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Muscle Na+/K+-ATPase isoform regulation in rainbow trout (Oncorhynchus mykiss) following burst and sustained swimming challenges

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Thesis Abstract

The sodium-potassium ATPase (Na^+/K^+-ATPase) is an important ion transporter which pumps sodium out of cells in exchange for potassium, thereby generating large gradients of these ions across the plasma membrane. In skeletal muscle, maintaining these gradients is essential for the induction of action potentials that lead to muscle contraction. Mammalian model systems have been examined to understand the physiology of muscle Na^+/K^+-ATPase regulation during exercise and have shown isoform-specific responses depending on exercise type and duration. Despite this progress many questions remain regarding Na^+/K^+-ATPase dynamics, specifically how different subunits of this protein (α, β) are transcriptionally regulated during and after exercise. To better understand the role and regulation of Na^+/K^+-ATPase in muscle contraction, I examined the response of rainbow trout to sustained and burst swimming challenges. Rainbow trout are often recognized for their great 'athleticism,' with lifecycles including extensive seaward and spawning migrations, often in the face of a variety of environmental obstacles.

Using a swim tunnel, juvenile rainbow trout were repeatedly swum at either sustainable (3 body lengths/s) or burst (8 body lengths/s) speeds based on preliminary swimming trials. Red muscle, white muscle, and heart samples were collected at various time points during training and recovery and relative mRNA levels for the α1c, α2 and α3 isoforms of the Na^+/K^+-ATPase were determined. Plasma levels of chloride as well as total osmolality were also measured. I found that the expression of individual Na^+/K^+-ATPase α- subunit isoforms is differentially regulated between muscle types in rainbow trout during both burst and sustained swimming. Red muscle was more responsive to

these exercise challenges, in contrast to little to no change observed in mRNA expression in white muscle and heart tissues. After four consecutive days of the sustained swim protocol, the α2 isoform increased two-fold in red muscle, with a trend towards an upregulation of α1c and α3. No significant change in ion balance was observed following this low-intensity sustained swim protocol. These results indicate that there is moderate demand for increased transcription of the Na^+/K^+-ATPase between two and four consecutive days of swimming at 3 BL/s, able to be regulated to normal levels by eight days. In contrast, burst swimming significantly increased mRNA for all three isoforms, decreased plasma chloride, and significantly reduced total osmolality. This study is the first to show an upregulation of Na^+/K^+-ATPase α isoform expression in teleost fish muscle following exercise challenges, suggesting this enzyme plays an important role in muscle contraction.

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CHAPTER 1: Structure and Function of the Na^+/K^+-ATPase: A Comparative Review

Introduction

All animal cells constitutively express the essential protein commonly referred to as the sodium (Na^+) pump, or more formally the Na^+/K^+ -stimulated adenosine triphosphatase (Na^+/K^+-ATPase). This multi-subunit protein is critical for cell as well as whole body homeostasis, playing a role in many physiological processes. Its most basic function is the active transport of sodium ions (Na^+) out of the cell and active uptake of potassium ions (K^+) into the cell in a 3:2 ratio respectively, using the energy of one ATP molecule (Lingrel and Kuntzweiler 1994). The ion gradients maintained by the Na^+/K^+-ATPase are used by excitable cells for many functions, including the regulation of action potential and maintenance of membrane potential. In addition, these gradients play a crucial role in the secondary transport of many other molecules across the plasma membrane, and are essential for the regulation of cell volume and whole body ion and water balance (Blanco and Mercer 1998).

The Na^+/K^+-ATPase is composed of an alpha (α) and a beta (β) subunit, forming a heterodimer. In some studies a third, gamma (γ), subunit has been identified but its presence does not appear to be required for the normal functioning of the enzyme. To date, four α- (α1, α2, α3, and α4), four β- (β1, β2, β3 and β4 /β_M) and one γ subunit isoforms have been identified in mammals. Many organisms express these Na^+/K^+-ATPase subunit isoforms, with amino acid sequences being highly conserved across species. Additionally, these isoforms appear to be expressed in a tissue-specific pattern suggesting individual isoforms may have specific cellular functions. The Na^+/K^+-ATPase heterodimer can be formed from various combinations of α and β subunit isoforms, thereby forming a variety of isozymes that may exhibit distinct molecular or biochemical properties (Blanco and Mercer 1998). Despite a significant amount of

research on mammalian Na^+/K^+-ATPase physiology, our understanding of the potential functions and roles of various Na^+/K^+-ATPase isoforms is still quite limited.

In skeletal muscle, the Na^+/K^+-ATPase has been identified as an important active ion transporter. Muscle contraction requires regulation of many ion gradients across the sarcolemma and T-tubules. These gradients must be maintained for proper membrane potential and induction of action potentials along the T-tubule. Muscle contraction during exercise is a demanding physiological challenge, which causes rapid shifts in major ions across the cell membrane, including the inward flooding of sodium, which eliminates membrane depolarization needed for future action potentials in active muscle.

It is known that multiple isoforms are present in mammalian and fish skeletal muscle, with α2 showing muscle-specific expression, however our current understanding of their roles here are very limited and it is uncertain whether certain isoforms may be involved in specific physiological challenges during exercise. My thesis took a comparative approach by examining the expression of three distinct α alpha isoforms in skeletal muscle (red and white fiber types) and heart tissues of rainbow trout (*Oncorhynchus mykiss*) following either sustained (endurance-type) or burst (sprint-type) swimming. Rainbow trout are excellent models for exercise physiology research in that most populations naturally exhibit extensive seaward and spawning migrations, involving both sustained and burst swimming behaviors. By investigating expression patterns for these isoforms in response to various demands on teleost muscle, it is possible to put these results in the broader context of what is known for other species. As studies such as this one continue, it may be possible to elucidate potential isoform-specific functions for the Na^+/K^+-ATPase.

Structure and Function of the Na^+/K^+-ATPase

The Na^+/K^+-ATPase is part of a large family of phospho-intermediate type (P-type) ATPases found in both prokaryotic and eukaryotic cells. 'P-type' ATPases are named for the unique formation of a phosphorylated aspartyl residue during the catalytic reaction (Blanco and Mercer 1998), and are classified by conserved features of their structure and function, including conformational changes during their reaction cycle, and the presence of a catalytic membrane-bound α-subunit with binding sites for ATP and specific cations (Mobasheri, Avila et al. 2000). Each P-type ATPase may possess non-conserved sequences, believed to be evolutionarily derived, thought to be for specialized functions related to transport of specific ions, regulation, and tissue-specificity.

Two main polypeptides, the α- and β-subunits, assemble to form the Na^+/K^+-ATPase in a 1:1 stoichiometric ratio. The α- subunit is a large trans-membrane protein that contains the ATP, Na^+, K^+ and ouabain (a Na^+/K^+-ATPase -specific inhibitor) binding sites and maintains the catalytic and transport function of the ATPase (**Figure 1**). The smaller β- subunit is essential for normal functions of the Na^+/K^+-ATPase, including control of enzyme affinity for sodium and potassium ions, and may act as a chaperone protein for the enzyme's proper folding and transport (Geering 1991). A third unique polypeptide sequence has also been observed in association with the α- and β-subunits, providing strong evidence for the existence of a gamma (γ) subunit (Collins and Leszyk 1987). At present, little is known about this very small, hydrophobic protein; however, homology among γ across species indicates a potentially important role in Na^+/K^+-ATPase function (Blanco and Mercer 1998).

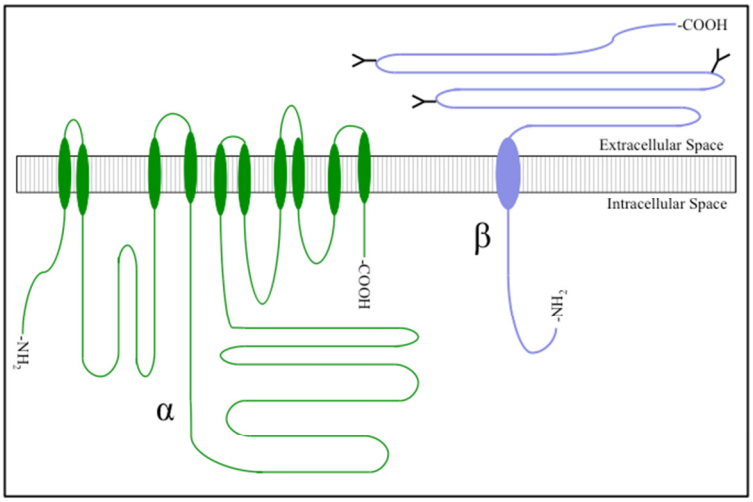

Figure 1. Schematic diagram representing the molecular protein structure of the Na^+/K^+-ATPase alpha (α) and beta (β) subunits within the plasma membrane of a cell. Adapted from Blanco and Mercer (1998).

Na^+/K^+-ATPase Subunit Isoforms

A notable 1978 discovery in Na^+/K^+-ATPase research described a doublet-banding pattern which was observed after electrophoretic separation of the Na^+/K^+-ATPase α subunit protein from the brine shrimp (*Artemia salina*) (Peterson 1978). Each of these proteins not only had unique amino acid sequences, but also individual enzymatic properties, confirming at least two types of the α subunit polypeptide, termed isoforms. One year later, work done on rat brains identified more than one Na^+/K^+-ATPase α isoform in mammals (Sweadner 1979). Techniques allowing the separation of two cell types in the brain, myelinated neurons and non-neuronal astrocytes, also provided the opportunity to examine the sodium pump in biochemically specialized cells. Sweadner's research revealed that the α isoform was only found in astrocytes while the α^+ was only found in the myelinated axons (Sweadner 1979). Since then, these isoforms have been renamed α1 and α2. After the development of successful cloning procedures

and the isolation of a third α- isoform, named α3 (Shull et al. 1986), it became clear that more Na^+/K^+-ATPase isoforms may exist. To date, a total of four α- (α1, α2, α3, α4) and four β- (β1, β2, β3, and β4/βM) subunits have been identified (Mobasheri et al. 2000).

The α1 and β1 isoforms appear to be constituitively expressed in all animal cells. Often called the "housekeeping" forms, these proteins are most likely involved in day-to-day cell and organism homeostasis. Strikingly, the remaining isoforms exhibit a more restricted tissue-specific expression. The α2 isoform mRNA and protein is expressed most prominently in muscle, adipose tissue, and myelinated axons of the brain, while α3 is abundant in the central nervous system with smaller amounts found in cardiac muscle, cartilage, and bone (Mobasheri, Avila et al. 2000). The α4 isoform has been isolated in the testes and localized in flagella of human sperm cells (Sanchez, Nguyen et al. 2006). A tissue-dependent distribution is seen in β-subunit isoforms as well, where β2 is found in skeletal muscle and nervous tissues and β3 is localized to testes, retina, liver, and lung (Blanco and Mercer 1998). This leads to the two principal questions guiding this study: Why is there such a diverse collection of Na^+/K^+-ATPase isoforms in animals and what are the physiological functions that underlie their differential expression?

Role of the Na^+/K^+ ATPase in Skeletal Muscle

Like all animal cells, skeletal muscle fibers have an outer plasma membrane, called the sarcolemma. The sarcolemma has many areas of invagination, known as transverse tubules (T-tubules), which extend deep into the cell and greatly increase the membrane surface area exposed to the extracellular space. Muscle fiber contraction requires that the sarcolemma become depolarized through rapid influx of sodium ions and

efflux of potassium ions (Clausen 2003). This membrane depolarization allows action potentials to travel along the T-tubules, activating voltage-gated ion channels, and triggering release of calcium ions from the sarcoplasmic reticulum which ultimately leads to muscle contraction.

The action potential-induced excitation of skeletal muscle contraction causes increased extracellular potassium and increased intracellular sodium. Once this occurs, cell excitability is reduced. Repeated contraction requires that both ions be actively transported such that the plasma membrane can promptly become depolarized again in preparation for another action potential. This is complicated by an increase in muscle water content during strenuous exercise, thereby reducing the volume of water in the blood plasma. This phenomenon, coupled with the movement of ions during action potentials, causes a significant increase in extracellular potassium and intracellular sodium. While high levels of potassium outside the sarcolemma have been suggested to have a role in muscle fatigue (Peterson 2005; McKenna 2008), low extracellular sodium has also been seen to reduce the amplitude of the action potential (Bezanilla 1972; Cairns 2003). Because the Na^+/K^+-ATPase is essential for restoring the sodium and potassium gradients after excitation and may therefore be expected to be a major regulatory target of activity and distribution. Expression of specific Na^+/K^+-ATPase isoforms may provide an additional level of regulation in that they may possess varying degrees of activity or specialized functions depending on the muscle activity required.

Na^+/K^+- ATPase Isoform Expression and Activity During Exercise

To date, nearly all research on Na^+/K^+-ATPase expression and function during exercise uses mammalian models. These experiments attempt to identify patterns of isoform-specific responses following muscle work, during/after recovery, as well as in different muscle types. Both mRNA and protein levels of individual isoforms have been measured, and the relative expression of each isoform appears dependent on the exercise regimen. Murphy *et al.* (2004) found a 3.5 fold increase in α3 mRNA immediately following knee extensor exercises in humans. Expression levels declined again by three hours post-exercise when an equivalent increase in α2 mRNA was detected. Following one day of recovery, both α2 and α3 expression returned to near resting levels, while α1 mRNA levels increased 2.5 fold (Murphy 2004), suggesting a time-dependent response of these isoforms. In general, these studies typically show an increase in Na^+/K^+-ATPase isoform mRNA relative to non-exercised controls immediately following intense, 'sprint-type' exercise, but little or no shift in expression after low intensity exercise (Nordsborg 2003; Murphy 2004; Murphy 2006; Rasmussen 2011). Interestingly, the rapid upregulation of Na^+/K^+-ATPase mRNA is not always accompanied by an immediate increase in protein expression and instead it may show a slightly delayed increase (Juel 2001; Murphy 2004; Rasmussen 2011). In other cases, no increase in isoform-specific protein abundance is seen after the specific challenge imposed (Murphy 2004).

The Na^+/K^+-ATPase maintains proper ion balance on both sides of the sarcolemma, enabling repetitive induction of action potentials. This event may occur at different firing frequencies dependent on whether the muscle is a "fast-twitch" (glycolytic) or "slow-twitch" (oxidative) fiber. It has been shown that fibers with greater

oxidative potential (*i.e.,* red muscle in comparison to white muscle) have higher levels of the Na^+/K^+ -ATPase protein, and higher expression of the α2 isoform (Fowles 2004). Two isoforms of the β- subunit have fiber-specific localization in rats, with β1 being highly expressed in red, oxidative muscle and β2 being highly expressed in white, glycolytic muscle (Hundal, Marette et al. 1993).

Na^+/K^+ -ATPase has been identified to play a significant role in muscle endurance and force recovery. After treatment with the Na^+/K^+-ATPase specific inhibitor ouabain, muscle fatigue increases and contractile force decreases (Nielsen O.B. 1996; Harrison 1997). Thus, shifts in Na^+/K^+-ATPase activity are proposed to improve the ability of the organism to properly regulate ion movement in muscle cells and minimize cellular damage. The up-regulation in the number of Na^+/K^+-ATPase pumps and overall enzyme activity is now a generally accepted physiological response to muscle activity, first observed in dogs after six weeks of treadmill running (Knochel 1985). While Na^+/K^+ -ATPase activity initially increases during muscle work (Nielsen 2004; Broch-Lips 2010; Juel 2011), the maximum attainable enzyme activity may actually be reduced after exhaustive exercise challenges, a phenomenon known as "Na^+/K^+-ATPase inactivation" (Leppik 2004; Peterson 2005; McKenna 2007; McKenna 2008). This decreases sarcolemmal excitability and therefore increases fatigue. Overall, these observations suggest that regulation of overall activity and expression of specific Na^+/K^+ -ATPase isoforms in certain muscles/fiber types is central to the physiological strain of muscle contraction.

The influence of exercise training on expression, activity and ion balance in regards to the sodium pump has been thoroughly investigated in mammalian systems,

including humans. Exercise can lead to the entry of water into muscle cells, thereby increasing extracellular ion levels (Kjellmer 1965; Watson PD 1993; McKenna 1995). Increases in extracellular potassium ions are of particular importance, as the Na^+/K^+-ATPase transports K^+ back into the muscle cell (Clausen 2003). Training has been shown to alter Na^+/K^+-ATPase isoform expression. For example, significant increases in the α- isoforms have been seen post-'interval' training, a change accompanied by an increase in Na^+/K^+-ATPase activity (Aughey 2007). Combining the concepts of ion maintenance with that of enzyme expression, cells which successfully up-regulated the Na^+/K^+-ATPase have been associated with lower levels of extracellular K^+ during exercise challenges relative to those that did not (McCutcheon 1999; Nielsen 2004), as well as tolerance of muscle to elevated plasma potassium (McCutcheon 1999; Broch-Lips 2011). One study examined the effect of immobilized limbs on muscle sodium pump protein levels, showing a significant decrease in Na^+/K^+-ATPase enzyme activity which was then restored when regular exercise levels were allowed to resume (as reviewed by Clausen 2003). Muscle from active rats compared to those kept sedentary have been shown to have a 22% increase in Na^+/K^+-ATPase pumps (as determined through measurement of ouabain binding), a finding well supported by several studies in representing diverse taxa from trout to humans (Madsen 1995; McKenna 1995; Nielsen 2004). Finally, rates of muscle action potential firing depend directly on the concentration gradient of sodium ions across the sarcolemma (Broch-Lips 2011), strongly supporting an important role for Na^+/K^+-ATPase in regulating muscle contraction.

Examination of the isoform-specific expression patterns of sodium pump sub-units following training regimens has provided somewhat more variable results. A two-

fold increase in Na^+/K^+-ATPase α1 and α2 mRNA has been described following high intensity exercise in untrained human subjects, a change not seen in those subjects repeatedly trained (Nordsborg 2003; Nordsborg 2010). Despite a significant amount of research on the role of Na^+/K^+-ATPase in mammalian exercise physiology, there are still many questions that have not been answered. Most importantly, why are multiple isoforms of the Na^+/K^+-ATPase expressed in muscle, and what influences the shifts in these isoforms observed under various exercise challenges?

A Comparative Physiology Approach

To better understand the individual roles and regulation of each Na^+/K^+-ATPase isoform my thesis takes a comparative approach, examining one species in the context of the existing knowledge in others. Although most physiological systems and mechanisms are highly conserved across all animal species, examining the variation that does exist between organisms can be a powerful tool to help biologists understand how animals work. Small physiological differences between species often lead to great advantages in nature, allowing certain animals to survive in different, often harsh and changing environments. Many physiologists traditionally study a small number of select model organisms which are well suited for a particular area of research. This strategy has proven to be quite effective as illustrated above, where studies on mice, dogs and humans have increased our understanding of Na^+/K^+-ATPase physiology. However we can also take advantage of studying other organisms that may employ similar or alternative solutions to common biological challenges (Somero 2000). August Krogh provided the foundation of this theory in his well-known principle stating that, "*For a large number of problems,*

there will be some animal of choice or a few such animals in which it can be most conveniently studied" (Krogh 1929). This concept allows researchers to compare biological mechanisms in the "ideal" organism (*i.e.*, well-adapted to a specific physiological challenge) to that in others in an attempt to tease apart the biological basis for why certain species are able to succeed in specific, sometimes extreme, environmental conditions. With this in mind, my thesis examined the regulation of muscle Na^+/K^+-ATPase in one of nature's notable athletes, the rainbow trout.

Salmonid Fishes

The Salmonidae family of fishes includes the salmon, trout and chars. Species in this family are naturally presented with and successfully acclimate to a wide range of physiological obstacles during their life cycle, such as changing elevation, salinities, temperatures and enduring long distances (Quinn 2005). By examining a species that encounters extreme challenges to its physiology, we aim to uncover a stress response that requires a change in muscle Na^+/K^+-ATPase isoform complement. This approach may help shed light on potential functional roles for individual Na^+/K^+-ATPase isoforms as well as improve our basic understanding of this essential enzyme.

Due to their remarkable ability to acclimate to changing salinity, much of the research on salmonids has focused on understanding their osmoregulatory capacity. Prior studies have shown that during salinity acclimation there is a switch in the dominant gill Na^+/K^+-ATPase isoform between α1a and α1b, where α1b mRNA levels increase and α1a levels decrease upon entrance to seawater. This indicates that the α1b isoform may be responsible for increased seawater tolerance (Richards 2003; Bystriansky 2006;

Bystriansky 2007; Bystriansky 2011), while the α1a isoform may be responsible for increased freshwater tolerance (Bystriansky 2007). No mammalian study has shown such a drastic change in Na^+/K^+-ATPase complement in response to stress, suggesting salmonid fishes may be an ideal group to study Na^+/K^+-ATPase regulation. Similar changes in Na^+/K^+-ATPase isoform expression may also be exhibited in response to other physiological challenges such as those imposed during exercise. Because the Na^+/K^+-ATPase α2 isoform has been identified as the dominant isoform in mammalian as well as fish skeletal muscle, its expression may be linked to exercise performance and training.

Rainbow trout (*Oncorhynchus mykiss*) naturally face numerous environmental conditions and physiological challenges during their life cycle. Like most other salmonid species, many rainbow trout populations are anadromous, hatching in freshwater, migrating to sea, and then returning to freshwater after several years to spawn. Both the seaward and spawning migrations may involve extended periods of temperature extremes, salinity shifts, and high energy demand. The water conditions these fish may encounter range from gentle currents of estuaries, rapids, reversing tides and waterfalls that require incredible strength to overcome. For example, approximately 84% of total energy used by sockeye salmon during simulated migration was consumed by swimming activity and locomotion (Rand 1998). Experimental studies that mimic various exercise challenges, such as maximal speed or endurance, would be beneficial in broadening our understanding of how the Na^+/K^+-ATPase responds to muscle exertion.

Exercise and Swimming Challenges in Teleost Fishes

Fish make excellent models for studying exercise physiology as swimming is a critical aspect of their life history and survival strategy. Three general categories of swimming style are typically examined, dependent on the species and based on an average of individuals: sustained, burst, and prolonged. Sustained swimming is generally assumed to be an aerobic activity in which metabolic demands are matched by the available physiological energy supply. Because of this, sustained swimming speeds are considered to be those at which a fish could potentially maintain indefinitely (*i.e.,* many hours, days or months). In contrast, high-intensity burst swimming can only be maintained for 30 seconds or less (Jones 1981), due to quick depletion of intracellular energy stores and reliance on anaerobic metabolism (Beamish 1978). Between these two extremes is a somewhat gray area termed prolonged exercise, an endurance-based activity similar to sustained swimming in intensity, but resulting in eventual exhaustion. In nature, this type of activity is probably most common. It consists of mostly relaxed swimming, with periods of high intensity swimming when necessary; however, the unpredictability of these patterns means that experimentally this pattern of exercise would be very difficult to replicate (Wilson 1994; Hammer 1995).

Critical swimming speed (U_{crit}) is perhaps the most common means of measuring the swimming ability of fishes. Often cited, Brett's 1964 study on salmon was the first to develop and publish this method, which is still used today. The method takes advantage of the opto-motor response, a behavior in which fish instinctively tend to maintain their position and swim into the water current. His established U_{crit} protocol introduces fish into a flume/tunnel at a very low water velocity (0.5 body lengths (BL) per second) to

acclimate to the conditions within the swimming space. Following acclimation, the water velocity is incrementally increased in set time intervals during which the fish is forced to swim against calibrated water velocities until it becomes fatigued and can no longer maintain its position against the flow. Based on the times and water velocities reached, the U_{crit} value for that particular fish can be calculated (Brett 1964). This value is often used to reflect the effect of certain variables, such as changing temperature, salinity, or oxygen availability, on swimming ability and is correlated to ecological or physiological effects that may exist in nature. Studies of this kind are perhaps the best-standardized mode of analyzing performance, especially in migratory species, and much work has been directed at identifying effects of different environmental factors on a species' maximum aerobic swimming capacity (Kieffer 1998; Plaut 2001).

Muscle Types and Metabolism

In addition to their 'athleticism', salmonids are an ideal model for studying skeletal muscle physiology because they possess anatomically separate red and white muscle fibers. This separation into discrete fiber types has been a useful attribute in examining red and white muscle responses to various swimming challenges. In general, red muscle is utilized at steady, low intensity swimming speeds and may also be referred to as "slow-twitch". This type of activity occurs at an exercise intensity in which adequate oxygen is available for cellular respiration, and is therefore termed as aerobic exercise. White muscle is generally recruited when the demands of intense physical activity surpass the capacity of red muscle during very intense but brief swimming periods, as may be seen during escape from predators, hunting, or migration in rough or

turbulent waters. Relative to red muscle, white muscle possesses approximately one-third of the electron transport chain enzymes and lower concentrations of mitochondria (Franzini-Armstrong 1964; Johnston 1982); however, at intermediate swimming intensities it is recruited in some species to help sustain aerobic energy supply (Johnston 1980; Holk 1998). The lack of lactate produced in white muscle indicates that these fibers have some aerobic capacity, albeit low, and that anaerobic energy production is only used at intense levels of activity.

Based on these distinct muscle types and an interest in furthering understanding of the physiological limits of fish species, a great deal of work has attempted to identify the individual species' threshold between aerobic and anaerobic energy use. Most frequently, common metabolites and phosphogens (such as glycogen, ATP and phosphocreatine) and glycolytic enzymes (*e.g.,* lactate dehydrogenase) are measured to describe their response to performance under certain conditions. After just 8 minutes of forced swimming, three species of juvenile salmonid species (Atlantic salmon, rainbow trout and brook trout) showed significant decreases in ATP, phosphocreatine, and glycogen, and an increase in lactate, with amounts of each of these molecules leveling off in all species after only four minutes (D. G. McDonald 1997). These changes tend to be greater following burst swimming, namely that which results in physiological exhaustion, compared to sustained swimming. When white and red muscle types were analyzed independently, Dobson and Hochachka (1987) found that at the point of exhaustion, white muscle glycogen (the main energy source) diminished by 90%, glucose dropped 2-fold, and muscle ATP declined from 5.24 to 0.54 μmol/gram. Aerobically fueled red muscle, on the other hand, showed no significant change in ATP at exhaustion and only a

10% decrease in phosphocreatine (PCr). This emphasizes the differences in energy supply for the two distinct muscle types and the greater efficiency at which red muscle can be maintained (Dobson 1987). Lactate concentrations are also greater in white muscle relative to red during swimming, a fact that persists even at rest (Milligan 1986). This increase in lactate results from depletion of PCr stores and a reliance on gluconeogenesis for anaerobic production of ATP, yielding lactic acid as a byproduct.

While white muscle can supply energy rapidly, there may be a trade-off in which individuals showing a large anaerobic scope (high maximum speed at exhaustion) also have a longer recovery period, compared to those with a greater aerobic scope (S. Marras 2010). This conclusion was partially supported by work done on Atlantic cod (*Gadus morhua*) in which a modified exercise protocol, called "U_{burst}", attempted to measure sustained anaerobic swimming ability in comparison to the traditional U_{crit} test (which is accepted to measure aerobic scope). The U_{crit} and U_{burst} results showed a negative correlation with one another, consistent with a trade-off between the physiological basis of anaerobic and aerobic activity. However, further analysis revealed that fish which had strong performance in the U_{crit} test and showed a positive correlation with aerobic scope, also showed a positive correlation with the results of a brief 'sprint', or burst swimming bout. This presents the idea that although some physiological variables can be regulated to ease the challenge of exercise, there may simply be individuals who are better adapted to exercise in general (Reidy 2000). A combination of these two extremes may be the reality, but further research on the physiological basis for the differences are certainly vital to our understanding. The underlying physiology of red and white muscle is very different, and each has important roles under different exercise challenges.

Muscle Physiology and Ion Balance in Fishes

Studies of skeletal muscle fatigue, defined by McKenna *et al.* (2008) as a "transient and recoverable decline in muscle force and/or power with continuous muscle contraction," have identified that the proper balance of sodium and potassium ions during exercise may contribute to higher levels of endurance in some organisms or. Because muscle cells have an extensive network of T-tubules, there is a large surface area for passive diffusion of ions; however, the small volume within these tubules increases the effect of altered ion concentrations (Nielsen O.B. 1996). Measurements made at early time points during exercise show an increase in Na^+/K^+-ATPase activity, maintaining ion levels intra- and extracellularly. However, following exhaustive exercise there is a rapid decline of Na^+/K^+-ATPase activity that depresses muscle excitation (Fraser 2002; Peterson 2005). This may be due in part to the inhibitory effect of high extracellular potassium and/or low intracellular sodium ion concentrations within muscle tissue (McKenna 1995; McKenna 2008).

Because many fish species have a high osmoregulatory capacity, disturbances and effects on ion balance in response to swimming have been extensively studied. Pink salmon (*Oncorhynchus gorbuscha*), known for very early smolting period in their life cycle, were swum to fatigue and concentrations of plasma sodium, chloride, and osmolality were measured (M. Sackville 2012),. Both whole body sodium and chloride concentrations were significantly higher in exercised fish compared to resting controls; however, most of these differences were believed to be associated with dehydration from muscle work rather than net ion gain at the gills (Nendick 2009). The gill is the main site of oxygen absorption in fishes. The high level of oxygen consumption that occurs during

exercise can be accompanied by a change in gill morphology such that the surface area is increased to maximize oxygen absorption from the surrounding water (Cameron 1972; Brauner 2011). This increase in gill surface area may also alter internal sodium and chloride concentrations through diffusion. Postlethwaite and McDonald (1995) explored the impact of continual aerobic swimming on this ion maintenance issue, finding a significant drop in blood plasma sodium and chloride within three hours of exercise at only 1.8 BL/s in juvenile rainbow trout. This was followed by an increase in whole body sodium ion concentration that became significant at 12 hours of the same swimming intensity; there was no change in whole body chloride levels during the entire exercise period. Interestingly, shifts in these ions were about 5-20 fold greater from extracellular to intracellular spaces compared to their loss at the gills (Wood 1991). Aside from sodium and chloride levels, potassium ion concentrations have a suggested involvement in the onset of muscle fatigue. At a sustainable swimming speed of 1-1.5 BL/s, trout exhibited an increase in plasma potassium ion concentrations (Nielsen 1994). A follow-up experiment increased the swimming intensity until rainbow trout reached fatigue, and saw similar results with an increase in plasma potassium concentration at speeds greater than 1.5 BL/s (Holk 1998). Because their work examined a range of swimming speeds, it was possible to suggest a sequence of change in plasma potassium: no strain on oxidative phosphorylation at less than 1.5 BL/s, with complete maintenance of potassium and lactate concentrations, slight strain and increase in potassium up to 2.0 BL/s, and marked increase in both potassium and lactate concentrations above 2.0 BL/s. These results indicate potassium loss from both fiber types and a strong reliance on anaerobic metabolism. An interesting route of research related to all of this would be in the

expression as well as activity of the Na^+/K^+-ATPase during swimming challenges as it has an essential ion balancing function, especially in actively contracting muscle cells.

Training Effects in Fishes

As in many mammalian studies, possible training effects have been explored in fishes with regard to their ability to maintain ion balance, muscle contraction, or improve U_{crit} performance with repetitive swim protocols. In rainbow trout, two months of swim training shifted the initial onset of increasing extracellular potassium and lactate to higher speeds, indicating that trained fish improved their ability to maintain homeostasis during demanding swim challenges (Holk 1998). Additionally, regularly exercised Coho salmon (*Oncorhynchus kisutch*) were better able to regulate ion levels than non-exercised fish upon 24-36 hours of seawater exposure (Woodward 1985) showing successful "multi-tasking" of internal physiological processes with training. Work on Chinook salmon concluded that training at high swim speeds results in a higher maximum oxygen consumption (M_{O2max}) and lessens increases in plasma osmolality that would initially be observed following exercise (Gallaugher 2001). ATP turnover during high-speed anaerobic swimming was not different between trained and untrained rainbow trout; however, the relative contributions of the energy supply in each case did differ such that trained fish were able to rely more heavily on glycolysis than those untrained (Pearson 1990). During a monitored six-hour recovery period, trained fish also were able to maintain higher levels (89% higher 1 hour post-exercise) of energy stores and replenish glycogen and high-energy phosphate molecules more efficiently relative to untrained. All of this together suggests that a physiological and biochemical training effect is seen in

fish, which minimizes energy depletion, moderates ion imbalances and accelerates successful recovery. The Na^+/K^+-ATPase is likely to be involved in some of these processes as a major ion-transporting enzyme in muscle, either through altered expression or activity.

Thesis Objectives

The conservation of a collection of Na^+/K^+-ATPase isoforms across different species suggests that the function of these individual polypeptides must be significant or essential. Subtle variation in amino acid sequences and biochemistry may have evolved to allow the Na^+/K^+ ATPase to perform specific physiological roles in individual cell types under certain conditions. The existence of the skeletal muscle-specific α2 isoform (Blanco and Mercer 1998; Mobasheri, Avila et al. 2000), suggests this isoform may play an important role in skeletal muscle and exercise physiology. Despite decades of research on exercise physiology in fishes, the expression, regulation and role of muscle Na^+/K^+-ATPase has never been examined in response to swimming challenges. This thesis investigates the tissue-specific regulation of Na^+/K^+-ATPase α- subunit isoforms in response to both sustained and sprint exercise challenges over a variety of durations.

Two parallel experiments were set up to examine the immediate effects of sustained (aerobic) and burst (glycolytic) swimming, as well as during training and recovery time courses. Salmonids are accepted to be "generalist" swimmers, maintaining swimming speeds that are less than 50% of their estimated U_{crit}, (Beamish, 1978) but also able to reach high speeds under natural or experimental conditions. To assess both aerobic and anaerobic challenges, a modified version of a "ramp-U_{crit}" protocol (Jain 1997) was developed to push the trout smoothly in brief

increments to a specific (sustainable or burst) swimming speed in a short period of time with minimal stress. Resting water speed within the swim tunnel was kept at 0.5 BL/s based on previous research and a need for unidirectional water flow within the swimming space (Wilson and Egginton 1994). Electromyography and metabolic analyses have suggested that white muscle is recruited at speeds ranging between 70% (Burgetz *et al.* 1998; Kieffer *et al.* 1998) to 94% (Wilson and Egginton 1994) of a species' U_{crit} value. Based on this, the sustained swimming (low intensity, extended duration) speed used in this research was approximately 3.0 BL/s (~50% U_{crit}) and the burst swimming speed (high intensity, short duration) was 8.0 BL/s (~ 100% U_{crit}) (based on previous studies with the same strain of rainbow trout used in this study (Scott 2012). Training regimens included two, four, or eight swimming bouts to examine any effects of repeated exercise on muscle isoform expression. Red muscle, white muscle and heart samples were assayed independently to determine relative mRNA levels of Na^+/K^+-ATPase isoforms (α1c, α2, α3). To assess whether the exercise type affected overall osmoregulatory status, blood plasma was analyzed for total osmolality as well as chloride (Cl^-) ion concentrations. It was hypothesized that there would be a shift in the skeletal muscle α Na^+/K^+-ATPase isoform in response to imposed swimming challenges. Overall, the results of this thesis will help provide a more complete understanding of Na^+/K^+-ATPase regulation in fish as a response to exercise, a natural challenge in the vast and demanding migration patterns of many species, but also crucial in broadening knowledge about the general physiology of this important enzyme.

CHAPTER 2: Muscle Na^+/K^+-ATPase isoform regulation in rainbow trout (*Oncorhynchus mykiss*) following burst and sustained swimming challenges

Abstract

Rainbow trout, *Oncorhynchus mykiss*, are often recognized for their physiologically demanding migration patterns. Shifts in relative expression of specific Na^+/K^+-ATPase isoforms have been identified during salinity acclimation and similar changes in expression may also occur in response to other physiological challenges. The Na^+/K^+-ATPase plays a crucial role in the maintenance of membrane potential and excitability during muscle contraction through prompt restoration of sodium and potassium gradients. Additionally, a tissue-specific isoform (α2) has been identified within skeletal muscle, with localized expression conserved across multiple species but the specific function is unknown. This study examined mRNA expression of α isoforms in hatchery-reared juvenile rainbow trout in response to aerobic (3 BL/s) and anaerobic (8 BL/s, near U_{crit}) swimming challenges, including an analysis of oxidative vs. glycolytic muscle types and potential training effects involving isoform expression. We observed differential regulation of Na^+/K^+-ATPase isoforms between red and white muscle, with significant transcriptional upregulation in red muscle and no shifts in white muscle or heart tissues. Generally, mRNA increases in red muscle were greatest following 4 days of training, with equivalent or larger increases after 4 days of recovery. This trend was seen after both burst and sustained swimming regimens; however, high-intensity swimming induced greater upregulation. During training, burst swimming near U_{crit} significantly increased mRNA levels for all α isoforms measured (1.5-fold for α1c, 2–fold for α2, and 2.5-fold for α3) while swimming at 3 BL/s resulted in upregulation only in α2. Repeated burst swimming reduced plasma osmolality and chloride, persisting into recovery, suggesting

intense exercise simultaneously compromises osmoregulatory function during periods of high-energy demand.

I. Introduction

All animal cells constitutively express the dynamic protein commonly referred to as the sodium (Na^+) pump, or more formally the Na^+/K^+ -ATPase, which actively transports sodium ions out of the cell in exchange for potassium ions in a 3:2 ratio, using the energy of one ATP molecule in the process (Lingrel and Kuntzweiler 1994). The resulting electrochemical gradients are used to power the secondary transport of many other molecules across the plasma membrane, regulating cell volume, and maintaining resting membrane potential. In excitable cells, the electrochemical gradients generated are especially important for the generation and regulation of action potentials (Blanco and Mercer 1998).

The sodium pump is made up of one catalytic alpha (α) subunit and one beta (β) subunit. In mammals, four α- and four β- subunit isoforms have been identified to date, with the α1 and β1 proteins being expressed in all cells. The resulting α1β1 heterodimer is often referred to as the "housekeeping" enzyme, and is thought to be involved in day-to-day cellular homeostasis (Blanco and Mercer 1998; Kaplan 2002). Strikingly, the remaining isoforms exhibit a more restricted, tissue-specific expression. The α2 isoform is expressed most prominently in muscle, adipose tissue, and myelinated axons of the brain, while α3 is abundant in the central nervous system, with smaller amounts found in cardiac muscle, cartilage, and bone (Mobasheri, Avila et al. 2000). The α4 isoform has been isolated from the testes and very specifically in the flagellum of human sperm cells

(Sanchez, Nguyen et al. 2006). In addition to their tissue distributions, alpha isoform may differ in enzymatic characteristics such as cation- and ATP-binding affinity (Mobasheri, Avila et al. 2000). A tissue-dependent distribution is also seen in β isoforms, where β2 is found in skeletal muscle and nervous tissues and β3 is localized to testes, retina, liver, and lung (Blanco and Mercer 1998). Overall, the functional difference between these isoforms is not well understood. The tissue specific nature of their expression and the fact that each isoform has been shown to possess unique physiological characteristics suggest they have a specialized function or role to play in certain tissues. This study examines the potential role of individual α subunit isoforms in muscle tissue, where the Na^+/K^+-ATPase plays a pivotal role in muscle contraction.

Action potentials involved in skeletal muscle contraction cause a loss of potassium from the cell and a gain of sodium. This reduction in sodium and potassium gradients across the sarcolemma reduces cell excitability. Repeated myofibril contraction requires that both sodium and potassium ions be actively transported back across the sarcolemma so the membrane can depolarize again in preparation for induction of another action potential, and the Na^+/K^+-ATPase plays a crucial role in restoring these gradients (Clausen 2003). Exercise physiology research on mammalian systems has shown shifts in muscle Na^+/K^+-ATPase α-subunit mRNA and protein content, as well as enzyme activity, in response to exercise challenges in several species (Hundal, Marette et al. 1993; Tsakiridis 1996; McCutcheon 1999; Nordsborg 2003; Murphy 2004; Peterson 2005; Nordsborg 2010).

Despite this research, our understanding of the potential functions and roles of various Na^+/K^+-ATPase isoforms is still quite limited. To broaden our current

understanding of the individual function and regulation of Na^+/K^+-ATPase α isoforms in muscle, this study takes a comparative approach by examining the exercise physiology of rainbow trout and putting results in the context of what is known for other species. Rainbow trout and other salmonid fishes serve as ideal models to study muscle physiology and exercise. Anadromous salmonid species (*e.g.,* steelhead rainbow trout) migrate from their native freshwater streams to the ocean as juveniles before returning to spawn as adults. For some populations this migration can be thousands of kilometers long, during which they display incredible endurance while swimming upstream, traversing rapids, and climbing waterfalls. Salmonid fishes are truly one of nature's great 'athletes'. Rainbow trout also express a similar range of α- isoforms (α1a, α1b, α1c, α2 and α3), with a similar tissue distribution as is seen in mammals (Richards 2003). The conservation of a collection of Na^+/K^+-ATPase isoforms across different species suggests that the molecular capacity of these unique polypeptides may be functionally significant. Subtle variability in amino acid sequences and biochemistry may have evolved to allow the Na^+/K^+ ATPase to perform specific physiological roles in individual cell types under certain conditions. Interestingly, rainbow trout α-subunit isoforms have been shown to respond dramatically to other environmental stressors. Following exposure to changing salinity, the dominant Na^+/K^+-ATPase isoform in the gill 'switches' between α1a and α1b (Richards 2003; Bystriansky 2006; Bystriansky 2007; Bystriansky 2011). Similar changes may occur in muscle during exercise. This is the first study to examine the potential role of specific Na^+/K^+-ATPase isoforms in muscle physiology in response to both intensity and duration of swimming in fishes. The α subunit was chosen for the focus of this study as this is the catalytic subunit seen to possess variable physical or

chemical properties, such as ion affinities and enzyme kinetics (Mobasheri, Avila et al. 2000). This study aims to investigate the tissue-specific localization and regulation of Na^+/K^+-ATPase α isoforms in teleost skeletal muscle and heart tissue in an attempt to uncover the physiological roles for which each may have evolved.

II. Materials and Methods

1. *Fish Husbandry* Juvenile rainbow trout (*Oncorhynchus mykiss*) used were a hatchery reared strain originally collected from the Blackwater River, British Columbia, Canada. Trout were raised in freshwater under natural photoperiod and fed daily *ad libitum* at the Abbotsford Fish Hatchery to approximately six months of age before being transported to the University of British Columbia, Vancouver, Canada. Trout were raised to approximately one year of age in large (160 gallon) indoor tanks supplied with fresh de-chlorinated tap water maintained at 15°C. Water oxygen levels where maintained near saturation with air stones and photoperiod was set to mimic a natural light cycle. Rainbow trout were sorted into twelve groups of eight fish and kept in individual five-gallon containers floating in a large (160 gallon) tank. Containers were open to the larger tank by numerous mesh openings allowing for ample water exchange. Fish were randomly sorted into groups (n = 8). The average size of trout used in this study was 13.54 ± 0.49 grams with a standard length of 11.6 ± 0.1 centimeters. There were no significant differences in fish weight or length between individual groups. All fish were allowed to acclimate to their containers for at least 12 hours prior to their first designated swimming protocol. Trout were fed to satiation daily up until two days prior to the start of the experiments. They remained unfed for the duration of the ten-day swimming

studies to eliminate potential differences in individual performance based on physiological feeding effects.

2. ***Swim Tunnel and Calibration.*** Swim trials were conducted using a Brett style (Logilo, 90.15 L) swim tunnel. Prior to each swim trial, the swim tunnel was filled with fresh, de-chlorinated tap water. Oxygen levels within the swim tunnel were maintained near saturation using two air stones, placed in opposite ends of swim tunnel outer tank. Water temperature was maintained at 15°C using an external circulating water chiller (VWR, Digital Model 1166D). To ensure uniform water temperature and oxygen levels, a submersible water pump was used to circulate water through the enclosed tunnel area.

The flow of water through the swim tunnel was controlled by an electric motor attached to a submerged propeller located inside the tunnel. The rotational speed of the motor was expressed in Hertz (Hz), so an insertion flow probe (Höntzsch, Vane Wheel Sensor, HFA-Ex Hand Unit) was used to determine the corresponding water velocities used in meters per second. The flow probe was placed in the middle of the swimming chamber, equidistant from the top and bottom of the tunnel. Three different positions within the swim tunnel were calibrated to account for possible differences in water velocity depending on the swimming locations of the fish: center of swimming area, inner swimming area and outer swimming area (**Fig. 2A** & **B**). At the water velocities used in this study (maximum of 7.5 cm/s) there were minimal differences in calibrated water velocities observed at each location within the swim tunnel.

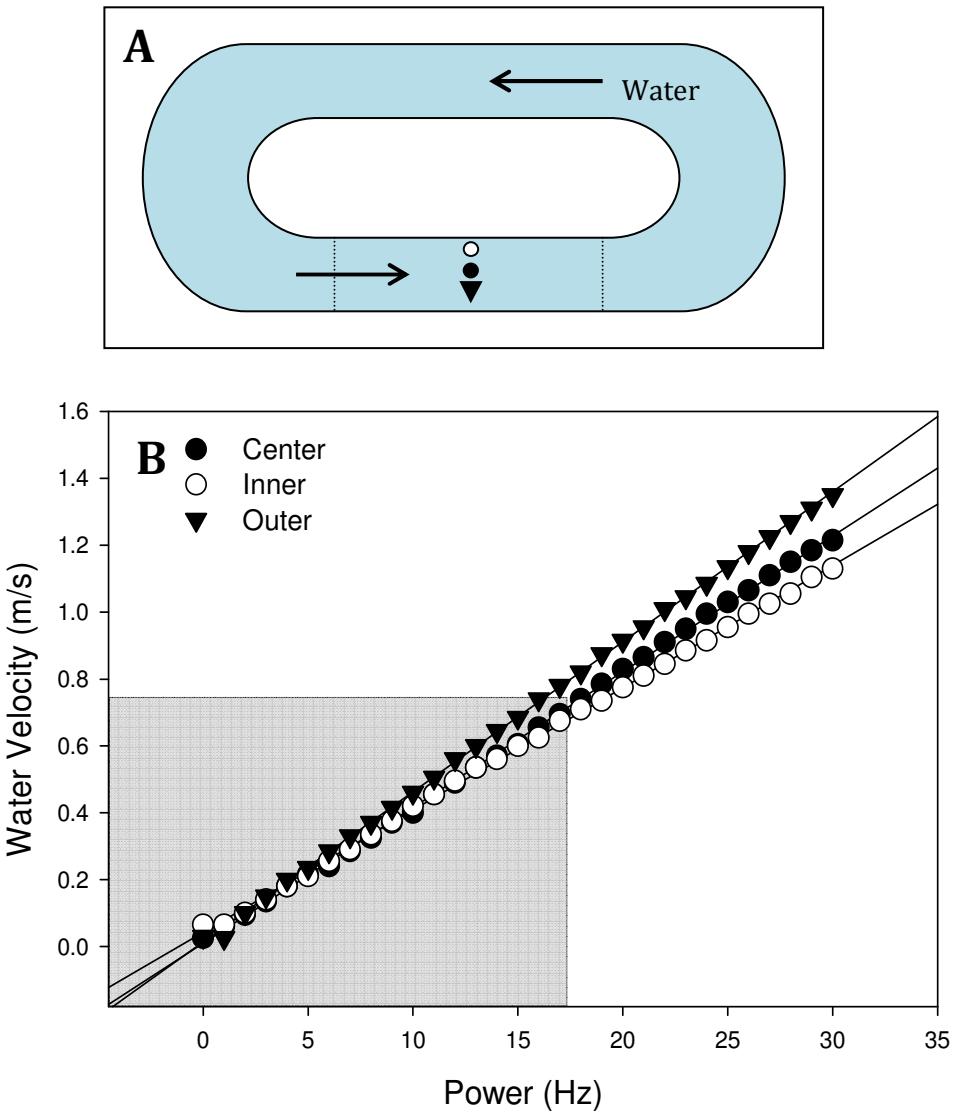

Figure 2. **(A)** Diagram of the swimming tunnel with arrow indicating the counter-clockwise flow of water. **(B)** Motor rotational speed (Hz) to water velocity (m/s) calibration. This was determined using a submersible flow probe in three different positions (inner, center, and outer) within the swimming area. The shaded area represents the range of water velocities used in these experiments, where differences in location within the tunnel do not experience notable differences in water velocity.

Fish were transferred from their holding tank to the swim tunnel in a transfer container the same size and shape as their holding container. Fish were gently poured directly into the transfer container filled with water from the large holding tank. This was a quick process in that it moved all fish at once, minimizing handling stress. Fish were then gently transferred to the swim tunnel chamber using a dip net. The swimming chamber was closed and covered with black plastic shields in order to reduce stress from external stimuli. Fish were allowed to acclimate to the tunnel conditions for one hour at 0.5 BL/s. This speed was used to create a water velocity with just enough force to cause water to flow in one direction rather than randomly fluctuate within the swim tunnel. The unidirectional movement of water through the tunnel also caused the trout to orient themselves against the flow of water during the initial one-hour acclimation period.

Two different exercise regimens were designed to examine effects of aerobic (sustained) and glycolytic (burst) swimming during both training and recovery time courses (n=8) (**Table 1**). Tissue samples were collected immediately following two bouts of exercise to analyze any "immediate" effects of the particular swimming intensity and training regimens included four (4x) or eight (8x) days of repeated swimming bouts. Following two bouts of exercise, some groups were allowed 1 or 4 days of recovery at "rest" before sampling (**Fig. 3A, 4A**).

Control Groups	Experimental Groups	
	Sustained (3 BL/s)	**Burst** (8 BL/s)
Time zero (0 day); sacrificed prior to start of experiments	**2 days** (2x); 2 swimming bouts over two consecutive days	**2 days** (2x); 2 swimming bouts over two consecutive days
Handling (4 day); transferred in/out of swim tunnel for 2 consecutive days and allowed to recover for 4 days	**4 days** (4x); 4 swimming bouts over four consecutive days	**4 days** (4x); 4 swimming bouts over four consecutive days
	8 days (8x); 8 swimming bouts over eight consecutive days	**8 days** (8x); 8 swimming bouts over eight consecutive days
Developmental (8 day); unswum for eight day duration of experiments	**4 days of recovery** following 2x challenge	**1 day of recovery** following 2x challenge
		4 days of recovery following 2x challenge

Table 1. Summary of control and experimental treatment groups for both the sustained and burst swimming experiments. Both sustained and burst experiments were analyzed compared to the same three controls above.

3. ***Sustained (Aerobic) Swim Trials.*** Physiological effects of endurance-type swimming, which is low in intensity but long in duration, were analyzed after repetitions of the following sustained swim protocol. Fish were swum in groups of 8 and at the same time each day on consecutive days. Following a one-hour acclimation to 0.5 BL/s, the water velocity was increased gradually at a rate of 1.0 BL/s every 30 seconds, until a maximum swimming speed of 3 BL/s was reached. Because rapid and constant acceleration may require anaerobic contribution at less than maximal swimming speeds (Farrell 2008), water velocity was increased gently until the designated speed was reached. Trout were left to swim at this speed for 3 hours (**Fig. 3B**). Throughout the exercise protocol, water temperature was maintained at 15°C. Upon completion of the three-hour exercise bout, the motor was immediately turned off and the propeller allowed to gradually slow to rest. This reduction in water velocity occurred over approximately 1 min. Fish were then removed by net from the swimming chamber to a holding container filled with fresh, aerated water taken directly from the tunnel. If the fish were to be exercised again on a

following day or to be examined during recovery, they were returned to their holding tank. Fish that had completed their designated exercise regimen were quickly moved to a five gallon holding container and immediately anesthetized.

4. ***Burst (Anaerobic) Swim Trials.*** A distinct burst swimming protocol, which is high in intensity and only maintained for seconds, was developed to assess physiological changes in response to an anaerobic challenge. Fish were swum in groups of 8 and at the same time each day on consecutive days. Following a one-hour acclimation at 0.5 BL/s, the water velocity flowing through the tunnel was increased as detailed above. The increase in water velocity was done very gradually creating continual change in swimming speed of approximately 1.0 BL/s every 30 seconds to keep consistency between sustained and burst protocols. This gradual increase continued until maximum swimming speed of 8 BL/s was reached, a speed determined to be near the average U_{crit} for fish utilized in this experiment (Scott 2012) (**Fig. 4B**). Fish were left to swim at U_{crit} for one minute. After one minute, water velocity was rapidly increased by 1 BL/s and held for an additional minute without allowing gradual acclimation for a total burst swimming duration of 2 minutes. This was done in order to push the fish to the brink of exhaustion as well as account for individual variation in volition and achievable U_{crit} in an effort to ensure activation of anaerobic means of metabolism. Temperature was maintained at 15°C within the swim tunnel throughout the exercise protocol.

Upon completion of the two-minute exercise bout, the motor was immediately turned off and the propeller allowed to gradually slow to rest. This reduction in water velocity occurred over approximately 1 min. Fish were then removed by net from

swimming chamber to their holding container filled with fresh, aerated water taken directly from tunnel. If the fish were to be exercised again on a following day or to be analyzed post-recovery, they were taken back to the large holding tank and placed in their individual container. If the fish completed their designated exercise regimen, they were quickly transferred to a five-gallon container and immediately anesthetized.

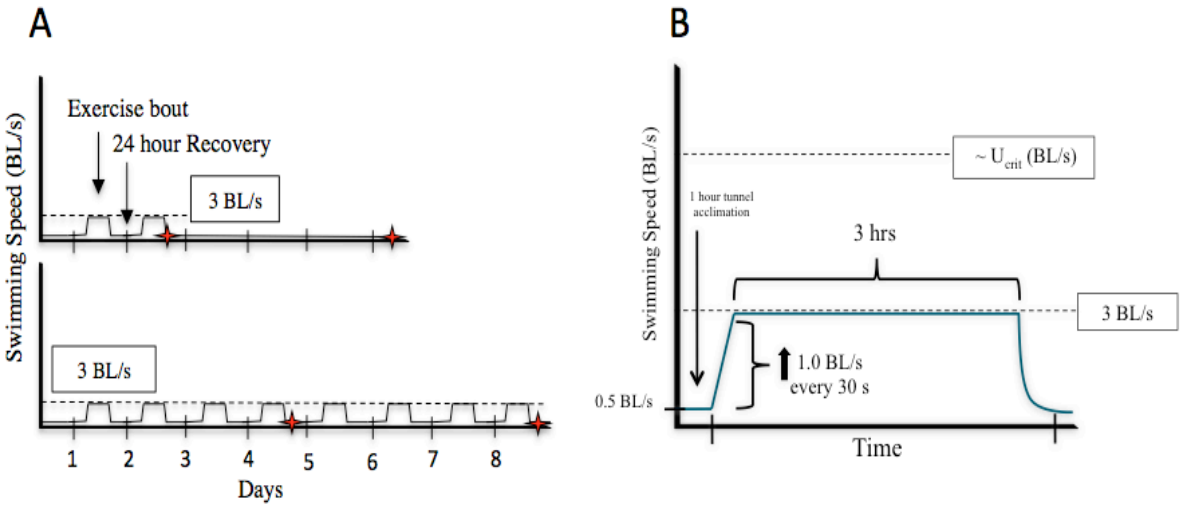

Figure 3. Sustained swim (3 BL/s) protocol. (**A**, top) Fish were exposed to 2 exercise bouts and either immediately collected or collected after 4 days of recovery from the same challenge (**A**, bottom) Other fish were exposed to 4 or 8 consecutive days of swimming. Sampling points (✦) indicate collections for analysis. (**B**) Schematic diagram of an individual sustained swimming bout.

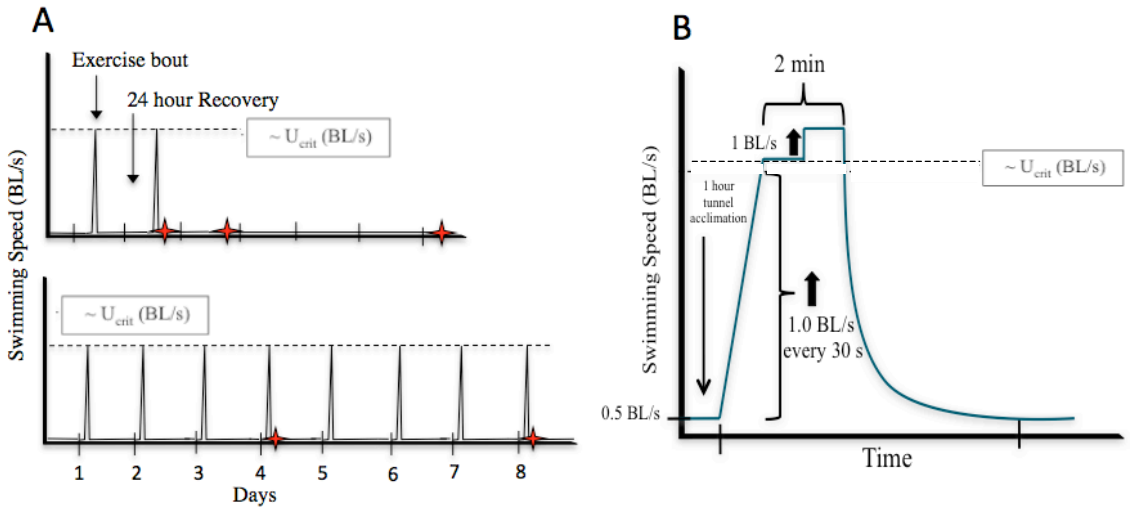

Figure 4. Burst swim (8 BL/s, near U_{crit}) protocol. (**A**, top) Fish were exposed to 2 exercise bouts and either immediately collected or collected after 4 days of recovery from the same challenge (**A**, bottom) Other fish were exposed to 4 or 8 consecutive days of swimming. Sampling points (✦) indicate collections for analysis. (**B**) Schematic diagram of an individual sustained swimming bout.

5. ***Tissue Sampling.*** Fish were anesthetized in a five-gallon container containing MS-222 (50mg/L) buffered with sodium bicarbonate (50mg/L). After approximately two minutes of anesthesia, fish lost equilibrium and did not respond to external stimuli. Anesthetized fish were then blotted dry and weight (grams) and fork length (centimeters) were measured. Blood was sampled by caudal severance using heparinized capillary tubes, then fish were quickly euthanized by severing the spinal cord posterior to the head. Blood was immediately centrifuged for five minutes at 3000g and plasma collected and frozen in liquid nitrogen. Samples of gill, heart, red and white muscle were quickly excised and flash frozen in liquid nitrogen. Samples were stored at ¯80 degrees Celsius until analysis.

6. ***Total RNA Extraction and cDNA Synthesis.*** Approximately 50 mg of red muscle, white muscle and heart tissue were ground separately using the bead-mill homogenization method (Biospec Mini-BeadBeater-1, Bartlesville, OK, USA). Samples were added to 1.5 ml reinforced, screw-cap tubes filled to the 1.0 mL mark with sterile zirconium beads (0.7 mm-1mm in diameter) and 1.0 mL of Trizol isolation reagent (Invitrogen, Carlsbad, CA, USA). Tubes were inverted to minimize air within the tube and submerge tissue. All samples were homogenized at 46,000 rpm for 30 seconds and then immediately placed on ice. Homogenates were removed from the top of the beads by pipette (1 mL) and transferred to a new 1.5 mL Eppendorf tube. Total RNA was extracted from samples using TriZol using the guanidine thiocyanate method (Chomczyncski 1987). Concentration and purity of isolated total RNA was determined spectrophotometrically (ThermoScientific NanoDrop2000c). All total RNA samples

used were found to be of very high purity (260:280 absorbance ratio of >1.8). RNA quality and concentration was further confirmed in several random samples by running 2 µg on an agarose gel (1%) and confirming the separation of the 28s and 18s ribosomal subunits. First strand cDNA was synthesized from 2 µg of total RNA (in 20µL total reaction volume) using a High Capacity cDNA Reverse Transcription Kit including MultiScribe™ reverse transcriptase and random hexamer primers (#4368814, Applied Biosystems, Foster City, CA, USA) in an Applied Biosystems 2720 Thermal Cycler. All cDNA was stored at ¯80 degrees Celsius until used for quantitative real-time PCR.

7. ***Quantitative Real-Time PCR (mRNA Expression).*** Quantitative RT-PCR (qRT-PCR) was performed on an ABI Step One Plus Real-Time PCR sequence analysis system (Applied Biosystems). PCR reactions contained 2 µL of cDNA in a total 22 µL reaction volume including 150 pmol of each primer, 10 µL of Universal SYBR-Green Master Mix (Applied Biosystems) and 9.2 µL of RNase-free water. Forward and reverse primers used for each gene were isoform specific (verified by melt curve analysis, Richards *et al.* 2003). Primer sequences used are listed in **Table 2**. Multiple random RNA samples (non-template controls, NTCs) that had not been reverse transcribed were also analyzed to confirm low genomic DNA contamination and these, as well as experimental samples, were run in technical duplicates. For the expression of each gene measured, the relative quantity of mRNA was normalized to an endogenous control gene (eukaryotic elongation factor 1 alpha, EF1α) and expressed relative to the mean value for rainbow trout in the '0 day' control group. EF1α expression was found to remain stable for all experimental and control groups indicating it was a suitable reference gene for this study. The relative

standard curve method was used for Cq determination and standard ramp speed used for amplification (Applied Biosystems, StepOne Plus real-time PCR system, StepOne Software v. 2.1).

qRT-PCR Primer Sequences (5'-3')			
Gene	**Accession #**	**Forward**	**Reverse**
Na^+/K^+ ATPase α2	NP001117930	-GGA AAC TGT TGA GCG TGA AAA-	-GTC CAC CGG TTT GTG TCA AGA-
Na^+/K^+ ATPase α1a	NP001117933	-GGC CGG CGA GTC CAA T-	-GAG CAG CTG TCC AGG ATC CT-
Na^+/K^+ ATPase α1b	NP001117932	-CTG CTA CAT CTC AAC CAA CAA CAT T-	-CAC CAT CAC AGT GTT CAT TGG AT-
Na^+/K^+ ATPase α1c	NP001117931	-GAG AGG GAG ACG TAC TAC TAG AAA GCA-	-CAG CAA GAC AAC CAT GCA AGA-
Na^+/K^+ ATPase α3	NP001118102	-CCA GGT ATT GAG TTC CGT GTG A-	-CAG CCT GAA ATG GGT GTT CCT-
EF1-α	NP001117811	-GTC TAC AAA ATC GGC GGT AT-	-CTT GAC GGA CAC GTT CTT GA-

Table 2. Primer sequences used for quantitative real-time PCR (5'-3'). EF1-α was used as a reference gene for all other genes of interest.

8. ***Total Osmolality and Chloride Concentration.*** Total plasma osmolality (mmol/kg) was determined by vapor pressure osmometer (Wescor, Logan, Utah, USA) and plasma chloride (meq/L) measured using a digital chloridometer (HBI, Haake Buchler Instruments Inc.).

9. ***Statistical Analysis.*** All data are presented as means ± standard error of the mean (s.e.m). of a sample size of eight individuals, unless otherwise stated. Comparisons of plasma chloride levels, total osmolality, and relative Na^+/K^+-ATPase isoform mRNA levels for each of the genes tested were performed using one-way analysis of variance (ANOVA) followed by a Fisher's Least Square Differences (LSD) secondary test to make pair-wise comparisons (SYSTAT 9). For all comparisons, $p < 0.05$ was considered significant. For most parameters examined, unless otherwise noted, the control groups did not differ statistically.

III. Results

All fish appeared to be in good health at the beginning of the study and there were no mortalities as a direct result of the swimming methods used. This supports that the regimens used were suitable to challenge fish both with low intensity (aerobic) and high intensity (anaerobic) swimming, pushing their limits within their natural physiological range.

1. Sustained Swimming Challenge (3.0 BL/s)

1.1 Plasma Osmolality and Chloride

In general, sustained swimming had little effect on the overall levels of plasma ions and osmolality (**Fig. 5**). Plasma total osmolality was similar amongst unswum control groups (294 ± 0.6 mosmol/kg; n = 8), and levels did not change significantly between 2, 4 or 8 consecutive days of sustained swimming. Levels remained stable after 4 days of recovery (**Fig. 5A**). Similar results were seen in plasma chloride (meq/L) (**Fig. 5B**).

1.2 Na^+/K^+-ATPase Isoform Expression

Preliminary tissue distribution results showed that red and white muscle expressed considerable levels of the α1c, α2 and α3 isoforms, while heart muscle only expressed α1c and α3 (Richards *et al.* 2003). In red muscle, α1c mRNA levels were stable across unswum control groups. Relative levels of the α-1c isoform were unchanged following two bouts of sustained swimming, but increased significantly relative to 4 day control group by approximately 38% after four bouts of sustained swimming (4x) before

returning to control levels after eight bouts of exercise (8x). Following four days of recovery, α1c levels in red muscle were significantly higher than all control groups, but not different from the 4 day trained group (**Fig. 6A**).

Similar to α1c expression patterns, α2 and α3 mRNA levels were unchanged in control groups but did show a trend for increased expression after 4 repeated bouts of exercise, as well as during recovery. This trend was significant when examining α2 levels in fish exercised for four consecutive days (4x), which showed significantly higher levels (~80%) than the four day control group (**Fig. 6B**). Following four days of recovery, the observed increase was more than two fold compared to controls. Most of the changes observed in α3 mRNA expression following exercise training were not considered statistically different; however, following four days of recovery, more than a two fold increase in mRNA levels was maintained (**Fig. 6C**). None of these changes were observed in white muscle following the same exercise protocol (**Fig. 7**). In heart, which only expresses α1c and α3, sustained swimming trials and a recovery period induced no changes in mRNA expression (**Fig. 8**).

2. Burst Swimming Challenge (8.0 BL/s)

2.1 Plasma Osmolality and Chloride

Plasma total osmolality was similar across control groups (294 ± 0.6 mmol/kg; n = 8) but was affected by high intensity swimming near U_{crit}, showing a significant decrease following two consecutive days (2x) of burst swim trials (245 ± 9 mmol/kg).

Low osmolality persisted at 1 and 4 days of post-exercise recovery and nearly returned to control levels by the fourth day (**Fig. 9A**).

Plasma chloride concentration (meq/L) dropped after 2 days of burst swimming bouts, parallel to results seen in osmolality; however, this decline persisted for chloride in the 4 day trained exercise group (4x) returning to control levels by 8 days (8x) (**Fig. 9B**). Plasma chloride was maintained at control levels (140 ± 2 meq/L) after both 1 and 4 days of recovery post-exercise and levels did not differ across control groups.

2.2 Na^+/K^+-ATPase Isoform Expression

Isolated red muscle resulted in the most pronounced changes, revealing a pattern in expression across all three isoforms measured (α1c, α2, and α3) with significant increases in mRNA levels immediately following 4 days of burst swimming (4x) near U_{crit} (**Fig. 10**). This shift was greatest for α2 and α3, increasing 2 and 2.5-fold from the controls respectively, a change which was not seen in either the 2 or 8 day trained groups which remained at resting levels (**Fig. 10B, 10C**). Two recovery time points were analyzed for burst swimming experiments, both 1 day and 4 days post-exercise, and the expression of the three Na^+/K^+-ATPase isoforms following recovery period of one day after two bouts of the burst swim protocol did not show a difference from any controls for α1c, α2 or α3. Analysis after four days of recovery from the same challenge revealed that all three isoforms showed an increase equivalent or greater in magnitude to that seen after 4 days of burst swim training. No differences between control groups were observed.

In white muscle (**Figure 11**) and heart (**Fig. 12**) tissue, there were no significant changes in isoform expression in any of the experimental or control groups.

IV. Discussion

Studying the response to exercise is an important part of broadening our understanding of animal physiology. In fishes, swimming is connected not only to general locomotion but also many other biological processes including development, growth, escape, hunting, and reproductive behavior (Plaut 2001; Planas 2011). Most members of the Salmonidae family of fishes make extensive seaward and spawning migrations as part of their natural life cycle. These migrations pose many physiological challenges, which individuals must acclimate to and overcome, making successful migration a key component in the overall ecological system in which these fish are a part of (Wilcove 2008; Hecht 2012). This study examined the effects of sustained and burst swimming challenges on whole body ion balance and the expression patterns of specific Na^+/K^+-ATPase isoforms in working muscle of rainbow trout. To our knowledge, no previous work has examined Na^+/K^+-ATPase expression in the muscle of any teleost fish in response to various degrees of swimming challenges and thus existing work in this area on mammalian systems will be relied upon for discussion.

In the present study, relative mRNA levels for α1c, α2 and α3 isoforms in white muscle and heart tissues were not affected by two, four or eight consecutive days of repeated swimming at 3 BL/s. There was also no shift in mRNA expression following a recovery period of four days in these tissues. Relative mRNA expression remained stable across unswum control groups for all isoforms examined during the duration of the experiment, confirming that any developmental changes and handling of fish did not affect the variable measured. A significant increase in α1c, α2 and α3 mRNA did occur in oxidative red muscle, suggesting that the two major muscle fiber types (oxidative vs.

glycolytic) are differentially regulated following a low intensity swimming challenge (3 BL/s). While some teleost species exhibit a mosaic-like muscle type mixed with both red and white fibers ('pink muscle'), this mixed muscle type is not predominant in rainbow trout (Johnston 1982) and therefore does not complicate our analysis.

The finding that red muscle Na^+/K^+-ATPase expression is more responsive to exercise is supported by previous data in rats in which the relative levels of both α1 and α2 were higher in oxidative red muscle fibers compared to white, glycolytic fibers (Fowles 2004). It has also been shown that oxidative muscle (versus glycolytic) shows higher sensitivity to fluctuations in intracellular sodium ion (Thompson 1999). Interestingly, α2 was the only isoform to significantly increase following low-intensity exercise, observed following four days of the training regimen. Red muscle mRNA levels of all three Na^+/K^+-ATPase isoforms increased after four days of recovery following two days of low intensity training. These shifts were greatest in α2 and α3 isoforms (approximately three-fold) and more moderate in α1c (~1.5-fold increase). The smaller change in the Na^+/K^+-ATPase α1c isoform is consistent with the established idea that the α1 isoform plays more of a "house-keeping" role in the cell, maintaining general membrane potential and ionic balance (Mobasheri, Avila et al. 2000). Therefore, a major change in mRNA expression of this isoform might not be expected in response to the muscle specific physiological challenge of exercise. Immunofluorescence studies of the localization of alpha isoforms within human and rat skeletal muscle cells have reported a differing distribution between α1, α2 and α3. The α1 subunit isoform was observed mainly in the sarcolemma while α2 was more widespread, expressed in the sarcolemma as well as within the T-tubule system (Hundal 1994; Williams 2001). Additionally, α2

within red and white muscle purified plasma membranes has shown greater increases in protein expression in response to exercise challenges relative to α1 (Tsakiridis 1996). Interestingly, the contribution of isozymes including α2 to general resting membrane potential is quite minimal despite its broader expression and abundance and knockout mice lacking this isoform specifically in skeletal muscle show significantly reduced muscle force and greatly increased rate of fatigue (Radzyukevich 2013). Taken as a whole, these studies coupled with the current results may indicate that the α2 may be more immediately involved in regulation of Na^+ and K^+ near the sarcoplasmic reticulum that occurs specifically during and following muscle contraction during exercise, while α1 (and α3) isoforms may serve a more compensatory role in overall skeletal muscle cell ion and osmotic balance, upregulated to a lesser degree as needed.

Prolonged low-intensity exercise in humans has been shown to induce a time-dependent pattern of α1, α2 and α3 isoform expression, where α1 up-regulation is delayed until 24 hours after recovery, α2 shows only a slight (non-significant) increase immediately post-exercise, and α3 is immediately up-regulated more than three-fold (Murphy 2006). The present study examined two time points into recovery with the earliest being one day post-exercise. Although we do not have data regarding mRNA expression in the first 24 hours, our results show that following a sustained swim there was significantly higher expression of all three isoforms by 4 days post-exercise and therefore all must be involved in the acclimation to low-intensity exercise at this time point.

Repeated bouts of burst swimming near U_{crit} showed a trend across sampling points similar to those seen during sustained swimming. While white muscle and heart tissues

showed no significant shifts in mRNA expression in any of the three Na^+/K^+-ATPase isoforms measured, oxidative red muscle displayed a 1.5-fold increase in α1c, a two-fold increase in α2 and a 2.5-fold increase in α3 following high-intensity swimming. Overall, this suggests that during intense exercise there is a general increase in all red muscle Na^+/K^+-ATPase α isoforms instead of reliance on any particular isoform. This general increase may suggest that expression of Na^+/K^+-ATPase may increase as the intensity of the muscle workload increases and if pushed to their maximal aerobic capacity the response involves not only the muscle-specific isoform, but α1c and α3 to a significant degree as well. It would have been interesting to monitor isoform expression under moderate exercise, to determine if expression of one isoform is seen prior to the other two. An additional time point into recovery was included in the design of the burst swim protocol, with samples measured at 1 day post-exercise for fish that were challenged in the event that higher intensity activity may induce a quicker change seen during recovery. No increase in mRNA levels of any of the isoforms was seen after 24 hours but a significant increase in mRNA expression was observed after 4 days as in low-intensity swimming. This upregulation does not appear to be isoform specific, and we can conclude that, whether low or high intensity, α1c, α2 and α3 isoforms show a delayed transcriptional up-regulation in response to exercise.

The observation of increased mRNA levels of individual Na^+/K^+-ATPase isoforms suggests there may be a corresponding increase in isoform-specific protein translation. It has been shown that even a one-hour bout of running in rats can induce correlated increases in α1 and α2 mRNA and protein within red and white muscle plasma membrane isolates (Tsakiridis 1996) showing that even acute stress involved in muscle activity can

affect Na^+/K^+-ATPase regulation. Although an overall increase in Na^+/K^+-ATPase content is often seen following training (Mohr 2007; Edge 2013), attempts to quantify changes in protein levels in the current study were not successful. At the current time, primary antibodies specific for teleost α1c, α2 and α3 isoforms do not exist. Available antibodies are being assessed to determine if they can be used to gauge total Na^+/K^+-ATPase α subunit protein levels in rainbow trout muscle. Additionally, attempted analyses of Na^+/K^+-ATPase enzyme activity have not been successful. Unfortunately, there are no published methods used to determine Na^+/K^+-ATPase activity in fish skeletal muscle. Standard methods used to determine muscle Na^+/K^+-ATPase activity in mammals, using ouabain inhibition and detection of liberated inorganic phosphate (P_i) (Brotherus 1981) or the 3-O-methylfluoroscein phosphate (3-O-MFPase) method (Huang 1975; Fraser 1998; Fowles 2002; Sandiford 2004)) did not yield detectable Na^+/K^+-ATPase levels in rainbow trout muscle. It is likely that the high relative amount of other ion-motive ATPases within muscle cells (*e.g.*, calcium-ATPase and sarco-endoplasmic reticulum ATPase, SERCA) may mask the specific detection of Na^+/K^+-ATPase activity. Despite not being able to measure this activity directly, we hypothesize that higher-intensity swimming would demand a higher activity of the enzyme to accommodate more frequent or more extreme shifts in ion movement within the muscle.

Swimming is a metabolically costly activity for fishes. Under low intensity exercise, it is expected that the animal can deliver the necessary ATP for muscle contraction without compromising other energy requiring systems. However, as ATP demand increases with increased exercise intensity, we might expect to see energetic trade-offs where the animal preferentially delivers energy to one physiological system

over another. One common energetic tradeoff seen in fishes is between locomotion and osmoregulation. Measurements of total plasma osmolality following sustained swimming at approximately 3 BL/s did not induce any large shifts in muscle Na^+/K^+-ATPase expression or lead to notable impairment of ion balance. Rainbow trout are generalist swimmers and a swimming speed of 3 BL/s is considered low intensity for salmonid fishes (Quinn 2004; Quinn 2005) and it is expected that this speed can be maintained without disrupting physiological homeostasis. In contrast, burst swimming did result in a reduction in plasma osmolality from approximately 300 mmol/kg to 250 mmol/kg after just two days of burst-type swimming near U_{crit}. It has been generally established that exhaustive swimming in teleosts disturbs ionic and osmotic balance, due in part to the increase of lactate intracellularly (and therefore corresponding uptake of water in the muscle) (Milligan 1986; Wood 1991). This loss of osmotic homeostasis may reflect an overall energy reallocation as a result of a trade-off scenario between energy demand for muscle contraction and that needed for osmoregulation. In addition, gill remodeling which has been shown to occur in some fish species in response to limits on oxygen supply may be simultaneously easing oxygen demand during strenuous activity, while creating large surface area for ion flux (Postlethwaite 1995; Randall 1998; Brauner 2011). This concept of an osmo-respiratory compromise has also been identified in rainbow trout through experiments involving the measurement of oxygen consumption and ion loss during exercise and recovery. Gonzalez and McDonald (1992) showed that Na^+ loss increased while oxygen consumption increased during exercise. Although initially depressed, plasma osmolality and chloride levels returned toward control levels following four days of training, and recovered completely by eight. This indicates there

may be a compensatory reaction by the osmoregulatory tissues to enhance ion maintenance during this period, or that there is an acclimation-like training effect seen in fish muscle, which may improve the animal's ability to provide adequate energy for osmoregulation (or both). Indeed, properly maintained levels of Cl^- have been suggested to actually serve as a driving force for potentially fatiguing extracellular K^+ during intense exercise (Wallinga 1999).

V. **Conclusions**

Expression of individual Na+/K+ ATPase α- subunit isoforms is differentially regulated between muscle types in rainbow trout during both burst and sustained swimming. Changes in Na^+/K^+-ATPase isoform expression were most pronounced in red muscle and were not evident in either heart or white muscle tissues sampled in these experiments. These results display that swimming speed does not target upregulation of the Na^+/K^+-ATPase in "fast-twitch" or "slow-twitch" muscle fiber types at swimming intensities where aerobic or glycolytic energy is expected to dominate. In terms of a training effect, levels of α1c, α2 and α3 isoforms were generally elevated in fish trained for four consecutive days at both burst as well as sustained swimming speeds, with burst swimming inducing greater and statistically significant upregulation and α2 showing the greatest increase. An increase in expression of all three α- isoforms examined was also seen in fish allowed to recover for four days following two bouts of their swimming protocol. This suggests all three isoforms are involved in correcting any muscle intracellular ion disturbances by this time point and may also be a general response to the overall decrease in plasma osmolality seen within 2 days of intense exercise.

Salmonids are noted to be able to maintain their total plasma osmolarity between 290-340 mosmol/L regardless of the environmental salinities or challenges they may be exposed to (McCormick 1987). Repeated burst swimming was seen to reduce total plasma osmolality as well as chloride levels. Since chloride returned to control levels during recovery and total osmolality did not, it is likely that a decrease in plasma sodium also occurs and is not regulated back to normal as efficiently as chloride. In this study, intense exercise appears to compromise osmoregulatory function in rainbow trout, possibly due to an energy re-allocation to muscle (for locomotion) and away from the gills (for ion balance). Alternativley, this is a side effect of increased surface area at the gills in response to increased oxygen demands.

Further examination of the roles of individual Na^+/K^+-ATPase isoforms under changing swimming conditions would better our understanding of their function in muscle and influence on overall physiological capacity. Mammalian research on muscle physiology has provided a great deal of knowledge to the field and a broader phylogenetic perspective can attempt to tackle the questions that remain. As emphasized, teleost fishes provide a useful model system for studies of exercise, and offer a chance to evolutionarily connect the adaptive physiologies across the many diverse species in nature.

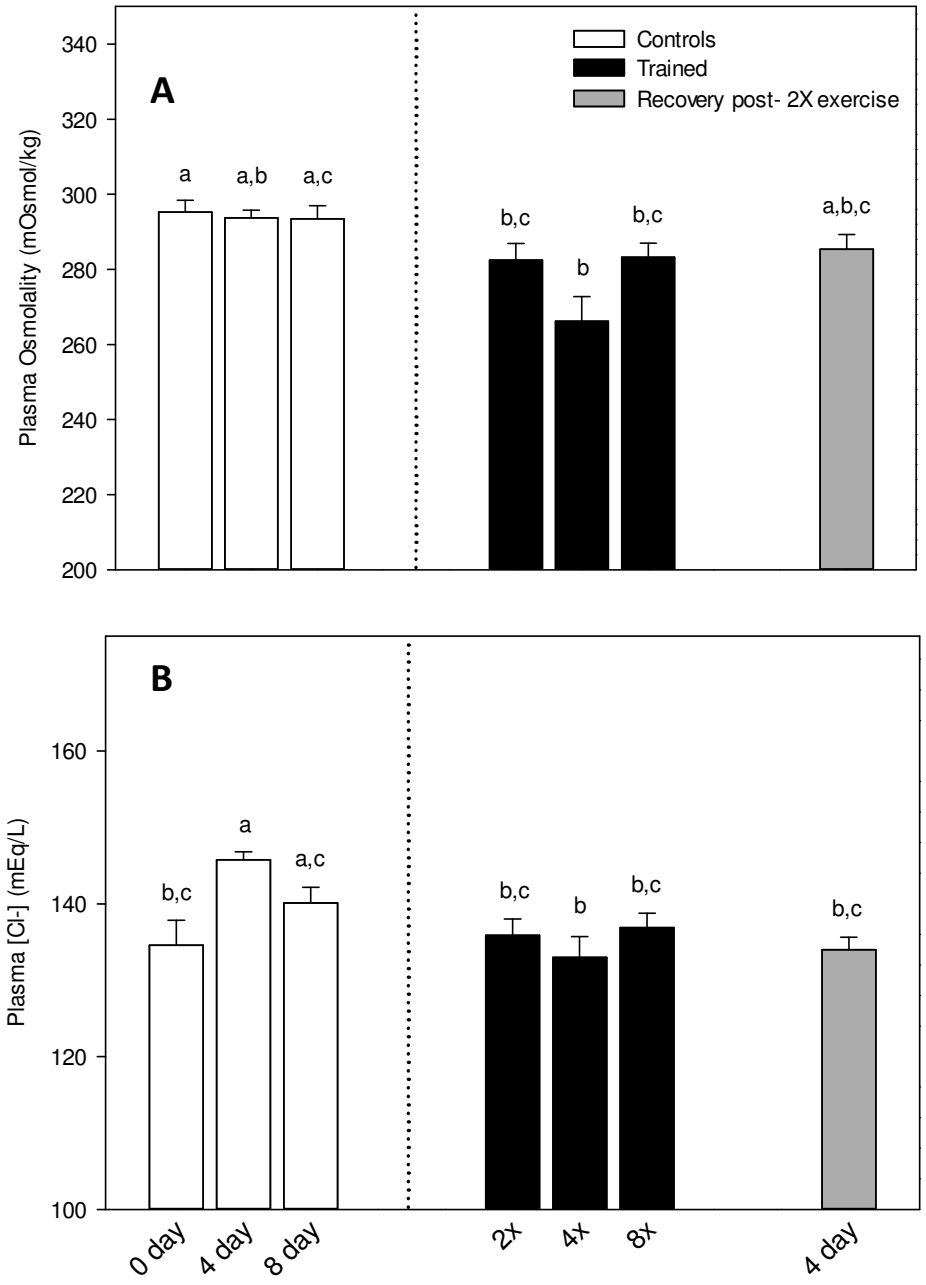

Figure 5. Measurement of total ion content (**A**) and chloride ion levels (**B**) in the blood plasma during sustained swimming, 3 BL/s. Bars that do not share a common letter are significantly different, $p < 0.05$ while figures lacking letters show no differences.

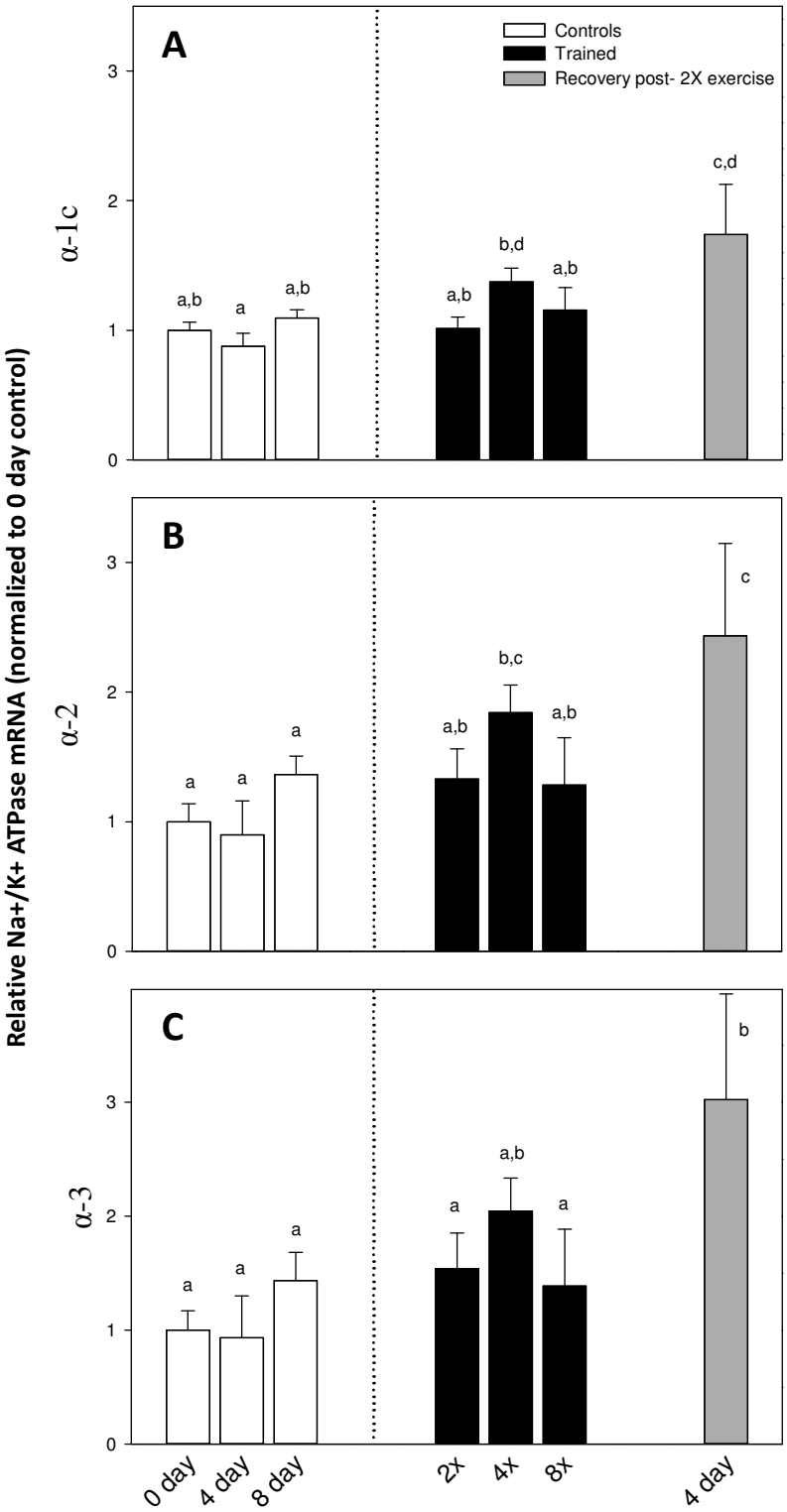

Figure 6. Red muscle Na^+/K^+-ATPase mRNA levels for α1c (**A**), α2 (**B**) and α3 (**C**) during sustained swimming in rainbow trout. Mean expression has been normalized to control gene (EF1α) mRNA expression, and all groups normalized to the 0 day control group. Bars that do not share a common letter are significantly different, $p < 0.05$, while figures lacking letters show no differences.

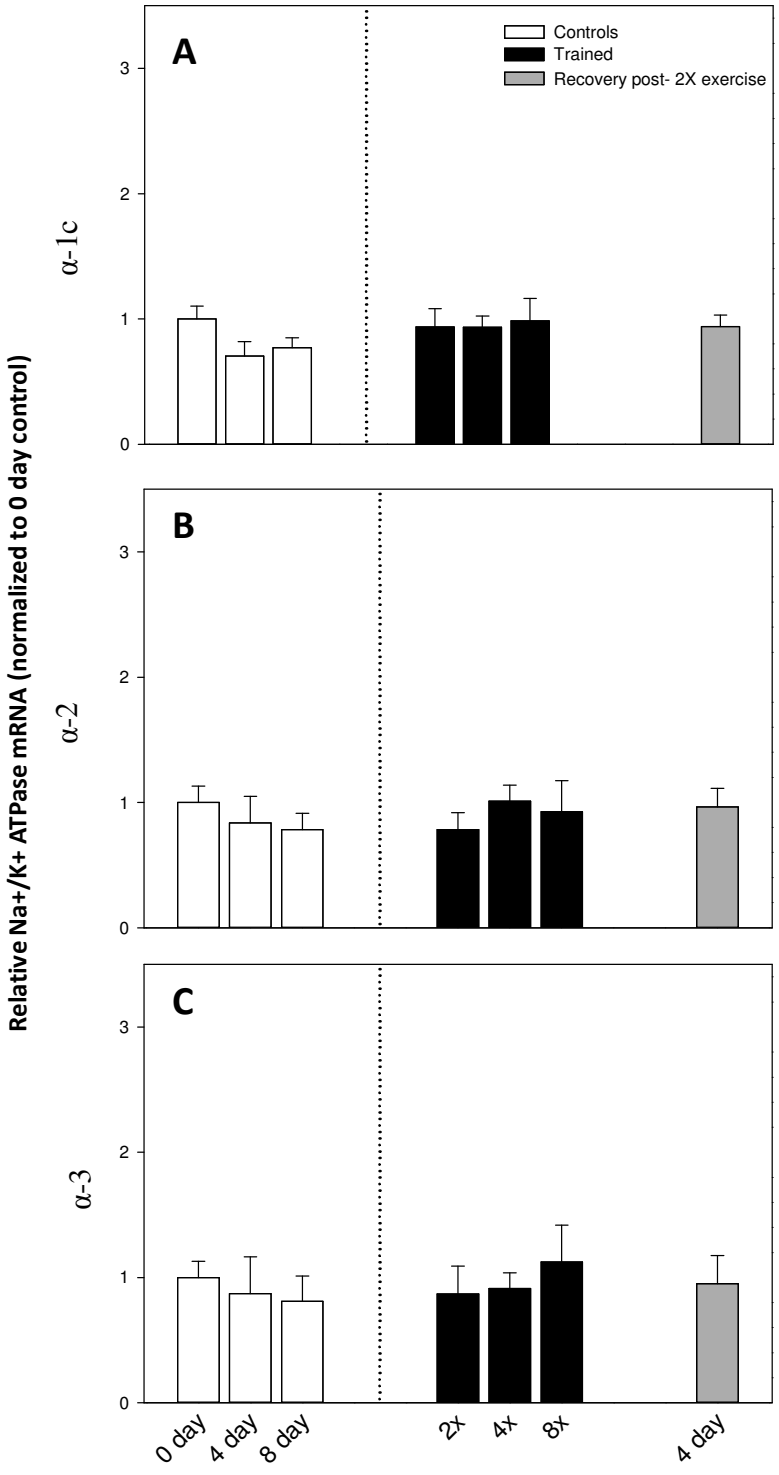

Figure 7. White muscle Na^+/K^+-ATPase mRNA levels for α1c (**A**), α2 (**B**) and α3 (**C**) during sustained swimming. Mean expression has been normalized to unchanging control gene (EF1α) mRNA expression, and all groups normalized to the 0 day control. Bars that do not share a common letter are significantly different, $p < 0.05$, while figures lacking letters show no differences.

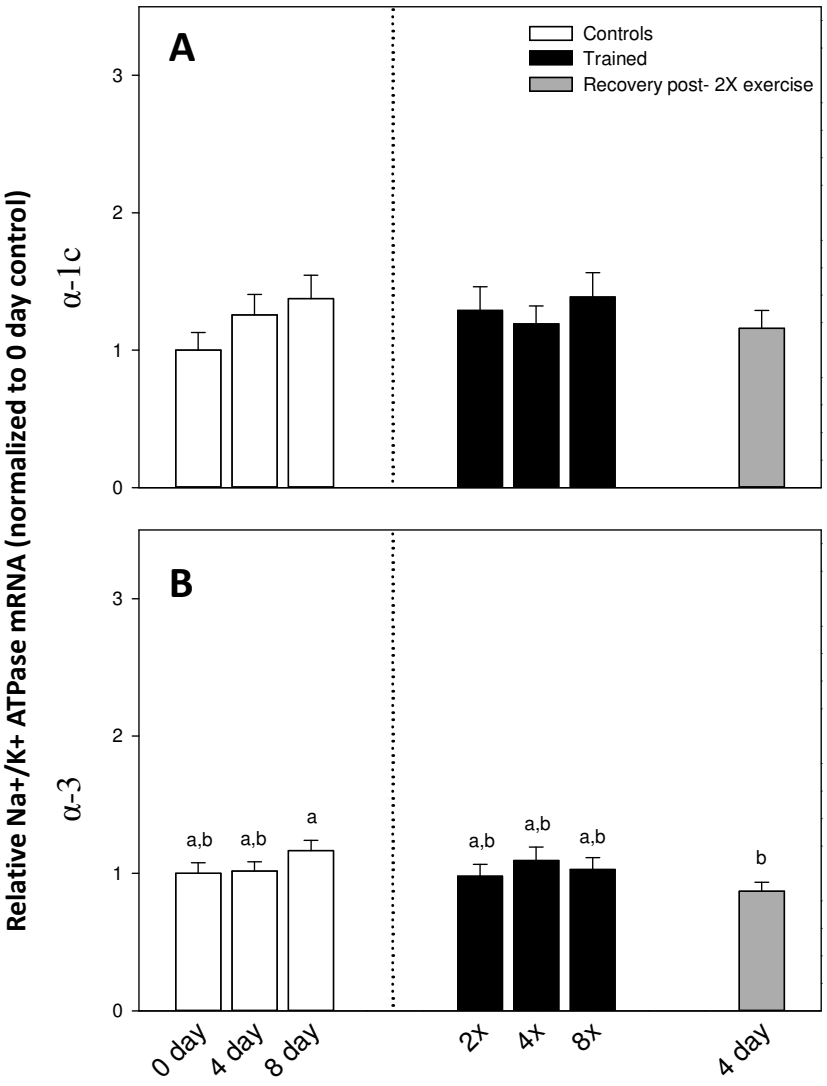

Figure 8. Heart tissue Na^+/K^+-ATPase mRNA levels for α1c (**A**) and α3 (**B**) during sustained swimming. Mean expression has been normalized to unchanging control gene (EF1α) mRNA expression, and all groups normalized to the 0 day control. Bars that do not share a common letter are significantly different, $p < 0.05$, while figures lacking letters show no differences.

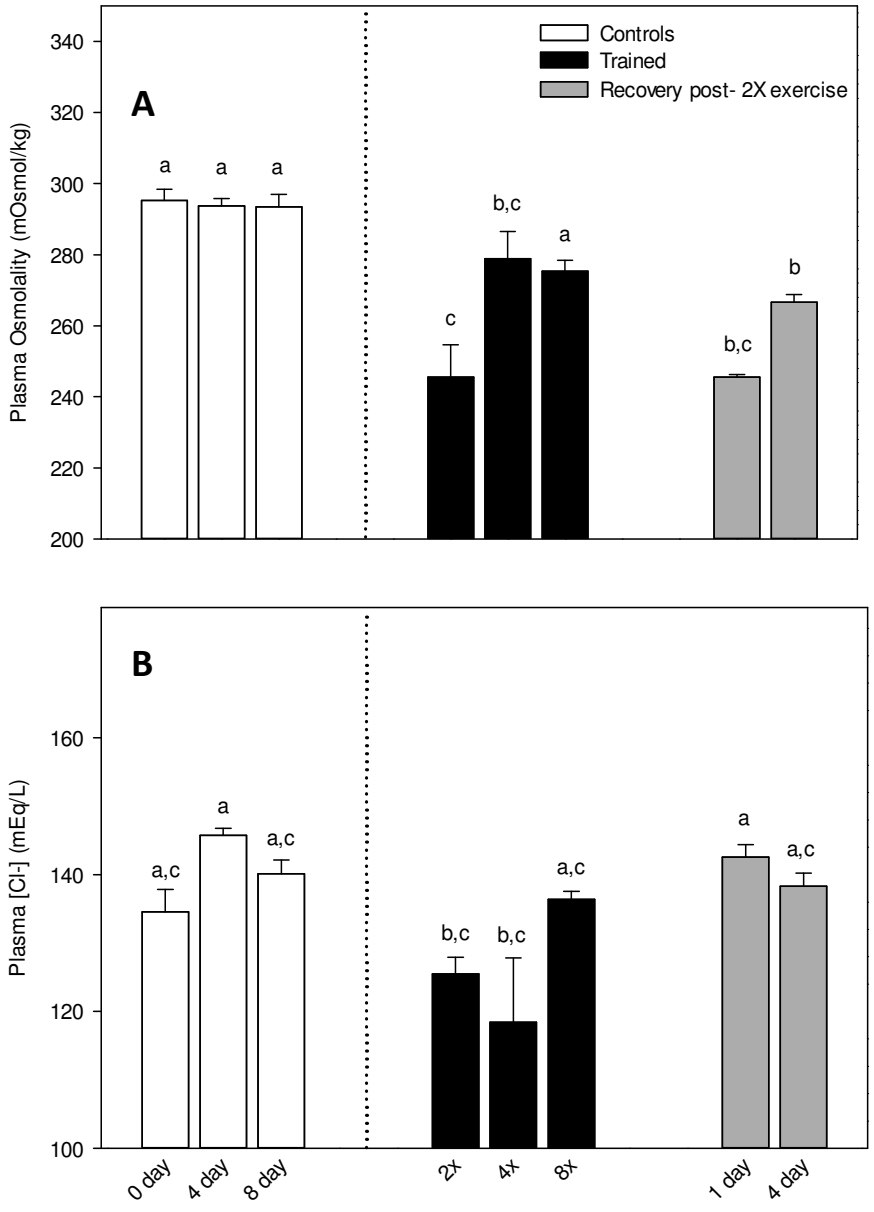

Figure 9. Measurement of total ion content (**A**) and chloride ion levels (**B**) in the blood plasma during burst swimming, 8 BL/s, near U_{crit}. Bars that do not share a common letter are significantly different, $p < 0.05$, while figures lacking letters show no differences.

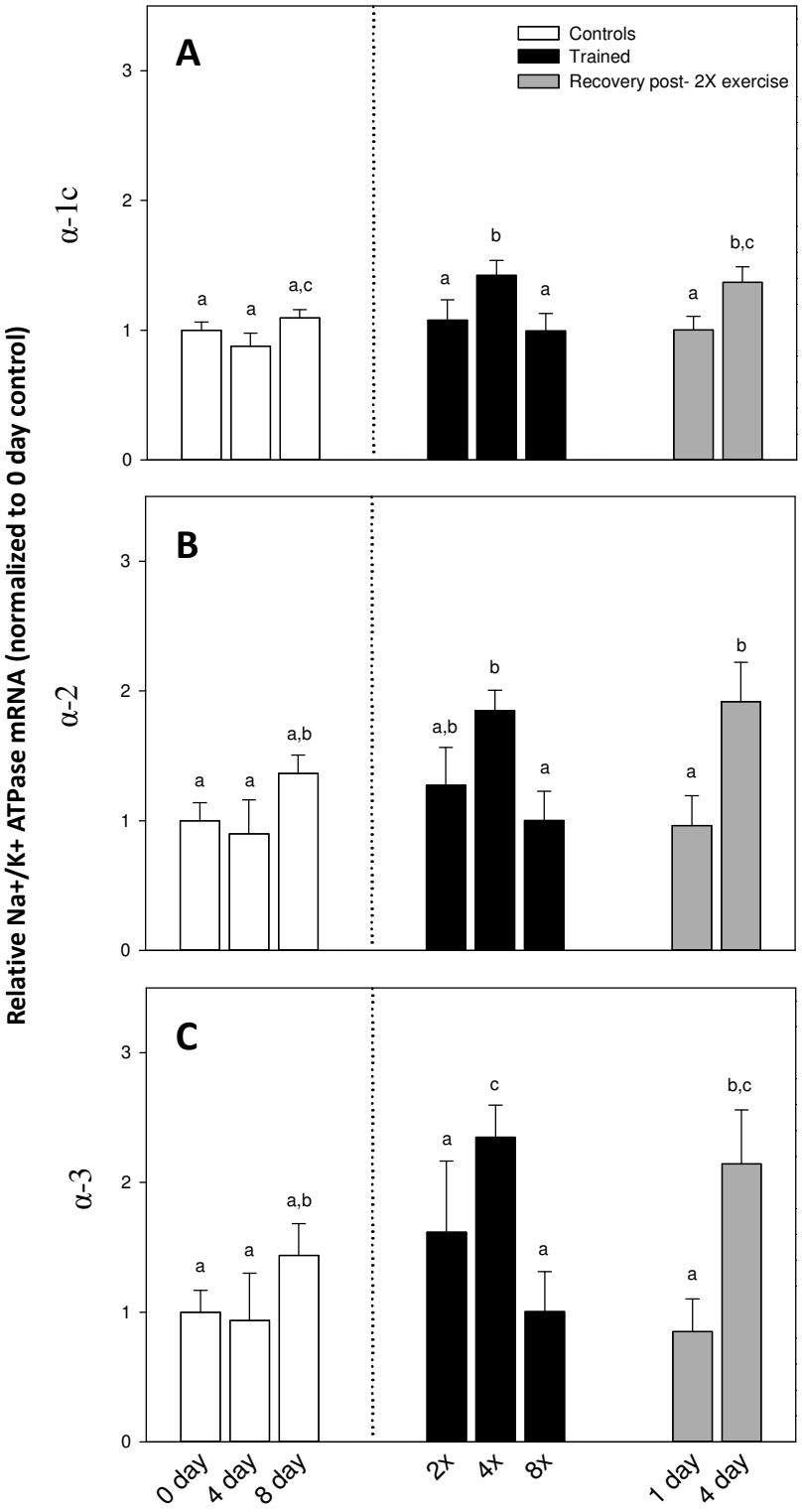

Figure 10. Red muscle Na^+/K^+-ATPase mRNA levels for α1c (**A**), α2 (**B**) and α3 (**C**) during burst swimming. Mean expression has been normalized to unchanging control gene (EF1α) mRNA expression, and all groups normalized to the 0 day control. Bars that do not share a common letter are significantly different, $p < 0.05$, while figures lacking letters show no differences.

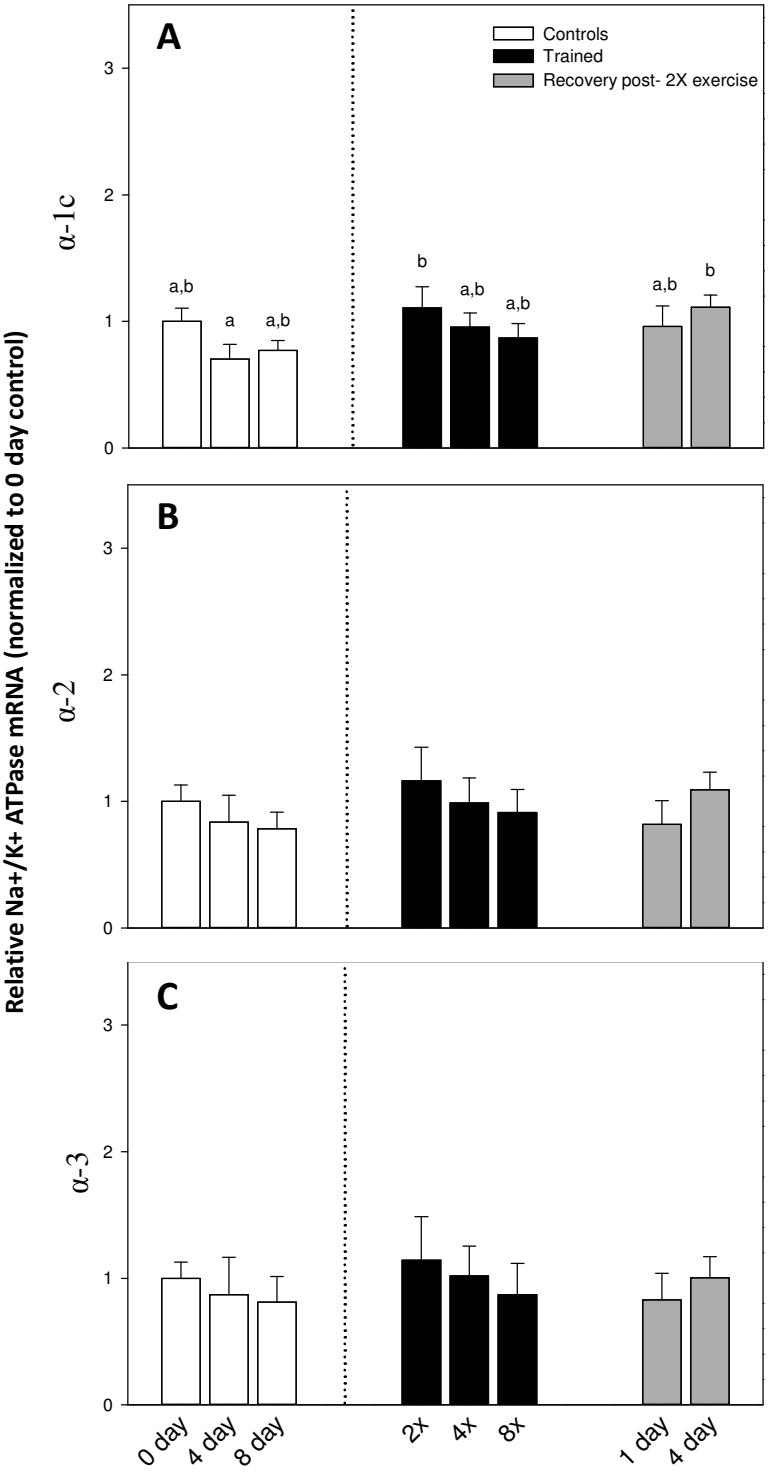

Figure 11. White muscle Na^+/K^+-ATPase mRNA levels for α1c (**A**), α2 (**B**) and α3 (**C**) during burst swimming. Mean expression has been normalized to unchanging control gene (EF1α) mRNA expression, and all groups normalized to the 0 day control. Bars that do not share a common letter are significantly different, $p < 0.05$, while figures lacking letters show no differences.

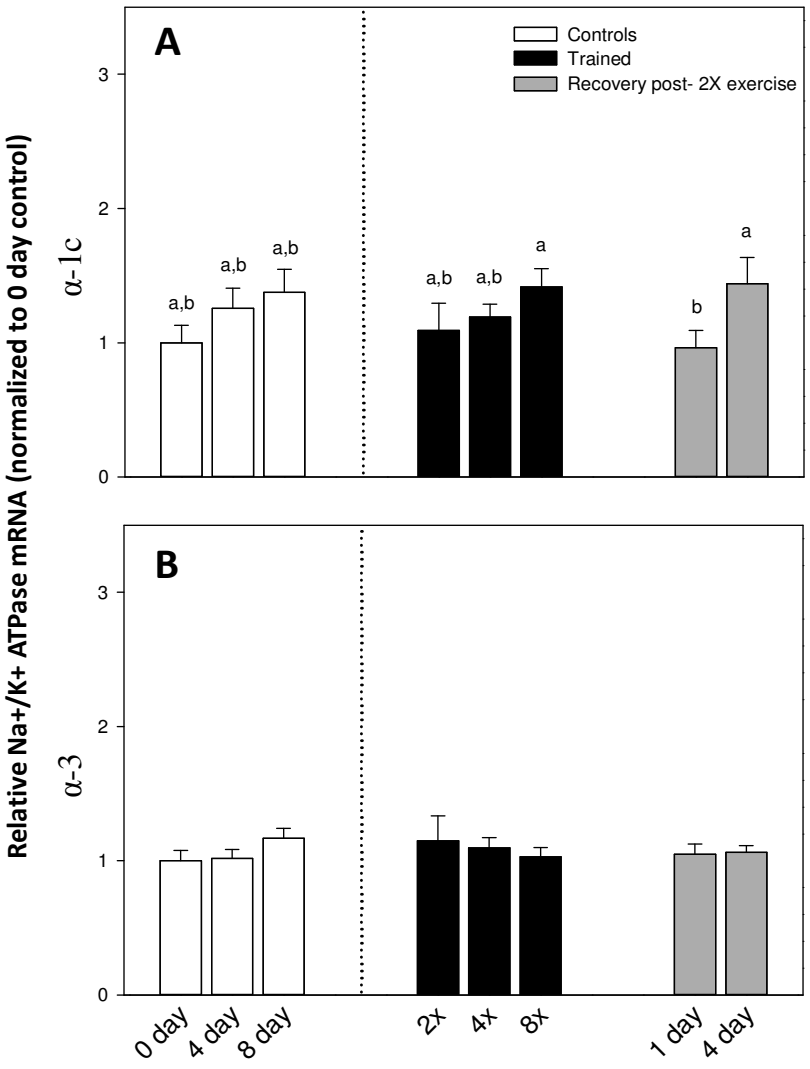

Figure 12. Heart tissue muscle Na^+/K^+-ATPase mRNA levels for α1c (**A**) and α3 (**B**) during burst swimming. Mean expression has been normalized to unchanging control gene (EF1α) mRNA expression, and all groups normalized to the 0 day control. Bars that do not share a common letter are significantly different, $p < 0.05$, while figures lacking letters show no differences.

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Appendix I: *Holding tank and swim tunnel set-up*

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Appendix I: *Holding tank and swim tunnel set-up*

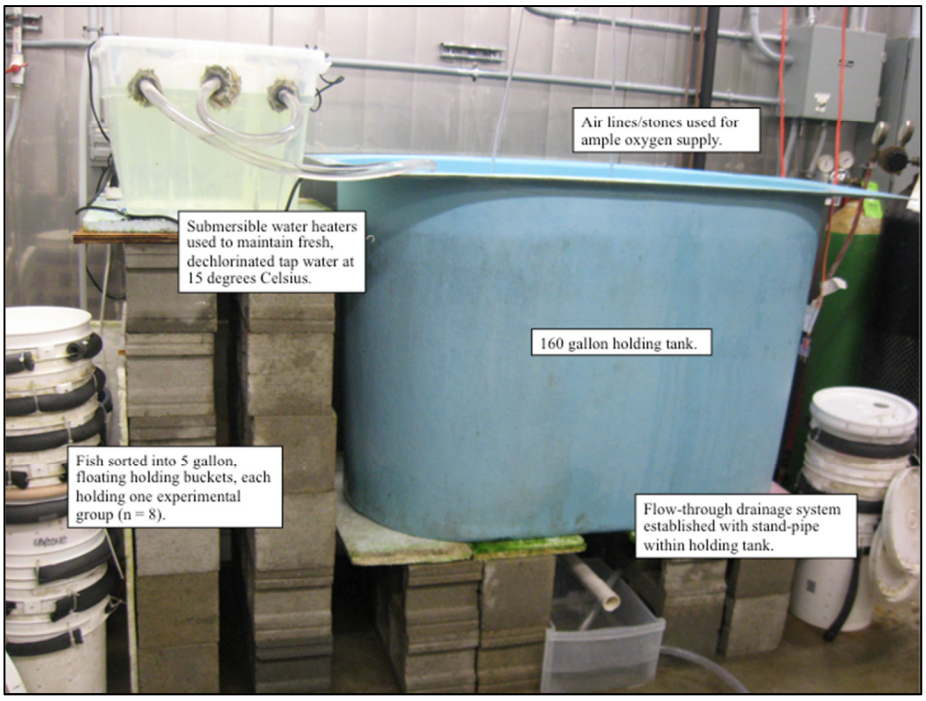

Figure A. Indoor flow-through tank where fish were held between swimming challenges, maintained within a 15 degrees Celsius cold room.

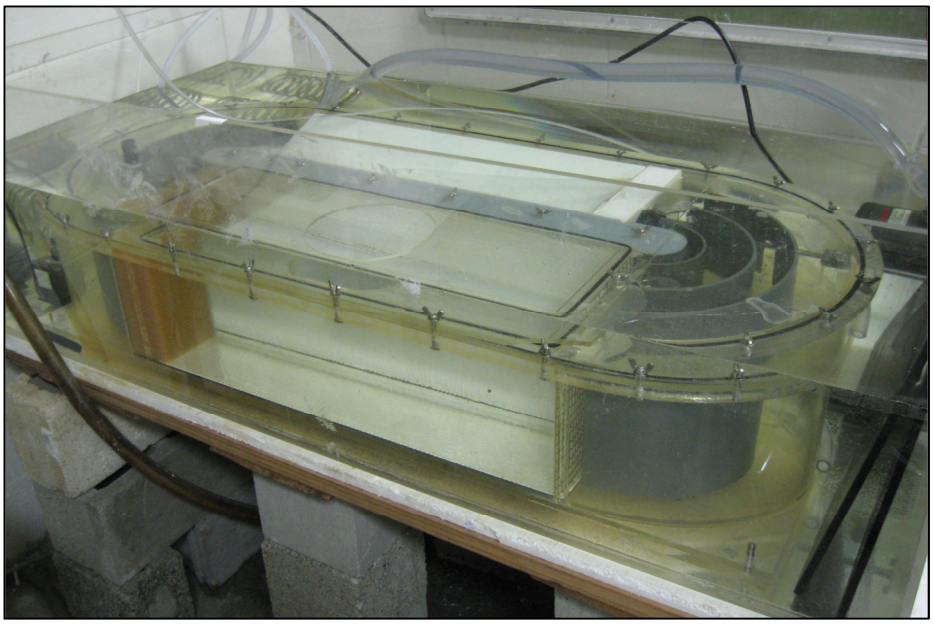

Figure B. Logilo, flume-style swim tunnel (90.15 L maximum volume) used for both burst and sustained swimming experiments.

Appendix II: *Quantitative real-time PCR parameters and validation*

EXPERIMENTAL DESIGN	Importance	Checklist
Definition of experimental and control groups	E	X
Number within each group	E	X
Assay carried out by core lab or investigator's lab?	D	X
Acknowledgment of author's contributions	D	X
SAMPLE		
Description	E	X
Volume/mass of sample processed	D	X
Microdissection or macrodissection	E	X
Processing procedure	E	X
If frozen - how and how quickly?	E	X
If fixed - with what, how quickly?	E	N/A
Sample storage conditions and duration	E	X
NUCLEIC ACID EXTRACTION		
Procedure and/or instructions	E	X
Name of kit and details of any modification	E	X
Source of additional reagents used	D	X
Details of DNase or RNase treatment	E	X
Contamination assessment (DNA/RNA)	E	X
Nucleic acid quantification	E	X
Instrument and method	E	X
Purity (A260/A280)	D	X
Yield	D	X
RNA integrity method/instrument	E	X
RIN/RQI or Cq od 3' and 5' transcripts	E	
Electrophoresis traces	D	X
Inhibition testing(Cq dilutions, spike or other)	E	N/A
REVERSE TRANSCRIPTION		
Complete reaction conditions	E	X
Amount of RNA and reaction volume	E	X
Priming oligonucleotides and concentration	E	X
Reverse transcriptase and concentration	E	X
Temperature and time	E	X
Manufacturer of reagents and catalogue numbers	D	X
Cqs with and without RT*	D	N/A
Storage conditions of cDNA	D	X
qPCR TARGET INFORMATION		
If multiplex, efficiency and LOD of each assay	E	N/A
Sequence accession number	E	X
Location of amplicon	D	
Amplicon length	E	
In silico specificity screen (BLAST, etc), pseudogenes, retropseudogenes, or other homologs?	E	X
Sequence alignment	D	
Secondary structure of amplicon	D	
Location of each primer by exon or intron (if applicable)	E	N/A
What splice variants are targeted?	E	N/A
qPCR OLIGONUCLEOTIDES		
Primer sequences	E	X
RT PrimerDB Identification Number	D	
Probe sequences**	D	N/A
Location and identity of any modifications	E	X
Manufacturer of oligonucleotides	D	
Purification method	D	

qPCR PROTOCOL		
Complete reaction conditions	E	X
Reaction volume and amount of cDNA/DNA	E	X
Primer, probe, Mg++ and dNTP concentrations	E	X
Polymerase identity and concentration	E	X
Buffer/kit identity and manufacturer	E	X
Exact chemical constitution of the buffer	D	N/A
Additives (SYBR Green, DMSO, etc.)	E	X
Manufacturer of plates/tubes and catalog number	D	
Complete thermocycling parameters	E	X
Reaction setup (manual/robotic)	D	
Manufacturer of qPCR instrument	E	X
qPCR VALIDATION		
Evidence of optimisation (from gradients)	D	
Specificity (gel, sequence, melt or digest)	E	X
For SYBR Green, Cq of the NTC	E	X
Standard curves with slope and y-intercept	E	X
PCR efficiency calculated from slope	E	X
Confidence interval for PCR efficiency or standard error	D	
R^2 of standard curve	E	X
Linear dynamic range	E	X
Cq variation at lower limit	E	X
Confidence intervals throughout range	D	
Evidence for limit detection	E	X
If multiplex, efficiency and LOD of each assay	E	N/A
DATA ANALYSIS		
qPCR analysis program (source, version)	E	X
Cq method determination	E	X
Outlier identification and disposition	E	X
Results of NTCs	E	X
Justification of number and choice of reference genes	E	X
Description of normalization method	E	X
Number and concordance of biological replicates	D	X
Number and stage (RT or qPCR) of technical replicates	E	X
Repeatability (intra-assay variation)	E	X
Reproducibility (inter-assay variation, % CV)	D	
Power analysis	D	
Statistical methods for result significance	E	X
Software (source, version)	E	X
Cq or raw data submission using RDML	D	

Table A. MIQE checklist indicating all essential (E) and desirable (D) quantitative real-time PCR information (Bustin *et al.* 2009).

*Assessing the absence of DNA using a no RT assay is essential when first extracting RNA. Once the sample has been validated as RDNA-free, inclusion of a no-RT control is desirable but no longer essential.

**Disclosure of the probe sequence is highly desirable and strongly encouraged. However, since not all commercial pre-designed assay vendors provide this information, it cannot be an essential requirement.

A.

Plate	*Gene*	*Std Curve Slope*	*R^2*	*Baseline*	*Threshold*	*Efficiency*
1	EF1α	-3.274	0.986	4-14	0.6	102%
2	EF1α	-3.274	0.986	4-14	0.6	102%
7	NKA α2	-3.251	0.991	3-8	1.0	103%
8	EF1α	-3.185	0.991	4-14	0.6	106%
9	NKA α2	-3.367	0.969	3-8	1.0	98%
12	NKA α2	-3.62	0.984	3-8	1.0	89%
13	NKA α1c	-3.247	0.996	0-22	1.0	103%
14	NKA α1c	-3.34	0.998	0-22	1.0	99%
19	NKA α1c	-3.668	0.997	0-22	1.0	87%
20	NKA α3	-3.195	0.993	6-18	1.0	105%
21	NKA α3	-3.152	0.999	6-18	1.0	107%
26	NKA α3	-3.208	0.999	6-18	1.0	104%

B.

Plate	*Gene*	*Std Curve Slope*	*R^2*	*Baseline*	*Threshold*	*Efficiency*
27	EF1α	-3.387	0.993	4-18	1.5	97%
28	EF1α	-3.401	0.976	4-18	1.5	97%
10	NKA α2	-3.425	0.991	8-10	1.0	96%
11	NKA α2	-3.598	0.989	8-10	1.0	89%
12	NKA α2	-3.425	0.98	8-10	1.0	96%
15	NKA α1c	-3.158	0.98	8-24	2.0	107%
16	NKA α1c	-3.188	0.986	8-24	2.0	107%
19	NKA α1c	-3.563	0.995	8-24	2.0	90%
22	NKA α3	-3.146	0.998	3-8	2.0	107%
23	NKA α3	-3.393	0.996	3-8	2.0	97%
26	NKA α3	-3.334	0.998	3-8	2.0	99%

C.

Plate	*Gene*	*Std Curve Slope*	*R^2*	*Baseline*	*Threshold*	*Efficiency*
29	EF1α	-3.303	0.994	3-18	2.0	100%
30	EF1α	-3.592	0.985	3-18	2.0	90%
17	NKA α1c	-3.238	0.997	3-15	1.5	103%
18	NKA α1c	-3.153	0.996	3-15	1.5	107%
19	NKA α1c	-3.480	0.987	3-15	1.5	94%
24	NKA α3	-3.148	0.993	12-23	2.0	108%
25	NKA α3	-3.390	0.994	12-23	2.0	97%
26	NKA α3	-3.347	0.985	12-23	2.0	99%

Table B. Quantitative real-time PCR (qRT-PCR) analysis data for red muscle (**A**), white muscle (**B**), and heart (**C**) tissue samples.

Appendix III: *Plasma and white muscle lactate levels*

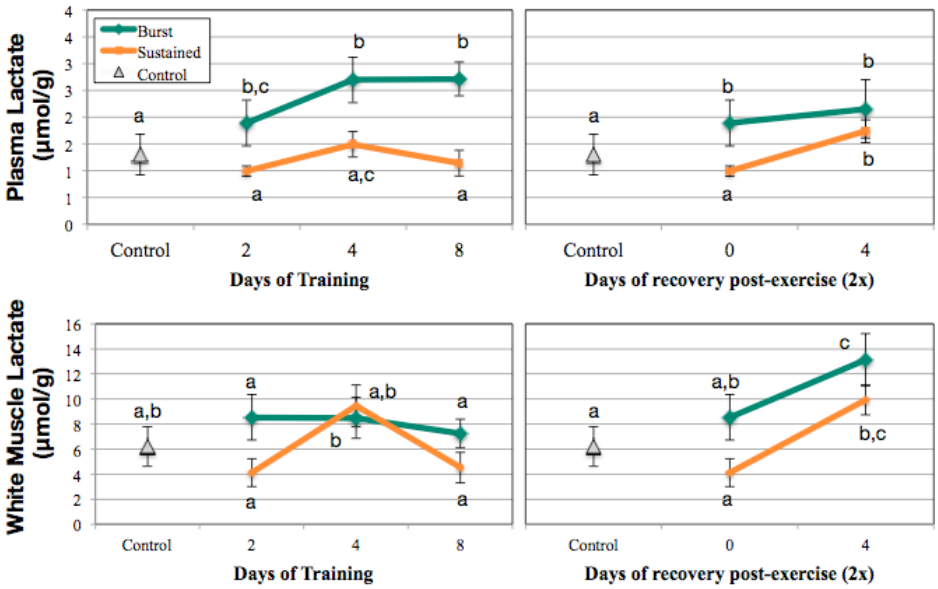

Figure C. Plasma (**top**) and white muscle (**bottom**) lactate levels during repeated bouts of burst and sustained swimming, as well as recovery with '4 day' handling control used for comparison. Points that do not share a common letter are significantly different, $p < 0.05$ (one-way ANOVA). Method used was modified from Wieland and Bergmeyer (1965) and based on Sigma Lactate Assay Kit (#MAK064) and the resulting data was used to validate the experimental design in which burst swimming speeds would induce anaerobic energy production.