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PHYSIOLOGICAL EFFECTS OF SUSTAINED SWIMMING ON SALINITY ACCLIMATION IN CHINOOK SALMON (Oncorhynchus tshawytscha)

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PHYSIOLOGICAL EFFECTS OF SUSTAINED SWIMMING ON SALINITY ACCLIMATION IN CHINOOK SALMON (*Oncorhynchus tshawytscha*)

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THESIS ABSTRACT

Juvenile chinook salmon (*Oncorhynchus tshawytscha*) encounter a variety of environmental conditions on their migration from freshwater to the ocean. These migrations are physiologically demanding, requiring salmon to reverse their osmoregulatory strategies to successfully acclimate to changing salinity. This reversal occurs primarily at the gills (main osmoregulatory organ) where respiratory and osmoregulatory functions are occurring simultaneously. When subjected to a physiological stressor, the gills will compromise in design to alleviate the most limited function. This tradeoff is known as the 'osmorespiratory compromise'. This thesis examined whether an osmorespiratory compromise in chinook salmon induced by sustained exercise (a gas exchange stressor) would lead to secondary changes in gill function that would enhance the fish's ability to acclimate from freshwater to saltwater (an osmoregulatory stressor). In this thesis, plasma total osmolality and ion concentrations, white muscle water content, gill Na^+/K^+-ATPase (NKA) and gill NKA α-subunit isoform mRNA expression were measured on exercised and non-exercised fish in freshwater, as well as exercised and non-exercised fish exposed to a 7-day salinity challenge. Exercise did not induce any notable impairment to ion balance or gill NKA activity after 20 days of being swum at ~1 body length/second (BL/s) in fish that remained in freshwater, but exercised fish did have a significantly higher white muscle water content than non-exercised fish. Both exercised and non-exercised fish exposed to saltwater experienced a characteristic rise in total plasma osmolality and ion concentrations, followed by a recovery to freshwater levels, with exercised fish demonstrating a clear trend of lessened osmoregulatory disturbances compared to non-exercised fish. Interestingly, exercised fish were able to significantly upregulate gill NKA activity and the proposed saltwater α-subunit isoform (α1b) mRNA levels quicker than non-exercised fish, which may explain why exercised fish

experienced less disturbance to osmoregulatory status. These results imply that a sustained exercise training period prior to saltwater exposure improves saltwater acclimation in chinook salmon.

CHAPTER 1: GENERAL INTRODUCTION

1.1 Introduction

Chinook salmon (*Oncorhynchus tshawytscha*) are an anadromous salmonid species that migrate from freshwater to the ocean as juveniles, then return to freshwater to spawn as adults. During these migrations they are exposed to a wide range of biotic and abiotic conditions (e.g. salinity, exercise, predators) which often require significant physiological, morphological, and behavioral acclimations (McCormick and Saunders, 1987). Both the seaward and spawning migrations require salmon to greatly increase their swimming activity (Neave, 1955), all while physiologically preparing to acclimate to a change in salinity. Survival largely depends on a salmon's ability to successfully osmoregulate once they reach their new environment. This is primarily regulated by the gills, which work to regulate whole-body ion concentrations at homeostatic levels, regardless of the external environment (Moyes and Schulte, 2008).

The fish gill is a multifunctional organ that simultaneously works to control gas exchange, ion regulation, acid-base balance and nitrogenous waste excretion (Evans et al., 2005). The gills are capable of remodeling their structure and physiology to optimize function in response to different environmental stressors (e.g. salinity, pH, temperature), allowing many fish species to acclimate to a variety of environmental conditions. Often these morphological and physiological gill modifications that help the fish cope with one environmental condition can be counter-productive with respect to acclimating to other stressors. One example of this can be seen when comparing mechanisms for gas exchange (oxygen uptake and CO_2 excretion) and ionoregulation. Gas exchange can be maximized by increasing surface area, reducing diffusion distance across the gill epithelia, and matching high blood flow to high water ventilation rates. However, these same modifications can be disadvantageous to the ionoregulatory function, as the gill epithelia becomes more prone to ion leakage, leading to problems with effective ion and water regulation

(Evans et al., 2005; Sardella and Brauner, 2007). This tradeoff is known as the 'osmorespiratory compromise' (Randall et al., 1972; Nilsson, 1986; Sardella and Brauner, 2007). Although both gas exchange and osmoregulation have been relatively well studied in a variety of salmonid species, few studies have examined them together. Since salmon are likely to experience multiple simultaneous stressors during migrations, it is important to examine the physiological responses to a multiple stressor scenario. My thesis research investigates whether an osmorespiratory compromise in chinook salmon induced by sustained exercise (a gas exchange stressor) will lead to secondary changes in gill function that will enhance the fish's ability to acclimate from freshwater to saltwater (an osmoregulatory stressor). To better introduce this topic and rationalize my experimental approach, I will first provide some background on the life history of chinook salmon and what is known about their osmoregulatory abilities and the effects of exercise on their physiology.

1.2 *Oncorhynchus tshawytscha* Life History

Chinook salmon (*Oncorhynchus tshawytscha*) are a euryhaline salmonid species originally from the Pacific coast, now found in temperate regions across North America (Crespi and Teo, 2002). All salmon spawn in freshwater and most chinook salmon migrate to the ocean as juveniles to grow, returning to freshwater as sexually mature adults and ready to spawn. This life history pattern is known as anadromy (Crespi and Teo, 2002). All salmon are anadromous, but some species of salmon have nonandromous populations that can live exclusively in freshwater throughout their life cycle. Juvenile chinook salmon have two basic patterns of freshwater residency, an 'ocean-type' and a 'stream-type' (Quinn, 2018). 'Ocean-type' chinook salmon begin their downstream migration to the ocean immediately after hatching, residing in freshwater or estuarine environments for only a short period of time. 'Stream-type' chinook salmon spend a

longer period in freshwater, typically a year, and then begin their downstream migration to the ocean (Quinn, 2018).

In preparation for their seaward migration, juvenile salmon undergo a transition known as the 'parr-smolt transformation' or smoltification which includes behavioral, morphological and physiological changes from a stream-dwelling parr to a seaward-migrating smolt (McCormick and Saunders, 1987; Hoar, 1988). Smoltification occurs seasonally (usually in spring) and can be triggered by abiotic factors such as changing photoperiod and water temperature. Behavioral changes that occur during smoltification include active, downstream swimming (Neave, 1955). The average salmon swimming speed has been recorded as 1.0 body length/second (BL/s), while a study showed during downstream migration sockeye salmon swam at 3 BL/s, virtually their maximum sustainable speed (Groot, 1972). Smoltification occurs while salmon are migrating (sometimes thousands of km) downstream to the ocean, all the while encountering a variety of water conditions such as rapids, waterfalls, and reversing tides that require a strong swimming ability and speed and large amounts of energy expenditure. For example, a study showed 84% of the total energy expenditure of sockeye salmon during simulated migration was used by locomotion and swimming activity (Rand and Hinch, 1998). Therefore, experimental studies that mimic swimming behavior downstream would be beneficial in broadening our understanding of how swimming activity during migration prepares salmon for seawater acclimation. Further, morphological modifications juvenile salmon experience during smoltification include a loss of the distinctive 'parr' color pattern, where salmon become silvery and more streamlined to enhance ocean survival (Hoar, 1988). The physiological changes that occur greatly enhance a salmon's capacity to osmoregulate in seawater (McCormick and Saunders, 1987). Having the

ability to osmoregulate in a wide range of salinities makes chinook salmon an ideal species to study osmoregulation.

1.3 Osmoregulation and Ionoregulation

Any organism that controls the osmolality of their extracellular fluid independent of the osmolality of their external environment is considered an osmoregulator (Moyes and Schulte, 2008). Similarly, any organism that controls the levels of ions in their extracellular fluids, employing a combination of ion absorption and excretion strategies is considered an ionoregulator (Moyes and Schulte, 2008). Chinook salmon are osmoregulating ionoregulators, meaning that regardless of the environment that salmon are found in (freshwater or seawater), their total plasma osmolality is maintained within a narrow range of 290-340 mOsmol/L (McCormick and Saunders, 1987). Ion transport proteins found in the gills allow salmon to 'hyperosmoregulate' in freshwater and 'hypoosmoregulate' in saltwater (Marshall, 2002). Salmon rely on the primary activity transporter, Na^+/K^+-ATPase (NKA) enzyme, primarily in the gills, to help maintain whole-body ion concentrations at homeostatic levels, regardless of their external environment. In general, salmon upregulate gill NKA activity during smoltification (while still in freshwater), as a preparatory mechanism for upcoming seaward migration and subsequent salinity acclimation (Zaugg and Wagner, 1973; McCormick and Saunders, 1987).

In freshwater, salmon hyperosmoregulate to counter the osmotic gain of water and diffusive loss of ions to the surrounding environment (Marshall, 2002). Salmon actively uptake ions (Na^+, Cl^-, and Ca^{2+}) across the gill epithelium and excrete large amounts of dilute urine to decrease loss of salts to the external environment (Marshall, 2002). In freshwater the active influx of ions occurs at the gill epithelium, which is composed of mitochondria-rich chloride cells (MRCs). The Na^+ and Cl^- uptake mechanisms in freshwater is linked to an apical Na^+ channel and a Cl^-

/$HCO3^-$ exchanger, respectively (**Fig. 1.1**) (Avella and Bornancin, 1989). Protons are pumped out of the cell into the external environment through an H^+-ATPase located on the apical membrane of MRCs, creating an electrical gradient. The electrochemical gradient created moves Na^+ into the cell through a proposed epithelial Na^+ channel (ENaC). A basolateral membrane located NKA then pumps the Na^+ into the blood in exchange for K^+. Salmon regulate NKA activity to keep a low intracellular gill Na^+ concentration to aid in apical Na^+ uptake from the external environment (Avella and Bornancin, 1989). H^+-ATPase causes acidification intracellularly and will lower $HCO3^-$ activity enough to drive $HCO3^-$ out of the cell and in turn uptake Cl^-. This exchange occurs through the $Cl^-/HCO3^-$ exchanger located on the apical membrane of MRCs (Marshall, 2002). The mechanism by which Cl^- enters the blood plasma is still not fully understood, but a basolateral Cl^- channel is proposed (Evans et al., 1999).

In seawater, salmon hypoosmoregulate to counter the osmotic loss of water and gain of ions from the concentrated external environment. Salmon drink seawater to combat water loss, and in turn absorb excess ions that must be actively secreted from the blood across the gill epithelium (Marshall, 2002). The driving force for ion secretion in seawater-acclimated salmon is a basolateral-located NKA transporter (**Fig. 1.2**). The NKA generates Na^+ and K^+ gradients across the plasma membrane, which in turn drives the action of a basolateral Na^+, K^+, $2Cl^-$ co-transporter (NKCC), as well as an apical Cl^- channel (Silva et al., 1977). NKA works to keep a low intracellular Na^+ concentration, keeping the MRCs at a highly negative charge and generating an electrochemical gradient to drive Na^+ molecules through the basolateral membrane into the blood in exchange for K^+ (McCormick, 1995). The electrochemical gradient drives two Cl^- molecules, coupled with Na^+ and K^+, into the chloride cell via the basolateral NKCC co-transporter, increasing intracellular Cl^- concentration. Once in the MRCs, Cl^- molecules are

thought to passively diffuse down their electrochemical gradient through an apical chloride channel to the external environment. Na^+ follows Cl^- by passively diffusing across its electrochemical gradient through leaky paracellular pathways between MRCs and accessory cells (Evans et al., 1999).

The gill is the primary site for osmoregulation and ionoregulation in salmon and will transform from an ion-absorbing epithelium to an ion-secreting epithelium as salmon migrate from freshwater to seawater (Marshall, 2002). When salmon reach the ocean they must go through an acclimation period, which is characterized by osmoregulatory adjustments to maintain homeostasis. To successfully hypoosmoregulate, salmon reduce the number and activity of ion transporters and channels used to absorb ions and increase the number of proteins associated with ion secretion. The acclimation period in salmon is thought to take four to five days depending on the species (Leray et al., 1981) and includes multiple phases until they are able to fully acclimate to salinity (Bath and Eddy, 1979). An initial 'crisis phase' occurs when salmon begin to drink seawater to replace lost water and reduce urine excretion, which leads to a characteristic rise in plasma osmolality and ion levels, as the gills have not yet been modified to start secreting ions (Gordon, 1959). Chloride cells increase in both quantity and size during the crisis phase, as well as an increase in gill NKA activity (McCormick and Saunders, 1987). As has been discussed, gill NKA activity plays a vital role in both freshwater and seawater as it is the main driver of blood-ion homeostasis and maintain electrochemical gradients. The gradients that are produced act as the sole energy source for the active influx (Avella and Bornancin, 1989) and efflux of ions across the gill (Silva et al., 1977). Following the crisis phase salmon enter the 'stabilization phase' where slower physiological changes occur, and plasma osmolality and ion levels stabilize to a new steady state. Inability to regulate ions and properly osmoregulate will

likely result in mortality, making plasma osmolality and ion levels, and gill NKA activity effective indicators of osmoregulatory status and water balance in fish.

1.4 Structure of Na^+/K^+-ATPase

Na^+/K^+-ATPase is an energy-dependent, ion-translocating protein found in virtually all animal cells and acts as the driving force for ion flux in MRCs (McCormick, 1995). NKA is a multi-subunit enzyme that is embedded in the plasma membrane and hydrolyzes one molecule of ATP for the coupled exchange of two extracellular K^+ ions for three intracellular Na^+ ions (Blanco and Mercer, 1998). NKA maintains a low intracellular Na^+ concentration and high intracellular K^+, to produce steep gradients for other plasma membrane embedded proteins performing essential cellular functions. NKA is typically composed of three subunits: the catalytic α-subunit that acts as the main binding site for ions, ATP, ouabain inhibitor, and the phosphorylation site, the regulatory β-subunit that is glycosylated and thought to assist in the folding and placement of the α subunit, and finally a third non-essential γ-subunit whose function is still not fully known (Blanco and Mercer, 1998; Mobasheri et al., 2000). The α-subunit and β-subunit noncovalently bond to form the αβ-heterodimer. Multiple subunit isoforms have been identified in mammals (Mobasheri et al., 2000) and identification of at least five α-subunit isoforms (α1a, α1b, α1c, α2, and α3) and one β-subunit have been found in salmonids (Richards et al., 2003). Subunit isoform configuration is expressed differently throughout tissues, and can exhibit different enzyme kinetics, based on different combinations (Blanco and Mercer, 1998). Slight differences in kinetics and configurations allow for isoform switching to occur in order to best suit the organisms specific physiological need.

1.5 Na^+/K^+-ATPase Subunit Isoform Switching

The importance of gill NKA activity when acclimating to seawater is widely accepted, and in Richards et al. (2003), a pioneering finding was made on the regulation of NKA isoforms α1a and α1b. In this study, five α-subunit isoforms (α1a, α1b, α1c, α2, and α3) were sequenced in varying rainbow trout tissues. Isoforms αla and α1b were expressed in the gills, with α1b also expressed in the brain, eye, and kidney, α2 expressed in red and white muscle, and α1c and α3 expressed ubiquitously throughout tissues. More specifically, this study found a switch in the dominant gill NKA isoform from α1a to α1b when rainbow trout are acclimated to saltwater. Later studies confirmed that upon exposure to seawater, gill NKA α1a mRNA levels are down-regulated, while gill NKA α1b mRNA levels are upregulated in a variety of salmonid species (Richards et al., 2003; Bystriansky et al., 2006; Bystriansky et al., 2007; Bystriansky and Ballantyne, 2007). Furthermore, the opposite isoform switch was seen when Atlantic salmon acclimated from seawater to freshwater (Bystriansky and Schulte 2011). These studies suggest that the α1a isoform is thought to be responsible for freshwater tolerance, while the α1b isoform is responsible for seawater tolerance. Stressors, such as salinity, induces a functional switch in gill NKA α1 isoforms, but more information is needed to understand if stressors like sustained swimming also induce these functional changes.

1.6 Osmorespiratory Compromise

The osmorespiratory compromise is a crucial component in understanding the physiological limits of fish performance when exposed to different environmental challenges (e.g. salinity, exercise, hypoxia, etc.). Respiratory and osmoregulatory functions present a tradeoff of optimal gill designs that is referred to as the ‘osmorespiratory compromise’ (Randall et al., 1972; Nilsson, 1986; Sardella and Brauner, 2007). Gas exchange function occurs in the gills at the

lamellar epithelium via diffusion of oxygen and carbon dioxide across pavement cells (PVCs). In contrast, ion regulation occurs at the filament epithelium within MRCs (Sardella and Brauner, 2007). MRCs are functionally specialized to the ionic concentration of the environment the fish is in and will undergo morphological and physiological changes to acclimate to the environments ionic composition (Sardella and Brauner, 2007). These changes to the gill are mostly short-term and reversible and are indicative of the function that is most limited by the acclimation to a new environment. When a stressor is mediated by the gill, the gill will return to a design that compromises between the minimum rate of oxygen consumption needed for metabolism and the maximum rate of ion and water flux that can be tolerated. Stressors, such as exercise and salinity, present a challenge to the gills and require opposing gill designs to achieve homeostasis. Exercise requires increased gill perfusion when oxygen demand is high, at the expense of ion flux, while salinity requires reduced functional surface area to defend ion balance, at the expense of gas exchange.

Upon entering seawater, salmon must switch osmoregulation strategies to successfully acclimate. Osmoregulation is strained in seawater due to excess ion influx internally from diffusion of ions from the concentrated environment and excess drinking of seawater to balance body water content. A disturbance to total plasma osmolality and plasma ion concentrations, due to excess ions, mobilizes the osmorespiratory compromise to defend osmotic balance. Osmotic imbalances have been linked to reductions in swimming performance in salmonids (Houston, 1959; Brauner et al., 1992) and it is suggested that the upper limit of aerobic exercise may be dictated by the need to defend ion balance, rather than the capacity of gas exchange mechanisms (Gonzalez and McDonald, 1992). While limiting ionoregulatory costs may inhibit swimming capacity in salmon, it was also found that exercise-trained fish enabled plasma osmolality to be

better maintained despite elevated water loss across the gills in seawater-adapted chinook salmon (Gallaugher et al., 2001). The relationship between exercise training and salinity acclimation is still not fully understood. Understanding this relationship using the context of the osmorespiratory compromise can shed light on the physiological mechanisms salmon use to acclimate to their external environments.

Exercise presents a physiological challenge to the gas exchange function of the osmorespiratory compromise. Oxygen demand is elevated during exercise, increasing water ventilation and perfusion rates of blood across the gills. Studies have found that increased perfusion leads to the recruitment of lamellae, which increase the functional surface area of the gills for oxygen uptake and reduce the blood to water diffusion distance (Booth, 1978). Previous studies have shown that exercise will remodel the gills to favorable conditions of gas exchange in both crucian carp (*Carassius carassius*) and goldfish (*Carassius auratus*) (Brauner et al., 2011; Fu et al., 2011; Perry et al., 2012). These studies found that when fish are swum close to U_{crit} (their maximum critical swimming speed), exercise significantly increased protruding lamellae basal length and lamellae surface area. Interestingly, all three studies found that metabolic rate increased with increasing swimming speed. As the exercise became more strenuous, fish had a higher demand for oxygen uptake, the probable cause of the observed gill remodeling in both species to accommodate increased oxygen consumption. Studies have shown that when salmon are exercised in freshwater, there is an increase in ion efflux across the gill epithelium (Randall et al., 1972; Wood and Randall, 1973; Gonzalez and McDonald, 1992; Postlethwaite and McDonald, 1995; Onukwufor and Wood, 2018). The relationship between metabolic rate and chronic exercise was previously studied by Gonzalez and McDonald (1992), when they investigated the relationship between Na^+ efflux and oxygen consumption of

freshwater-acclimated rainbow trout. They found that when rainbow trout were exercised, loss of Na^+ across the gills increased substantially. The authors found that although exercise caused an acute increase in Na^+ efflux, with chronic exercise the ion/gas ratio returned to routine levels. In a similar study, rainbow trout exhibited a 70% increase in Na^+ efflux when subjected to strenuous exercise while in freshwater (Randall et al., 1972). Such a dramatic increase in ion efflux is due to an increase in functional surface area caused by an increased need for oxygen uptake during exercise. An increase in functional surface area leads to an increase in Na^+ permeability and a decrease in diffusion distance from the internal to external environments (Onukwufor and Wood, 2018) and an increase in ion permeability between paracellular pathways (Rodnick and Planas, 2016). It is hypothesized that ion efflux is due to an increase in ion transport carriers (Randall et al., 1972) for Na^+ and Cl^- found in MRCs post-exercise (Postlethwaite and McDonald, 1995).

Although an increase in ion excretion in freshwater may cause a disturbance to homeostasis, it may be beneficial for ionoregulation in seawater where salmon must actively excrete excess ions into their hyperosmotic environment.

1.7 Exercise Training

Chinook salmon serve as an ideal species to use for exercise training studies as swimming is a critical aspect of their life history strategy and survival. Salmon can swim incredible distances in nature, all while simultaneously acclimating to their external environment. Being anadromous, chinook salmon can experience a variety of water conditions, from gentle flowing streams and estuaries, to strong ocean currents, rapids, and waterfalls, with the latter requiring strenuous swimming for prolonged periods of time. Some salmon are raised in hatcheries and large aquaculture facilities in high density, low flow conditions that differ from their natural habitat,

that can lead to poor survival after being released into the wild (Woodward and Smith, 1985). Understanding the effects modified flow conditions have on hatchery-reared salmon can help produce fish that could potentially have a higher probability of survival after release.

Swimming in fish can be aerobic (sustained), anaerobic (burst) or prolonged, depending on the intensity and length of the exercise. Sustained swimming is described as aerobic activity that can be maintained for 200 minutes or longer in which metabolic demands are matched by the available physiological supply of energy (Beamish, 1978). Swimming at a speed of 1.0-1.5 BL/s is considered aerobic activity that can be maintained indefinitely. These low swimming speeds have been shown to cause an increase growth in salmon (Davison, 1997) while high-intensity swimming speeds of 2.0-3.0 BL/s showed a negative training effect, resulting in a decrease in physiological condition and growth in rainbow trout (Farrell et al., 1991). Anaerobic (burst) swimming is a high-intensity challenge that can only be tolerated for short periods of time (~20-30 seconds), due to fast depletion of intracellular energy stores and reliance on anaerobic metabolism (Beamish, 1978). Prolonged swimming falls in-between aerobic and anaerobic swimming with duration of swimming lasting anywhere from 20-200 minutes, terminated by fatigue (Brett, 1964). Prolonged swimming in fish is described as unpredictable patterns of high-intensity swimming speeds to relaxed 'cruising' swimming speeds (Hammer, 1995).

Only a few studies have looked at the effects of long-term (> 200 minutes) sustained swimming exercise on salmon with subsequent salinity transfer (Jørgensen and Jobling, 1993; Khovanskiy et al., 1993; Esbaugh et al., 2014). Both Jørgensen and Jobling (1993) and Khovanskiy et al. (1993) assessed whole-body plasma ion and osmolarity levels, but no parameters were assessed regarding gill ion regulation or gill NKA activity. Khovanskiy et al. (1993) assessed osmoregulatory status and found that when chum salmon (*Oncorhynchus keta*)

were exercised, plasma ion and water concentrations following salinity exposure returned to homeostatic levels quicker than in non-exercised fish. This study indicated that exercise helped in acclimation by decreasing osmoregulatory disturbances associated with salinity transfer, but the mechanism for the improvement was not determined. Esbaugh et al. (2014) conducted an experiment measuring branchial gene expression of osmoregulatory pathways in Atlantic salmon (*Salmo salar*) following a 10-week exercise training period, a one-week recovery from flow period, and an eight-day salinity transfer. The acclimation period usually lasts four to five days and by waiting to sample eight days after salinity exposure, the authors may have missed the window to understand how exercise training effected salinity acclimation. However, they did find that when exercised continuously at 1.53 BL/s for 10 weeks, salmon gill NKA α1b isoform was upregulated to seawater level expression. The gill NKA α1b isoform is thought to be responsible for salinity tolerance (Richards et al., 2003; Bystriansky et al., 2006; Bystriansky et al., 2007). The study infers exercise has an effect on gene expression that is favorable to osmoregulatory pathways associated with salinity acclimation; although more information regarding how sustained exercise training effects the initial 'crisis phase' of salinity acclimation is needed. In contrast, a study conducted by Alonge (2013) has shown that exercise in freshwater juvenile rainbow trout induced an increase in gill NKA isoform α1a expression and a general increase in both gill NKA and H^+-ATPase activity. Researching the activity and expression of gill NKA during exercise training, as it is necessary for ion balance, may shed light on the physiological processes that play a role in the osmorespiratory compromise.

1.8 Thesis Objectives

The osmorespiratory compromise is affected differently by both exercise and salinity. While these stressors have been researched extensively separately, studying the combined effect of how

exercise effects salinity acclimation can help to understand important information on the osmoregulatory and ionoregulatory strategies of anadromous fish. This study was designed to assess whether a 20-day aerobic sustained swimming challenge affected osmoregulatory mechanisms related to salinity acclimation in chinook salmon.

This thesis addresses the questions: (1) Is there a preparatory effect of exercise on osmoregulatory mechanisms in chinook salmon suitable for saltwater acclimation; and (2) do exercised and non-exercised trained chinook salmon respond to disturbances incurred by saltwater differently? It was predicted that sustained exercise will improve salinity acclimation, as exercise will lead to difficulties maintaining plasma ion homeostasis, resulting in a gill design (increased gill NKA activity, increased expression of the NKA α1b isoform, and increased gill ion flux) that is optimal for osmoregulation and ionoregulation in saltwater. Overall, the results of this thesis provide insight into the effects of sustained exercise followed by salinity acclimation, both of which are natural challenges that juvenile anadromous fish, like chinook salmon, must overcome to complete their life cycle.

1.9 Figures

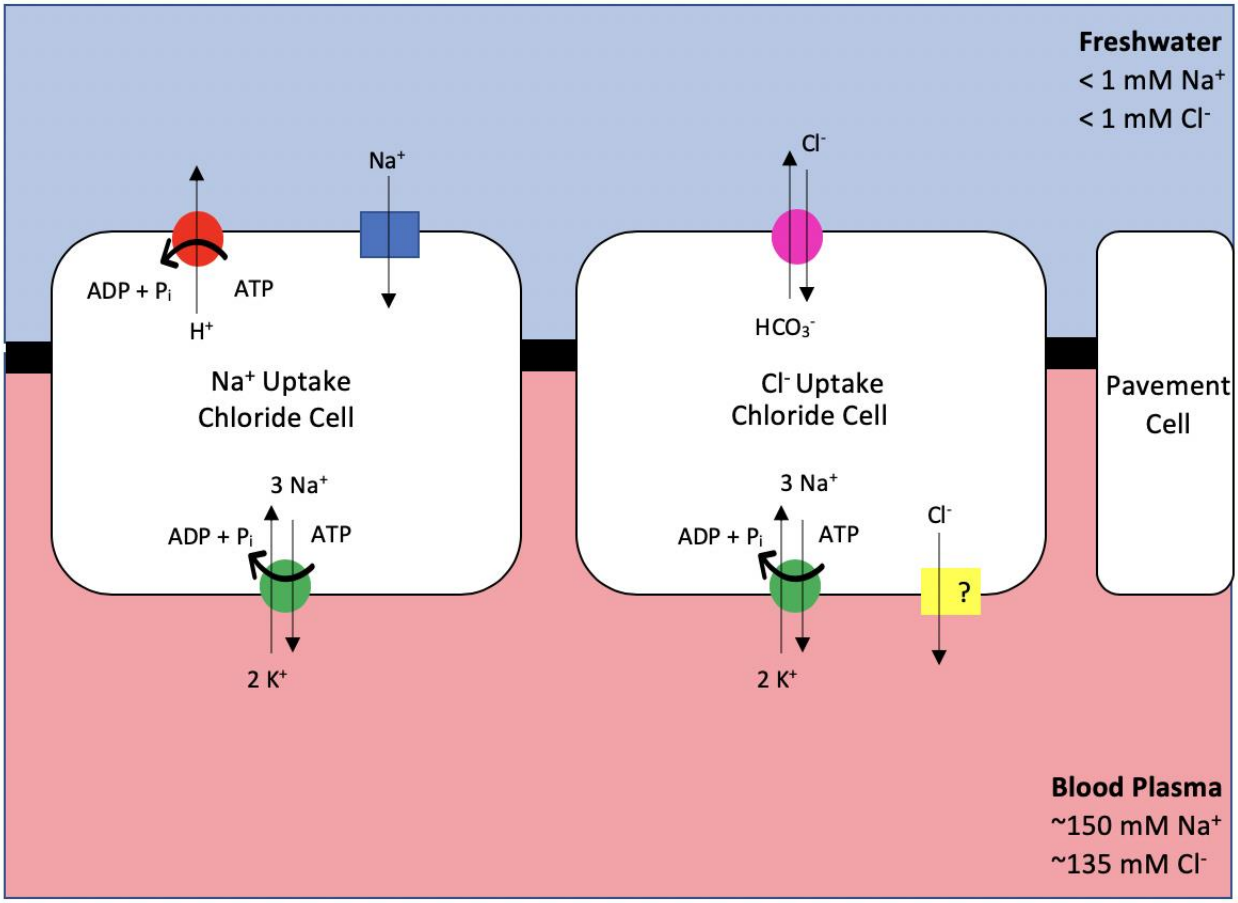

Figure 1.1: Schematic diagram of mechanism for net uptake of Na^+ and Cl^- ions from freshwater into the blood across the fish gill epithelium (adapted from Evans et al. (1999)).

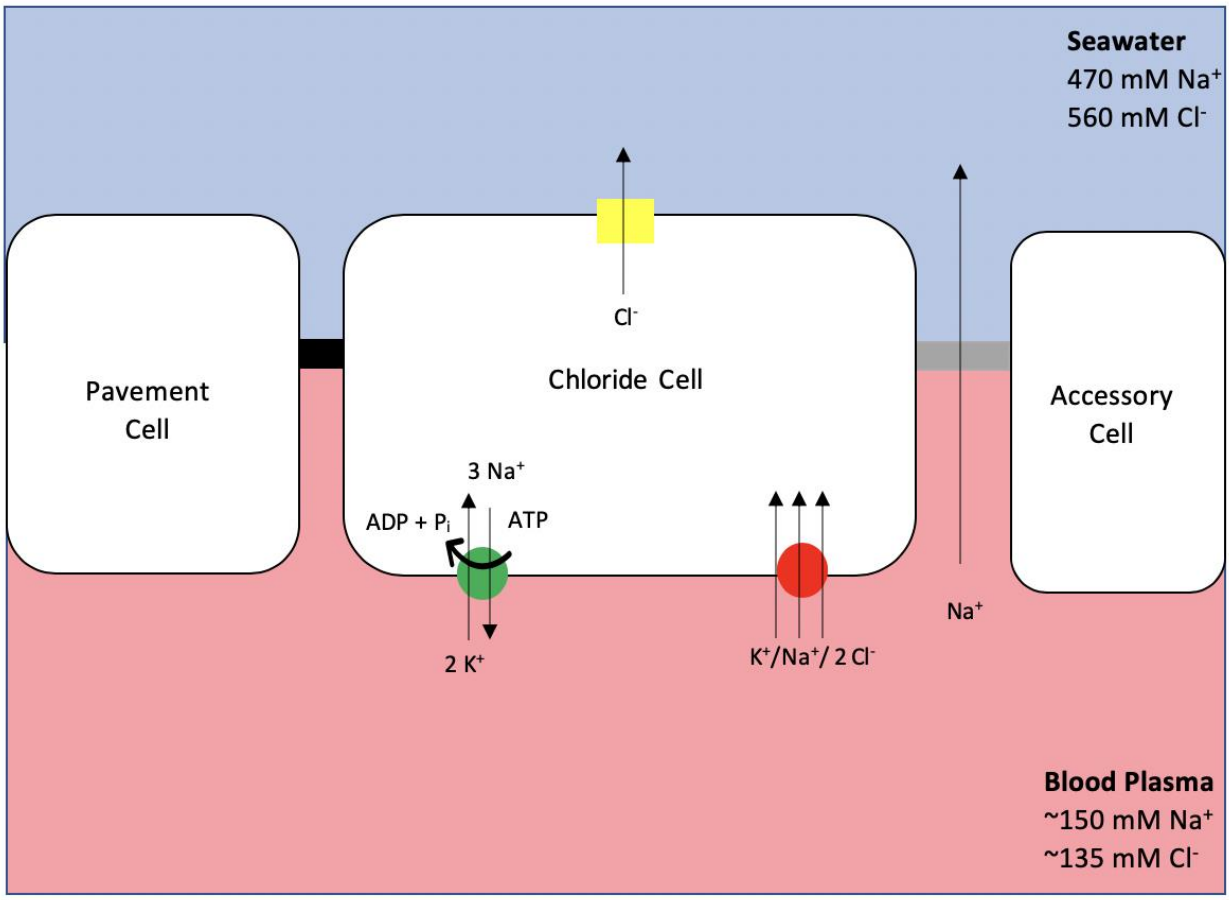

Figure 1.2: Schematic diagram of mechanisms for net excretion of Na^+ and Cl^- ions from the blood into seawater across the gill epithelium (adapted from Evans et al. (1999)).

CHAPTER 2: PHYSIOLOGICAL EFFECTS OF SUSTAINED SWIMMING ON SALINITY ACCLIMATION IN CHINOOK SALMON (*Oncorhynchus tshawytscha*)

2.1 Abstract

The osmorespiratory compromise (a physiological trade-off between respiratory and osmoregulatory functions) has been well studied in response to exercise in salmonids. During exercise, the functional surface area of the gills in increased to meet oxygen demand which leads to perturbations in ion and water fluxes. A remodeled gill, due to exercise, may enhance salmon acclimation to saltwater as it would improve ion excretion at the gills to counter the gain of ions from saltwater. This study examined whether an osmorespiratory compromise in chinook salmon induced by sustained exercise would lead to secondary changes in gill function that would enhance the fish's ability to acclimate from freshwater to saltwater. Plasma osmolality, plasma [Na^+] and [Cl^-], white muscle water content, gill Na^+/K^+-ATPase (NKA) and gill NKA α-subunit isoform expression were measured on exercised and non-exercised fish that remained in freshwater and in fish exposed to saltwater. Exercise did not induce any notable impairment to ion balance or gill NKA activity after 20 days of exercise at ~1 body length/second (BL/s) in freshwater, but exercised fish did have a significantly higher muscle water content than non-exercised fish. Both exercised and non-exercised fish exposed to saltwater experienced a rise in plasma osmolality and ion concentrations, followed by a recovery to freshwater levels, with exercised fish experiencing a "lessened" osmoregulatory disturbance compared to non-exercised fish. Interestingly, exercised fish were able to significantly upregulate gill NKA activity and NKA α1b mRNA levels quicker than non-exercised fish, which may explain why exercised fish experienced less disturbance to osmoregulatory status. The osmoregulatory plasticity seen in this study provides additional evidence to how salmon use the osmorespiratory compromise to mediate competing stressors.

2.2 Introduction

The fish gill is a multifunctional organ that works to control gas exchange, ion regulation, acid-base balance and nitrogenous waste excretion (Evans et al., 2005). These functions of the gill occur simultaneously across a single structure and require certain conditions for optimal performance, often that differ from one another (Sardella and Brauner, 2007). For example, conditions advantageous to gas exchange such as increased surface area, reduced diffusion distance across the gill epithelia, and high blood flow matched to high water ventilation rates directly oppose conditions favorable to osmoregulation. When subjected to a situation that challenges the physiological capacity of either function (respiratory or osmoregulatory), a fish gill will compromise in design to alleviate the most limited function. This tradeoff is known as the 'osmorespiratory compromise' (Randall et al., 1972; Nilsson, 1986; Sardella and Brauner, 2007). Under normal conditions, fish will compromise between the minimum rate of oxygen consumption needed for metabolism and the maximum rate of ion and water flux that can be tolerated. Gills exhibit a certain degree of functional plasticity when exposed to stressors (exercise, salinity, pH, temperature, etc.) to alleviate the effects the stressors impose. By utilizing the osmorespiratory compromise, the gills will compromise between opposing demands when alleviating stressors.

When fish are exercised, the need for improved oxygen uptake increases, leading to greater surface area of the gills for oxygen uptake and reduced blood to water diffusion distance (Booth, 1978). Conditions to increase gas exchange during exercise result in an unfavorable efflux of ions, therefore when exercised in freshwater ionic disturbance is increased (Randall et al., 1972; Wood and Randall, 1973; Gonzalez and McDonald, 1992; Postlethwaite and McDonald, 1995; Onukwufor and Wood, 2018). Exercise can be detrimental to a salmon in freshwater because

salmon are hyperosmotic to the freshwater environment. In freshwater, salmon rely on the active influx of Na^+ and Cl^- from the environment while producing large amounts of dilute urine to counter the osmotic gain of water and diffusive loss of ions to the surrounding environment (Marshall, 2002). The active influx of ions occurs primarily at the gills and rely on Na^+/K^+-ATPase (NKA) activity to fuel the process (Avella and Bornancin, 1989). If exercise is not mediated by the osmorespiratory compromise in freshwater, ion regulation can be severely perturbed. Alternatively in saltwater, salmon are hypoosmotic to their environment and rely on active excretion of Na^+ and Cl^- while producing small amounts of concentrated urine to combat the osmotic loss of water and gain of ions from the surrounding concentrated environment (Marshall, 2002). Therefore, saltwater acclimation favors reduced functional surface area and permeability to reduce ion influx from the surrounding environment. A remodeled gill, due to exercise, may enhance salmon acclimation to saltwater as it would improve net ion excretion at the gills to counter the gain of ions from saltwater. This study aims to evaluate how the osmorespiratory compromise alleviates the physiological challenge that exercise poses on a fish's osmoregulatory and respiratory capacity, while also determining if exercise remodels the gill to an optimal design for subsequent ion regulation in saltwater.

Juvenile salmon undergo a physiological, morphological, and behavioral transition from a stream-dwelling parr to a seaward-migrating smolt, known as 'smoltification' (McCormick and Saunders, 1987). Smoltification is a preparatory phase for saltwater tolerance, that occurs in freshwater, when a salmon gains the ability to osmoregulate in saltwater and is considered the optimal window for migration to the ocean (McCormick et al., 2013). Gill NKA activity is increased during smoltification (Tipsmark and Madsen, 2001) to prepare for osmoregulation in saltwater, along with a switch in dominant NKA α-subunit isoform expression from NKA α1a to

NKA α1b (Richards et al., 2003; Bystriansky et al., 2006; McCormick et al., 2013). In combination with the natural smoltification process, this study hypothesizes that exercise may also have a preparatory effect on juvenile chinook salmon, enhancing saltwater tolerance and therefore marine survival. Exercise has mostly been studied in the context of locomotory performance, with fewer studies examining exercise and osmoregulation while in freshwater (Randall et al., 1972; Wood and Randall, 1973; Gonzalez and McDonald, 1995; Postlethwaite and McDonald, 1995; Onukwufor and Wood, 2018) and even fewer examining exercise and osmoregulation after transfer to saltwater (Jørgensen and Jobling, 1993; Khovanskiy et al., 1993; Esbaugh et al., 2014). In the few studies examining the effects of exercise on osmoregulation after transfer to saltwater, no study has performed a comprehensive analysis of osmoregulatory status throughout the entire time-course of saltwater acclimation.

Based on previous literature, a study was designed with conditions expected to invoke the osmorespiratory compromise. The first objective was to determine if there was a preparatory effect of exercise on osmoregulatory mechanisms in chinook salmon suitable for saltwater acclimation. To address this objective, chinook salmon were swum at ~1 BL/s (body length/second), a known speed to induce aerobic activity, for 20 days in freshwater. Following the 20 days, exercise training was halted, and a subset of salmon were sampled to assess the effect of exercise while in freshwater. The second objective was to determine if exercised and non-exercised chinook salmon respond to disturbances incurred by saltwater differently. To address this objective, chinook salmon that had been exercised and that were not exercised were exposed to saltwater and sampled throughout the acclimation period. My hypothesis, given the osmorespiratory compromise, was that exercised salmon would experience improved capacity to acclimate to saltwater compared to non-exercised salmon.

2.3 Materials and Methods

Animal Rearing Conditions

All experiments were performed at an indoor research support facility (RSF) at DePaul University (DPU), Chicago, IL, USA during June of 2019. This experiment used 138 juvenile chinook salmon (1-year post-hatch; 11.95 ± 0.13 cm; 16.83 ± 0.57 g) procured from a local fish hatchery (Jake Wolf Fish Hatchery, USA origin). There were no significant differences in fish weight or length between treatments ($P > 0.05$) prior to the start of the experiment. Salmon were acclimated in two 70-gallon dechlorinated freshwater tanks at ~15°C under simulated natural photoperiod (14L:10D) for two weeks prior to experimentation. Oxygen was kept at/near saturation using aerators (Medo LA-120, Roselle, IL). Fish were fed to satiation every other day. All procedures were performed according to Animal Use Protocols investigated and approved by the DePaul University Institutional Animal Care and Use Committee (IACUC). Experimental design was executed to minimize handling stress.

Experimental Design

This experiment consisted of two phases. In the first phase, half of the salmon were randomly subjected to an exercise training treatment. Selected individuals were removed directly from the rearing tanks and distributed across ten 15-gallon oval fiberglass raceways (3.25 kg/m^3 stocking density per tank), where the flow rate was kept at 0.99 BL/s for 20 days. The remaining half of salmon were not exercised and were removed from the rearing tanks and placed back into the two 70-gallon rearing tanks (3.06 kg/m^3 stocking density per tank), to keep handling stress of both treatments consistent, where the flow rate was kept at ~0 BL/s for 20 days. All tanks and raceways were maintained at ~15°C and kept on a recirculating filtration system. During the second phase of the experiment, fish from both treatments were divided into four experimental

treatments to test two variables (effects of prior exercise training on saltwater acclimation). On day 20 of the first phase (day 0 of the second phase), exercise training was halted, and flows were minimized in both treatments to ~0 BL/s. Eleven individuals from both treatments, exercised (*N*=6) and non-exercised (*N*=5) were sampled, to assess the effect of an exercise training period prior to saltwater exposure. On day 0 of the second phase, after the 11 fish had been sampled, salmon from both treatments (exercised and non-exercised) either remained in freshwater or were transferred to 30‰ saltwater. Therefore, the resulting four experimental treatments were: (1) non-exercised fish that remained in freshwater (freshwater control; FW-No Exercise); (2) exercised fish that remained in freshwater (FW-Exercise); (3) non-exercised fish exposed to saltwater (saltwater control; SW-No Exercise); and (4) exercised fish exposed to saltwater (SW-Exercise). Over the course of the seven-day saltwater exposure, fish from each treatment (*N* = 5-7) were sampled at each of the following time points: 1, 2, 4, and 7 days. A period of seven days was chosen as most studies suggest salmon acclimate to saltwater in four to five days of exposure (Leray et al., 1981; Prunet and Boeuf, 1985; Bystriansky, 2006). The following two to three days allowed for the potential impact of exercise on saltwater acclimation to be analyzed. Food was withheld from the salmon 24 hours prior to sampling to exclude feeding/appetite as a confounding variable.

Sampling Protocol

During each sampling time point (0, 1, 2, 4, and 7 days) chinook salmon were anesthetized in a bath of MS-222 (~200 mg/L; buffered with 200 mg/L $NaHCO_3$) until the salmon lost equilibrium and did not respond to external stimuli (~2 minutes). Salmon were blotted dry and fork length (cm) and weight (grams) were measured. Blood was extracted via caudal puncture in a chilled, heparinized (500 U sodium heparin/mL) syringe. The blood was centrifuged at 5000 g

at 4°C for 5 minutes. Plasma was extracted and frozen in liquid nitrogen, then stored at -80°C until analysis. After blood was collected, salmon were euthanized by a severance at the spinal cord. Gill tissue was excised and frozen in liquid nitrogen, then stored at -80°C until analysis. A white muscle sample was biopsied, blotted dry, and weighed to determine wet weight, then transferred to a drying oven (60°C) until dried white muscle weight was consistent. Wet and dry weights were used to calculate percent white muscle water content.

Osmoregulatory Status

Blood plasma was analyzed for plasma total osmolality using a vapor pressure osmometer (VAPRO 5600, Wescor, Inc., Logan UT). Plasma ion concentrations were analyzed using a flame photometer (Jenway PFP7, Bibby Scientific Ltd., Staffordshire, UK) for [Na^+] and a ChloroChek chloridometer (Wescor, Inc., Logan, UT) for [Cl^-]. White muscle samples were dehydrated to a stable weight (dry weight) and percent water content was calculated as: [(wet weight − dry weight) x 100] / wet weight (Shaughnessy et al., 2015).

Gill Na^+/K^+-ATPase Activity

To quantify gill NKA activity extracted gill filaments were homogenized on ice in SEID buffer (150mM sucrose, 10mM EDTA, 50mM imidazole, and 0.1% sodium deoxycholate; pH 7.5) by hand, using a ground glass homogenizer, and then centrifuged at 4000 g for 1 minute at 4°C to remove filaments and other insoluble materials from the supernatant, which was used directly in the assay. Gill NKA activity was determined spectrophotometrically (Agilent Cary 60 UV-Vis, Agilent Technologies, Inc., Santa Clara, CA) at 15°C using an enzyme (NADH)-linked assay as described in Bystriansky et al. (2006), modified from the methods of (Gibbs and Somero, 1989) and (McCormick, 1993). This modified method enzymatically couples ADP (formed from the hydrolysis of ATP by Na^+/K^+-ATPase) to the oxidation of reduced NADH

using commercially prepped pyruvate kinase (PK) and lactate dehydrogenase (LDH). Gill samples were assayed for ATPase activity in the presence and absence of the NKA-specific inhibitor ouabain (1mM). Gill homogenates were run in duplicate in the presence and absence of ouabain and the difference in NADH oxidation (ε_{340}=6.22) rate between the two conditions was used to calculate NKA activity. Gill NKA activity was expressed as μmol ATP/mg protein/hr. Gill homogenate protein concentration was determined spectrophotometrically (λ = 595 nm, SPECTRAmax PLUS 384, Molecular Devices Corp., Sunnyvale, CA) (Bradford, 1976).

Total RNA Isolation and cDNA Synthesis

Gill tissue samples were used to isolate total RNA and determine Na^+/K^+ ATPase α-subunit isoform mRNA expression. Total RNA was isolated using TriZol isolation reagent (Invitrogen, Carlsbad, CA, USA) and the guanidine thiocyanate method determined by Chomczynski and Sacchi (1987). Isolated total RNA was quantified spectrophotometrically (ThermoScientific NanoDrop2000c) and all samples used were found to be of very high purity (260:280 absorbance ratio of >1.8). RNA was stored at -80°C until cDNA was synthesized. First strand cDNA was synthesized from 4 μg of total RNA (in 20 μL total reaction volume) using a high capacity cDNA reverse transcription kit, including MultiScribe™ reverse transcriptase and random hexamer primers (Applied Biosystems, Foster City, CA, USA) in an Applied Biosystems 2720 Thermal Cycler. All cDNA was stored at -80°C until used for quantitative real-time PCR.

Quantitative Real-Time PCR (mRNA Expression)

Quantitative RT-PCR (qRT-PCR) was performed on a QuantStudio 7 Flex Real-Time System (ThermoFisher Scientific). qRT-PCR reactions contained 1 μL of cDNA in a total 11 μL reaction volume including 0.2 μL of each primer, 5 μL of Universal SYBR-Green Master Mix (Applied Biosystems) and 4.6 μL of RNase-free water. All qRT-PCR reactions were performed

under the following conditions: 2 mins at 50°C, 10 mins at 95°C, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Melt curve analysis was performed following each reaction to confirm the presence of only a single product of the reaction. Forward and reverse primers used for each gene were isoform specific. To confirm primers designed for rainbow trout Na^+/K^+-ATPase α1b (Richards et al., 2003) were appropriate for chinook salmon, Grant et al. (2010) partially cloned a sequence of chinook salmon Na^+/K^+-ATPase α1b 3' untranslated region of the gene and found that the chinook salmon and rainbow trout 3' UTR sequences were 100% identical; therefore primers designed by Richards et al. (2003) were used in this current study. Primer sequences used are listed in **Table 2.1**. Non-template controls (NTCs) were performed on a subset of random samples that did not undergo reverse transcription to confirm low genomic DNA contamination. One random control sample was selected to develop a standard curve relating threshold cycle to cDNA amount for each primer set. All results are expressed relative to these standard curves and relative quantity of mRNA was normalized to day 0 of the FW-No Exercise control condition. All samples were run in duplicate.

Statistical Analysis

All values are represented as means ± standard error of the mean (s.e.m.) (*N*=5-7). Comparisons of total plasma osmolality, plasma $[Na^+]$ and $[Cl^-]$, white muscle water content, gill NKA activity, and relative gill NKA α -subunit isoform mRNA levels were performed using a one-way analysis of variance (ANOVA), followed by a Holm-Šídák *post hoc* analysis to make pair-wise comparisons (Holm, 1979). We anticipated to see a temporal change (slowly deviating and returning to control levels) in osmoregulatory status parameters, and expected to observe significant changes in all of the parameters measured during the 'crisis phase' in response to exposure to saltwater. It is for that reason that a two-step statistical analysis approach was

chosen. A series of one-way ANOVAs were first conducted on the four conditions (FW-No Exercise, FW-Exercise, SW-No Exercise, and SW-Exercise) to assess changes in any of the parameters, over the duration of the seven-day salinity exposure. Another series of one-way ANOVAs were then conducted to make pairwise-comparisons between the four groups at each sample period (0, 1, 2, 4, 7 days). Normality and equal variance tests failed and could not be normalized using reciprocal or log transformation on the following groups: total plasma osmolality, plasma [Na^+] and [Cl^-], white muscle water content, gill NKA activity, and gill NKA α -subunit isoform mRNA levels. Linear regressions were conducted to determine the relationships between log-transformed gill NKA activity against total plasma osmolality and white muscle water content. Significance was determined at $P < 0.05$. All statistical tests and graphs were performed using R Studio version 1.2.1335 (RStudio, Inc., Boston, MA, USA).

2.4 Results

Fish Health and Survival

Non-exercised fish that remained in freshwater (FW-No Exercise) and exposed to saltwater (SW-No Exercise) and exercised fish that remained in freshwater (FW-Exercise) and exposed to saltwater (SW-No Exercise) appeared to be in good health throughout the 7-day experiment, with the exception of 17 fish that became moribund between days 1-7.

Total Plasma Osmolality

At time point 0, total plasma osmolality levels did not significantly differ between fish in the FW-Exercise and FW-No Exercise conditions (**Fig. 2.1**). The FW-No Exercise fish's total plasma osmolality changed over time with a significant increase in levels at days 4 and 7, compared to the beginning 2 days of the experiment ($P < 0.01$) (**Fig. 2.1**). The SW-No Exercise fish's total plasma osmolality was significantly elevated ($P < 0.001$) in the first 4 days of the

experiment compared to day 7 within the condition. In both exercised conditions (FW-Exercise and SW-Exercise) there was no significant differences over time. There was no significant difference in total plasma osmolality levels between FW-No Exercise and FW-Exercise fish for the duration of the experiment, as well as no significant differences between fish in the SW-No Exercise and SW-Exercise conditions throughout the experiment. Total plasma osmolality of fish in the SW-No Exercise condition experienced the characteristic rise in total plasma osmolality, with maximum disturbance after 24 hours, followed by a gradual recovery to levels similar to FW-No Exercise by day 7. Fish in the SW-Exercise condition demonstrated similar trends but remained significantly elevated ($P = 0.005$) at day 7 from the FW-Exercise fish.

Plasma Sodium Concentration

At time point 0, plasma sodium concentrations did not significantly differ between fish in the FW-Exercise and FW-No Exercise conditions (**Fig. 2.2**). Over the course of the experiment, FW-No Exercise and FW-Exercise fish had similar plasma sodium concentrations except for at day 4 where the FW-Exercise fish had reduced levels ($P < 0.001$), (**Fig. 2.2**). The SW-No Exercise fish's plasma sodium was significantly elevated ($P < 0.001$) over the first 4 days of the experiment compared to day 7 within the condition. The FW-Exercise fish had a significant decrease in plasma sodium at day 4 ($P < 0.05$), compared to day 1 and 2, but not from day 7. The SW-Exercise fish at day 1 and 4 were significantly increased from day 7 ($P < 0.01$), and at day 2 the fish experienced a reduction in plasma sodium concentrations that were similar to stabilized levels at day 7. FW-Exercise fish had significantly lower plasma sodium levels 4 days after the start of the experiment ($P = 0.003$), compared to the FW-No Exercise fish at the same day, but recovered to FW-No Exercise levels by the end of the experiment. After 2 days, the SW-Exercise fish had significantly decreased plasma sodium levels compared to the SW-No Exercise fish ($P =$

0.0005) but did not significantly differ from the FW-Exercise fish at the same time point. Similar to total plasma osmolality, the SW-No Exercise fish also experienced a characteristic rise in plasma sodium, with maximum disturbance after 48 hours, followed by a gradual recovery to FW-No Exercise levels by day 7.

Plasma Chloride Concentration

At time point 0, plasma chloride concentrations did not significantly differ between fish in the FW-Exercise and FW-No Exercise conditions (**Fig. 2.3**). The FW-No Exercise and FW-Exercise fish's plasma chloride concentrations remained stable throughout the experiment (**Fig. 2.3**). The fish in the SW-No Exercise condition had significantly elevated ($P < 0.001$) plasma chloride concentrations for the first 4 days of the experiment compared to day 7. Fish in the SW-Exercise condition had significantly elevated ($P < 0.05$) plasma chloride concentrations during the first 2 days of the experiments compared to days 4 and 7. There was no significant difference in plasma chloride concentrations between fish in the FW-No Exercise and FW-Exercise conditions, as well as no significant difference between fish in the SW-No Exercise and SW-Exercise conditions throughout the experiment. Similar to total plasma osmolality and sodium, fish in the SW-No Exercise condition experienced an elevated disturbance to concentrations that appeared to be returning to near freshwater control levels, but was still slightly elevated at day 7 from FW-No Exercise fish ($P = 0.02$). Fish in the SW-Exercise condition did not significantly differ from the FW-Exercise fish after 4 days but increased significantly from the freshwater condition by the end of the experiment ($P < 0.001$).

Overall, although there was no significant differences between the SW-No Exercise and SW-Exercise fish (excluding plasma sodium concentrations of SW-Exercise at day 2), but there was a clear trend that fish in the SW-Exercise condition experienced a lessened "disturbance" to total

plasma osmolality and plasma ion concentrations. This potentially supports the idea that a sustained exercise training period prior to saltwater exposure improves saltwater acclimation.

White Muscle Water Content

At time point 0, FW-Exercise fish had significantly increased white muscle water content compared to the FW-No Exercise fish ($P = 0.0006$) (**Fig. 2.4**). The FW-No Exercise and FW-Exercise fish's white muscle water content remained stable throughout the 7-day experiment (**Fig. 2.4**). The SW-No Exercise fish had a significant rehydration of white muscle water content by day 7 ($P < 0.01$), that was significant from days 1 and 4, but not from day 2. The fish in the SW-Exercise condition had significantly decreased ($P < 0.005$) white muscle water content in the first 4 days of the experiment compared to day 7. Fish in the FW-Exercise condition were significantly dehydrated compared to the FW-No Exercise fish after 24 hours ($P = 0.01$) but recovered to FW-No Exercise levels throughout the rest of the experiment. After 24 hours and 7 days, white muscle water content was significantly increased in the SW-Exercise fish compared to the fish in the SW-No Exercise condition ($P < 0.001$). Fish in the SW-No Exercise condition were significantly dehydrated ($P < 0.001$) throughout the experiment compared to the FW-No Exercise fish. By day 4, SW-Exercise fish recovered to levels not significantly different than fish in the FW-Exercise condition and remained similar through the end of the experiment.

Gill Na^+/K^+ ATPase Activity

At time point 0, FW-Exercise fish's gill NKA activity did not significantly differ from the fish in the FW-No Exercise condition (**Fig. 2.5**). The FW-No Exercise and FW-Exercise fish's gill NKA activity remained stable throughout the experiment (**Fig. 2.5**). The SW-No Exercise fish had significantly decreased ($P < 0.005$) gill NKA activity in the first 4 days of the experiment compared to day 7. By day 4, gill NKA activity in the SW-Exercise fish was

significantly elevated compared to the first 2 days of the experiment and remained significantly elevated through the duration of the experiment. There was no significant difference in gill NKA activity between fish in the FW-No Exercise and FW-Exercise conditions, as well as no significant difference between fish in the SW-No Exercise and SW-Exercise conditions throughout the experiment. SW-Exercise fish had significantly increased gill NKA activity after 4 days compared to the FW-Exercise fish ($P = 0.02$), while SW-No Exercise fish was not significantly different from the FW-No Exercise fish until day 7 ($P = 0.0001$).

As a follow-up analysis, a regression of log-transformed gill NKA activity against total plasma osmolality revealed significant negative relationships for the SW-Exercise fish ($P = 0.01$, $r^2 = 0.23$) and SW-No Exercise fish ($P = 0.001$, $r^2 = 0.38$) (**Fig. 2.6**). A regression analysis between log-transformed gill NKA activity against white muscle water content revealed significant positive relationships for SW-Exercise fish ($P = 0.005$, $r^2 = 0.27$) and SW-No Exercise fish ($P = 0.001$, $r^2 = 0.37$) (**Fig. 2.7**).

Gill Na^+/K^+-ATPase α-subunit Isoform mRNA expression

At time point 0, FW-Exercise fish's α-subunit isoform mRNA expression was not significantly different from fish in the FW-No Exercise condition (**Fig. 2.8**). For fish in the FW-No Exercise and FW-Exercise conditions, α1a (**Fig. 2.8A**) and α1b (**Fig. 2.8B**) mRNA levels remained stable throughout the duration of the experiment, except at day 7 in the FW-No Exercise condition where α1a mRNA levels decreased significantly ($P = 0.008$) from the beginning of the experiment. Fish in the SW-No Exercise and SW-Exercise conditions had significantly lower α1a mRNA levels by day 7 compared to day 1 ($P < 0.01$). SW-Exercise fish's α1b mRNA levels remained stable through the duration of the experiment, while SW-No Exercise fish's α1b mRNA levels became significantly elevated by day 2 ($P < 0.001$) and

remained elevated through the duration of the experiment. Fish in the FW-Exercise condition had significantly decreased α1a and α1b mRNA levels compared to the FW-No Exercise fish at days 2 and 7 ($P < 0.05$), as well as day 4 ($P = 0.002$) for α1b mRNA levels. Fish in the SW-Exercise and SW-No Exercise conditions both experienced a decrease in α1a mRNA levels, with SW-Exercise fish decreasing these levels significantly by day 2 ($P = 0.0002$), and SW-No Exercise fish by day 4 ($P = 0.04$). Interestingly, fish in the SW-Exercise condition began the experiment with increased α1b mRNA levels that were significantly elevated compared to the SW-No Exercise fish ($P = 0.001$), but by day 2 SW-No Exercise fish were able to upregulate α1b mRNA to levels similar to the SW-Exercise fish. SW-Exercise fish did not have significantly different α1a mRNA levels at any time point from the FW-Exercise fish but did have significantly elevated α1b mRNA levels at all time points ($P < 0.001$) compared to the FW-Exercise fish. The SW-No Exercise fish had significantly decreased α1a mRNA levels at day 4 ($P = 0.009$) compared to FW-No Exercise fish; whereas FW-Exercise fish had significantly increased α1b mRNA levels compared to FW-No Exercise fish after day 1 ($P < 0.001$) and remained elevated through the duration of the experiment.

2.5 Discussion

Overview

Studying the response to exercise in juvenile chinook salmon, whose life cycle includes strenuous migrations, is important in understanding anadromous fish physiology. This study is one of the first to describe the effects of exercise on saltwater acclimation in salmon. The following discussion will outline the following objectives of this study: (1) There was a preparatory effect of exercise training on osmoregulatory mechanisms in chinook salmon

suitable to aid in saltwater acclimation; and (2) exercise and non-exercised fish appeared to respond to disturbances incurred by saltwater differently.

The Effect of Exercise on Chinook Salmon while in Freshwater

Moderate (< 1.5 BL/s) exercise training has been shown to improve growth (Davison, 1997), aggression (Postlethwaite and McDonald, 1995), food conversion efficiency (Jørgensen and Jobling, 1993), and cardiovascular function (Gallaugher et al., 2001) in multiple species of salmon. Most studies have focused on the benefit of exercise training on locomotory performance, while fewer studies have examined the effect of exercise on osmoregulatory status (Randall et al., 1972; Wood and Randall, 1973; Gonzalez and McDonald, 1992; Jørgensen and Jobling, 1993; Khovanskiy et al., 1993; Postlethwaite and McDonald, 1995; Gallaugher et al., 2001; Esbaugh et al., 2014; Onukwufor and Wood, 2018). In the present study, total plasma osmolality and plasma $[Na^+]$ and $[Cl^-]$ were not affected by exercise training in chinook salmon that remained in freshwater through the duration of the experiment. Similar to total plasma osmolality and plasma $[Na^+]$ and $[Cl^-]$, there was also no significant difference in gill NKA activity between exercised and non-exercised fish that remained in freshwater, contrary to what was predicted. Although gill NKA activity was not affected, exercised and non-exercised fish that remained in freshwater did differentially express α1a and α1b mRNA levels, with exercised fish that remained in freshwater expressing decreased α1a and α1b mRNA levels throughout the duration of the experiment compared to the non-exercised fish that remained in freshwater. In a study examining different training regimens on branchial gene expression, Esbaugh et al. (2014) found no significant difference in training regime on relative α1a and α1b mRNA levels while Atlantic salmon were in freshwater. In this present study, chinook salmon were exercised for 20 days, while in Esbaugh et al. (2014) the training regime lasted 10 weeks. This difference in

duration of training could be why this study found a significant difference between expression levels of α1a and α1b mRNA levels in exercised and non-exercised fish that remained in freshwater, while the Esbaugh et al. (2014) study did not. While 20 days of training may have been enough time to induce transcriptional changes in mRNA expression, it may also have been enough time for the fish to fully recover from the osmotic stress that exercise may have induced, thereby negating any significant differences we would likely have seen in osmoregulatory parameters had we sampled in the first few hours/days of exercising. Interestingly, we did see a significant difference in white muscle water content between exercised and non-exercised salmon that remained in freshwater. After 20 days of exercise training and at time 0 of sampling, exercised salmon had significantly higher muscle water content than non-exercised fish that remained in freshwater. Previous studies have found that exercise can induce an increase in lactate intracellularly and a subsequent uptake of water in the muscle (Milligan and Wood, 1986; Wood, 1991), which may explain the results of this study.

Saltwater Acclimation of Chinook Salmon

When salmon enter saltwater, they experience an influx of ions and passive loss of water. To successfully acclimate, salmon begin drinking immediately to offset the osmotic loss of water (Bath and Eddy, 1979). Ingesting highly concentrated saltwater activates the active transport of ions into the bloodstream, resulting in excess Na^+ and Cl^- concentrations in the blood plasma. Being ionoregulators, salmon normally maintain total plasma osmolality within a narrow range of 290-340 mOsmol/L (McCormick and Saunders, 1987). Prolonged disturbances of total plasma osmolality caused by saltwater can result in mortality if a salmon is not able to successfully acclimate, making total plasma osmolality and plasma ion concentrations good indicators of osmoregulatory status. Both exercised and non-exercised fish exposed to saltwater experienced a

rise in total plasma osmolality and plasma ion concentrations, followed by a recovery to freshwater levels. This increase is characteristic to the 'crisis phase' of acclimation, when a fish transitions from hyperosmoregulating to hypoosmoregulating (Gordon, 1959). Disturbance and recovery of total plasma osmolality coincides with dehydration and rehydration of white muscle water content, which were both seen in this study in the exercised and non-exercised fish exposed to saltwater. An increase in total plasma osmolality likely triggered the significant increase in gill NKA activity seen in fish exposed to saltwater, as well as the decreased expression of α1a mRNA levels and increased expression of α1b mRNA levels. The α-subunit isoform switching that occurred in this study is common to anadromous fish during saltwater acclimation (Richards et al., 2003; Bystriansky et al., 2006; Bystriansky et al., 2007; Bystriansky and Ballantyne, 2007). The response in osmoregulatory status, gill NKA, and α-subunit isoform switching seen in this study, confirms that salmon underwent a disturbance following saltwater transfer and were able to successfully acclimate, regardless if exercised or not.

The Effect of Exercise on Chinook Salmon during Saltwater Acclimation

Anthropogenic factors in the wild (contaminants, increase in temperature, dams, etc.) have been found to affect saltwater survival of salmon smolts (McCormick et al., 2009). Previous laboratory studies have examined exercise on osmoregulatory status on Atlantic salmon 24 hours post saltwater transfer (Jørgensen and Jobling, 1993), 8 days post saltwater transfer (Esbaugh et al., 2014), 3 days post saltwater transfer on chum salmon (Khovanskiy et al., 1993), and already saltwater-adapted chinook salmon (Gallaugher et al., 2001). The present study is unique in that it is the first study to focus on the effects of exercise over the entire time course of saltwater acclimation in chinook salmon. A preparatory effect was observed of exercise training on osmoregulatory mechanisms in chinook salmon suitable to improve saltwater acclimation and

therefore marine survival. Exercised fish exposed to saltwater had an overall lower disturbance to their total plasma osmolality, plasma [Na^+] and [Cl^-], and white muscle water content compared to non-exercised fish exposed to saltwater. A prior exercise training period seems to have improved the salmon's ability to switch from hyperosmoregulating to hypoosmoregulating, allowing for a reduced osmotic disturbance during the 'crisis phase' of saltwater acclimation.

Exercised fish exposed to saltwater were able to significantly upregulate gill NKA activity after 4 days in saltwater, while it took non-exercised fish 7 days to significantly upregulate gill NKA activity. An upregulation of gill NKA activity is essential in saltwater acclimation as it is the main driver of blood-ion homeostasis and maintains electrochemical gradients (Silva et al., 1977). The gradients that are produced act as the sole energy source for the active efflux of ions across the gill in saltwater (Silva et al., 1977). Exercised fish were able to upregulate gill NKA activity earlier than non-exercised, potentially allowing for improved saltwater acclimation. As a follow-up analysis, a significant negative relationship was found between log-transformed gill NKA activity against plasma osmolality in both exercised and non-exercised fish exposed to saltwater. Interestingly, individuals with the highest log-transformed gill NKA activity also had the lowest disturbance to plasma osmolality, similar to what was found by McCormick et al. (2009). Furthermore, a significant positive relationship was found between log-transformed gill NKA activity and white muscle water content and to our knowledge is the first study to examine this relationship. Individuals with the highest gill NKA activity experienced less dehydration when exposed to saltwater compared to individuals that had decreased gill NKA activity, therefore improving saltwater acclimation.

It is common for salmon to differentially express gill NKA α-subunit isoform mRNA levels when acclimating to saltwater, and the α1b isoform is thought to be responsible for seawater

tolerance (Richards et al., 2003). After 24 hours of being exposed to saltwater, exercised fish had significantly upregulated α1b mRNA levels compared to non-exercised fish. Although we did not see any significance in α1b mRNA expression in salmon at day 0, exercised salmon were able to upregulate α1b mRNA levels after just 24 hours of being exposed to saltwater, while non-exercised fish needed two days to significantly upregulate α1b mRNA levels. These results provide further evidence for differential expression of α-subunit isoforms in chinook salmon and directly supports the hypothesis that exercise improves saltwater acclimation. The more limited disturbance in plasma osmolality in exercised fish exposed to saltwater may be due to the earlier upregulation of α1b mRNA levels, which in part may be why exercised fish were able to upregulate gill NKA activity faster than non-exercised fish exposed to saltwater.

Osmorespiratory Compromise in Exercised Fish

It is important to discuss the results in the context of the osmorespiratory compromise, as salmon use the osmorespiratory compromise to alleviate the stress imposed by exercising and salinity acclimation. Exercise did not induce any notable impairment to ion balance or gill NKA activity after 20 days of being swam at 0.99 BL/s in fish that remained in freshwater. While exercise may initially have caused a disturbance to ion balance, by the time exercise training was completed the stress induced by exercise may have already been mediated by the gill through remodeling to optimize oxygen consumption for exercise and minimize ion flux. It is assumed that exercise induced a remodeling of gill physiology to better compensate for the additional stress of saltwater acclimation. In every parameter measured, exercised fish showed improved saltwater acclimation compared to non-exercised fish, supporting the objective that exercise training remodels the gill to conditions that improve saltwater acclimation.

2.6 Conclusion

This study has described the time course of saltwater acclimation by juvenile chinook salmon following a prior exercise training period. The first objective was supported, indicating that there was a preparatory effect of exercise training on osmoregulatory mechanisms in chinook salmon suitable for saltwater acclimation. While no ionic parameters were significantly different in exercised and non-exercised fish that remained in freshwater, white muscle water content was significantly elevated in the exercised fish that remained in freshwater, potentially allowing for a less disturbed acclimation to the dehydrating effects of saltwater. The second objective was also supported in that exercised and non-exercised chinook salmon appeared to respond to disturbances incurred by saltwater differently. Exercised fish exposed to saltwater were able to significantly upregulate gill NKA activity and the proposed saltwater isoform α1b mRNA levels quicker than non-exercised fish, which may explain why exercised fish experienced less disturbance to osmoregulatory status. The osmoregulatory plasticity seen in this study provides additional evidence to how salmon use the osmorespiratory compromise to mediate competing stressors.

Further examination of the effects of exercise during the smoltification window would provide a better understanding if exercise induces these osmoregulatory changes, or if it simply amplifies the natural smoltification process. It would also be of interest to examine gill morphology of salmon after exercise to see if exercise induces morphological changes to the interlamellar cellular mass of salmon gills.

2.7 Tables

Table 2.1: Na^+/K^+-ATPase α1a and α1b primer sequences used for qRT-PCR (5'-3') (Richards et al., 2003).

Primer	Accession #	Sequence
α1a forward	AY319391	5′ GGC CGG CGA GTC CAA T 3′
α1a reverse	AY319391	5′ GAG CAG CTG TCC AGG ATC CT 3′
α1b forward	AY319390	5′ CTG CTA CAT CTC AAC CAA CAA CAT T 3′
α1b reverse	AY319390	5′ CAC CAT CAC AGT GTT CAT TGG AT 3′

2.8 Figures

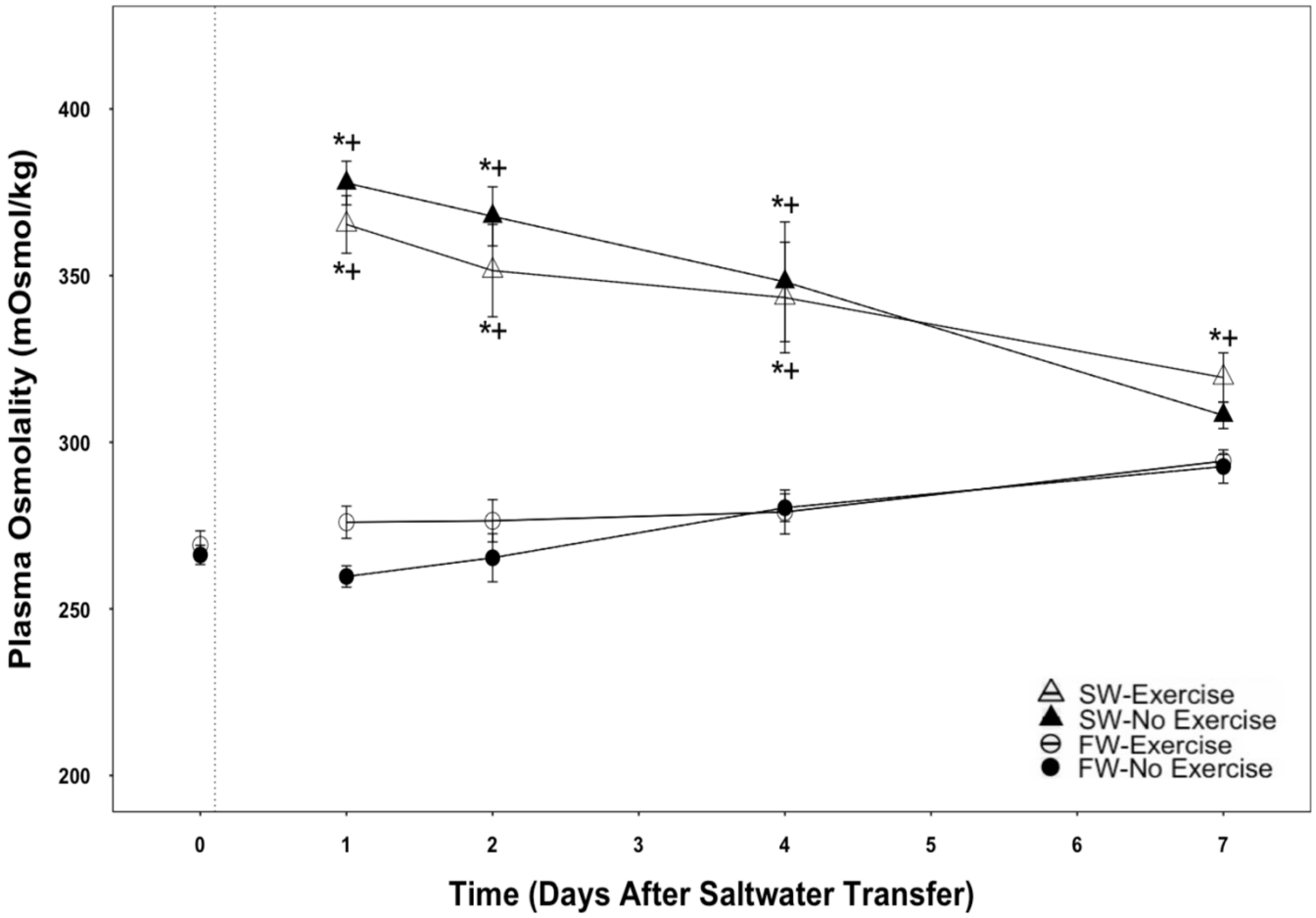

Figure 2.1: Total plasma osmolality levels in non-exercised fish that remained in freshwater (FW) (filled circle) or exposed to saltwater (SW) (filled triangle) and in exercised fish that remained in FW (open circle) or exposed to SW (open triangle). The vertical broken line represents the halt of exercise and subsequent transfer of fish to FW or SW at time 0 (line is slightly offset as not to obscure data). Statistical comparisons made over time within each experimental group are stated in the results. For statistical comparisons made between groups at each time point (one-way ANOVA; $P < 0.05$): * denotes significantly different from FW-No Exercise group; + denotes significantly different from FW-Exercise group. No differences were seen between SW-Exercise and SW-No Exercise groups at any time point.

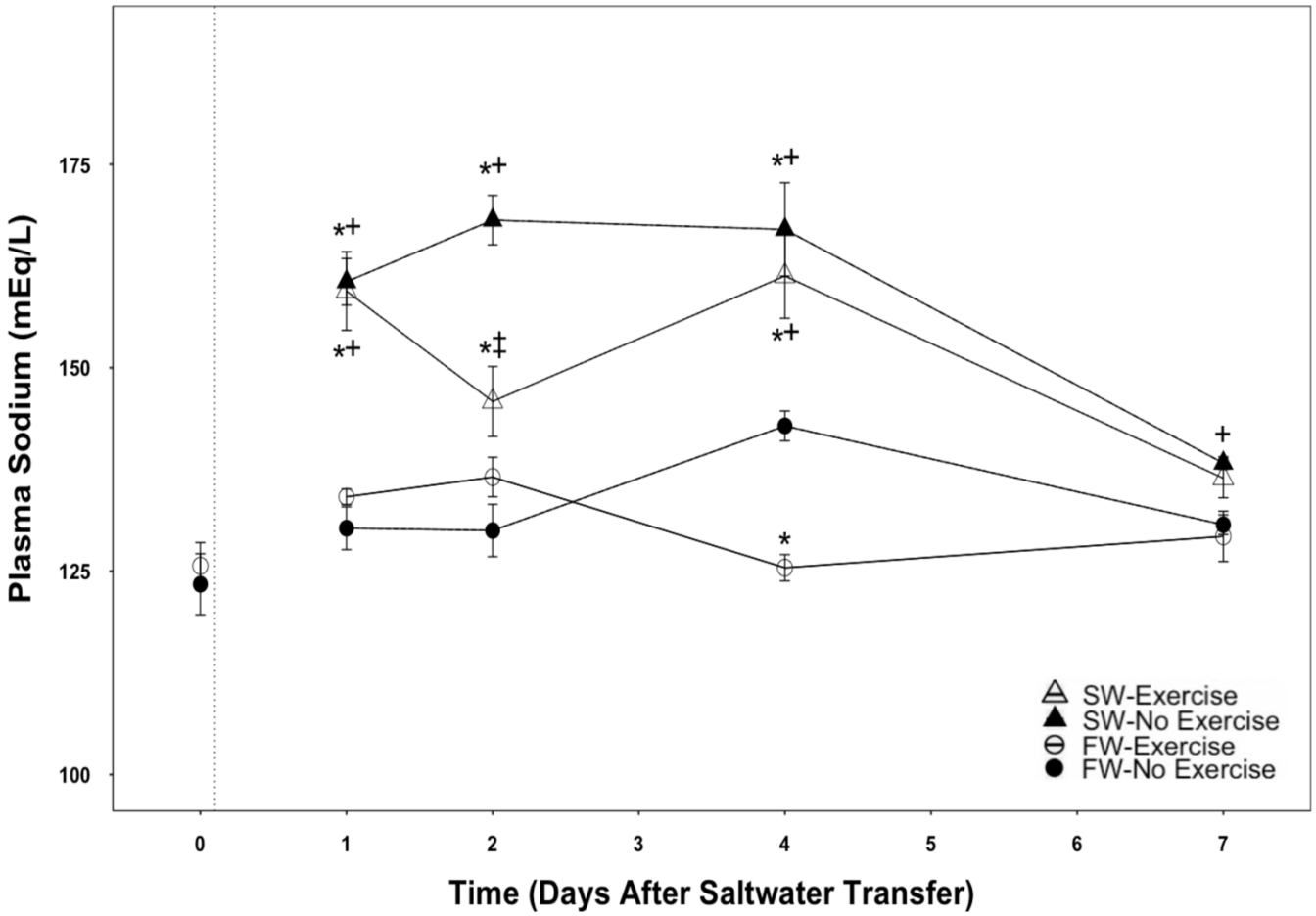

Figure 2.2: Plasma [Na^+] in non-exercised fish that remained in freshwater (FW) (filled circle) or exposed to saltwater (SW) (filled triangle) and in exercised fish that remained in FW (open circle) or exposed to SW (open triangle). The vertical broken line represents the halt of exercise and subsequent transfer of fish to FW or SW at time 0 (line is slightly offset as not to obscure data). Statistical comparisons made over time within each experimental group are stated in the results. For statistical comparisons made between groups at each time point (one-way ANOVA; $P < 0.05$): * denotes significantly different from FW-No Exercise group; + denotes significantly different from FW-Exercise group; ‡ denotes significantly different from SW-No Exercise group.

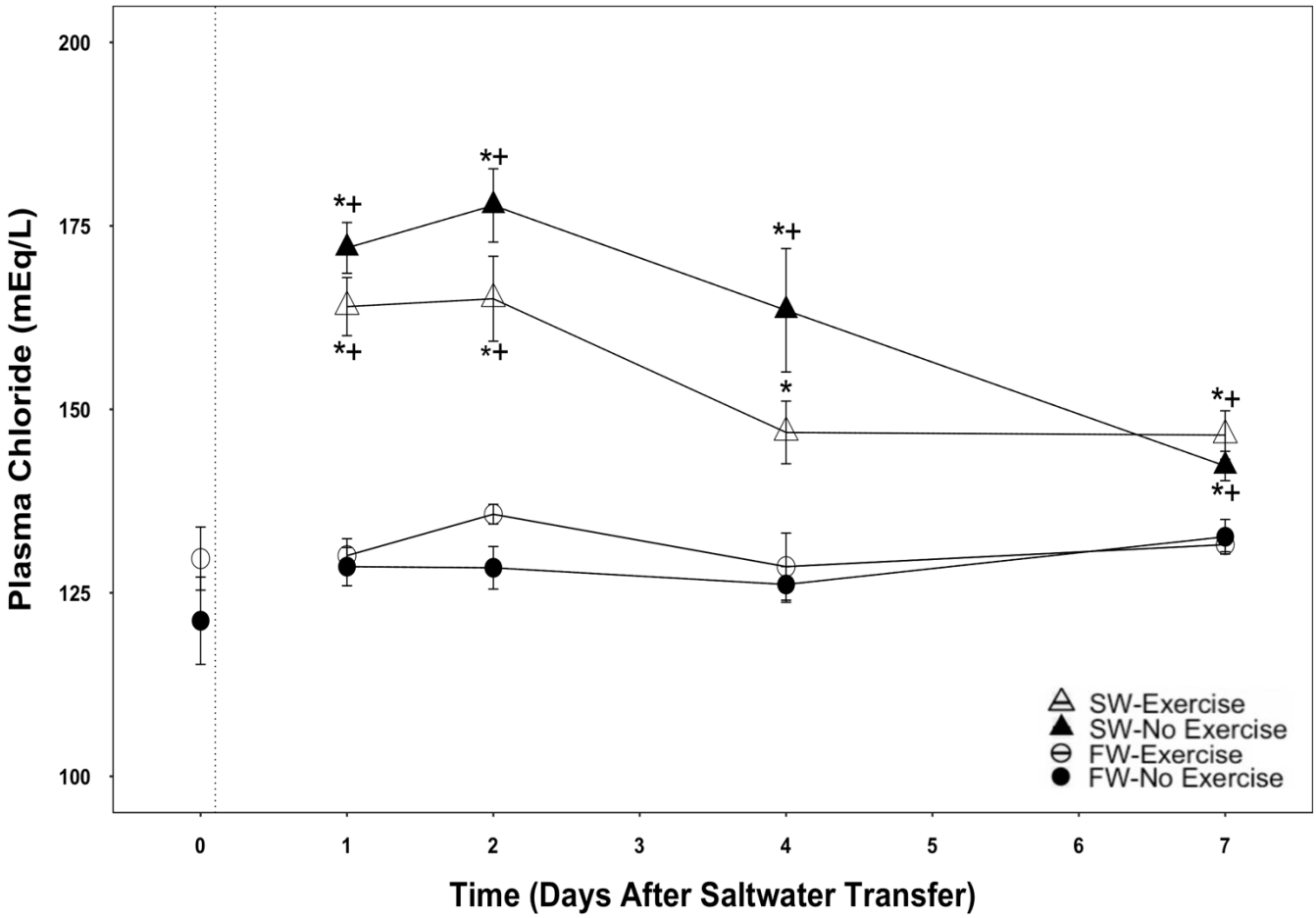

Figure 2.3: Plasma [Cl^-] in non-exercised fish that remained in freshwater (FW) (filled circle) or exposed to saltwater (SW) (filled triangle) and in exercised fish that remained in FW (open circle) or exposed to SW (open triangle). The vertical broken line represents the halt of exercise and subsequent transfer of fish to FW or SW at time 0 (line is slightly offset as not to obscure data). Statistical comparisons made over time within each experimental group are stated in the results. For statistical comparisons made between groups at each time point (one-way ANOVA; $P < 0.05$): * denotes significantly different from FW-No Exercise group; + denotes significantly different from FW-Exercise group. No differences were seen between SW-Exercise and SW-No Exercise groups at any time point.

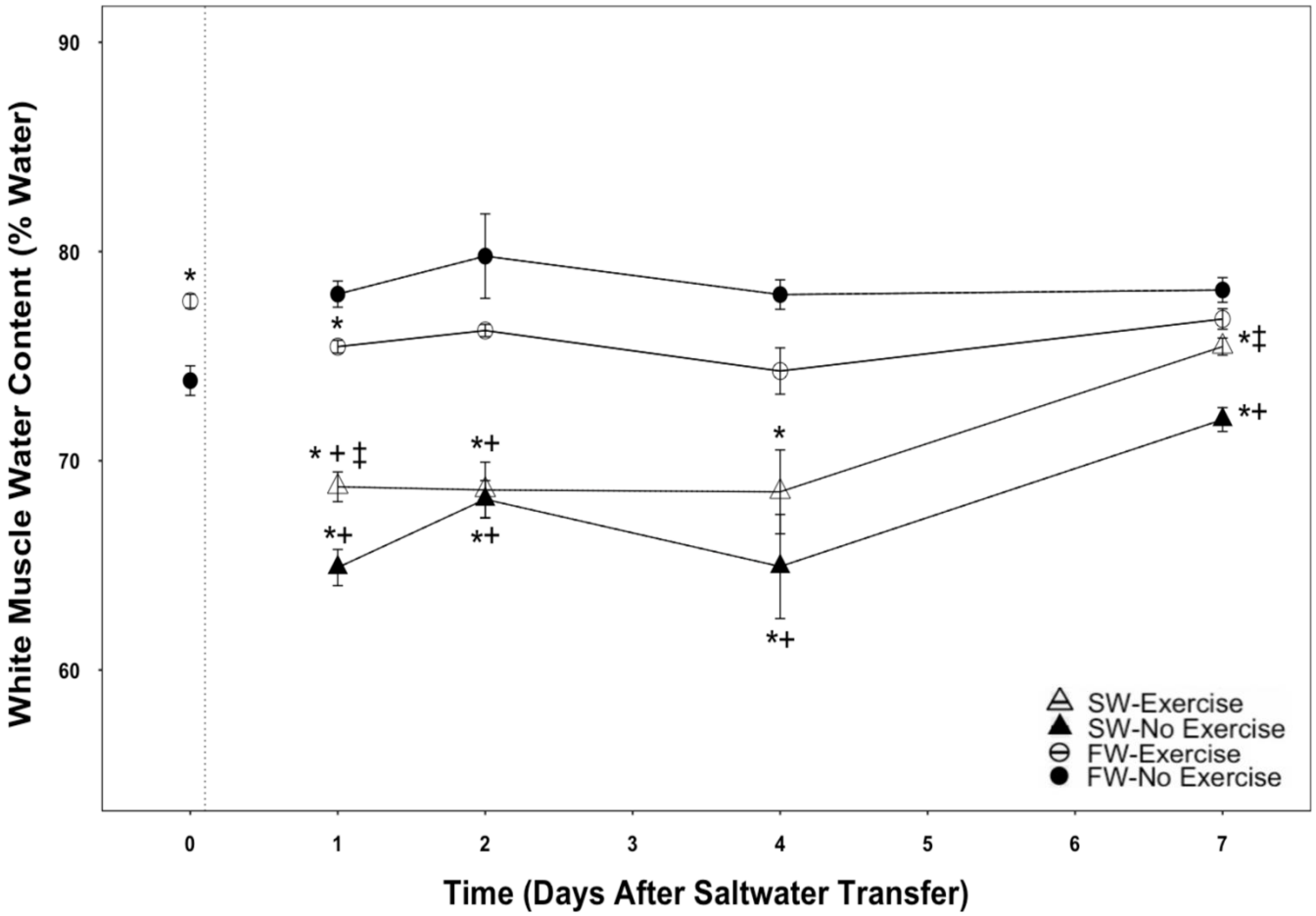

Figure 2.4: White Muscle Water Content in non-exercised fish that remained in freshwater (FW) (filled circle) or exposed to saltwater (SW) (filled triangle) and in exercised fish that remained in FW (open circle) or exposed to SW (open triangle). The vertical broken line represents the halt of exercise and subsequent transfer of fish to FW or SW at time 0 (line is slightly offset as not to obscure data). Statistical comparisons made over time within each experimental group are stated in the results. For statistical comparisons made between groups at each time point (one-way ANOVA; $P < 0.05$): * denotes significantly different from FW-No Exercise group; + denotes significantly different from FW-Exercise group; ‡ denotes significantly different from SW-No Exercise group.

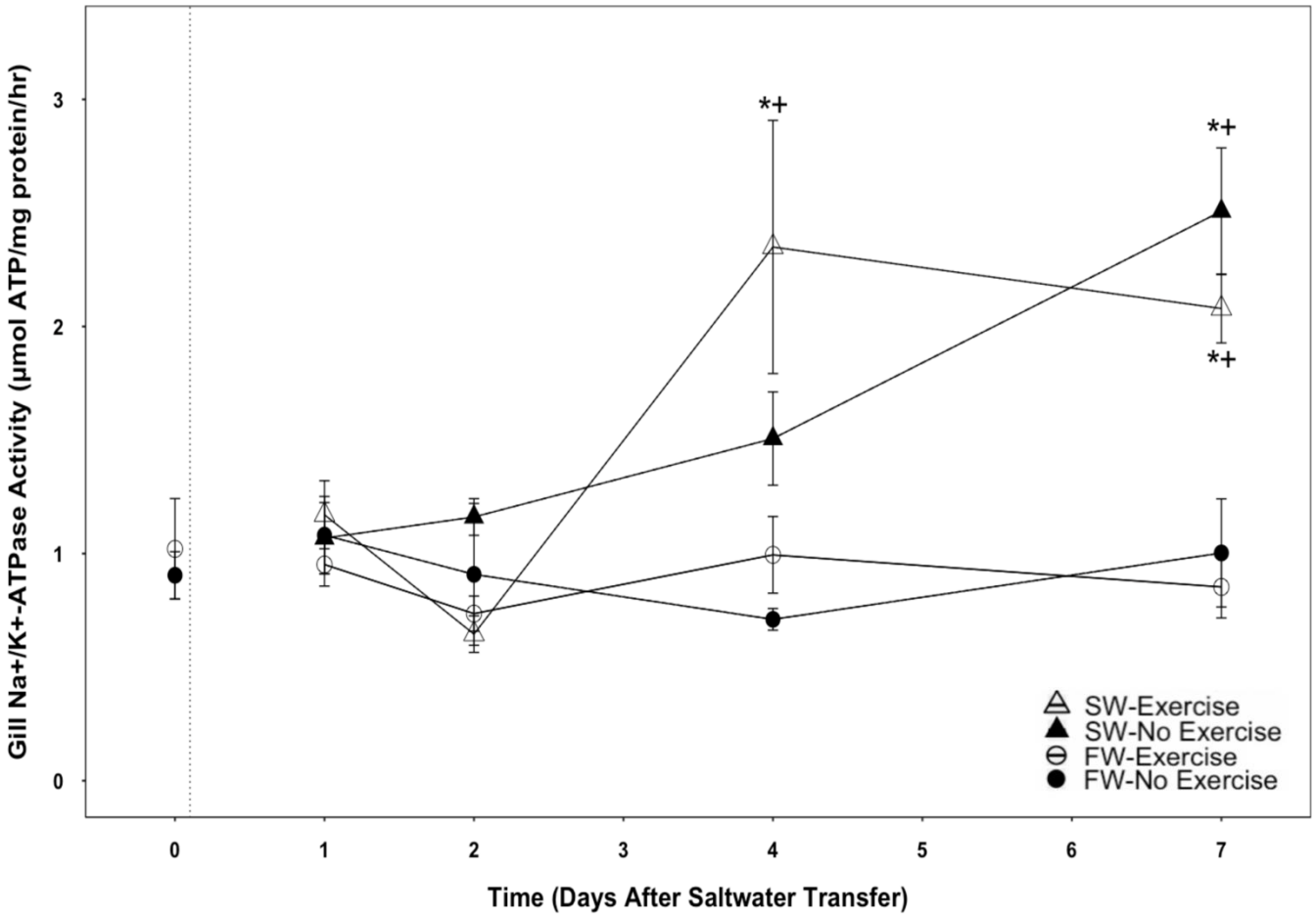

Figure 2.5: Gill NKA activity in non-exercised fish that remained in freshwater (FW) (filled circle) or exposed to saltwater (SW) (filled triangle) and in exercised fish that remained in FW (open circle) or exposed to SW (open triangle). The vertical broken line represents the halt of exercise and subsequent transfer of fish to FW or SW at time 0 (line is slightly offset as not to obscure data). Statistical comparisons made over time within each experimental group are stated in the results. For statistical comparisons made between groups at each time point (one-way ANOVA; $P < 0.05$): * denotes significantly different from FW-No Exercise group; + denotes significantly different from FW-Exercise group. No differences were seen between SW-Exercise and SW-No Exercise groups at any time point.

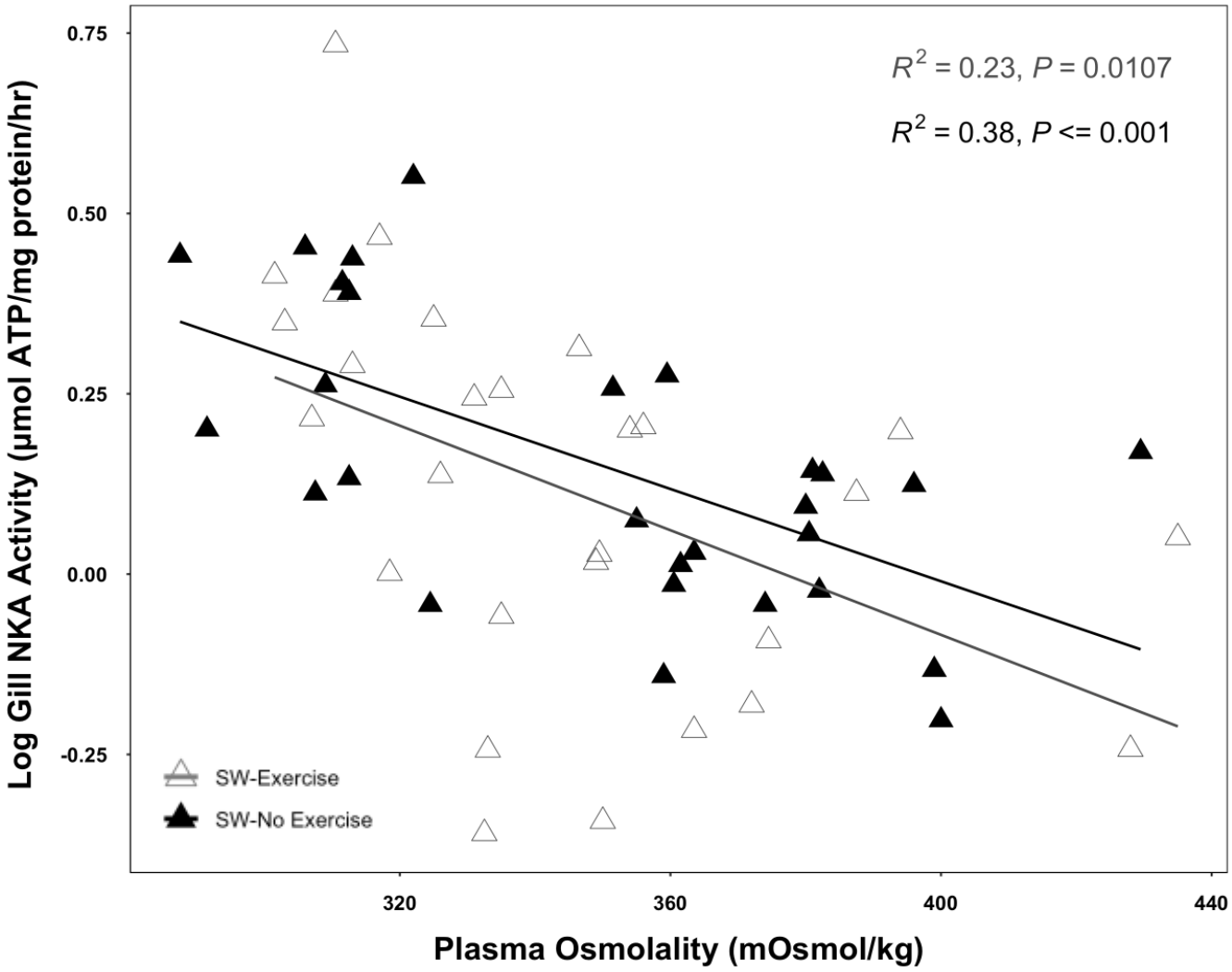

Figure 2.6: Log-transformed gill NKA activity plotted against plasma total osmolality for exercised (open triangle) and non-exercised (filled triangle) fish exposed to saltwater at all time points; n = 28 for both groups. Regressions for both groups were significant (P < 0.05).

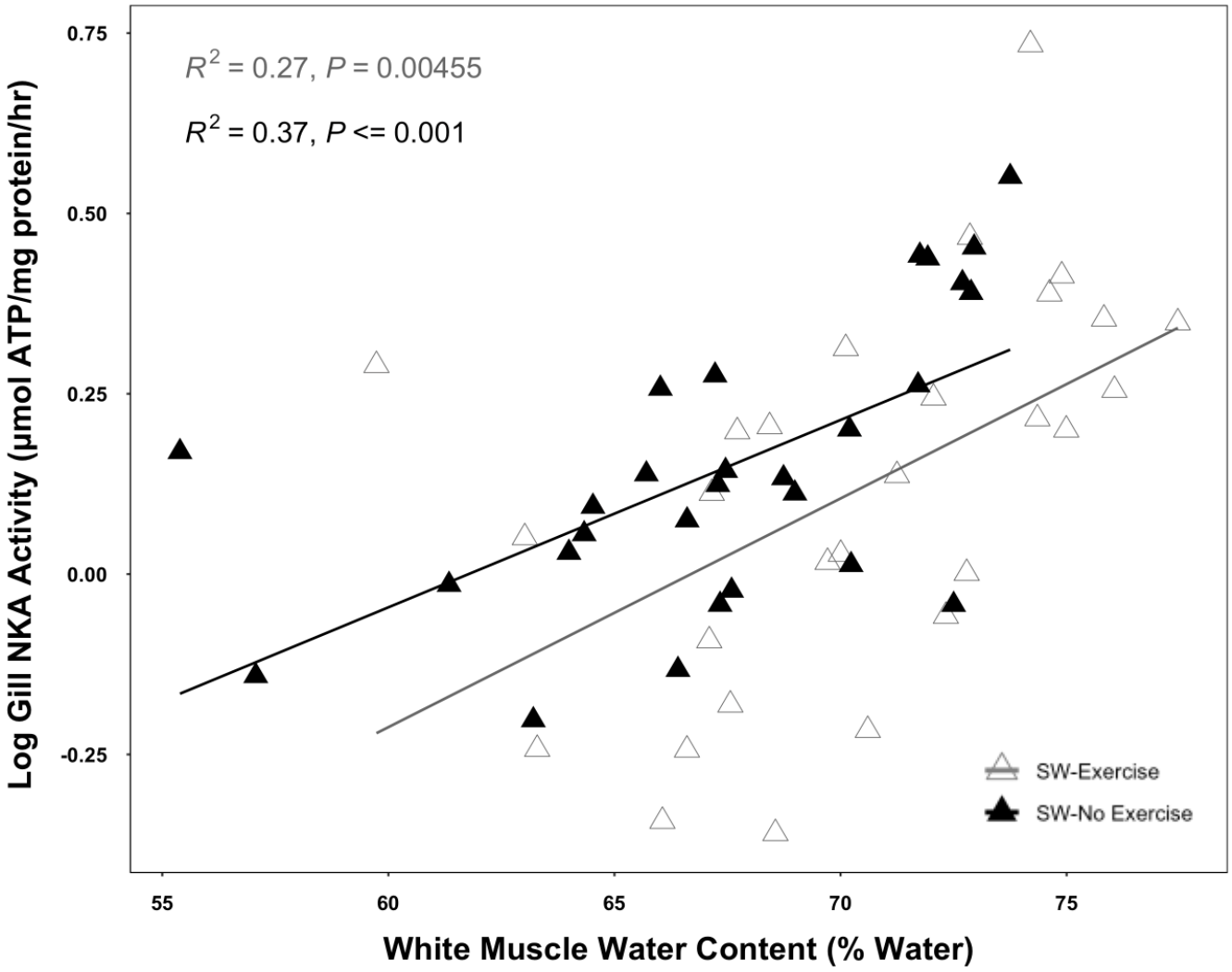

Figure 2.7: Log-transformed gill NKA activity plotted against white muscle water content for exercised (open triangle) and non-exercised (filled triangle) fish exposed to saltwater at all time points; n = 28 for both groups. Regressions for both groups were significant ($P < 0.05$).

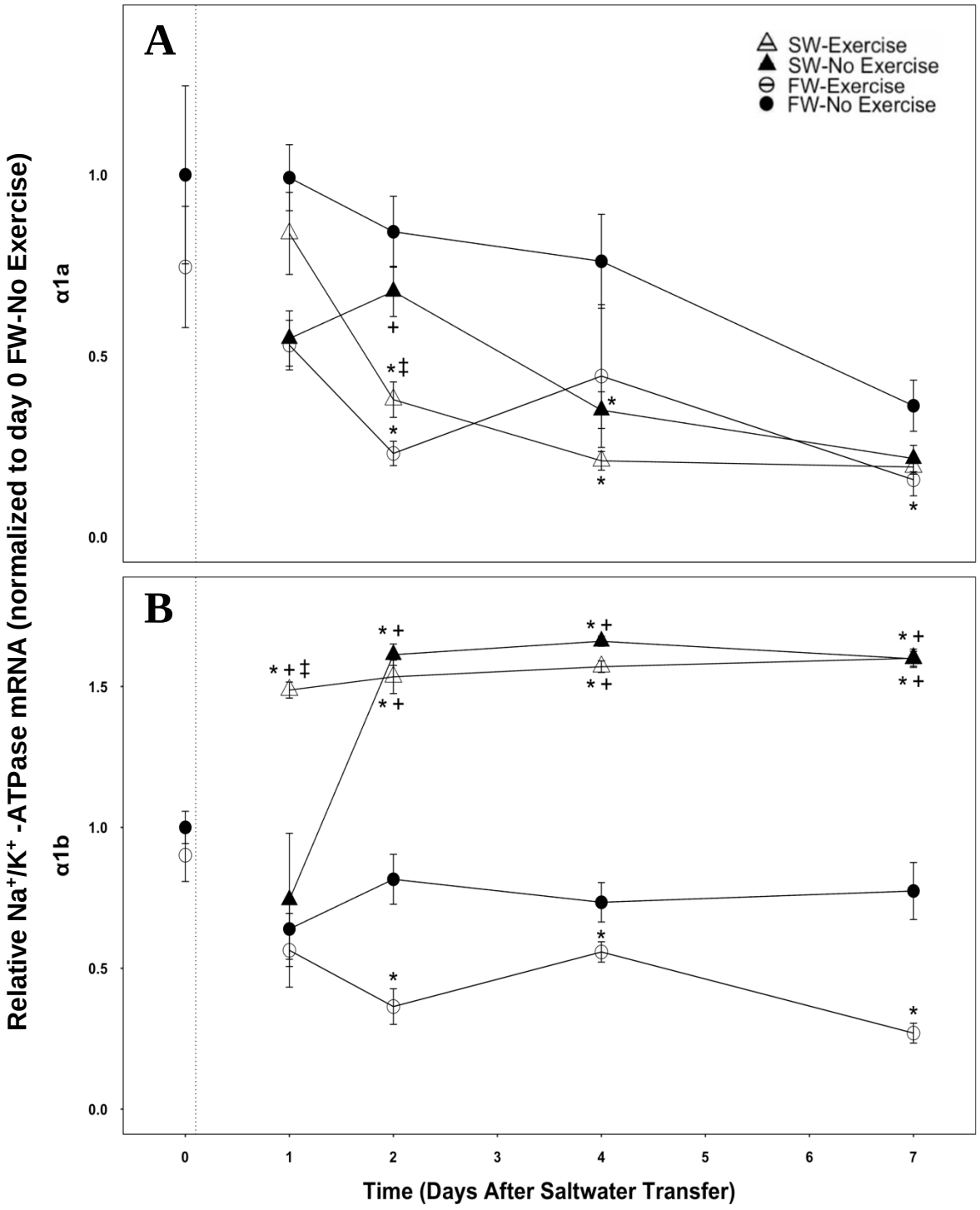

Figure 2.8: Gill NKA mRNA expression for α1a (**A**), and α1b (**B**) in non-exercised fish that remained in freshwater (FW) (filled circle) or exposed to saltwater (SW) (filled triangle) and in exercised fish that remained in FW (open circle) or exposed to SW (open triangle). Mean expression of all groups has been normalized to day 0 FW-No Exercise. The vertical broken line represents the halt of exercise and subsequent transfer of fish to FW or SW at time 0 (line is slightly offset as not to obscure data). Statistical comparisons made over time within each experimental group are stated in the results. For statistical comparisons made between groups at each time point (one-way ANOVA; $P < 0.05$): * denotes significantly different from FW-No Exercise group; + denotes significantly different from FW-Exercise group; ‡ denotes significantly different from SW-No Exercise group.

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