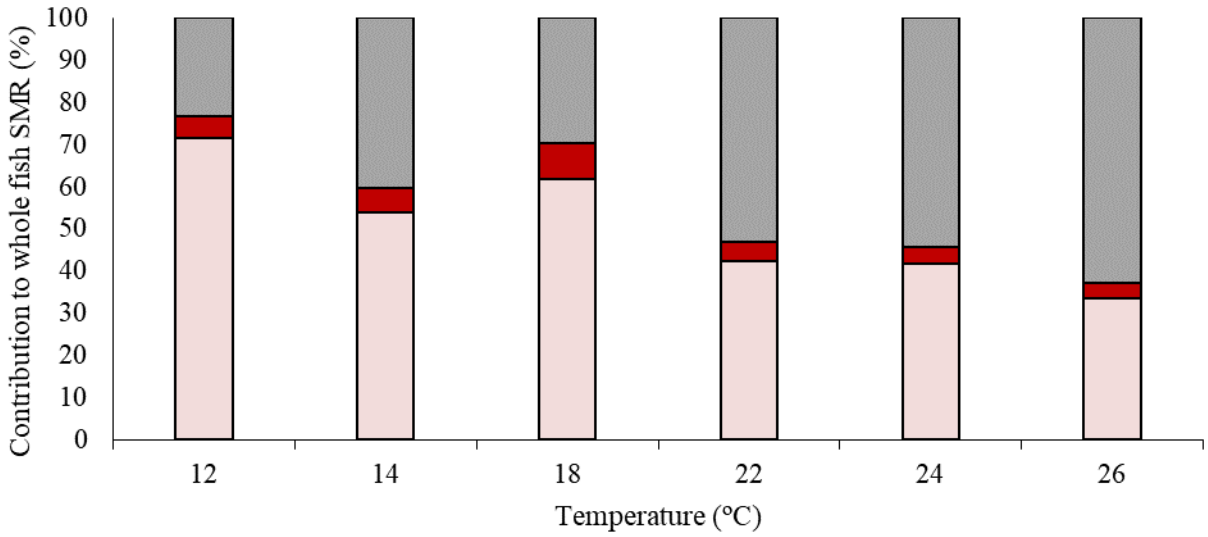

Figure 4.1 Percentage contribution of muscle tissue to whole fish standard metabolic rate (SMR) in rainbow trout *(O. mykiss*) acclimated to 12ºC and tested across a range of temperatures. Beige bars are white muscle, red bars are red muscle, grey bars are the remaining resting metabolic cost of other, non-skeletal muscle metabolism. Skeletal muscle contributions were calculated from the mean values at each testing temperature divided by the means of the whole fish at each temperature.

Red muscle AAS was 3.5 – 7.6% of the whole fish AAS across all temperatures (Table 3.1, Figure 4.3). It was initially anticipated that a larger portion of the whole fish metabolism would be accounted for by red muscle metabolic rate, primarily because of the highly oxidative nature of red muscle, and because it is almost exclusively powering locomotion during sustained swimming where MMR is suggested to be maximal during maximal swimming (Altringham and Ellerby, 1999; West et al., 1993). Thus, it appears that red muscle makes a relatively small contribution to whole fish metabolism, even during vigorous swimming, and thus that red muscle is not determining either the resting or active aerobic metabolism of fish. However, considering that only 2.7% of the whole fish is red muscle, and the average contribution of red muscle to total aerobic metabolic rate of the fish across all temperatures was 5.65 ± 0.53%, which is nearly

double what would be expected based on the relative volume of red muscle alone, red muscle appears to be contributing a disproportionately high amount to whole fish metabolism, which is consistent with the relatively high metabolic rate of working muscle.

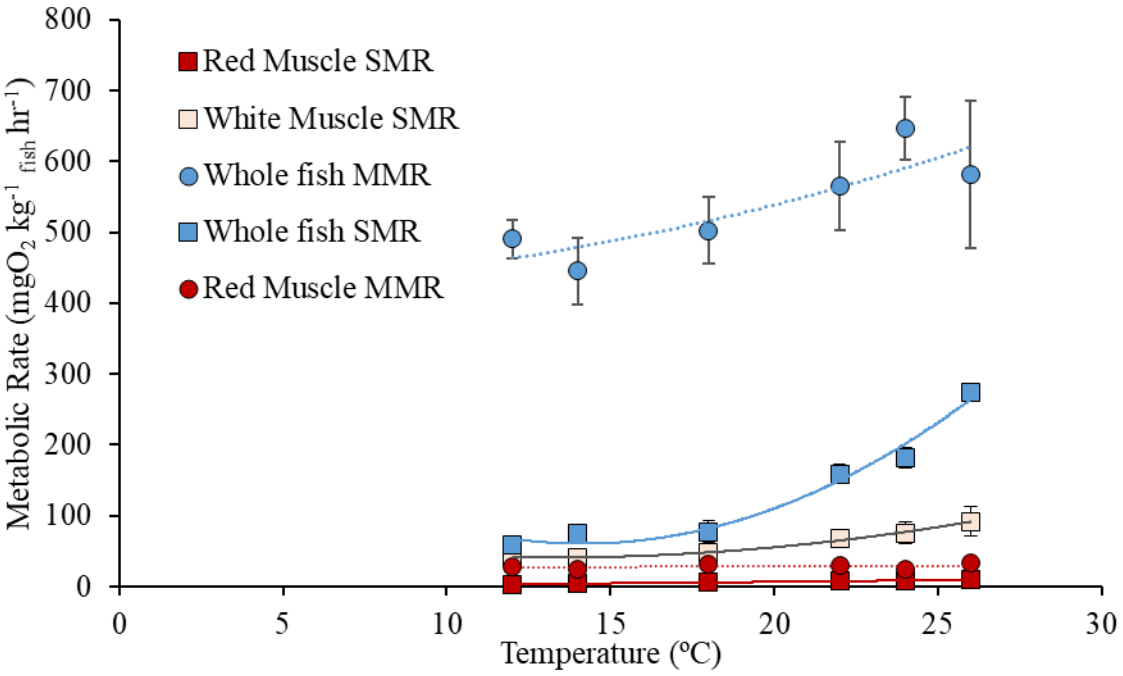

Figure 4.2 The aerobic metabolic rate of whole fish, red muscle and white muscle of rainbow trout (*O. mykiss*) acclimated to 12ºC at various temperatures. Red muscle and white muscle are in relation to their total contribution to whole fish (assuming 2.73% and 70.8% of fish mass, respectively). Red muscle standard metabolic rate (SMR) (red, square) fitted regression $y = 0.0076x^2 + 0.155x + 0.488$; $R^2 = 0.900$. Red muscle maximum metabolic rate (MMR) (red, circle) fitted regression $y = -0.0127x^2 + 0.695x + 20.3$; $R^2 = 0.105$), white muscle SMR (beige, square) fitted regression $y = 0.300x^2 - 7.78x + 91.2$; $R^2 = 0.996$, whole fish SMR (blue, square) fitted regression $y = 1.45x^2 - 41.1x + 356$; $R^2 = 0.975$, whole fish MMR (blue, circle) $y = 0.304x^2 - 0.333x + 424$; $R^2 = 0.746$. Values are means ± SEM.

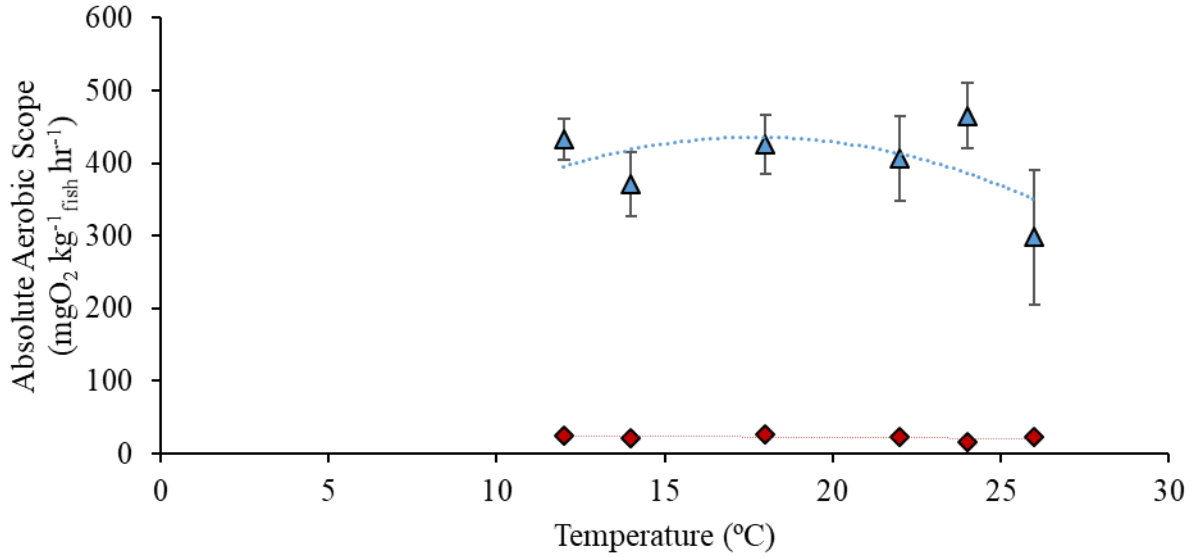

Figure 4.3 The absolute aerobic scope (MMR – SMR) of whole fish and red muscle of rainbow trout acclimated to 12ºC across a range of acutely tested temperatures. Whole fish AAS (blue, triangle) fitted regression $y = -1.25x^2 + 44.3x + 43.7$; $R^2 = 0.261$, and red muscle AAS (red, diamond) fitted regression $y = -0.0203x^2 + 0.540x + 19.8$; $R^2 = 0.18$. Values are means ± S.E.M.

Conversely, white muscle SMR contributed about 10-fold more to whole fish SMR than that of red muscle, being between 33 and 71% of the whole fish's metabolism (Table 4.1). Even though white muscle is largely anaerobic, the substantial contribution of white muscle to whole fish SMR was not surprising, considering white muscle was 70.8% of the whole fish mass. Thus, even a relatively low aerobic metabolism in white muscle becomes significant given how much white muscle is in the fish. However, despite the high contribution of white muscle to whole fish metabolism, the mass-specific rate of oxygen consumption of red muscle tissue at rest was still 1.95 times greater than white muscle tissue (Table 3.2). This higher rate of metabolism in red muscle was to be expected for several reasons: mitochondria counts, muscle capillarization and blood perfusion for red muscle are all at least double that of white muscle (Cameron and Cech, 1970; Davie et al., 1986; Johnson et al., 1991; Johnston et al., 1985; Neumann et al., 1983).

When considered together, red and white skeletal muscle contributed 37 – 77% of the whole fish resting metabolism, a very substantial proportion.

4.1.2 Temperature effects on the contribution of muscle to whole fish aerobic metabolism

Both red and white muscle SMR increased with increasing temperature, similar to what was seen in whole fish SMR (Figure 4.2). One explanation could be that there is an increase in mitochondrial respiration leak (Kraffe et al., 2007; reviewed by Khan et al., 2014). Mitochondrial proton leak would increase with acute increases in temperature, and thus the respiratory rate of mitochondria increases (Fangue et al., 2009; Sappal et al., 2014; Sappal et al., 2015). However, SMR increased at a greater rate for red muscle than for white muscle. It is possible that this is related to the greater proton conductance and lower membrane fluidity in white skeletal muscle over red muscle (Khan et al., 2014; Leary et al., 2003). Other factors could include oxygen availability in the tissues, which in this study was based on diffusion but in living fish would also be dependent on perfusion, but the effect of acute temperature changes on the ability to transport oxygen into each tissue type is unknown.

White muscle's contribution to whole fish metabolic rate decreased by about half as temperature increased from 12ºC to at 26ºC, while that of red muscle did not change notably (Figure 4.1), so that skeletal muscle contributed about 77% to whole fish metabolism at cool temperatures, but only about 37% at the warmest temperatures, and most of this was due to white muscle at all temperatures. This suggests that at low temperatures white muscle accounted for the majority of resting metabolism in fish, but as temperature increased beyond 22ºC, metabolic activity of other tissues dominate metabolism of the fish. Thus, white muscle, not red muscle,

tends to determine resting metabolism in fish, but temperature plays an important role in affecting this relationship.

Red muscle MMR, much like whole fish MMR, was not affected by temperature (Figure 4.2), and SMR of muscle and whole fish increased with temperature. Thus, the effects of temperature on the AAS and FAS were similar between whole fish and red muscle (Figure 4.3, Figure 4.4). FAS of both muscle and whole fish declined with increasing temperature, suggesting that an increase in temperature affects metabolism of skeletal muscle similarly to the whole fish, as might be expected in a poikilotherm. However, red muscle FAS at 26ºC was 1.7 times greater than whole fish FAS, suggesting that even though SMR increased with temperature for both muscle and whole fish, the 'resting' component of metabolism was a greater fraction of MMR in whole fish. This would also suggest that aspects of metabolism in addition to red muscle are also a significant contributor to whole fish metabolism.

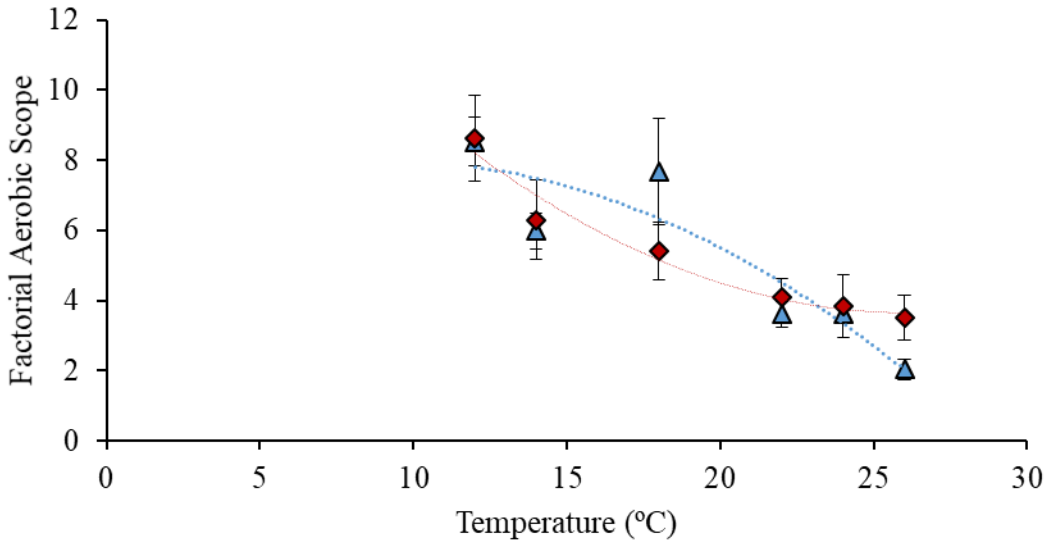

Figure 4.4 The factorial aerobic scope (MMR/SMR) of whole fish and red muscle of rainbow trout acclimated to 12ºC across a range of acutely tested temperatures. Whole fish FAS (blue, triangle) fitted regression $y = -0.021x^2 + 0.385x + 6.21$; $R^2 = 0.834$, and red muscle FAS (red, diamond) fitted regression $y = 0.0229x^2 - 1.20x + 19.2$; $R^2 = 0.960$. Values are means ± S.E.M.

Of note, the metabolic contribution of working red muscle to whole fish AAS increased substantially to 7.6% at 26ºC (Table 4.1). This disproportionate rise between 24ºC and 26ºC (i.e. double that of any other temperature) could be a result of the low sample size of whole fish metabolic measures at 26ºC (n = 2), but could also be attributed to the notable fall in power output and efficiency of muscle at high temperatures (Table 3.5), and a sustained high MMR. A loss in power could mean the fish must increase recruitment of red muscle to sustain swimming at high temperatures, and along with a high metabolic rate would lead to a loss of aerobic scope in the muscle and the fish.

4.1.3 Comparing blood flow to metabolism of muscle

If the rate of blood flow to muscle is an accurate measure of metabolic rate, then given the number of studies conducted on rainbow trout blood flow and cardiac output (e.g. (Cameron and Cech, 1970; Clark et al., 2008; Davie et al., 1986; Farrell, 2002; Gerry and Ellerby, 2014; Heath and Hughes, 1973; Neumann et al., 1983), some estimates of the relative contributions of red and white muscle to metabolism in intact fish can be made. Gerry and Ellerby (2014) examined blood flow in resting and swimming rainbow trout acclimated to 15ºC and saw that red muscle received ~13% of the total blood flow when at rest while white (mosaic) muscle received nearly 40% (at 15ºC). Similarly, the present study showed that white muscle SMR (14ºC) was 53.9% of the whole fish SMR, while red muscle was about 6%. Both observations support the contention that a large portion of the resting metabolic rate of trout is attributed to skeletal muscle, and specifically white muscle.

With respect to MMR, when the fish swam at ~75% U_{crit} (i.e. approaching $\dot{V}O_{2max}$), Gerry and Ellerby (2014) observed blood flow to red muscle increased to 45% of total blood flow, a

3.5-fold increase over flow at rest. Similarly, at 14ºC, the present study observed red muscle MMR to be 5 times greater than muscle SMR, although red muscle contributed only ~5.7% to whole fish MMR, considerably less than blood flow would predict. Therefore, this suggests that the contribution of muscle metabolism to whole fish metabolism changes considerably with exercise.

4.2 Conclusion

White muscle SMR increased with increasing temperature, and was a major contributor to whole fish SMR. Although this contribution decreased substantially with increased temperature, the metabolic cost of white muscle maintenance remained a significant component of fish SMR even at high temperatures. While red muscle SMR showed similar patterns of increase with increased temperature, it remained a relatively small component of whole fish SMR. Further, red muscle MMR also did not dominate aerobic scope of swimming fish as predicted, being only 4-6% of total MMR in the whole fish. However, red muscle power output fell substantially at temperatures approaching CT_{max}, while the rate of oxygen consumption did not, resulting in a fall in muscle efficiency but a maintained contribution to MMR. This impediment of increasing temperature on red muscle contractile capacity is likely an important limiter to the survival of fish at high temperatures, and Seebacher et al. (2014) put forth the prediction that the relationship between ATP usage and power output with temperature likely cannot be offset by acclimation, whereby warm temperatures appear to be an insurmountable limit to fish survival over acute and acclimation time periods. However, some studies have shown that strains of rainbow trout occupying warmer climates have a slightly higher CT_{max} than those at cooler climates, such as the ones used in this study, and thus there must be some cellular adaption to account for this improved thermal success (Chen et al., 2015; Gamperl et al., 2004). Munoz et al. (2015) also discusses the concerns of increasing environmental temperature and a lack of genetic plasticity in the context of the onset of cardiac arrhythmia in salmonids. Cardiac arrhythmia in salmonids is considered a strong indicator of nearing CT_{max} and is frequently observed at 26ºC (Chen et al., 2015; Farrell, 2002; Gamperl and Farrell, 2004; Heath and Hughes, 1973; Scott et al., 2014). Thus, some fish may have the genetic advantage of being adapted to warmer temperatures, but

the rate of global temperature increase could exceed the rate of adaptive response for many salmonids, even fish in general (Muñoz et al., 2015), which would likely have significant effects on their muscle performance and thus survival.

It was predicted that whole fish aerobic scope would narrow significantly with increasing temperature, and that the temperature at which SMR and MMR converged would be near CT_{max}, which would suggest that aerobic scope might limit CT_{max}. However, we observed fish death at 26ºC when aerobic scope remained substantial, in both the whole fish and the red muscle, suggesting that CT_{max} is a result of other physiological impairments beyond a reduced aerobic scope. MacMillan (2019) pointed out that it is likely not a single factor determining CT_{max}, and many variables have the potential of altering CT_{max} for individuals and species. It is likely that there is no one specific cause of death at high temperatures in fish, but cumulative negative effects, likely including increasing SMR in skeletal muscle as was seen in this study. Although muscle metabolic rate was not identified as the likely determinant for CT_{max}, this study revealed that the physiological impediments of temperature on muscle metabolism and contractile capacity likely add to the breakdown of function at high temperatures for these poikilothermic ectotherms. Knowing the metabolic rate of skeletal muscle also adds to the collective understanding of temperature effects on salmonid physiology, and adds one more piece to the unfolding puzzle of why extremes of temperature can be detrimental to the survival of an organism.

4.3 Future Directions

Although it was suggested that acclimation to warm temperatures may not impact the ability of muscles to function when warm, this remains to be tested, particularly how thermal acclimation impacts metabolism of muscle. Future work to replicate this study on fish acclimated to warmer temperatures, rather than being only acutely warmed, could reveal if there exists plasticity to allow shifts in skeletal muscle metabolic rate, which can be useful in identifying the success of certain species with increasing changes of environmental temperatures. Also, this study needs to be replicated for different species with different thermal niches, to expand our understanding of the effects of temperature on muscle metabolism and its contribution to metabolism of the whole fish beyond just rainbow trout. Additionally, identifying the aerobic metabolic rate of white muscle doing work and the relative contribution to aerobic scope would improve our understanding of contributors to total aerobic scope, since white muscle is the dominant tissue in most fish species, and we also need to know more about the relative amount of white muscle contributing to power production at any given swim speed, which is currently not known.

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