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The Effect of Cadmium Exposure on Repeat Swimming Performance and Recovery in Rainbow Trout (*Oncorhynchus mykiss*), Brown Trout (*Salmo trutta*) and Lake Whitefish (*Coregonus clupeaformis*)

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THE EFFECT OF CADMIUM EXPOSURE ON REPEAT SWIMMING PERFORMANCE AND RECOVERY IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*), BROWN TROUT (*SALMO TRUTTA*) AND LAKE WHITEFISH (*COREGONUS CLUPEAFORMIS*)

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Abstract

Swim performance provides a measure of the integrated metabolic costs of sub-lethal exposure to stressors, including Cd. While the impacts to sustained swimming performance in Cd exposed fish have already been characterised, effects to repeat swimming ability have not. This thesis identifies the effects of sub-lethal waterborne Cd exposure on ionoregulatory function, repeat swimming ability, anaerobic metabolism and stress response in rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*) and lake whitefish (*Coregonus clupeaformis*).

Repeat swim trials included an initial swim to 85% of the U_{crit} of control fish and a recovery period of varying duration, followed by a second, exhaustive, swim trial. Fish were terminally sampled prior to and directly following each of the two swim challenges so as to evaluate fluctuations in metabolic (glycogen, lactate, ATP) and stress (cortisol) parameters as a result of exercise and Cd exposure. All exposures were performed in duplicate with an exposure concentration of 18 nM Cd in moderately hard water (120 mg $CaCO_3$ L^{-1}) and a control group.

Repeat swimming ability was assessed in brown trout and lake whitefish over a 30 day period. Cd exposure resulted in 31% and 38% decreases in secondary swimming ability of each species, with the greatest decreases observed after one week of exposure. This reduction in swimming ability was associated with ionoregulatory disruption and reduced resting ATP stores in the white muscle. As a result, a second study was performed in order to identify the role of the recovery period. Recovery ability was evaluated in rainbow trout following 6 d exposure to 0 or 18 nM Cd. Three recovery periods were employed; 0.5, 1.5 and 6 h so as to identify the effect of Cd exposure on the

recovery of metabolic parameters. As anticipated, Cd exposed fish had decreased swimming ability when given a short, 0.5h recovery period. However, a 1.5 hour recovery period resulted in significantly increased swimming performance in Cd exposed fish compared with controls. Improved swimming ability in Cd exposed fish corresponded to increased rates of recovery of muscle and liver glycogen and ATP and reductions in lactate, compared with control fish.

Authors Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

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Cheers.

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Chapter 1

Introduction

1.1 Waterborne Cadmium Toxicity

Cadmium is an uncommon element that is not found in a pure state in nature (Johnson, 1997). It is present in the environment as a result of both natural (weathering of phosphate rock, erosion and volcanic activity) and anthropogenic sources (mining and smelting of Zn, Pb, and Cu ores, industrial activity and fuel combustion; Wilson, 1988; International Cadmium Association, 2000; ATSDR, 2008). In freshwater, Cd concentrations can be less than 0.02 nM (0.002 µg Cd L^{-1}) in pristine sites. Typical waterborne Cd concentrations are typically no greater than 4.5 nM (0.5 µg Cd L^{-1}) with significantly contaminated sites reachings levels as great as 13 nM (1.5 µg Cd L^{-1}: Pan *et al*., 2010). Commercially, 83% of Cd use is in the production of Ni-Cd batteries (Panagapko, 2007), while use in pigments and as a stabilizer to reduce ultraviolet degradation in plastics occupies the other major proportion of use at approximately 15%. Minor commercial uses of Cd include: plating, non-ferrous alloys and photovoltaic devices (International Cadmium Association, 2000).

Unlike other metals such as Zn or Cu, Cd is a non-essential element and thus fish do not required it in their diet. Cd can have deleterious and potentially lethal effects on aquatic organisms when present at elevated concentrations (Hollis *et al.*, 2000). Waterborne cadmium first targets the gills of fish where it passes from the external environment to the blood stream. The presence of Cd in various body tissues results in a host of negative physiological effects including ionoregulatory disturbance (Larsson *et al*., 1981, Pratap *et al*., 1989; McGeer *et al*., 2000a), oxidative damage (Livingstone, 2001), reduced reproductive capacity (Brown *et al*., 1994), decreased hatch success (Lizardo-Daudt and Kennedy, 2008), reduced growth and skeletal abnormalities (Hansen

et al., 2002). Salmonids, including trout species, are amongst the most sensitive teleost species to waterborne Cd exposure with LC_{50} values in the low to mid μg L^{-1} range (Chapman, 1978; Chapman and Stevens, 1978; Roch and Maly, 1979; Kumada *et al*., 1980; Majewski and Giles, 1981).

Acutely, the toxic effects of waterborne Cd are generally a result of the divalent cation form Cd^{2+}, which competes with free calcium ions (Ca^{2+}) for high affinity calcium binding and uptake sites on the gill (Niyogi *et al*., 2008). In fresh water fish, Ca^{2+} ions must be actively taken up across both the gill and to a lesser extent the intestinal epithelia. At the gill, Ca^{2+} must be imported via active transport because of significant electrochemical gradients (Wood, 1991; Perry, 1997: Perry *et al.,* 2003; Moyes and Schulte, 2008). Ca^{2+} is transported into the blood via PNA^{+} MR cells at the gills (Galvez *et al*., 2006). Uptake across the apical surface occurs via a lanthanum-sensitive voltage-independent epithelial Ca^{2+} channels (ECaC: Goss *et al*., 2001; Perry *et al*., 2003; Galvez *et al*., 2006), facilitated by low intracellular Ca concentrations (Marshall, 2002). At the basolateral membrane active transport of Ca^{2+} occurs via Ca^{2+}-ATPase although Na^{+}-Ca^{2+} exchange also contributes (Marshall, 2002). Through non-competitive inhibition, Cd^{2+} irreversibly blocks the Ca^{2+}-ATPase transport enzyme (Hollis *et al*., 2000), ultimately leading to Cd rather than Ca being translocated into the blood (Verbost *et al.,* 1989) and then on to various tissues of the body (Szebedinsky *et al*., 2001). This displacement of Ca is the primary mechanism of the acute toxic effects of Cd, which can result in hypocalcaemia, a fatal reduction in whole body calcium (Hollis *et al*., 2000). Chronic toxicity as a result of Cd exposure is less well understood, but can be linked to its distribution to sensitive tissues via the circulation (McGeer *et al.*, 2011).

Swimming ability (in a singular U_{crit} challenge) in rainbow trout is not affected by chronic exposure to Cd (Hollis *et al.,* 2000; McGeer *et al.,* 2000a; Cunningham and McGeer, 2009). Additionally, Cd exposure does not produce variations in either resting or maximal oxygen consumption in comparison to control fish (Hollis *et al.,* 2000).

1.2 Damage Repair Model

McDonald and Wood (1993) identified a pattern of response to chronic metal exposure and developed a model to describe the phases of acclimation. Termed the damage-repair model, it is characterized by a general series of events, divided into three phases. The first of these is the initial shock phase. Here, primary damage is first observed at the gills, where metals accumulate both on and in the gill tissue. This can result in homeostatic disruptions such as alterations in ionoregulatory function and increased oxygen consumption amongst other homeostatic disruptions which ultimately characterize this phase (McDonald and Wood, 1993). This period generally lasts several days before transitioning into the second stage, the recovery phase. This period is associated with increased biosynthetic processes such as mitosis and protein synthesis, and increased metabolic activity which is required to repair the initial physiological damage (McDonald and Wood, 1993). Most important to this phase is the mobilization of metal-binding proteins such as methallothionein, which aid in metal detoxification and storage (McDonald and Wood, 1993).

As exposure to metal shifts from acute to chronic, the gills begin to function as a barrier to cadmium, reducing internal Cd loading (Hollis *et al.,* 2000). As these actions continue the final phase (acclimation phase) is reached. Here, the internal physiology will either return to pre-exposure conditions or else a new, functional steady state will be

established. This can ultimately result in a reduction of negative physiological effects associated with chronic accumulation of metals; however homeostatic costs may still be incurred (McDonald and Wood, 1993).

1.3 Stress Physiology

Despite applications in the majority of biological fields, an overarching definition of stress has proven difficult. For the purpose of this thesis, stress is defined as a condition in which the dynamic equilibrium of an organism (homeostasis) is threatened or disturbed as a result of the actions of intrinsic or extrinsic stimuli. Such stimuli are termed stressors and have two primary roles in the establishment and reaction to stress. The first is to produce effects which disturb the homeostatic equilibrium. This then elicits the second role, which is to produce a response to deviations from normal homeostatic control. More specifically, the application of a stressor will produce a coordinated set of behavioural and physiological responses to allow the animal to overcome the threat (Chrousos and Gold, 1992).

The current integrated stress response model in fish is developed from Cannons' (1929) study on the role of catecholamines in the fight or flight response and Selyes' (1936) general adaptive syndrome which identified the role of glucocorticoids in the response. While a number of other hormones have subsequently been implicated in the response to the application of a stressful stimulus, these hormones represent the primary messengers of the two major pathways which coordinate the stress response: the hypothalamic-sympathetic chromaffin cell axis and the hypothalamic-pituitary-adrenal axis (Chrousos and Gold, 1992). Through these pathways organisms are able to cope with

stressors by adjusting their biological activities to allow for reallocation of energy to accommodate the stress condition (Wendelaar Bonga, 1997). Although initially described in terrestrial animals, the stress response of fishes has been shown to employ similar primary neuroendocrine control mechanisms associated with the administration of stress response, and thus fall under a general pattern of stress response in vertebrates (Donaldson, 1981; Mazeaud and Mazeaud, 1981).

The first of the two general pathways associated with the coordination of the stress response in the hypothalamic-sympathetic chromafin cell axis. This pathway is responsible for the mobilization of cathecholamines (epinephrine and norepinephrine). In mammals, catecholamines are released from the adrenal medulla, in teleosts, the homologue of this tissue is the head kidney. Specifically, catecholamines are mobilized from the chromaffin cells of the head kidney. Following a stressful stimulus, a significant and rapid increase in catecholamine levels typically occurs. Following an acute stressor, the observed peak in plasma catecholamines decreases rapidly with catecholamines having a short biological half-life (<10 min; McDonald and Milligan, 1992; Randall and Perry, 1992). The presence of elevated caetcholamines in the circulation is correlated with rapid increases in oxygen uptake rate at the gill as a result of increased ventilation rate, stimulation of branchial blood flow and oxygen diffusing capacity as well as increased oxygen transport capacity of the blood (Randall and Perry, 1992; Wood and Munger, 1994). Elevated catecholamines are also associated with increases in cardiac output (Randall and Perry, 1992) and hyperglycemia (Barton and Iwama, 1991).

The second pathway, hypothalamic-pituitary-adrenal axis, is ultimately responsible for the production of the stress hormone cortisol. Within the brain,

specifically, the hypothalamus, a response to a stressor is instigated through the stimulatory and inhibitory effects of a variety of hormones. These hormones are transported to the pituitary gland stimulating the release of MSH and ACTH. These hormones then act on the interrenal cells of the head kidney resulting in the production of cortisol. Cortisol release from the head kidney is controlled by a negative feedback loop (Wendelaar Bonga, 1997). The mobilization of ACTH has been associated with increases in plasma cortisol in coho salmon (*Oncorhynchus kisutch*), rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*: Pickering *et al*., 1986; Sumpter *et al*., 1986: Balm and Pottinger, 1995). Cortisol plays a key role in the regulation of hydromineral balance and energy metabolism (Wendelaar Bonga, 1997).

Following exposure to a stressful stimulus, fish will often exhibit alterations in behaviour, physiology and/or biochemistry. These changes result in one of two possible outcomes; a negative outcome, termed an effect, or a positive outcome, termed a response. A response combines adaptive changes which work to return homeostasis to pre-exposure conditions or to re-establish homeostasis at a new, functional level. The ability of an organism to respond and adapt to stressors is key to its survival and evolutionary fitness (Beitinger and McCauley, 1990).

The reaction of a fish to a stressful stimulus can be categorized into a framework known as the general adaptive syndrome (GAS). In this model, responses associated with stimuli are categorized as primary, secondary and tertiary (Wedemeyer *et al*., 1990). Primary responses generally involve the endocrine system and the release of stress hormones. During a primary response to a stressful stimulus, hormones such as cortisol and epinephrine are released from the head kidney into the blood stream to increase the

magnitude of the stress response (Beitinger and McCauley, 1990). Secondary responses are characterized by changes to the blood and tissues. In the blood, a typical stress response involves hyperglycemia, hyperlacticemia, and a reduction in blood clotting time. At the tissue level, a rapid depletion of nutrients, including glycogen and vitamin C, is observed (Beitinger and McCauley, 1990). Finally, tertiary effects reflect those that affect the entire organism or a population of organisms. To the individual, impairment can be observed in many processes including: growth, locomotion, resistance to disease, reproduction and survival. These impairments may combine to result in a decrease in the number of individuals present in a population (Beitinger and McCauley, 1990).

1.4 Stress Testing

Physiological impairment can have deleterious effects to an organism. There has been significant research into the responses of fish to different environmental stimuli (Beitinger and McCauley, 1990). In order to assess the reaction of an organism to a stressor, a series of tests can be performed. Such tests were first categorised by Brett (1958) into three distinct categories. The first of these allows for analysis of a loading stress and the decrease in performance associated with it. Tests of loading stress include exposure to extreme temperatures, hypoxia, hyperosmotic solutions and forced swimming (Beitinger and McCauley, 1990). The second category measures growth and the third category measures changes in internal physiological function, such as cough frequency. Any departures from anticipated norms will reflect an effect as a result of the stress (Beitinger and McCauley, 1990). The research presented in this thesis focusses primarily on the first category, specifically those that can be used to determine the effects of metal exposure on the physiology of individual organisms by measuring loading stress.

1.5 Swimming Performance

Swimming ability is central to fish health and survival and is important in many behavioural responses. As fish are in near constant motion, swim performance is one of the most observable and critical measures of the effects of loading stressors (Beitinger and McCauley, 1990). The ability of a fish to swim has important repercussions on its ability to feed, evade predators, migrate and avoid unfavourable environmental conditions (Beaumont *et al*., 1995; Drucker, 1996; Plaut, 2001). Swimming ability has been researched extensively in physiological and toxicological studies, however providing a fully ecologically relevant result still poses a challenge as lab based swimming tests do not fully replicate environmental conditions.

1.6 Classification of Swimming Types

Fish demonstrate various types of swimming involving the use of different metabolic strategies. There are three primary methods of swimming utilized by fish species as described by Beamish (1978); these are sustained, burst and prolonged swimming. These three swim types differ in the duration, intensity and type of metabolic activity required to support locomotion (Beitinger and McCauley, 1990).

Sustained swimming, the method used the most frequently by fish, represents a low level of activity which can be maintained for long periods of time. Sustained swimming generally lasts a minimum of 200 minutes but can allow fish to swim for days or even months without fatiguing (Hammer, 1995). Sustained swimming utilizes aerobic metabolic pathways to generate required ATP with anaerobic intramuscular fuel sources (ATP and creatine phosphate) being recruited only minimally. This causes few anaerobic

by-products (lactate) to be generated (Jobling, 1994). Sustained swimming does not result in the accumulation of oxygen debt, a measure of excess post-exercise oxygen consumption which is required to eliminate lactate formed through anaerobic metabolism (Beitinger and McCauley, 1990). Generally, it is though that swimming speeds below 80% of the critical swimming speed (U_{crit}: see section 1.7) is fuelled almost entirely aerobically; however it has been shown in goldfish (*Carassius auratus*) that anaerobic pathways may contribute to swimming below 80% U_{crit} in cyprinids (Jones, 1982). Richards *et al*., (2002b) identified 90% U_{crit} as the critical point at which, swimming becomes unsustainable for salmonids and requires anaerobic metabolic contributions. Although the most frequently employed, the study of sustained swimming has received relatively limited research interest as tests can be time and resource intensive. Evaluations can however, be useful in the examination of bioenergetic effects of aerobic exercise in fish species (Hammer, 1995).

The second swimming type, burst swimming, reflects an anaerobically fuelled sprint swimming speed typically employed during preditor prey interactions and at unsustainable swimming speeds. Burst swimming can only be maintained for very brief periods, typically no more than 15-20 seconds (Jones, 1982, Beitinger and McCauley, 1990; Hammer, 1995, Domenici and Blake, 1997). Peak swimming speeds obtained during burst swimming greatly exceed those attainable with the other two swimming methods (Beamish, 1978). For example, Farrell (2008) observed a constant acceleration (U_{max}) 57% greater than U_{crit} in rainbow trout.

Burst swimming can only be maintained for a brief period of time; as a result, peak swimming speed is decreased as time increases, due primarily to an exhaustion of

anaerobically derived intracellular fuel supplies and a corresponding accumulation of waste products (Brett, 1964; Jones, 1982). Rapid bursts of swimming are not impacted by oxygen or temperature (Beitinger and McCauley, 1990). Burst swimming ability is assessed by applying an external stimulus, such as a light electric shock to the caudal fin, to elicit rapid acceleration in order to allow for a fright response and escape. Maximum attained sprint velocity can be determined through several different forms of measurement (reviewed by Domenici and Blake, 1997) and provides an examination of ecologically relevant properties such as predator evasion as well as prey capture ability (Plaut, 2001).

The final type of swimming, prolonged swimming, is a term generally applied to swimming ability between burst and sustained levels, and represents the primary cruising speed of a fish (Plaut, 2001). Prolonged swimming can be maintained for long periods of time, generally between 2 and 200 minutes (Brett, 1964; Plaut, 2001), ending in exhaustion (Hammer, 1995). This category of swimming has been used extensively as a measure of fish swimming ability, as it combines both burst and sustained swimming (Brett, 1964; Beamish, 1978, Hammer, 1995).

Prolonged swim performance is typically studied in chambers where water velocity can be altered to suit the requirements of the given test. For the most part, fish will exhibit rheotropism and will instinctively swim against the administered current. Several methods have been developed in order to test prolonged swimming ability. The first is a fixed velocity test, in which the length of time that a fish can swim at a constant, elevated, speed is measured (Hammer, 1995; Plaut, 2001). As well, gate transition speed, which measures the time spent on both prolonged and burst swimming, can be

determined by observing the fish in the chamber and recording the length of time that each of the previously described swimming methods is employed (Plaut, 2001). The final type of swim test is an incremental velocity test, in which the water velocity is increased in steps of a prescribed duration, until exhaustion is achieved (Beamish, 1978; Hammer, 1995; Plaut, 2001). The time and velocity at exhaustion are used in order to calculate the critical swimming speed (U_{crit}; Brett, 1964), the most commonly used measure of fish swimming ability.

1.7 Critical Swimming Speed Tests

Prolonged swimming is measured most frequently by increasing water velocity in increments of consistent durations. This testing method is used to determine the critical swimming speed, U_{crit}, which quantifies how long a fish can swim before it becomes exhausted (Brett, 1964; Beitinger and McCauley, 1990; Hammer, 1995; Farrell, 2008). As critical swimming speed can be influenced by fish size, in particular fork length (Bainbridge, 1958; Beamish, 1978; Hammer, 1995), swim performance testing should be standardized to account for variations in body length (bl).

The formula for critical swimming speed is as follows:

$$\mathrm{Ucrit} = \mathrm{Ui} + \left(\frac{\mathrm{Ti}}{\mathrm{Tii}} \times \mathrm{Uii}\right)$$

Where:

U_i is the highest velocity (peak) velocity step completed (cm s^{-1})

U_{ii} is the velocity increment (cm s^{-1})

T_i is the time from the last velocity step increase to fatigue (s)

T_{ii} is the time between velocity increase steps (s)

1.8 Factors Effecting Critical Swimming Speed

A wide variety of physiological factors and environmental conditions can influence swimming performance (for reviews see Hammer, 1995 and Kieffer, 2000). Kieffer (2000) identified three physiological factors which limit anaerobic swimming: 1) levels of anaerobic fuel stores, 2) accumulation of metabolic end products, and 3) rate of recovery. Other factors such as nutritional status, water quality, training and body size are potential factors limiting anaerobic capacity and recovery pattern (Kieffer, 2000).

Temperature is one such factor which can have a significant effect on swimming ability and other physiological functions. Fish have an optimal temperature (T_{opt}) at which aerobic scope is maximized, with performance declining at temperatures above or below this point (Brett, 1964; Myrick and Cech, 2000; Lee *et al*., 2003; Sandblom and Axelsson, 2007). Steinhausen *et al*. (2008) observed a 50% failure of sustained swimming in sockeye salmon at 23.5 ^{o}C due to an inability to increase cardiac output with temperature, leading to an increase of lactate in the blood. The oxygen carrying capacity of water decreases as temperature increases leading to reductions in oxygen availability that potentially inhibit the aerobic capacity. Reductions in swimming performance at water temperatures below T_{opt} may be as a result of temperature related physiological alterations that occur in poikilotherms.

Prolonged swimming at speeds such as those observed during U_{crit} swimming requires both aerobic and anaerobic contributions, with a minimum of 75% of swimming being powered aerobically (Webb, 1971a). Reductions in oxygen availability can have significant effects on U_{crit} swimming performance. Reductions in U_{crit} as a result of decreased oxygen availability have been observed in sockeye salmon (Brett, 1964), rainbow trout (Jones, 1971; Bushnell *et al*., 1984) and Atlantic cod (*Gadus morhua*: Petersen and Gamperl, 2010).

U_{crit} performance varies across species, as lifestyle and morphology can have significant influence on swimming motion and performance. For example, U_{crit} of fish with pelagic lifestyles and significant migratory behaviour such as European perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*; Tudorache *et al*., 2008) and rainbow trout (Hollis *et al*., 1999; McGeer *et al*., 2000a; Waser *et al*., 2009) are higher than in demersal fish, or those with short migratory distances such as the stone loach (*Barbatula barbatula*) or bullhead loach (*Cottus gobio:* Tudorache *et al.,* 2008). Morphological differences can similarly affect swimming performance. Tail morphology can have a significant impact on swimming speeds and longevity. For example, salmonid tails are homocercal with broad, identical upper and lower lobes while sturgeon (*Acipenser spp*.) posses heterocercal tails with a smaller lower lobe which generates 66% less thrust than the lower tail lobe of a similarly sized trout, ultimately leading to a net reduction of 18% less thrust produced by sturgeon than trout of the same size (Webb, 1986; Peak *et al*., 1997). Salmonids also have smooth, streamlined bodies, while sturgeon possess rougher, less fusiform bodies, primarily due to the presence of heavy bony plates or scrutes causing 3.5 times more drag than in salmonids (Webb, 1986). In order to match

swimming ability of salmonid species, sturgeon must generate more thrust, with a less efficient tail, ultimately requiring a greater metabolic cost so as to supply increased energy demands (Webb, 1986; Peak *et al*., 1997). Salmonids typically have long migratory distances, and excel at both long term cruising and burst ability (Milligan, 2003). They have been used extensively throughout studies measuring swimming performance, and have provided significant information regarding swimming ability in teleosts.

1.9 Repeat Swimming

Another measure of prolonged swim performance evaluates the ability of an organism to swim in repeated challenges. This is achieved by performing a series of prolonged swimming tests separated by recovery periods of a designated length (Farrell *et al*., 1998). The performance of the fish in the second swim test (measured as U_{crit}) is of particular interest as it shows the extent to which an organism can recovery between swims. How frequently a fish can undergo bouts of high intensity exercise is determined by the recovery time required, particularly the amount of time necessary to restore energy reserves (Milligan, 1996). Impacts upon recovery of energy stores can be used to show impairment as a result of mechanical damage or environmental conditions (Jain and Farrell, 2003). The ability of a fish to perform successive bouts of exhaustive exercise is important for predator avoidance, feeding and migration. Impairment of this ability may have significant ecological impacts, as organisms with long recovery periods are more susceptible to predation and are disadvantaged in the fields of migration or foraging (Farrell *et al*., 1998). The aim of the present study is to characterize the influence of chronic Cd exposure on repeated swimming ability.

1.10 Exercise Metabolism

The ability to undergo high intensity exercise in fish requires a substantial contribution from trunk musculature. Muscle activity is dependent upon the synthesis and subsequent catalysis of adenosine triphosphate (ATP). This is achieved through a process known as phosphorylation in which inorganic phosphosphate (P_i) is added to adenosine diphosphate (ADP) in order to produce the ATP necessary for swimming. Production of ATP within the muscle occurs by two primary methods. The first, oxidative phosphorylation, is an aerobic metabolic pathway generally utilized at sustained swimming speeds, in the presence of oxygen. A second metabolic pathway, substrate level phosphorylation, is an anaerobic pathway in which ATP is produced in the absence of oxygen. This pathway is primarily employed during burst swimming activity and at unsustainable swimming speeds.

1.10.1 Fish Musculature

The muscle mass of teleost fish is estimated to account for approximately 60-85% of the total body mass (Hochachka and Somero, 2002). The two fibre types red (slow-twitch) and white (fast-twitch) are anatomically divided. Arranged along the lateral line on each side of the fish are the red muscle fibres, which account for 5-15% of the total muscle mass (Moyes and West, 1995). Red muscle derives its colouration from a high degree of vascularity. Red muscle contains a significant number of mitochondria (Moyes *et al*., 1992) and is recruited primarily during aerobic activity, such as foraging, and lower intensity swimming (below 80% $U_{cri)t}$. Red muscle tends to contain elevated ATP stores, with the majority being produced from the oxidation of intramuscular lipids

(Moyes and West, 1995) while carbohydrate metabolism can account for up to 10% of the available substrate (West *et al*., 1993).

The remaining 85-95% of the total muscle mass of teleosts is comprised of white fibres. Vascularisation and mitochondrial content are significantly lower when compared with red muscle (West *et al*., 1993). Both ATP stores, and particularly oxygen are limiting factors required for high intensity exercise. White muscle fibres are recruited for short burst type activity at high speeds as well as during acceleration. White muscle relies on anaerobic glycolysis which provides initially available intramuscular ATP stores and facilitates intramuscular glycogenolysis. White muscle is recruited when oxygen becomes limiting, such as at swimming speeds in excess of 80-90% U_{crit}. It is probable that white muscle also contributes to aerobic swimming; however it is unclear how significant this contribution may be (Moyes *et al*., 1992; Rajotte and Couture, 2002).

1.10.2 Fatty Acid β Oxidation

Triglycerides obtained through the diet are converted aerobically into metabolically useful compounds required for the generation of ATP through the tricarboxylic acid (TCA) cycle by fatty acid β-oxidation (Jobling, 1994). Fatty acids, produced from triglycerides are oxidized through a series of enzymatically catalyzed steps in which pairs of carbons are removed from the end of each fatty acid molecule. The shortened fatty acid returns to the beginning of the pathway, and the cycle continues until the entire fatty acid has been broken down into acetyl CoA (Moyes and Schulte, 2008). The rate limiting step is the conversion of β-Hydroxyacyl CoA to β-Ketoacyl CoA via the enzyme β-hydroxyacyl dehydrogenase (HOAD; Moyes and Schulte, 2008). Increased HOAD activity occurs in tissues with greater aerobic capacity and lipid metabolism, such as red

muscle, which is recruited primarily during non-exhaustive swimming, when oxygen levels are not limiting (Moyes *et al*., 1992).

1.10.3 Oxidative Phosphorylation

When oxygen is not limited, ATP can be generated through oxidative phosphorylation, which generates more ATP than can be generated by substrate-level phosphorylation (see section 1.10.4). Oxidative phosphorylation forms ATP through the catabolism of metabolites within the mitochondria. This conversion occurs within a series or complex, of membrane proteins associated with the mitochondria. In order to supply substrate for oxidative phosphorylation (NADH and FADH) the TCA, a series of enzyme-catalyzed reactions, is first employed. The required substrate for TCA is acetyl CoA, which can be generated through a variety of pathways, including fatty acid β-oxidation and aerobic glycolysis (Moyes and Shulte, 2008). It is converted to oxaloacetate and citrate by the rate limiting enzyme citrate synthase; this step is often regarded as a measure of aerobic efficiency (Rajotte and Couture, 2002).

The TCA then feeds required substrates, particularly NADH, into the electron transport chain (ETC) which provides cellular energy via oxidative phosphorylation. The ETC is a series of five multi-subunit protein complexes. ETC complexes are embedded in the inner membrane of the mitochondria. Three subunits act as proton pumps, which obtain energy from the passage of electrons. Protons are actively pumped into the intermembrane space. The membrane bound structures are connected by hoydrophilic and hydrophobic electron carriers. The proton gradient created within the ETC via the transfer of protons between complexes I, III, and IV is both electrical and chemical and

ultimately provides the ADP and Pi necessary to generate ATP (Moyes and Shulte, 2008).

1.10.4 Substrate Level Phosphorylation

When oxygen availability is limited, such as during burst activity or swimming speeds above 80% of U_{crit}, oxidative phosphorylation is no longer sufficient to supply the metabolic demands for high intensity exercise. During intense physical activity white muscle is recruited. When this occurs, oxygen is limited and anaerobic metabolism is employed. Anaerobic metabolism is driven by substrate level phosphorylation. ATP stores within the white muscle are limited (Moyes and Shulte, 2008) and thus substrate-level phosphorylation is required for in-situ ATP production (Richards *et al*, 2002a). White muscle is also poorly supplied with mitochondria, and as a result substrate level phosphorylation occurs within the cytoplasm of the cell. Although substantially less efficient, in terms of ATP production, substrate level phosphorylation can generate ATP more readily and faster than oxidative phosphorylation (Moyes and Schulte, 2008). Glycogen stores are, however, limited, which can result in decreased performance as fuel source availability declines (Moyes and Schulte, 2008).

Through the same mechanism of aerobic glycogenolysis, glycogen molecules are catabolised in individual glucose sub-units and then further to yield pyruvate through the process of anaerobic glycolysis. Aerobically, pyruvate is converted to acetyl CoA, but in the absence of oxygen pyruvate is ultimately converted to lactate via the enzyme lactate dehydrogenase. This conversion supplies ATP required to continue substrate level phosphorylation. Similarly, phosphocreatine can donate a phosphate group for the anaerobic synthesis of ATP, ultimately resulting in the accumulation of lactate. As a

result, high intensity exercise is impaired by the accumulation of lactate within the white muscle. Recovery occurs both as a result of *in situ* glycogen re-synthesis from lactate as well as a shuttling of lactate to the circulation and subsequent transfer to oxidative tissues such as the liver (Moyes and West, 1995; McDonald *et al*., 1998; Kieffer, 2010).

1.11 Physiology and Metabolism of Swimming and Recovery

Measures of exercise physiology are amongst the most obvious and important indicators of organism health and fitness. As fish are often required to undergo repeated bouts of high intensity exercise it is essential that recovery occur rapidly. High intensity exercise has a significant intensity on the entire organism. In fish, the metabolic disturbances associated with high intensity exercise are both greater and require a longer recovery period than in mammals. This is due to the homogeneous nature of the fish skeletal muscle (Johnston *et al*., 1975). The majority of a fishes' body is composed of white muscle fibres, which are used extensively during high intensity exercise. Thus, the majority of a fishs' body is recruited during a bout of strenuous activity, while in mammals white muscle represents a much smaller proportion of the body mass, and thus the metabolic debt incurred is less (Milligan, 1996). Recall, the musculature of salmonid species is divided into two regions: the first, red-oxidative fibres use ATP generated primarily from oxidative phosphorylation (Moyes and West, 1995) are recruited almost entirely independently up to swimming speeds of 80% of the critical swimming speed. Locomotion at these sustainable swimming speeds results in minor changes in cellular energy status with only minor utilization of intramuscular substrates (Richards *et al.,* 2002b). Sustained swimming is powered by the oxidation of approximately 45% carbohydrates, 35% lipids and 20% protein (Moyes and West, 1995; Richards *et al*.

2002b). At swimming speeds greater than 80% of U_{crit} the second muscle type, the white-glycolytic fibres are recruited (Hudson, 1973; Johnson, 1981; Wilson and Egginton, 1994; Burgetz *et al*., 1998). White muscle has a significantly lower capacity for mitochondrial ATP production, compared with red muscle, and as such is reliant upon creatine phosphate (CrP) hydrolysis and glycolysis for the production of ATP (Richards *et al*., 2002a).

As swimming speed increases to over 90% of U_{crit}, it can be described as non-sustainable swimming, which requires a greater amount of ATP production and results in significant depletions of intramuscular substrates with red muscle predominantly oxidizing carbohydrates, while the recruited white muscle relies on substrate level phosphorylation (Richards *et al*., 2002b). Exercise to exhaustion in salmonids has previously been characterized by decreases of white muscle ATP stores by 40-60%, as well as similar observations for creatine phosphate (CrP), as early stages of exercise rely heavily on these fuel sources for muscular contraction. Additionally, decreases of up to 90% have been observed for white muscle glycogen concentrations, leading to reciprocal increases in lactate accumulation (Wang *et al*., 1994). Reported decreases in each metabolite vary with studies reporting PCr decreases of as little as 40% (Wang *et al*., 1994) and as great as 90% (Milligan and Wood, 1986; Dobson and Hochachka, 1987; Schulte *et al*., 1992). This variability may be due to the sensitive nature of both ATP and PCr stores during the process of sampling and analysis leading to difficulty in determining precise concentrations of either pool (Wang *et al*., 1994).

The ability of an organism to repeatedly undergo bouts of high intensity exercise is dependent upon the amount of time required to restore energy reserves (Milligan,

1996). Central to recovery is the re-establishment of glycogen stores and the removal of lactate. In mammals, this process is facilitated through the Cori cycle, in which lactate produced in the muscles is transported to the liver, where it is converted to glucose which is then transported back to the muscle. In fish however, the role of the Cori cycle is not so well defined. The majority of muscle glycogen replenishment occurs *in situ* with approximately 80% of the total lactate produced during exercise being retained within the muscle (Milligan, 1996). Retained lactate acts as the primary substrate for the re-synthesis of glycogen. The mechanism of glycogen re-synthesis from lactate, and the energy sources involved remain somewhat unclear (Milligan, 1996). Typically it is only at elevated concentrations that lactate may pass into the circulation. Lactate present in the circulation will be transported to oxidative tissues, such as the liver, where it will then be used to resynthesize glycogen as is observed in the Cori cycle (Moyes *et al*., 2002).

1.12 Cortisol

Cortisol is a metabolic hormone which has been shown to increase metabolic rate in fish (Chan and Woo, 1978). Cortisol plays an important role in energy metabolism. Following exercise, cortisol levels can be increase up to 5 x from resting levels, and will remain elevated for more than 6 h (Kieffer, 2000; Milligan, 2003). Elevations in cortisol have been shown to mobilize lipids and to increase gluconeogenisis from amino acids to supply energetic demands (Mommsen *et al*., 1999: Vijayan *et al*., 1996). Cortisol also has a role in the regulation of anaerobic metabolism in teleosts (Milligan, 1996). Elevations in plasma cortisol appear to produce an inhibitory response on muscle glycogen synthesis, glycogenesis. While cortisol levels remain elevated, muscle glycogen synthesis cannot occur. Studies which inhibit the characteristic cortisol response post exercise have

established that in the absence of cortisol, muscle glycogen stores are replenished more quickly (Pagnotta *et al.*, 1994). Elimination of the cortisol response also altered post-exercise lactate dynamics in trout. Fish with an inhibited cortisol response exhibited increases in lactate, less than half of those observed in control fish (5 mmol L^{-1} and 12 mmol L^{-1} respectively) and returned to pre-exercise concentrations within two hours, while more than eight hours was required to re-establish resting lactate levels in control fish. This indicates that increasing plasma cortisol following exhaustive exercise has an inhibitory effect on metabolic recovery (Milligan, 1996).

Cadmium exposure has been shown to affect the characteristic cortisol response. Scott *et al*. (2003) observed that the anticipated peak in plasma cortisol following the application of a stressful stimulus or an alarm cue was decreased as a result of Cd exposure. In the field, an attenuated cortisol stress response was also observed in adult yellow perch in Cd contaminated lakes compared with reference lakes (Laflamme *et al*, 2000; Levesque *et al*., 2002, 2003). Lacroix and Hontella (2004) have shown that Cd exposure inhibits adrenocorticotropic hormone (ACTH) stimulated cortisol secretion from the interregnal cells of fish kidney. Similarly, Laflamme *et al*. (2000) observed that *in vitro* cortisol secretions of interrenal tissues were reduced as a result of Cd exposure. The effect of Cd exposure was further evaluated by Sandhu and Vijayan (2011) who observed a dose related suppression of ACTH-mediated cortisol production in the head kidney of rainbow trout. This suppression of the cortisol response correlated with significant reductions of melanocortin 2 receptor (MC2R), steroidogenic acute regulatory protein (StAR) and cytochrome P450 side chain cleavage enzyme (P450scc) RNA levels. Such inhibition indicates that Cd exposure disrupts expression of genes critical for the

synthesis of corticosteroid hormones, particularly that MC2R, the required gene for the ACTH induced first step of corticosteroidogenis is the target of Cd (Sandhu and Vijayan, 2011).

1.13 Objectives and Hypotheses

The work performed in this project measures whole organism response to sub-lethal waterborne Cd exposure by evaluating the highly relevant endpoint of repeat swimming performance. In addition to measures of swimming, ionic disruption, tissue specific metal accumulation, stress response and the metabolic costs incurred as a result of metal exposure will also be evaluated in three salmonid species (*Oncorhynchus mykiss, Salmo trutta, and Coregonus clupeaformis*).

The first objective of this research project was to evaluate the effect of acute and chronic Cd exposure on basic indicators of toxicity such as mortality, tissue specific Cd accumulation and alterations in plasma ion composition (Ca and Na). An exposure concentration of 18 nM Cd has been selected to provide sub-lethal exposure yet still cause physiological strain. I hypothesize that exposure to 18 nM Cd will result in minimal mortalities but will cause alterations in plasma ion composition, specifically an acute decrease in plasma Ca. It is also hypothesized that Cd present in the water will be taken up and stored within the tissues. The generally accepted pattern of chronic Cd accumulation in fish is kidney<gill<liver (SEE J-MILNE THESIS), thus it is hypothesized that such a pattern of accumulation will be observed.

The second objective was then to evaluate the effect of sustained, repeat swimming ability, and to determine the role in the rest period in the recovery of swimming performance. Previously, Cd exposure has been shown not to reduce the Ucrit of salmonid species in a single swim trial (Hollis *et al*., 1999; McGeer *et al*., 2000a; Cunningham and McGeer, 2009) and thus it is hypothesized that exposure to 18 nM Cd will not affect swim performance in a single swim trial. However, the effect of Cd exposure on recovery ability and performance in subsequent bouts of swimming has not been evaluated. Repeat swimming has been shown to act as a more sensive indicator of stress and impairment than the use of an individual swim challenge (Jain *et al*., 2003), and thus is more likely to identify an impairment to swimming ability as a result of Cd exposure.

The third objective was to identify the physiological mechanisms associated with variations in swimming performance, and to determine how they are affected by Cd. These include indicators of anaerobic metabolic stress (lactate, glycogen, glucose, ATP) and endocrine (cortisol) responses. Exposure to elevated Cd concentrations has been associated with increased substrait level phosphorylation, thus it is hypothesized that exposure to 18 nM Cd will result in increased lactate and decreased glycogen concentrations in resting fish. Based on previous measures it is also anticipated that the intital swim challenge to 85% U_{crit} will result in significant recruitment of white muscle for substrate level phosphorylation, and thus significant depletion of glycogen stores and accumulation of lactate, in both treatments. Sub-lethal Cd exposure has been shown not to affect basal cortisol levels, however, following a stressful stimulous, fish exposed to Cd show an attenuated cortisol response. The exposure concentration selected for these

experiments should cause a similar attenuation of the characteristic cortisol response. Additionally, as elevated cortisol levels have been shown to have an inhibitory response on the resporation of muscle glycogen it is anticipated that the recovery ability of Cd exposed fish, which experienced an attenuation of the cortisol response, will show a faster rate of metabolic recovery compaired with control fish.

Chapter 2

The effects of chronic cadmium exposure on repeat swimming performance and anaerobic metabolism in brown trout (Salmo trutta) and lake whitefish (Coregonus clupeaformis)

2.1 Abstract

This study investigated the effect of chronic Cd exposure on the ability of fish to undergo repeat swim challenges. Also, species specific effects were demonstrated by compairing the performance in brown trout (*Salmo trutta*) and lake whitefish (*Coregonus clupeaformis*). The goal was to develop an understanding of the linkages between exposure, bioaccumulation, physiological disruption and impairment of whole animal performance. Fish were exposed to waterborne Cd (18 nM Cd) in moderately hard water (120 mg L^{-1} $CaCO_3$) for up to 30 d with swim trials performed at various times over the course of the exposure. Trials consisted of swimming to 85% of the maximum sustained swimming capacity (U_{crit}) of unexposed fish, followed by a 30 minute recovery period, and then performing a second, exhaustive, swim challenge. Plasma, gill, liver, kidney and muscle samples were collected before and after each of the swim periods in all trials. Plasma ionic composition over the course of the 30 d exposure showed a classic damage-recovery-acclimation pattern of sub-lethal effects, particularly for Ca where a 20% reduction occurred within the first few days. The gill was the primary site of Cd accumulation with up to 30 fold increase after 30 days of exposure. The liver and kidney also accumulated a significant amount of Cd with approximately 20 fold increases in both tissues in both species. Muscle accumulated little to no Cd. In the first phase of the swim trials all fish successfully swam to 85% of the U_{crit} of exposed fish (4.0 body lengths per second [bl s^{-1}] in brown trout and 4.25 bl s^{-1} in lake whitefish). Performance in the second swim challenge was significantly reduced in Cd exposed fish, particularly after a week of exposure where it was reduced by 31% for brown trout and 38% for lake whitefish, compared with their respective controls. ATP and glycogen stores were significantly

reduced and lactate increased in the white muscle of all fish as a result of the first swim challenge. There was little metabolic recovery over the 30 min rest period between swims and fish chronically exposed to Cd were generally similar to controls, with the exception of a reduction in white muscle ATP stores after 1 week of exposure where ATP levels were lower in Cd exposed. The results show that Cd exposure results in an impairment of swimming ability in the second swim of a repeat swim challenge but this impairment is generally not related to metabolic processes in white muscle, with both species showing similar effects as a result of Cd exposure.

2.2 Introduction

Swim performance can be one of the most observable and relevant measures of the effects of stressors on fish (Beitinger and McCauley, 1990). Swimming has important ramifications on feeding, avoidance of unfavourable environmental conditions, reproductive behaviour and migration (Plaut, 2001). There are three primary swimming methods as described by Beamish (1978); these are sustained, prolonged, and burst swimming. These methods differ in the duration, intensity and type of metabolic activity required to support locomotion (Beitinger and McCauley, 1990).

Sustained swimming is the method used the most frequently. It represents a low level of activity which can be maintained for long periods of time (a minimum of 200 minutes), is supported entirely aerobically and does not result in the accumulation of oxygen debt. Oxygen debt is measured as the excess (or additional) oxygen consumption incurred after exercise that is required to eliminate the lactate formed through anaerobic metabolism (Beitinger and McCauley, 1990; Scarabello *et al*., 1991). The second type of swimming, prolonged swimming, describes the primary cruising speed of a fish. It can also be maintained for a long period of time (2 to 200 min), however, not as long as sustained swimming. Prolonged swimming is supported through both aerobic and anaerobic metabolism (Plaut, 2001). The final type of swimming is burst activity, which reflects a sprint swimming speed. This burst of activity can only be maintained for very brief periods lasting only a few seconds, and is supported entirely anaerobically (Beitinger and McCauley, 1990).

Prolonged swimming, the method studied most frequently by a fish, is usually measured by increasing water velocity in increments of consistent durations to determine

the critical swimming speed. The critical swimming speed, U_{crit} (Brett, 1964), measures the capacity for prolonged swimming by swimming fish to fatigue (Beitinger and McCauley, 1990; Farrell, 2008). U_{crit} swimming challenges have been used as a standard measure to demonstrate the integrated metabolic costs associated with exposure to contaminants (Little and Finger, 1990).

The effects of metal contamination on the swimming capacity of salmonids has been studied through the use of U_{crit} swim challenges (e.g. Beaumont *et al.*, 1995; McGeer *et al.*, 2000a; Pane *et al.*, 2004). Salmonids provide a good model for evaluations of swimming performance as they are active predators and spend much of their life in motion. Beaumont *et al*. (1995) showed that 96 h of copper exposure (11 nM Cu) caused reductions in swimming performance of brown trout (*Salmo trutta*). Cu exposed fish, acclimated to summer temperatures (15 ^{o}C), showed a 28% reduction in U_{crit} performance relative to controls at a similar temperature. Winter acclimated fish (5 ^{o}C) showed even greater reductions (approx. 50%) in performance as a result of Cu exposure. Warm water acclimated trout exposed to higher Cu concentrations (66 nM Cu) the mean U_{crit} was 0.3 m s^{-1} compared with 1.95 ± 0.13 body lengths s^{-1} (bl s^{-1}) and it was noted that only one of the six fish tested was able to swim steadily at the lowest speed while the rest swam for only a few minutes using a burst and glide method before becoming exhausted. The one fish that did complete the challenge had a low U_{crit} value of 0.89 bl s^{-1}. Nickel exposure has also been shown to reduce swimming performance. Pane *et al.* (2004) found that after exposure to 6.5 mM Ni swimming performance was uninhibited in rainbow trout (*Oncorhynchus mykiss*). However, at an exposure concentration of 35 mM Ni the U_{crit} was reduced by 42% and 35% on after 12 and 24 (respectively) d of exposure.

Reductions in swimming ability were linked with gill damage resulting in limited oxygen availability (Pane *et al*., 2004).

Swim performance is not always impaired during metal exposure. For example, both Hollis *et al.* (1999) and McGeer *et al.* (2000a) found that chronic sublethal Cd exposure did not intensity swimming ability (U_{crit}) even though other impairments were obvious (e.g. mortalities, reduced feeding and significant reductions in plasma ions). Therefore, while chronic exposure to some metals (e.g. Cu or Ni) can impact swim performance, others such as Cd do not appear to impair sustained swimming ability (i.e. U_{crit}) even though fish clearly exhibit physiological impairment.

Swim performance testing for the assessment of the potential impacts of contaminant exposure has primarily employed single swim challenges. Performance in repeated swim challenges provides a potentially more sensitive and ecologically relevant reflection of impacts of chronic sublethal exposure than single swim challenges do. For example, Jain *et al.* (1998) found that the use of a repeat swimming trial identified reduced performance in dehydroabietic acid (DHA) exposed fish that was not identified in a single swimming challenge alone. Repeat swim performance has also been used to assess the effect of altering dietary lipid sources in manufactured feeds for Atlantic salmon (Wagner *et al*., 2004). In that study, reduced recovery ratios (calculated as second U_{crit}/initial U_{crit}) occurred in fish fed a diet containing anchovy oil as the lipid source but not in those with plant-based lipid sources (Wagner *et al*., 2004). Temperature has also been shown to affect performance in repeated bouts of swimming. Jain and Farrell (2003) showed a significant reduction in recovery ratio of fish acclimated to warm temperatures (17 ^{o}C) compared with those acclimated to cold temperatures (5 ^{o}C).

The frequency with which a fish can undergo bouts of exhaustive exercise is determined by the recovery time, particularly the time necessary to restore metabolic fuel reserves such as glycogen, and to clear accumulated waste products such as lactate (Milligan, 1996). It is proposed that oxygen consumption by a fish remains elevated for several hours following high intensity exercise as accumulated lactate is oxidized to re-synthesize depleted glycogen stores (Scarabello *et al*., 1991). Originally described as the oxygen dept hypothesis (Hill and Lupton, 1923) this model has proven to be more complex than originally described, and is now more commonly referred to as excess post-exercise oxygen consumption (EPOC) model. The 'oxygen debt' incurred is the total amount of O_2 used to recover following exercise. The EPOC model is described by two phases. The initial fast component accounts for approximately 20% of the total oxygen debt. This component, also referred to as alactacid oxygen debt, is associated with the recovery of ATP, phosphocreatine (PCr) and internal O_2 stores.

The second phase of the EPOC model of recovery is the slow component (or lactacid oxygen debt: 80%) and it lasts for several hours following exercise. It is primarily involved in the recovery of glycogen. During this portion, expended glucose is restablished and is then converted to glycogen. The ATP required for this conversion is supplied by the oxidation of lactate (Margaria *et al*., 1933; Scarabello *et al*., 1991). The process of full metabolic recovery, relating to the re-synthesis of glycogen, clearance of metabolic waste products and reestablishment of ATP stores, can take upwards of 12 hours to complete (Milligan, 1996), however many fish species can successfully undergo successive bouts of swimming with much shorter recovery periods. Thus, a full

metabolic recovery, as just defined, is not required to sustain high intensity exercise (Hochachka, 1961).

The recovery of swimming ability in salmonids may take between 40 min and several hours. Randall *et al.* (1987) found that chinook salmon (*Oncorhynchus tshawytscha*) given a 60 minute recovery period performed equally as well in successive U_{crit} tests. Return to initial swim performance capacity was observed in juvenile coho salmon (*Oncorhynchus kisutch*) following a 2 hour recovery period (Brauner *et al*. 1994), while with a recovery period of only 45 minutes restored swim performance in three successive challenges in mature sockeye salmon (*Oncorhynchus nerka*; Farrell *et al.* 1998). Studies with rainbow trout suggest their responses are similar to Pacific salmon. For example, Jain *et al*. (1998) found that hatchery reared rainbow trout fully recovered their swimming ability after a 70 minute rest period. Even shorter recovery times of 40 (Jain and Farrell, 2003) and 45 minutes (MacNutt *et al*., 2004) were observed as sufficient to completely restore swimming ability following exhaustive exercise in mature fish. The ability to recover quickly from exhaustive exercise can have significant ecological implications as organisms with impaired or extended recovery periods may be more susceptible to predation.

In this study the effect of chronic waterborne Cd exposure on repeat swim performance was studied in, two fish species: brown trout (*Salmo trutta*) and lake whitefish (*Coregonus clupeaformis*). The responses to Cd exposure are not well studied in these fish. A chronic sublethal Cd exposure of 18 nM Cd was chosen based on studies with rainbow trout, a species where the physiological disruption and acclimation response has been characterized (Hollis *et al.* 1999; McGeer *et al.* 2000a, b). In those studies

chronic Cd exposure was shown not to impair swim performance (single U_{crit}) in rainbow trout. In the present study we selected an initial swim to 85% of the mean U_{crit} value of unexposed controls as a way to give a consistent, size adjusted, bout of exercise that would simulate naturally occurring conditions such as during migration. Repeated bouts of high intensity exercise may be required during migration (for example); however, it is unlikely that they will be fully exhaustive. We then sought to identify the effect of Cd exposure on recovery and performance in a subsequent bout of swimming. A 30 min recovery period was selected to ensure incomplete metabolic recovery and thus elucidate the potential effects of Cd exposure on the alactacid phase of recovery. In addition to focusing on anaerobic aspects of repeated swim challenges we also identified ionoregulation and bioaccumulation as markers of exposure.

2.3 Materials and Methods

2.3.1 Fish Culture

Juvenile brown trout (*Salmo trutta*) and lake whitefish (*Coregonus clupeaformis*) were donated by the Ontario Ministry of Natural Resources (White Lake Hatchery; Sharbot Lake, ON). Fish were transported to the aquatics facility at Wilfrid Laurier University where they were held in 200 L polyethylene tanks with flowing fresh water for at least 6 months prior to experimentation. Brown trout had attained a mean fork length of 13.4 cm ± 0.1 cm and a mean weight of 33.2g ± 0.9g (n = 256) while the lake whitefish had a mean fork length 15. 8 cm ± 0.1 cm and a mean weight of 45.5 g ± 1.2g (n = 144; Table 2.1). Fish were monitored daily and fed commercial fish food (Skretting, Moore Clarke Canada, Vancouver, BC) at 2% of body weight daily. Culturing and

experimentation was conducted in accordance with the Canadian Council on Animal Care as reviewed and approved by the Wilfrid Laurier University Animal Care Committee.

2.3.2 Chronic Metal Exposure

Fish were non-selectively distributed from the holding tanks to one of four exposure tanks at least 7 d prior to experimentation. Exposure water was a mixture of reverse osmosis processed water and dechlorinated Waterloo city tap water with a conductivity of 203 ± 2 µS and a hardness of approximately 115 mg $CaCO_3$ for brown trout (730 ± 2.7 µM Ca, 722 ± 5.3 µM Na, 415 ± 2.7 µM Mg, n = 68). For lake whitefish reverse osmosis processed water and well water were mixed with a conductivity of 210 ± 1.5 µS and a hardness of 121 mg $CaCO_3$ (786 ± 6.6 µM Ca, 374 ± 7.6 µM Na, 424 ± 5.9 µM Mg, n = 21). The pH was 7.2 ± 0.02 and temperature was 12 ± 1^{o}C for both exposures. Cd exposures involved metering (QG6 pump, Fluid Metering Inc., Oyster Bay NY) a concentrated $CdCl_2$ solution into a mixing head tank before delivery to exposure tanks (Fig. A1). To initiate the exposure a spike of concentrated metal solution was added to the head tanks and fish tanks to bring them to the desired Cd exposure concentration of 18 nM Cd. During the exposure all tanks were continuously aerated to ensure mixing. Exposures were done in duplicate and an unexposed (control) group was included for each species. Temperature, pH and conductivity of the water were measured daily and recorded. Water samples (10 mL) were collected every other day and acidified to 1% with 16N HNO_3 (trace metals grade; Fisher Scientific, Mississauga ON) and subsequently measured for total Cd concentration.

2.3.3 Swim Performance Testing

Swim performance was evaluated on days 1, 6, 14 and 30 of the brown trout exposure and on days 3, 7 and 30 for lake whitefish. Fish were starved 24 hours prior to the start of each swimming test to ensure a post absorptive state. Fish were non-selectively netted, in groups of three (lake whitefish) and four (brown trout), measured for fork length, and transferred into a 30 L swim flume (D30 Loligo Systems; Tjele, Denmark) with a swimming chamber of 55x14x14 cm. The mechanism for controlling water velocity within the swim flume had previously been calibrated using a flow meter (HFA-U276 & 278, Hüntzsch; Waiblingen, Germany) so that flow could be adjusted on a body length per second (bl s^{-1}) basis.

Prior to Cd exposure a group of unexposed fish underwent a standard, single, exhaustive U_{crit} swimming challenge. This swim challenge followed the protocol of Jain *et al*. (1997) and produced a mean U_{crit} value of 4.7 ± 0.16 body lengths per second (bl s^{-1} ; n=8) in brown trout and 5.0 ± 0.12 bl s^{-1} (n=6) in lake whitefish. This U_{crit} data allowed the first swim of Cd exposed (or control) fish to be done on a consistent basis, at 85% of the critical swimming speed of unexposed fish (i.e. 4.0 bl s^{-1} for brown trout and 4.25 bl s^{-1} for lake whitefish).

The repeated swim test protocol was adapted from Jain and Farrell (2003) and a schematic description is provided in Figure 2.1. Briefly, fish were given a 30 min acclimation to the swim flume, with an orientation velocity of 0.5 bl s^{-1}. Next, water velocity was increased to 1 bl s^{-1} and then ramped to 2.8 bl s^{-1} in brown trout and 3.0 bl s^{-1} in lake whitefish, representing 60% of U_{crit}, over a 10 min period as described in Jain and Farrell (2003). Water velocity was maintained at this speed for 30 minutes and was

then increased to 4.0 bl s^{-1} in brown trout and 4.25 bl s^{-1} in lake whitefish (85% of the U_{crit} of control fish) for a further 30 min. Fish were then given a 30 min recovery period, at a water velocity of 0.5 bl s^{-1}. Following the recovery period fish were swum as before, a 10 min ramp to 60% of U_{crit} and then swimming in 30 minute intervals with water velocity increases of 0.75 bl s^{-1}, continued until the fish reached exhaustion. Exhaustion was defined as the time when a fish could no longer hold its position in the swim chamber and was swept against the rear grid for the third time, after being returned twice within the swim chamber. The duration of the 30 min interval and water velocity at exhaustion was noted.

2.3.4 Sample Collection and Storage

Metabolic effects over the course of the repeat swim challenge were assessed at four distinct time points; before exercise (sampled directly from exposure tanks: TP 1), at the end of the first swim (TP 2), at the end of the recovery period (TP 3), and after the exhaustive second swim (TP 4). Fish were euthanized with an overdose of tricaine methanesulfonate (MS222: 0.6 g L^{-1}; buffered with $NaHCO_3$), blotted dry with paper towel, weighed and measured for fork length, maximum depth and maximum width. Blood was collected from the caudal vasculature after caudal severance into 1.5 ml centrifuge tubes containing 10 µl of heparinised saline (Li salt, Sigma Aldrich Canada, Oakville ON) and centrifuged (Spectrafuge 16M, Labnet International, Edison NJ) at 13,000 rpm. Plasma was isolated and stored at -80^{o}C. White muscle samples (~200mg) were removed from the right side of the fish from a region between the pectoral and dorsal fins. Skin and scales were removed and muscle samples were immediately frozen in liquid nitrogen and later stored at -80^{o}C. Gill samples were removed and rinsed

thoroughly in deionized water and blotted dry. Liver and kidney samples were also collected and, along with gills, were stored at -40^{o}C prior to analysis of bioaccumulation.

2.3.5 Analytical Techniques

2.3.5a Tissue Metal Bioaccumulation and Plasma Ion Composition

Gill, liver, kidney and muscle samples were digested and analysed for Cd accumulation following a protocol adapted from Janes and Playle (1995). Briefly, tissues were divided and amounts of approximately 100 mg, were digested with 5:1 (volume: weight) 1N trace metals grade nitric acid (Fisher Scientific Canada, Ottawa ON) and heated to 80^{o}C for 3 h. Digested samples were re-suspended by vortexing (30 s), left to settle for at least 12 h, and centrifuged at 10 000g for 2 min before the supernatant was appropriately diluted in 1% nitric acid and analysed by graphite furnace atomic absorption spectrophotometry (SpectraAA 880 with GTA100, Varian Inc., Palo Alto, CA). Reference standards (TM 26.3 and TM 28.3) certified by the National Water Research Institute (Environment Canada) were used to verify analyzed values. Plasma ion compositions (Ca and Na) were analysed by flame atomic absorption spectrophotometry.

2.3.5b Metabolic Analysis

Unless noted, all enzymes and reagents were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Sample processing for metabolic determination of glycogen, ATP and PCR was adapted from Wilkie *et al.* (1997, 2001) following the protocol of Wang *et al*. (1994a). Briefly, a mortar and pestle was used to grind 100 mg (approx.) of each muscle sample to a fine powder in liquid nitrogen,

followed by deproteination in 4 volumes of 8% PCA containing 1mM ethylenediaminetetraacetic (EDTA). Samples were left to deproteinate on ice for 10 min at which time a 100 µL sub-sample (sub-sample one) of the PCA slurry was removed, neutralized with 3M K_2CO_3, frozen in liquid nitrogen and stored at -80^{o}C for subsequent glycogen characterization. The remaining slurry (sub-sample two) was then centrifuged at 4^{o}C for 2 minutes at 10 000 x g. The supernatant was drawn off, weighed to the nearest 0.001g, and neutralized with 0.5 volumes of 2M KOH cocktail (0.4M imidazole and 0.4M KCl). The solution was vortexed and then centrifuged as before. The remaining supernatant was drawn off and stored at -80^{o}C for subsequent analysis for lactate and ATP.

Sub-sample one was further digested for the determination of muscle glycogen by adding one part 2M acetate buffer to one part PCA slurry, followed by 40 units (U) amyloglucosidase. It was then incubated at 37^{o}C for two hours, allowing the amyloglucosidase to convert the glycogen to glucose. Incubation was terminated by adding 70% PCA (25 µL) to the sub-sample and then neutralizing with 3M K_2CO_3. This sample was then measured for total glucosal units. An additional sub sample was collected prior to the addition of amyloglucosidase and was flash frozen immediately in liquid nitrogen without undergoing the incubation steps. This sub-sample was saved and used to determine free glucose. All samples were stored at -80^{o}C.

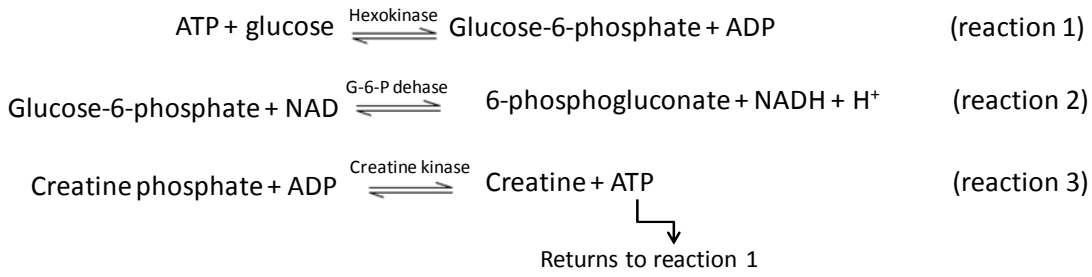
Glucose concentrations in the digests were determined following the protocol of Wang *et al*. (1994a), by adding 500 µL of a glucose cocktail solution comprised of 0.25 M TEA-HCl containing Mg, NAD, ATP and glucose-6-phosphate dehydrogenase to 20 µL of the tissue homogenate. Concentrations were determined spectrophotometrically

(Pharmacia LKB-Novaspec II) following the addition of hexokinase (5 units) to the glucose cocktail, by measuring the absorbance at 340 nm due to the appearance of NADH in the solution as a result of the conversion of glucose to glucose-6-phosphate. Both free glucose and total glucose were determined via this method and free glucose was subtracted from the total glucose concentration in the muscle tissue in order to yield white muscle glycogen concentrations.

Sub-sample two was analysed enzymatically for lactate following the method of Bergmeyer (1983). In the presence of saturating NAD, lactate is converted to pyruvate via lactate dehydrogenase (LDH) and the absorbance at 340 nm due to conversion of NAD to NADH is quantified. A 10 µL aliquot of subsample two was added to a NAD solution (0.2 M hydrazine buffer and NAD) followed by the addition of the enzyme, lactate dehydrogenase (5 units), which allowed for the determination of white muscle lactate based on the conversion of lactate to pyruvate resulting in the generation of NADH.

Sub-sample two was also used for the analysis of white muscle ATP. The basis of the ATP assay (Bergmeyer, 1983) uses the hexokinase catalyzed reduction of ATP and glucose to glucose-6-phosohate (G6P) and ADP (reaction 1). This G6P then acts as a substrate and combined with NAD to produce 6-phosphogluconate, H^+ and NADH in the presence of glucose-6-phosphate-dehydrogenase (G6P-dehase; reaction 2). The NADH produced is directly proportional (1:1) to the original concentration of ATP in sub-sample 2. ADP produced in the first reaction reacts with creatine phosphate, in the presence of creatine kinase to regenerate ATP (reaction 3), which is cycled back to power reaction 1.

By measuring the amount of ATP formed in this third reaction the levels of PCr in the sample can be determined using the previous two reactions.



2.3.5c Calculations and Statistical Analysis

Tissue Cd concentrations were combined with weight data to calculate accumulation on a weight adjusted basis (nmol g^{-1} tissue wet weight). The plasma ion concentrations are given in mM and muscle metabolites are presented as weight adjusted concentrations (mmol kg^{-1} wet tissue).

The critical swimming speed was calculated for each fish using the following formula:

$$Ucrit = Ui + \left(\frac{Ti}{Tii} \times Uii\right)$$

Where U_i is the last velocity maintained for the whole interval (cm s^{-1}), U_{ii} is the velocity increment (cm s^{-1}), here it was 0.75 bl s^{-1}, T_i is the time spent at fatigue velocity (min), and T_{ii} is the interval length (min), here 30 min (Brett, 1964). This formula yields a value in cm s^{-1} which is divided by the fork length of the fish in order to produce a U_{crit} value in bl s^{-1}.

All data are presented as the mean ± 1 SEM. All statistical analysis was performed using SigmaPlot (version 11.0, SPSS Inc., Chicago, IL). Comparisons of multiple groups, such as comparisons within a single treatment, were done by one-way ANOVAs followed by the Student-Newman-Keuls post-hoc test when required.

Comparisons between control and Cd exposed fish at a given point were made via Students T-Test. These methods were used for all pairwise comparisons of mean responses among the different treatment groups with $P<0.05$ used as the limit of significance.

2.4 Results

2.4.1 Mortality and Plasma Ion Concentrations

Brown trout exposed to 18 nM Cd had a 93% survival rate over 30 d of exposure with mortalities occurring during the first 4 d of exposure. In controls there were no mortalities. All lake whitefish involved in exposures survived (exposed and controls).

Plasma Ca^{2+} and Na^{+} concentrations showed a pattern of Cd induced ionic disruption. In the brown trout exposure, plasma Ca^{2+} concentrations were significantly lower in Cd exposed fish compared to unexposed controls, except at day 6 where control values fell slightly (Fig 2.2A). Plasma Na^{+} results were more variable with no clear difference between exposed and control measurements (Fig 2.2B). On day 6 the mean Na^{+} concentrations in plasma for both control and Cd exposed fish were very low, perhaps due to a sampling or measurement artefact.

Lake whitefish showed a pattern of plasma ion disruption and recovery. Plasma Ca^{2+} levels were significantly decreased by day 7 and returned to levels found in unexposed whitefish at day 30 (Fig 2.2C). Plasma Na was also significantly reduced in the first week of exposure to Cd with differences being eliminated by day 30 (Fig. 2.2D).

2.4.2 Tissue Cd Burden

Cd concentrations in the tissues of brown trout and lake whitefish increased significantly as a result of exposure (Fig. 2.3 and 2.4 respectively). In brown trout, significant Cd accumulation (compared to controls) occurred by day one in gills, liver and kidney. By day 30 gill tissue (Fig. 2.3A) had accumulated Cd to levels 30 fold higher than unexposed trout while for kidney (Fig. 2.3C) and liver (Fig. 2.3B) there were 19 fold and 17 fold increases respectively. White muscle accumulated little to no Cd with maximum concentration much lower than were observed in the other tissues tested (Fig. 2.3D).

In lake whitefish, Cd accumulated significantly within the gills, liver and kidney by the third day of exposure, and continued to increase, linearly, through the conclusion of the exposure (30d). Again, the gills (Fig 2.4A) showed the greatest Cd accumulation with a 30 fold increase in Cd concentration relative to controls. The liver (Fig. 2.4B) and kidney (Fig. 2.4C) accumulated similar quantities of Cd with 17 and 18 fold increases relative to controls observed respectively. No Cd accumulated within the white muscle of lake whitefish (Fig 2.4D).

2.4.3 Swimming Performance

Swimming performance was assessed by calculating the sustained swimming ability (U_{crit}) for the second swim of the repeat swimming protocol. In an initial single swim challenge brown trout had a mean U_{crit} value of 4.7 ± 0.2 bl s^{-1} while for white fish this value was 5.0 ± 0.2 bl s^{-1}.

Results for the repeat swimming trial with brown trout showed that the second swim U_{crit} values were relatively consistent over time with no significantly differences

across time (d 1, 6, 14, 30) as well as compared to the initial single swim U_{crit} value (Fig. 2.5A). The overall mean for control brown trout in the second swim challenge was 4.3 ±0.1 bl s^{-1}. After 24 hours of Cd exposure performance in the second U_{crit} challenge was significantly reduced and this trend persisted through the 6^{th} and 14^{th} day of exposure. However, by the 30^{th} day performance in the second swimming trial was no longer impacted by Cd exposure. In the lake whitefish exposure (Fig. 2.5B), control fish had a mean U_{crit} of 4.8 ± 0.2 bl s^{-1} in the second swim challenge. In Cd exposed fish, performance in the second swim trial was significantly reduced relative to controls on days 3, 7 and 30 of exposure.

2.4.4 Metabolic Analysis

2.4.4a White Muscle Glycogen

Brown trout had the highest glycogen concentrations prior to exercise. Following the first swim to 85% U_{crit} these stores were significantly reduced, with an average reduction of just under 50% from resting values (Fig 2.6). There was no clear pattern of glycogen recovery between the first and second swim challenges but rather, concentrations either remained low or continued to decline during the recovery period despite the termination of exhaustive exercise. There were two notable exceptions, d 1 and d 14 where controls and Cd exposed fish (respectively) showed increased glycogen concentrations. Glycogen levels following the second swim (time point 4) showed little change compared to concentrations observed following the recovery period (time point 3). Of note, on days 6, 14 and 30 of exposure, brown trout exposed to Cd had higher concentrations of glycogen in their white muscle at the end of the second swim than did controls.

In lake whitefish glycogen stores (Fig 2.7) were highest prior to exercise and were then depleted at the end of the first swim. Muscle glycogen levels remained low after the first swim. There were few differences between fish exposed to 18 nM Cd and unexposed controls. Unlike in brown trout, where Cd exposed fish showed higher glycogen concentration in the white muscle than did controls, in lake whitefish levels in both control and Cd exposed fish were similar at the completion of the second swim trial.

2.4.4b White Muscle Lactate

Lactate levels within the white muscle of brown trout (Fig. 2.8) showed a roughly inverse trend of that observed for glycogen stores. There was an increase, on average 37%, in white muscle lactate following completion of the first swimming challenge in both control and Cd exposed fish. Following the 30 minute recovery period there were limited decreases in white muscle lactate concentration, with the exception Cd exposed fish on days 6 and 14. During the final swim muscle lactate concentrations did not increase any further and remained similar to those observed after the first swim and recovery period. In lake whitefish the trends were consistent across days 3 (Fig. 2.9A), 7 (Fig 2.9B) and 30 (Fig. 2.9C). Lactate levels were lowest prior to the start of swimming. Concentrations increased significantly, nearly 50%, over the course of the first swim, and remained constant over the rest period, with levels remaining elevated through the close of the second swim.

2.4.4c White Muscle ATP

In both brown trout and lake white fish ATP levels were somewhat variable. In brown trout, ATP levels on day 1 (Fig. 2.10A) in control fish continued to decrease over the course of the 30 minute rest period, reaching a significant decrease in ATP stores from the pre-exercise condition prior to the start of the second swim (time point 3). In general the ATP stores in resting (time point 1) fish were greater in controls than in the Cd exposed group. This difference was most prevalent after six days of Cd exposure (Fig. 2.10B) where a significant, nearly 75 % reduction in white muscle ATP stores was observed. It can also be noted that, as a general pattern, with the exception of day one (Fig. 2.10A), control fish showed a greater recovery of muscle ATP stores during the 30 minute rest period, compared with Cd exposed fish which showed almost no improvement during this time. In lake whitefish a similar trend was observed (Fig. 2.11), with muscle ATP stores showing the most significant decrease after 7 days of Cd exposure (Fig. 2.11B). Corresponding with the largest decrease observed in brown trout, which occurred after 6 days of Cd exposure.

2.5 Discussion

2.5.1 Plasma Ion Concentrations

Ionic disturbances are frequently linked with waterborne metal exposure and often have been described in terms of the damage-repair-acclimation pattern discussed by McDonald and Wood (1993). In this study both plasma Ca^{2+} and Na^{+} were affected by chronic exposure to 18 nM Cd. Plasma Ca was significantly reduced in brown trout after 24h of exposure to Cd and remained so throughout the 30 day exposure (Fig 2.2A).

Plasma Na^+ showed a similar loss (Fig. 2.2B). Lake whitefish showed a decrease in plasma Ca^{2+}, however this was not observed until the 7th day of exposure and by day 30 levels had recovered (Fig. 2.2C). Plasma Na^+ levels showed a significant, acute, decrease by day 6, but were restored by day 30 (Fig. 2.2D).

Cd exposure has been shown to cause ionoregulatory disruption, specifically to Ca^{2+} homeostasis (Verbost *et al*., 1989). Verbost *et al.* (1989) showed that Cd^{2+} enters the gills through the same route as Ca^{2+} and observed that Cd accumulation was associated with inhibition of both Ca^{2+}-ATPase and Cd^{2+} uptake. Ca^{2+}-ATPase on the basolateral membrane has an extremely high affinity for Cd (I_{50}=3 $nmol^{-1}$) and thus, minute concentrations of waterborne Cd can inhibit Ca^{2+} transport (Verbost *et al*. 1988).

Pelgrom *et al.* (1995) showed disruption of plasma ion composition as a result of Cd exposure. Following 6 days of Cd exposure, tilapia exposed to the lowest concentration (178 nM Cd) showed a significant increase in plasma Ca^{2+} concentration. The intermediate concentration (311 nM Cd) did not cause a change in plasma Ca^{2+}, while exposure to the highest concentration (623 nM Cd) resulted in a reduction of plasma Ca^{2+}, causing concentrations to fall to roughly half of that observed in controls. These results were similar to those observed by Pratap *et al*. (1989) where tilapia exposed to 89 nM Cd in soft water showed a 40% and 31% reduction in plasma Ca^{2+} after 2 and 4 days respectively, while in higher Ca^{2+} concentrations the reductions were 32% and 20% on the same days. All disturbances were eliminated after 14 days. Additionally, Chowdhury *et al*. (2004) observed a 44% reduction in plasma Ca of mature rainbow trout exposed to 89 nM Cd for 72h.

In the present study, the acute reduction in plasma Ca^{2+} observed in Cd exposed lake whitefish was no longer present by the 30th day of exposure (Fig 2.2C). A similar result was observed by Hollis *et al*. (2000) where, after 30 days of exposure to 1 nM Cd in soft water (hardness=20 mg $CaCO_3$ L^{-1}) juvenile rainbow trout showed no variation in plasma Ca^{2+} levels between control and Cd exposed fish indicating a potential re-establishment of homeostasis. In the field, Van Campenhout *et al.* (2010) observed that gibel carp (*Carassius auratus gibelio*) inhabiting a river with elevated Cd concentrations (129 nM Cd) had significantly reduced serum Ca^{2+}, relative to fish caught in an uncontaminated river.

Additionally, Cd exposure has been linked to reductions in whole body Ca^{2+} concentrations (McGeer *et al*., 2000a; Niyogi and Wood, 2004). For example, whole organism Ca^{2+} was reduced in fingerling rainbow trout after 16 days of exposure to 27 nM Cd. The reduction in carcass Ca^{2+} observed in Cd exposed fish on day 16 of exposure was no longer observed on the 65th day of exposure, indicating that recovery had occurred (McGeer *et al*., 2000a).

Disturbances to Na^+ homeostasis are typically less prominent and have a shorter duration than do disturbances to Ca^{2+} concentrations (McGeer *et al*., 2000a). This was true of the present study where only acute reductions in plasma Na^+ were observed, with concentrations being restored in Cd exposed fish by day 30. Pelgrom *et al*. (1995) observed an acute reduction in plasma Na^+ in tilapa (*Oreochromis mossambicus*) exposed to 623 nM Cd for 6 days. While a significant increase in plasma Na^+ concentration was observed by Chowdhury *et al*. (2004) following 48 hours of Cd exposure (89 nM Cd) in

adult rainbow trout. Other studies have shown no change in plasma Na^+ concentration as a result of Cd exposure (Pratap *et al*., 1989, Verbost *et al*., 1989, Baldisserotto, 2004).

Several studies have shown that Cd inhibits activity of Na^+-K^+-ATPase at the basolateral membrane of the gill (Schoenmakers *et al*., 1992; Pratap and Wendelaar Bonga, 1993). Working with *in vitro* preparations of gill epithelia, Lionetto *et al*. (1998) showed that Cd strongly inhibits branchial Na^+-K^+-ATPase in the European eel (*Anguilla anguilla*), with maximum inhibition (~ 80%) attained after an hour of exposure to 44 nM Cd. Additionally, using a similar in vitro system a 1 h incubation at 60 µM Cd resulted in an 80% inhibition of carbonic anhydrase (CA; Lionetto *et al*., 1998), a sensitive exchanger which provides H^+ and HCO_3^- ions for apical exchanger sites (Morgan *et al*., 2004), in gill epithelia of European eel (*Anguilla anguilla*; Lionetto *et al.,* 1998). Schoenmakers et al. (1992) observed a similar inhibition of Na+-K+-ATPase in tilapia.

2.5.2 Tissue Cd Burdens

Both brown trout and lake whitefish accumulated Cd in a time and dose dependant manner but there were differences in the rate and extent of accumulation among tissues and between species. Cd accumulated in the gills in a linear fashion over the 30 days in both species (Fig 2.3A and 2.4A) and tissue burdens reached just less than 40 nmol g^{-1} in each species. This accumulation is consistent with the observations of Hollis *et al.* (1999, 2000) who similarly identified the gills as the site of the highest Cd accumulation compared to liver, gall bladder, carcass and whole body. Such a result was anticipated because the gill is the primary site of Cd uptake during a waterborne exposure.

The kidney also had elevated concentrations of (~ 25 nmol g^{-1}) Cd in each species; Fig 2.3C and 2.4C). Accumulation in the kidney was delayed, particularly in brown trout with the most significant increases in tissue Cd concentration not occurring until after the 14th day of exposure. The livers of lake whitefish accumulated roughly twice the concentration of Cd compared to those of brown trout (Fig. 2.3B and 2.4B). For all tissues in both species accumulation was ultimately linear and there appeared to be saturation of tissue burdens. This observation is supported by the results of McGeer *et al*. (2000b) where approximately linear increases in Cd concentration in both the liver and kidney over 65 days were observed in rainbow trout during exposure to 27 nM Cd. In the McGeer *et al*. (2000b) study, gill accumulations reached steady state within the first 5 days of exposure and then remained constant but the profile of accumulation in the current study was not like this. It was also observed that Cd concentrations within the gills were higher than all other tissues after 16 days but that by day 65 of exposure gill concentrations remained consistent while kidney accumulation continued in a linear fashion and thus concentrations in the kidney greatly exceeded those of all other tissues by day 65. Szebedinsky *et al*. (2001) similarly observed that the kidney was the primary site of Cd accumulation, with the gills representing the next greatest concentration of Cd, in rainbow trout as a result of 30 days of waterborne exposure to 18 nM Cd.

As waterborne Cd is taken up primarily at the gill, an initial, rapid, increase of Cd in this tissue is anticipated. The delayed increase in Cd concentrations in other tissues, such as the liver or kidney may reflect the transition from loading at the gills alone to distribution, via the blood stream, to other tissues. This was observed by McGeer *et al*. (2000b), who found that half-times for gill loading were lower than for internal tissues.

The rapid accumulation of Cd in the gills combined with (relatively) longer times before significant accumulations to other tissues indicates that the gills may act as a barrier to quantities of metal could restrict initial uptake of Cd to internal organs. Initial uptake and storage of large quantities of Cd in the gills could inhibit its' passage into the blood stream and to other more sensitive organs.

The relatively constant increase in tissue Cd concentrations over time suggests that elimination is not used as a physiological strategy for dealing with Cd accumulation. As a nonessential element, Cd, is typically less metabolically active (Shaikh and Lucis 1972; Cain and Holt, 1979; Monia *et al*., 1986). Wicklund Glynn (1991) observed that even after 60 days post exposure there was no elimination of accumulated Cd within the organs although there were shifts in distributions, from gill to the kidney and liver. This suggests that Cd was sequestered by metal binding proteins such as metalothionine, immobilizing it and leading to its detoxification.

Cd accumulated very little within the white muscle of brown trout and did not accumulate to significant levels in the white muscle of lake whitefish (Fig. 2.3D and 2.4D respectively). In both species Cd concentrations were negligible compared with accumulation in other tissues. This is consistent with the results of de Conto Cinier *et al*. (1999) where Cd accumulation within the white muscle of carp (*Cyprinus carpio*) was not significantly different from controls until more than 3 months of Cd exposure had elapsed. Once taken up at the gill, Cd is transported, via the bloodstream, to other organs. Both the liver and kidney are well perfused, especially when compared with the white muscle. This limited perfusion by blood vessels may also account for why Cd did not accumulate within the white muscle.

2.5.3 Repeat Swimming Ability

This study shows that chronic sublethal Cd exposure impairs the ability of both brown trout and lake whitefish to perform in the second swim of a repeat swim challenge. U_{crit} values for Cd exposed fish were significantly less than unexposed (control) fish in the second swim (Fig 2.5A and B). This impairment occurred early in exposure and persisted, particularly in lake whitefish (Fig 2.5B) although by day 30 the U_{crit} of brown trout had recovered to match the performance of controls (Fig 2.5A). This indicates that repair and acclimation applies to ionoregulation as well as swimming. On all days (i.e. 1, 6, 14 and 30 for brown trout and 3, 7 and 30 for lake whitefish) the performance of unexposed fish in the second swim were not significantly different from the pre-exposure single swim U_{crit} values, indicating that 30 min of recovery time between swims was sufficient. For Cd exposed fish the 30 min recovery time was not sufficient. Interestingly, however, the pattern of restoration to ionic disruption did not match that of the restoration of secondary swimming performance. In brown trout, plasma Ca^{2+} levels were significantly lower in Cd exposed fish compaired with controls after 30 d of exposure, however, following 30 d exposure the decrease in swim performance in the second swim trial observed acutely was restored to that of controls in Cd exposed fish. In lake whitefish the opposite was observed. At day 30 of exposure plasma Ca^{2+} concentrations were restored however swimming performance in the second swim trial remained significantly reduced in Cd exposed fish compaired with controls. This result was not anticipated, as significant homeostatic disruptions, such as alterations to plasma ion composition, can incur metabolically demanding costs of reparation, which inturn can

reduce performance in other capacities, such as swimming. As the pattern of repair of swimming ability does not correlate with restorations of plasma ions it can be concluded that other physiological parameters are affected by Cd which may result in the continued reduction in the secondary swim performance of lake whitefish, despite the restoration of plasma Ca^{2+}.

The effect of Cd on swimming performance in rainbow trout has previously been assessed (e.g. Farag *et al*., 1994; Hollis *et al*., 1999; McGeer *et al*., 2000a) and generally chronic Cd exposure does not result in reductions in U_{crit} values (Hollis *et al.*, 1999; McGeer *et al.*, 2000a) and swimming stamina (Hollis *et al.*, 1999). Similar effects have been observed in the field, where U_{crit} performance was similar in perch (*Perca flavescens*) from across a gradient of Cd contaminated lakes (Rajotte and Couture, 2002). Interestingly, in a 277 day sub-lethal exposure of 4 nM Cd to adult lake trout (*Salvelinus namaycush*) and fingerling rainbow trout swim performance, as represented by foraging of lake trout upon the juvenile rainbow trout, was impaired. However, the predator avoidance of the fingerling rainbow trout was unaffected (Scherer *et al*., 1997). Therefore, studies to date have been relatively conclusive in showing no effects on U_{crit} swimming ability as a result of Cd exposure. However, the impacts of Cd exposure on the results of other swimming evaluations have not been thoroughly evaluated. In the swim testing reported here, we assessed U_{crit} only after an initial challenge and short recovery. It is noteworthy that during the initial swim all fish (both species, Cd exposed and unexposed) were able to swim successfully at approximately 85% of the U_{crit} of unexposed fish. Prior to the start of the exposure, performance in a singular swimming challenge was assessed. The U_{crit} for control fish, in an individual challenge was

determined to be 4.7 bl s^{-1} in brown trout and 5.0 bl s^{-1} in lake whitefish. During the repeat swim challenges, U_{crit} values obtained for control fish did not differ from the values obtained in the single swim challenges, indicating that the 30 minute recovery period was adequate for the recovery of swimming performance in control fish. Cd exposed fish, however, showed a reduction in swimming performance in the second swimming trial (Fig. 2.5). This reduction was not chronic in brown trout, with swimming performance in repeated swim trials being restored by the 30^{th} day of exposure to Cd. Lake whitefish however, did not show this restoration of secondary swimming ability by day 30. This suggests a potential species difference between whitefish and brown trout in terms of acclimation processes¸ potentially related to species sensitivity to Cd exposure.

In the few studies that have measured repeat swimming ability it has proven to be a more sensitive indicator of physiological impairment than the use of a singular swim challenge alone. Exposure to varying concentrations of dehydroabietic acid (DHA), a toxic component of pulp-mill effluent, resulted in no variation in swim performance, relative to controls, in the first U_{crit} challenge. However, performance in the second swim challenge, following a 40 minute recovery period, was impacted as was evidenced by a decrease in the recovery ratio of contaminant exposed sockeye salmon (*Oncorhynchus nerka*) relative to controls (Jain *et al*., 1998). The recovery ratio is calculated by dividing the U_{crit} value from the first second swim by the U_{crit} value from the first. If swimming performance in the two trials is equal than a recovery ratio of 1 will be obtained; a value less than one represents a decrease in performance in the second trial, while a value greater than one represents an improvement is swimming ability. To calculate a recovery ratio, fish must be swum to exhaustion in both swimming trials. This study took a

different approach to the initial swim as it was not exhaustive but rather provided a consistent challenge for both control and exposed groups that allowed direct comparisons of the effects of Cd on swimming ability in the second swim. It is clear that in both species performance in the second swim challenge is significantly impacted by Cd exposure for at least the first few weeks of exposure in brown trout (Fig. 2.5A) and longer in lake whitefish (Fig. 2.5B). To our knowledge this is the first paper to examine the effects of waterborne metal exposure, particularly Cd, on the repeat swimming ability of salmonids and thus comparisons cannot be drawn to previous studies.

2.5.4 Initial Metabolic Effects

In the present study exposure to 18 nM Cd resulted in a reduction in resting white muscle ATP stores in both brown trout (day 6; Fig 2.10B) and lake whitefish (day 7; Fig. 2.11B). These reductions were not evident by the end of the 30 day exposure (Fig. 2.10D and 2.11C respectively). Metabolic fuel sources (white muscle glycogen; Fig. 2.6 and 2.7) and waste by-products (lactate; Fig. 2.8 and 2.9), showed few differences between Cd exposed and control fish. A similar result was observed by Lowe-Jinde and Niimi (1984) who observed no variations in muscle glycogen concentrations as well as several other metabolic parameters in rainbow trout exposed to low Cd concentrations. Exposure to much greater concentrations did result in acute (1-3 d) reductions in liver and muscle glycogen. Cicik and Engin (2005) observed that common carp (*Cypinus carpio*) exposed to high doses of Cd (up to 1.0 mg L^{-1}) had significantly reduced glycogen stores in the liver and muscle (24% and 29% for each tissue, respectively) while serum glucose increased in a dose dependant manner as a result of Cd exposure. Soengas *et al.* (1996) observed increased plasma glucose associated with increased liver glycogenolysis as a

result of Cd exposure. This increase in plasma glucose corresponded with a dose-dependent increase in the activity of glycogen phosohorylase (GPase *a*) and a decrease in glycogen synthase (GSase *a*) in Cd exposed fish. This ratio of GSase reduction to GPase activation is consistent with glycogen depletion. Such reductions could be as a result of stimulation of enzymes involved in glycogenolysis as a result of Cd exposure.

2.5.5 Metabolic Effects of High Intensity Exercise

In the present study, the initial swimming trial consisted of swimming the fish up to 85% of the U_{crit} value, representative of a swimming speed which would require recruitment of white muscle metabolism in order to meet energy demands. This 85% U_{crit} swimming velocity is only slightly lower than the 90% U_{crit} value which Richards *et al*. (2002b) described as unsustainable and associated with the significant depletion of white muscle fuel reserves. In both brown trout and lake whitefish the first swim resulted in significant reductions in glycogen (Fig. 2.6 and 2.7 respectively) and subsequent accumulation of lactate (Fig. 2.8 and 2.9 respectively) in the white muscle. This is consistent with the results of Richards *et al*. (2002b) who reported that oxidative phosphorylation of carbohydrates, such as glycogen, act as a major source of ATP during high intensity exercise.

Burgetz *et al*. (1998) studied metabolic effects at 70, 80 and 100% U_{crit} and showed that swimming at 70 and 80% U_{crit} required the recruitment of white muscle fibres and activation of anaerobic metabolism. Elevations in muscle lactate and decreases on phosphocreatine were observed at all three swimming speeds with increases at 80 and 100% U_{crit} being significantly greater. A decrease in muscle glycogen was also inferred based on a ratio of lactate accumulation to glycogen depletion (2 mol lactate = 1 mol of

glycosyl unit; Arthur *et al*., 1992; Moyes *et al*., 1990). In this study a sub-maximal initial swimming trial to 85% U_{crit}, as in the study by Burgetz *et al*. (1998) this resulted in significant depletions of metabolic fuel sources (glycogen, ATP) and accumulation of lactate.

2.5.6 Metabolic Recovery Following High Intensity Exercise

Recovery from exhaustive exercise in fish is a much slower process than in mammals (Kieffer, 2000). While replenishment of ATP and PCr stores occurs fairly rapidly, generally occurring within the first hour post exercise (Wood, 1991; Wang *et al*. 1994b; Kieffer, 1995), replenishment of glycogen, and subsequent removal of lactate can be a lengthy process. A full recovery of muscle glycogen stores can take anywhere from 6 to as many as 12 hours varying between species and studies (Wendt and Saunders, 1973; Milligan and Wood, 1986; Schwalme and Mackay, 1991, Kieffer *et al*., 1994; Wang *et al*., 1994b; Wilkie *et al*., 1997; Milligan, 2003). Milligan (1996) identified the recovery process for muscle ATP to be much faster than for the replenishment of glycogen, or clearance of lactate, with full recovery of pre-exercise ATP stores generally occurring within the first two hours of recovery. Consequently, it was anticipated that ATP would be recovered at a greater rate than glycogen. Although not significant on all days tested, increases in mean ATP concentration within the muscle were observed, particularly in control fish of both species (Fig. 2.10 and 2.11).

During recovery, the two primary fuel sources available to trout white muscle are the lactate accumulated from glycolysis and remaining lipid fuels and it has been shown that protein oxidation contributes little to exercise and recovery (Weber, 1997). Previously, the contribution of lipid oxidation to recovery from exhaustive exercise had

been thought to be minimal, however several studies have shown decreases in white muscle lipid concentrations (Milligan and Girard, 1993) and in nonesterified fatty acids (Dobson and Hochachka, 1987). The majority of lactate produced as a result of glycolysis is retained as the substrate for glyconeogenesis in the muscle, and is used primarily for oxidative phosphorylation (Schulte *et al*., 1992; Milligan and Girard, 1993). Following exhaustive exercise, lactate levels are generally highest, while glycogen levels in the white muscle are decreased with reductions as great as 90% (Wang *et al*., 1994b). A portion of the acid and lactate produced during the commission of exhaustive exercise is released from the muscle to the blood stream with lactate levels reaching their peak concentration in the plasma 2 h post exercise with clearance after 8h (Milligan, 1996). The majority of lactate, however, is retained within the white muscle.

In the present study significant reductions in muscle glycogen and subsequent increases in muscle lactate were followed by no significant recovery of either parameter. Using three successive swimming challenges, separated by 45 minute recovery periods Farrell *et al*. (1998) observed that lactate levels in normoxic sockeye salmon were significantly elevated from rest following the first swimming trial, but remained elevated over the course of the subsequent two swimming trials and recovery periods, indicating that 45 minutes was not sufficient for the clearance of muscle lactate. Here, it can be concluded that the shorter 30 minute recovery period was not sufficient for the clearance of lactate and subsequent replenishment of muscle glycogen stores as had been intended. In a review of metabolic recovery following exhaustive exercise in trout Milligan (1996) identified 8 h as the time required to return both muscle glycogen and lactate stores to pre-exercise conditions. The 30 minute recovery period was selected to allow for minimal

recovery to produce an effect similar to migration. The 30 min recovery period used in this study would not facilitate a significant change in either glycogen or lactate (Milligan 1996) as was desired.

Recovery ability can vary between species. Arthur *et al*. (1992) observed that high intensity swimming resulted in sharp increases (in excess of 100 mmol kg^{-1}) in muscle lactate, with a corresponding decrease in muscle glycogen, in skipjack tuna (*Katsuwonus pelamis*). These fluctuations were corrected very rapidly, with muscle lactate and glycogen concentrations returning to levels observed in unexercised fish within 90 minutes of recovery. Significant increases in plasma lactate were observed following exercise. Concentrations remained elevated until lactate and glycogen concentrations within the epaxial muscle were restored. This increased transfer of lactate to the blood may account for the fast recovery observed in tuna, which more closely resembles that of mammals. Other teleosts retain a much greater proportion of lactate within the white muscle, thus requiring a longer time for lactate clearance (Milligan and McDonald, 1988).

2.6 Conclusions

As salmonids, brown trout and lake whitefish are highly sensitive to metal exposure. The dose of 18 nM Cd utilized in this study resulted in minimal mortalities (7% brown trout; 0% lake whitefish), it was however, sufficient to cause a variety of physiological disturbances. In both species Cd exposure resulted in acute reductions in plasma Ca, with this reduction persisting chronically in brown trout. The two species also differed in their patterns or Cd accumulation. While the gill was observed to be the primary site of accumulation in both species, lake whitefish accumulated twice as much

Cd in the liver than did brown trout. The liver is associated with detoxification, so an increase in partitioning to the liver may infer a greater capacity for detoxification in lake whitefish compared with brown trout.

In both species, swimming to 85% of U_{crit} in the initial trial required significant recruitment of anaerobic metabolic pathways. The observed effects of high intensity exercise were very similar between control and Cd exposed fish of both species. Metabolic recovery and subsequent expenditure in the second swim challenge also showed little variation between control and Cd exposed fish. As such it can be concluded the reduction in U_{crit} performance in the second swim trial in Cd exposed fish was not as a result of alterations to anaerobic metabolism (glycogen, lactate, ATP) in the white muscle.

Little recovery of metabolic fuel sources or clearance of metabolic waste products was observed after the 30 minute recovery period. This indicates that another fuel source is required to support the second swim challenge. At sub-maximal swimming speeds (<70% U_{crit}) sustained activity is powered by aerobic metabolism. As the initial phases of the second swim trial are at a relatively low velocity, swimming is fuelled aerobically. As a result the initial portion of the second swim trial will not draw on the depleted anaerobic fuel sources such as glycogen. Although swimming is taking place, when at a low level, recovery of anaerobic parameters, such as those examined in this study, can occur.

As the recovery of swimming ability does not appear to be correlated with the recovery of anaerobic metabolic parameters, it may be the case that Cd exposure affects

aerobic fuel supplies and their allocation. As performance in the initial swim trial is unaffected by Cd exposure the metabolic fuel sources present are sufficient to support equal swimming in control and Cd exposed fish. The impairment of secondary swim performance observed here could be as a result of reduced aerobic fuel sources. If the substrate (lipids) for aerobic metabolism is limited, or not restored properly following the initial swim challenge then fish may be required to shift to anaerobic metabolism sooner, depleting those stores more rapidly, and ultimately decreasing swimming ability in the second swim challenge.

Table 2.1. Means for wet weight and body dimensions of fish by species. Values are shown with ± 1 SEM and n = 256 and 144 for brown trout and lake whitefish respectively.

	Mass (g)	**Fork Length (cm)**	**Maximum Depth (cm)**	**Maximum Width (cm)**
Brown Trout	33.7 ± 1	13.5 ± 0.2	2.9 ± 0.04	1.7 ± 0.02
Lake Whitefish	45 ± 1.2	15.8 ± 0.1	3.1 ±0.03	1.7 ±0.02

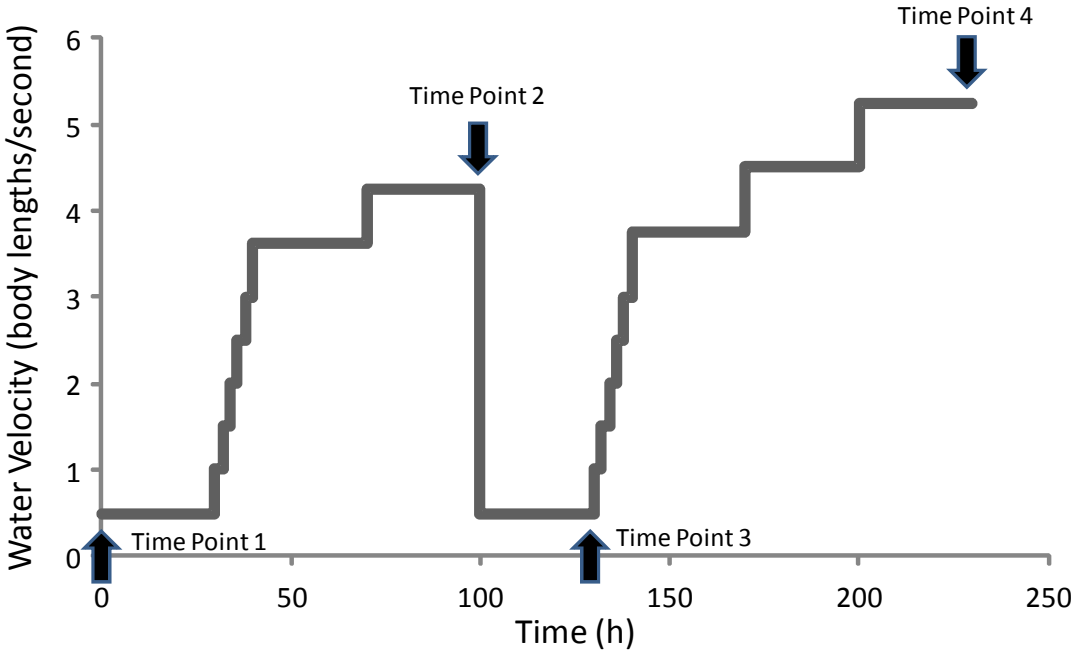

Water Velocity (body lengths/second)
6
5
4
3
2
1
0
Time Point 1
Time Point 2
Time Point 3
Time Point 4
0
50
100
150
200
250
Time (h)

Figure 2.1

Figure 2.1 A schematic diagram showing the velocity of water (in bl s^{-1}) in the swim flume over the course of the repeat swimming protocol using brown trout. Lake white fish used the same protocol, however water velocity increases were adjusted slightly to reflect a higher mean control U_{crit}. Fish were introduced into the swim flume and given a 30 min acclimation period at 0.5 bl s^{-1}. Following this water velocity was increased to 1 bl s^{-1} and then increased to 60% U_{crit} (2.8 bl s^{-1} in brown trout and 3 bl s^{-1} in lake whitefish). Following the ramp period water velocity was increased over two half hour increments in order to achieve 85% U_{crit} swimming (4 bl s^{-1} in brown trout and 4.25 bl s^{-1} in lake whitefish). Fish were then given a 30 min recovery period, again at 0.5 bl s^{-1}. The second swim challenge followed the steps described for the first challenge except that, once a swimming velocity of 60% U_{crit} was reached the flow was increased by 0.75 bl s^{-1} at 30 min intervals until exhaustion. Arrows show the four tissue sampling point, representing unexercised fish (point 1), the end of the first swim challenge (point 2), after recovery (point 3) and exhaustion after the second swim challenge (point 4).

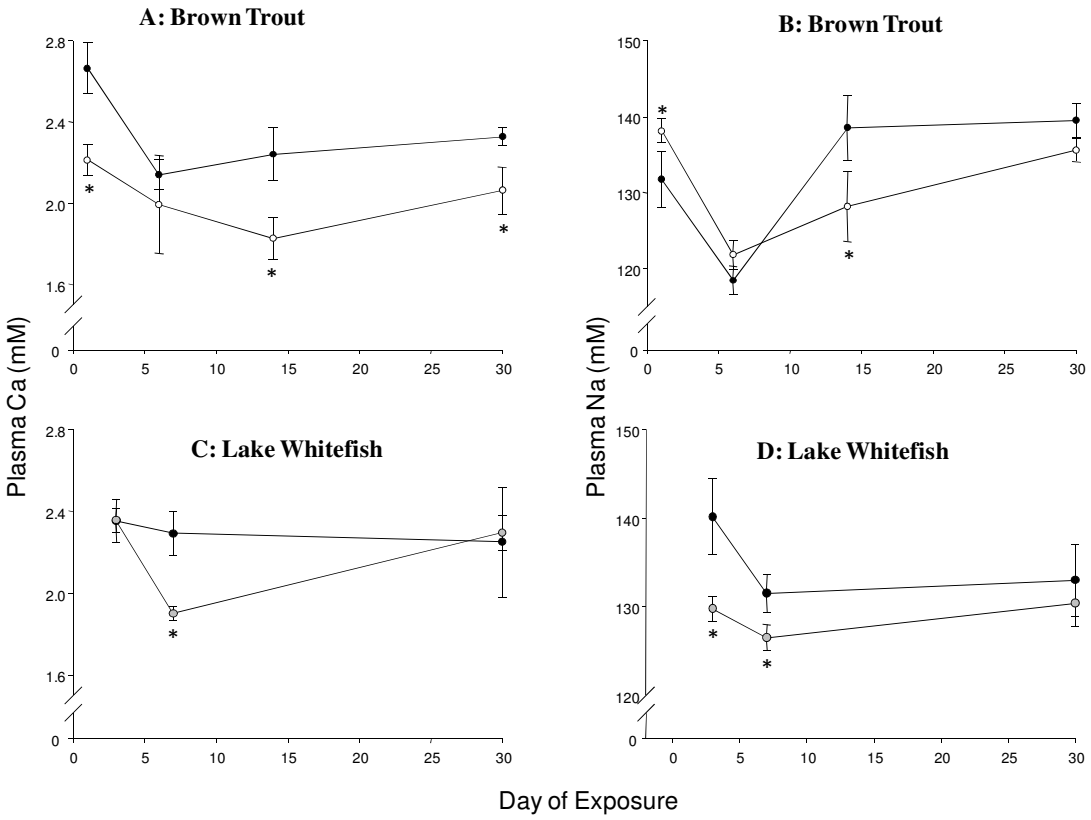

A: Brown Trout
B: Brown Trout
C: Lake Whitefish
D: Lake Whitefish
Plasma Ca (mM)
Plasma Na (mM)
Day of Exposure

Figure 2.2

Figure 2.2 The effect of chronic exposure to waterborne Cd on plasma Ca^{2+} (A) and Na^{2+} (B) in brown trout and on plasma Ca^{2+} (C) and Na^{2+} (D) in lake whitefish. Controls are shown as black points for both exposures while fish exposed to 18 nM Cd are shown as white points in brown trout and grey points in lake white fish. Values are mean ± 1 SEM, n=8 for brown trout and n = 6 for lake whitefish. A * indicates a significant difference compared to controls at that time point (P =0.05).

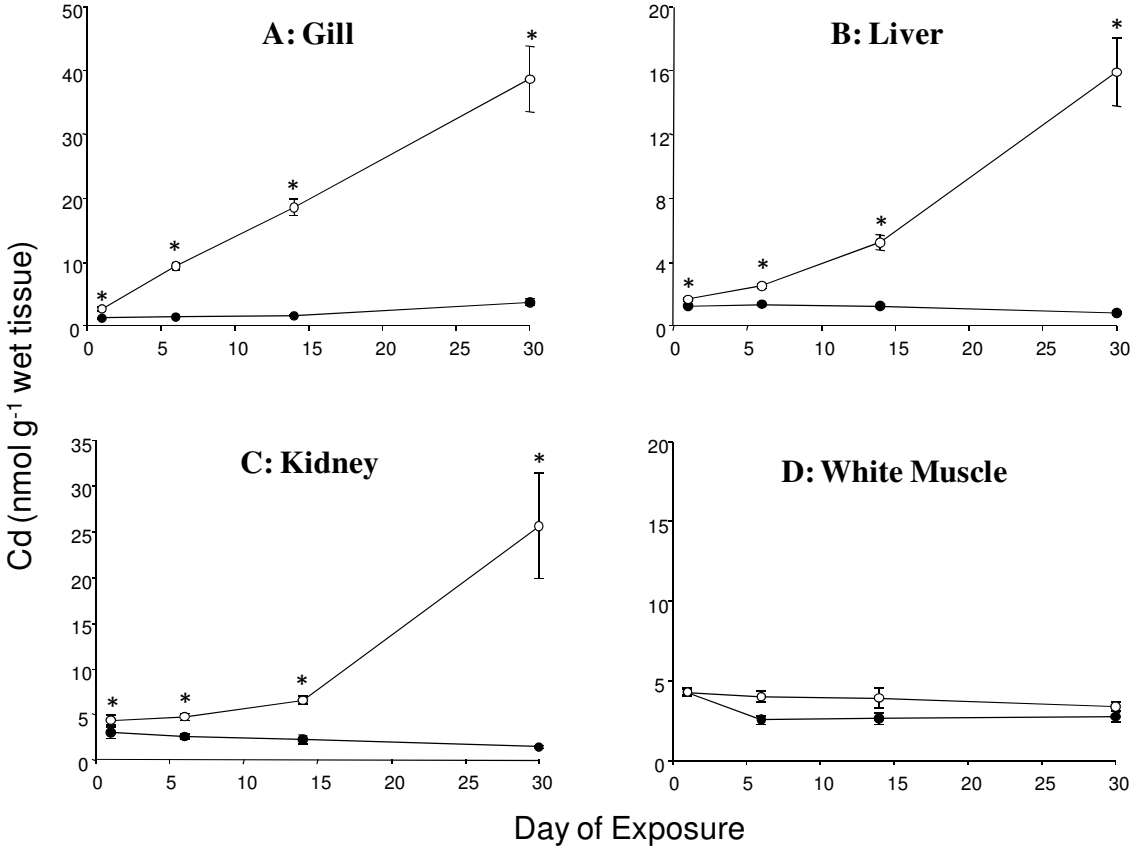

A: Gill
B: Liver
C: Kidney
D: White Muscle
Cd (nmol g-1 wet tissue)
Day of Exposure

Figure 2.3

Figure 2.3 Time course of Cd accumulation in the gills (A), liver (B), kidney (C) and white muscle (D) of brown trout during 30 days of exposure to 18 nM Cd (open symbols). Values are mean ± 1 SEM with n=8 fish and a group of unexposed (controls, filled symbols) are shown at each sampling time. A * indicates a significant difference from controls ($P<0.05$).

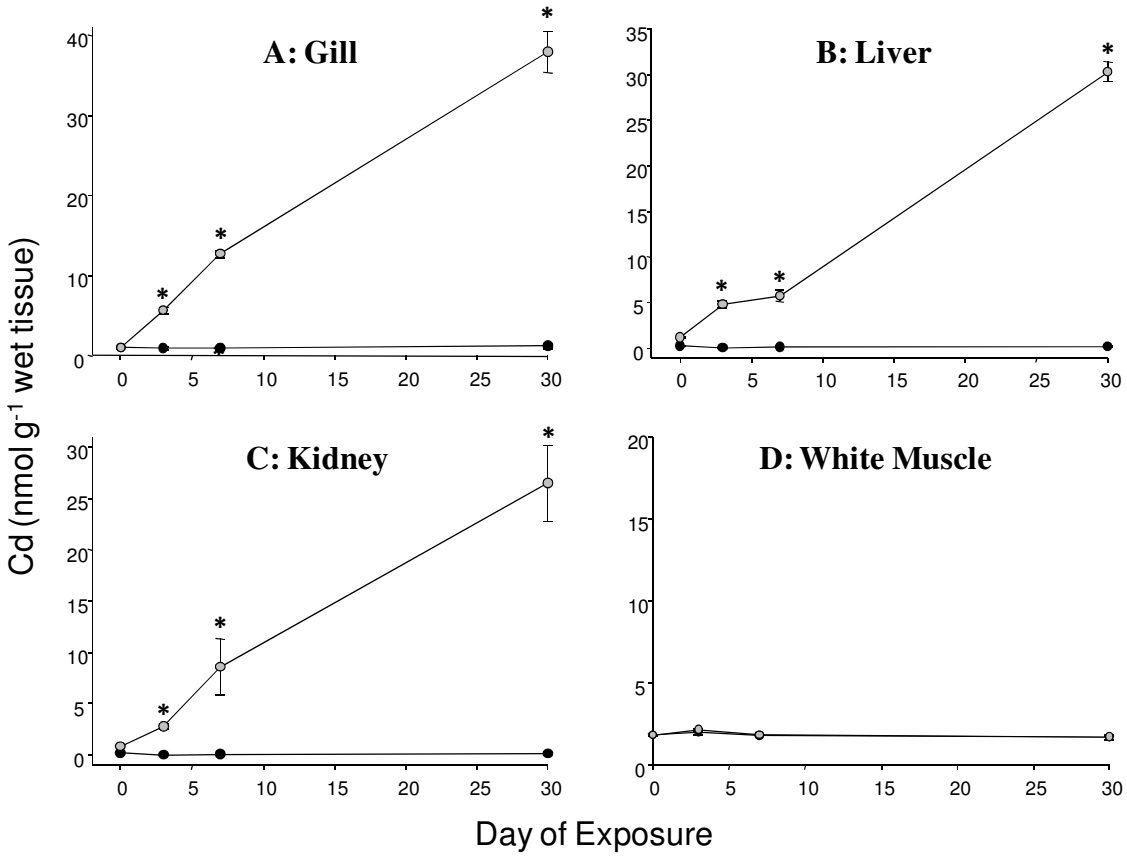

A: Gill
B: Liver
C: Kidney
D: White Muscle
Cd (nmol g-1 wet tissue)
Day of Exposure

Figure 2.4

Figure 2.4 Time course of Cd accumulation in gills (A), liver (B), kidney (C) and white muscle (D) of lake whitefish during 30 days of exposure to 18 nM Cd (grey symbols). Values are mean ± 1 SEM with n=6 fish and a group of unexposed (controls, black symbols) are shown at each sampling time. A * indicates a significant difference from controls ($P<0.05$).

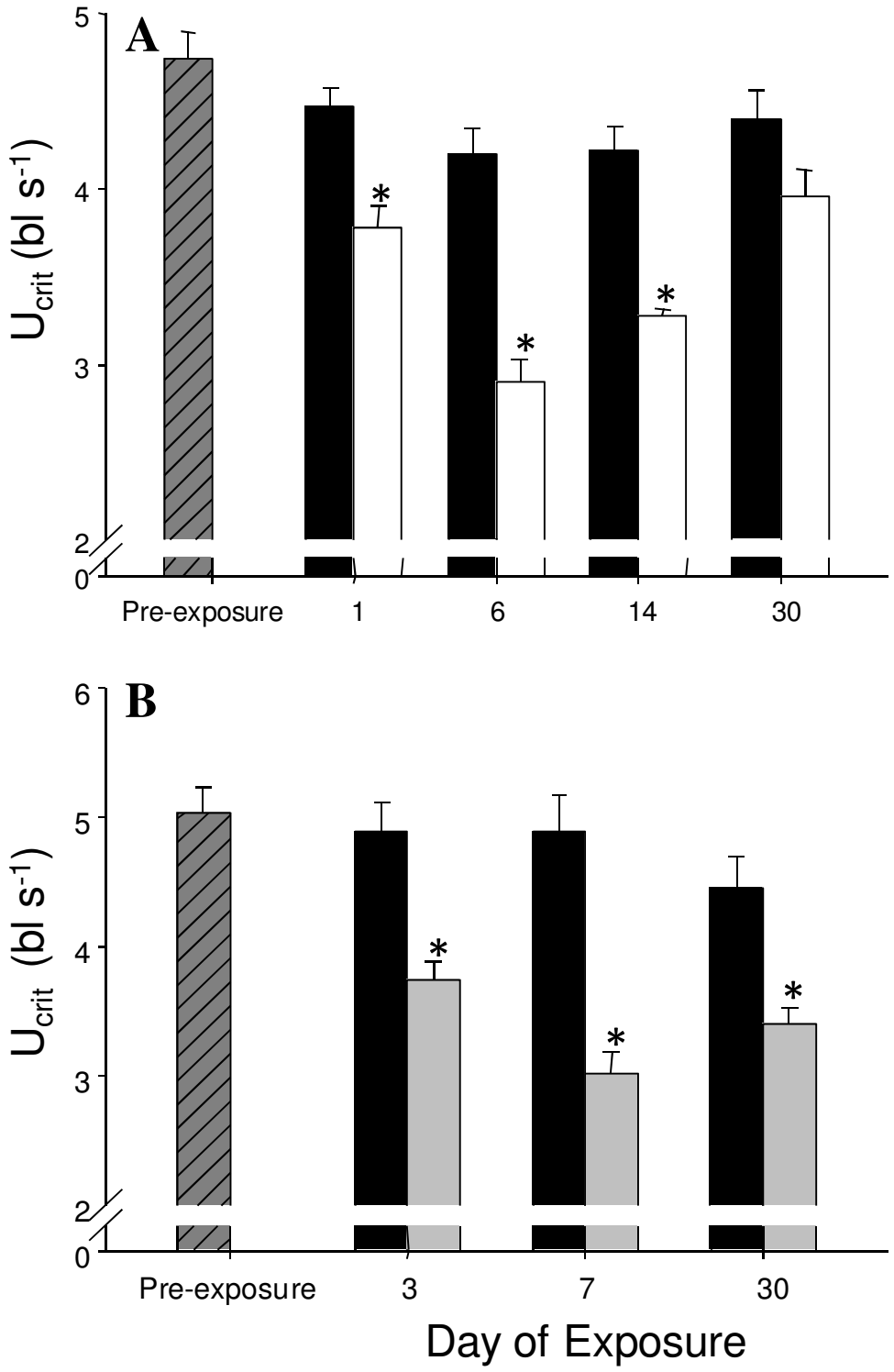


Figure 2.5

Figure 2.5 The effect of chronic waterborne Cd exposure (18 nM Cd) on U_{crit} following an initial swim challenge and 30 min rest period in brown trout (A) and lake whitefish (B). The U_{crit} for control fish of both species are shown in black while Cd exposed brown trout are shown in white and Cd exposed lake whitefish are shown as grey bars. Values are mean ± 1 SEM with n=8 for brown trout and n=6 for lake whitefish. A * represents a significant difference from controls for that day ($P<0.05$).

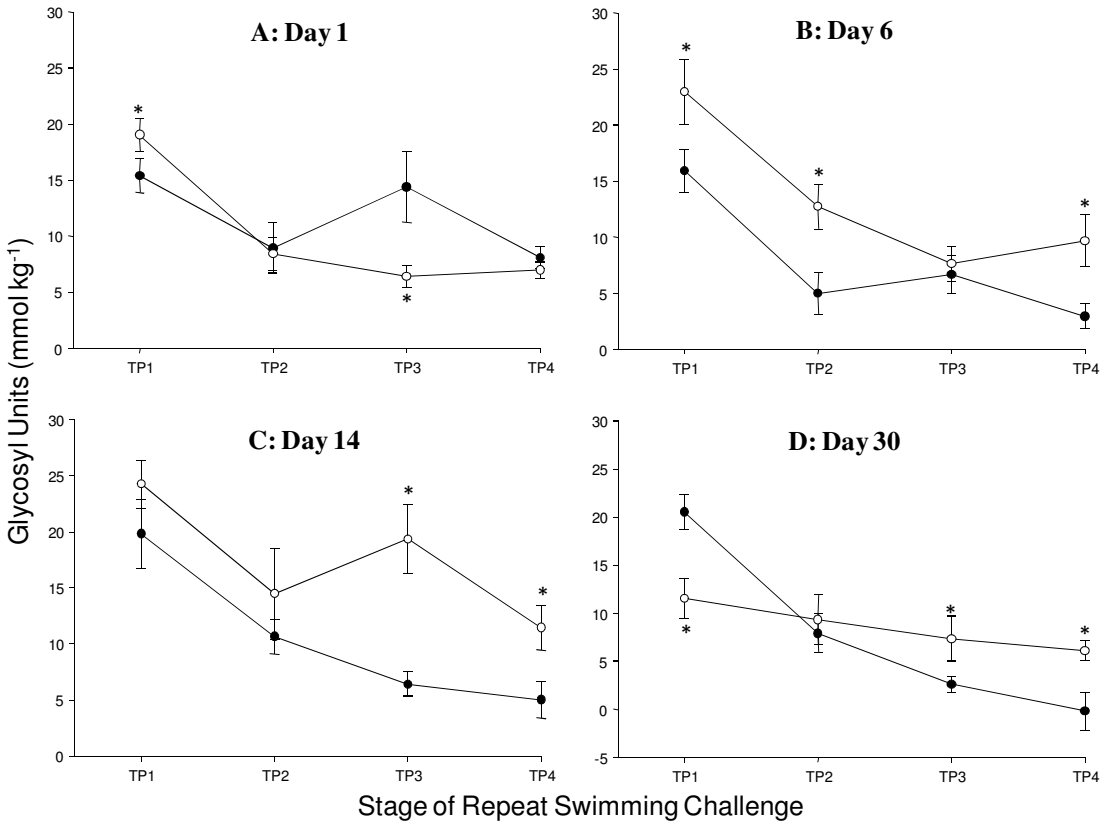

A: Day 1
B: Day 6
C: Day 14
D: Day 30
Glycosyl Units (mmol kg-1)
Stage of Repeat Swimming Challenge
TP1
TP2
TP3
TP4

Figure 2.6

Figure 2.6 Muscle glycogen levels in brown trout under both control (black points) and Cd exposed (18 nM Cd: white points) conditions on days 1 (A), 6 (B), 14 (C) and 30 (D) of exposure. Represented on the x axis are the four time points described previously. Briefly, time point 1 represents unexercised fish, time point 2 represents fish sampled following the first swim. Time point 3 represents fish that have been sampled after the first swim and subsequent 30 min recovery period. And finally time point 4 is representative of fish sampled immediately after completing two successive swims. Values are mean ± 1 SEM with n=8 and * represents a significant difference from controls at that time point ($P<0.05$).

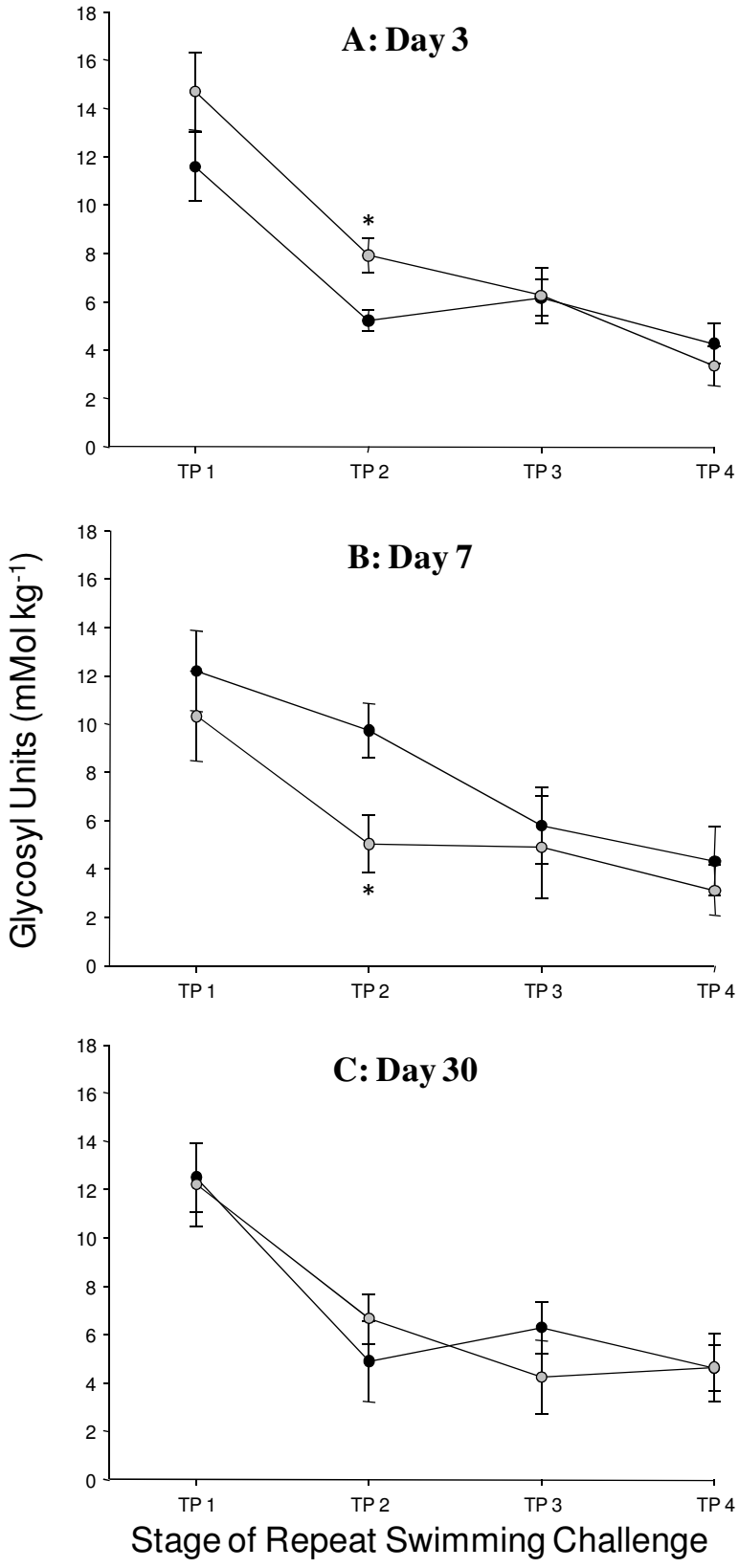

A: Day 3
B: Day 7
C: Day 30
Glycosyl Units (mMol kg-1)
Stage of Repeat Swimming Challenge
TP 1
TP 2
TP 3
TP 4
0
2
4
6
8
10
12
14
16
18
*
*

Figure 2.7

Figure 2.7 Muscle glycogen levels in lake whitefish under both control (black points) and Cd exposed (18 nM Cd: grey points) conditions on days 3 (A), 7 (B) and 30 (C) of exposure. Represented on the x axis are the four time points described previously. Briefly, time point 1 represents unexercised fish, time point 2 represents fish sampled following the first swim. Time point 3 represents fish that have been sampled after the first swim and subsequent 30 min recovery period. And finally time point 4 is representative of fish sampled immediately after completing two successive swims. Values are mean ± 1 SEM, with n=6. A * represents a significant difference from controls at that time point ($P<0.05$).

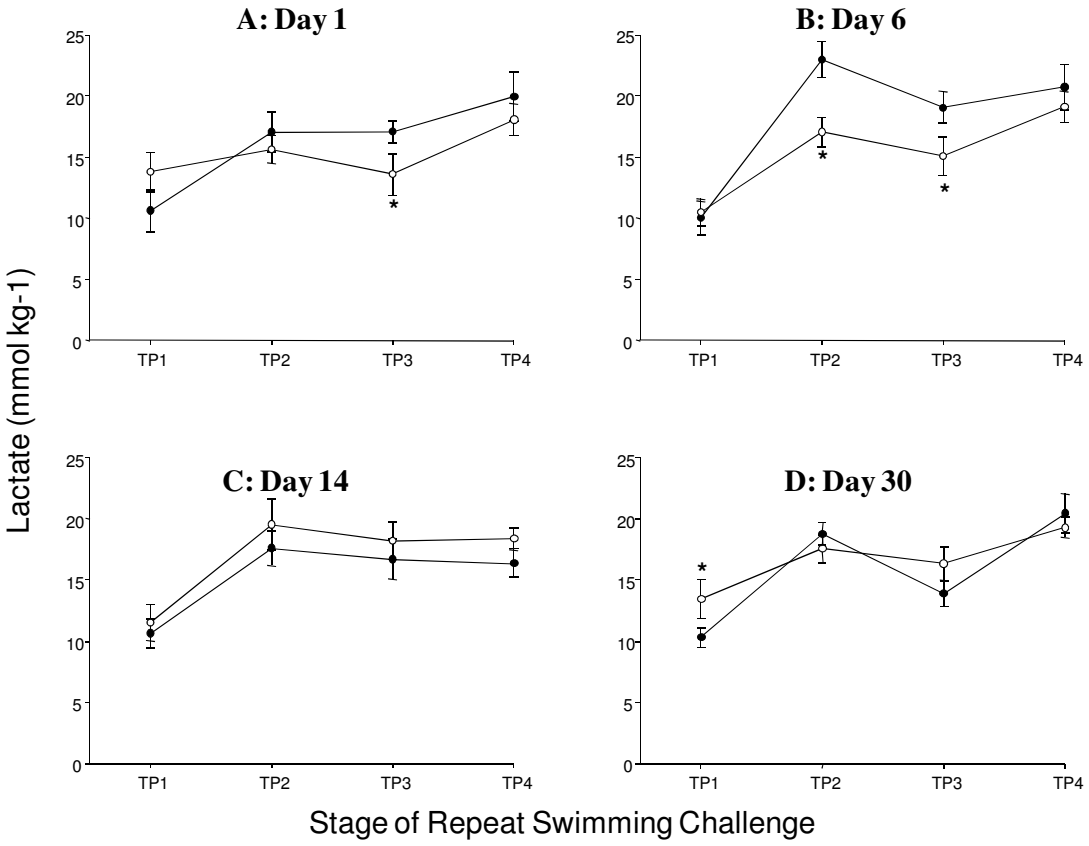

A: Day 1
B: Day 6
C: Day 14
D: Day 30
Lactate (mmol kg-1)
Stage of Repeat Swimming Challenge
TP1
TP2
TP3
TP4
0
5
10
15
20
25
*

Figure 2.8

Figure 2.8 Muscle lactate levels in brown trout under both control (black points) and Cd exposed (18 nM Cd: white points) conditions on days 1 (A), 6 (B), 14 (C) and 30 (D) of exposure. Represented on the x axis are the four time points described previously. Briefly, time point 1 represents unexercised fish, time point 2 represents fish sampled following the first swim. Time point 3 represents fish that have been sampled after the first swim and subsequent 30 min recovery period. And finally time point 4 is representative of fish sampled immediately after completing two successive swims. Values are mean ± 1 SEM, with n=8. A * represents a significant difference from controls at that time point ($P<0.05$).

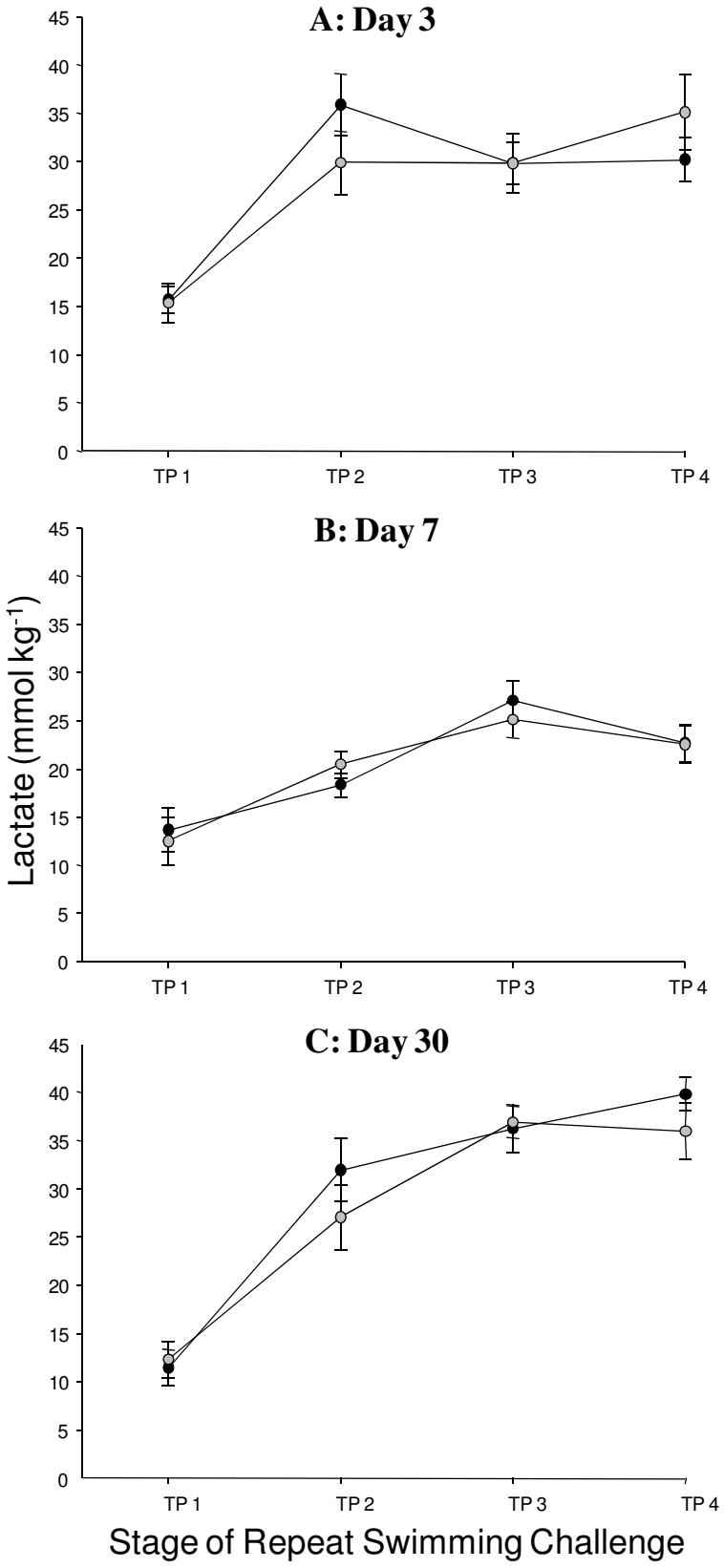

A: Day 3
B: Day 7
C: Day 30
Lactate (mmol kg-1)
0
5
10
15
20
25
30
35
40
45
TP 1
TP 2
TP 3
TP 4
Stage of Repeat Swimming Challenge

Figure 2.9

Figure 2.9 Muscle lactate levels in lake whitefish under both control (black points) and Cd exposed (18 nM Cd: grey points) conditions on days 3 (A), 7 (B) and 30 (C) of exposure. Represented on the x axis are the four time points described previously. Briefly, time point 1 represents unexercised fish, time point 2 represents fish sampled following the first swim. Time point 3 represents fish that have been sampled after the first swim and subsequent 30 min recovery period. And finally time point 4 is representative of fish sampled immediately after completing two successive swims. Values are mean ± 1 SEM, with n=6. A * represents a significant difference from controls at that time point ($P<0.05$).

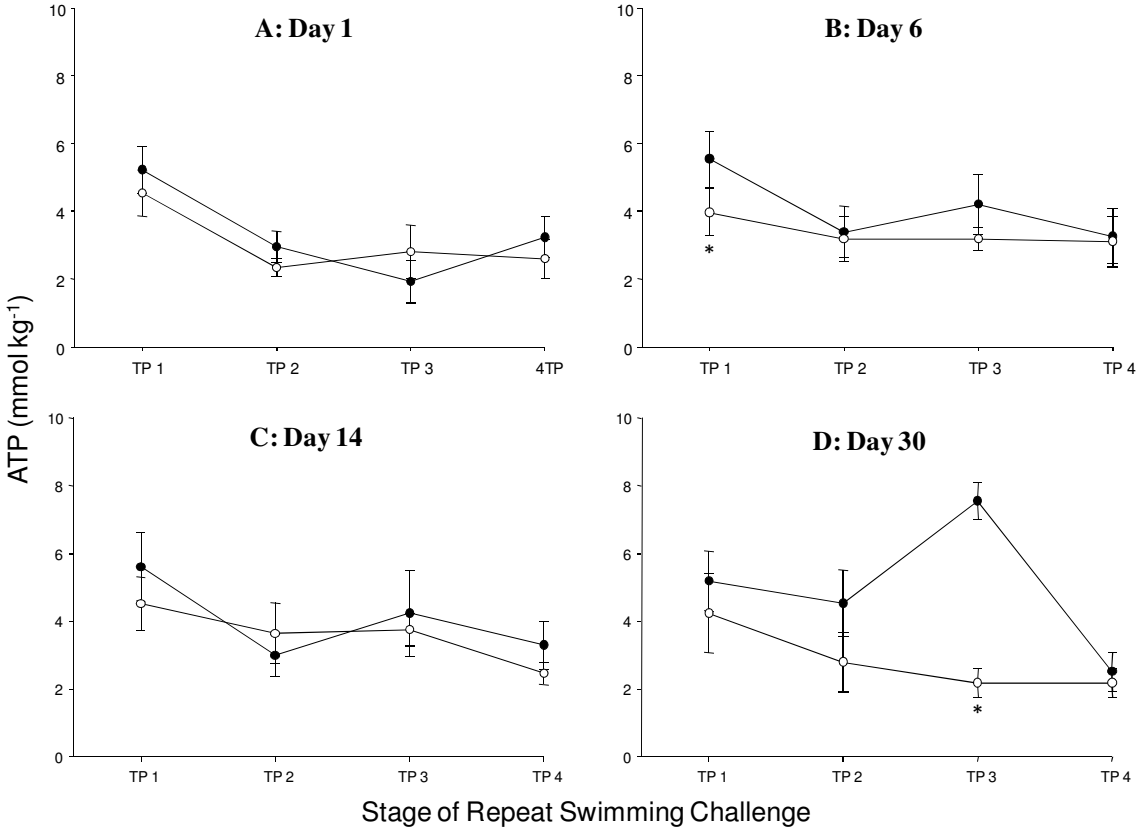

A: Day 1
B: Day 6
C: Day 14
D: Day 30
ATP (mmol kg-1)
TP 1
TP 2
TP 3
4TP
TP 4
Stage of Repeat Swimming Challenge

Figure 2.10

Figure 2.10 Muscle ATP levels in brown trout under both control (black points) and Cd exposed (18 nM Cd: white points) conditions on days 1 (A), 6 (B), 14 (C) and 30 (D) of exposure. Represented on the x axis are the four time points described previously. Briefly, time point 1 represents unexercised fish, time point 2 represents fish sampled following the first swim. Time point 3 represents fish that have been sampled after the first swim and subsequent 30 min recovery period. And finally time point 4 is representative of fish sampled immediately after completing two successive swims. Values are mean ± 1 SEM, with n=8. A * represents a significant difference from controls at that time point ($P<0.05$).

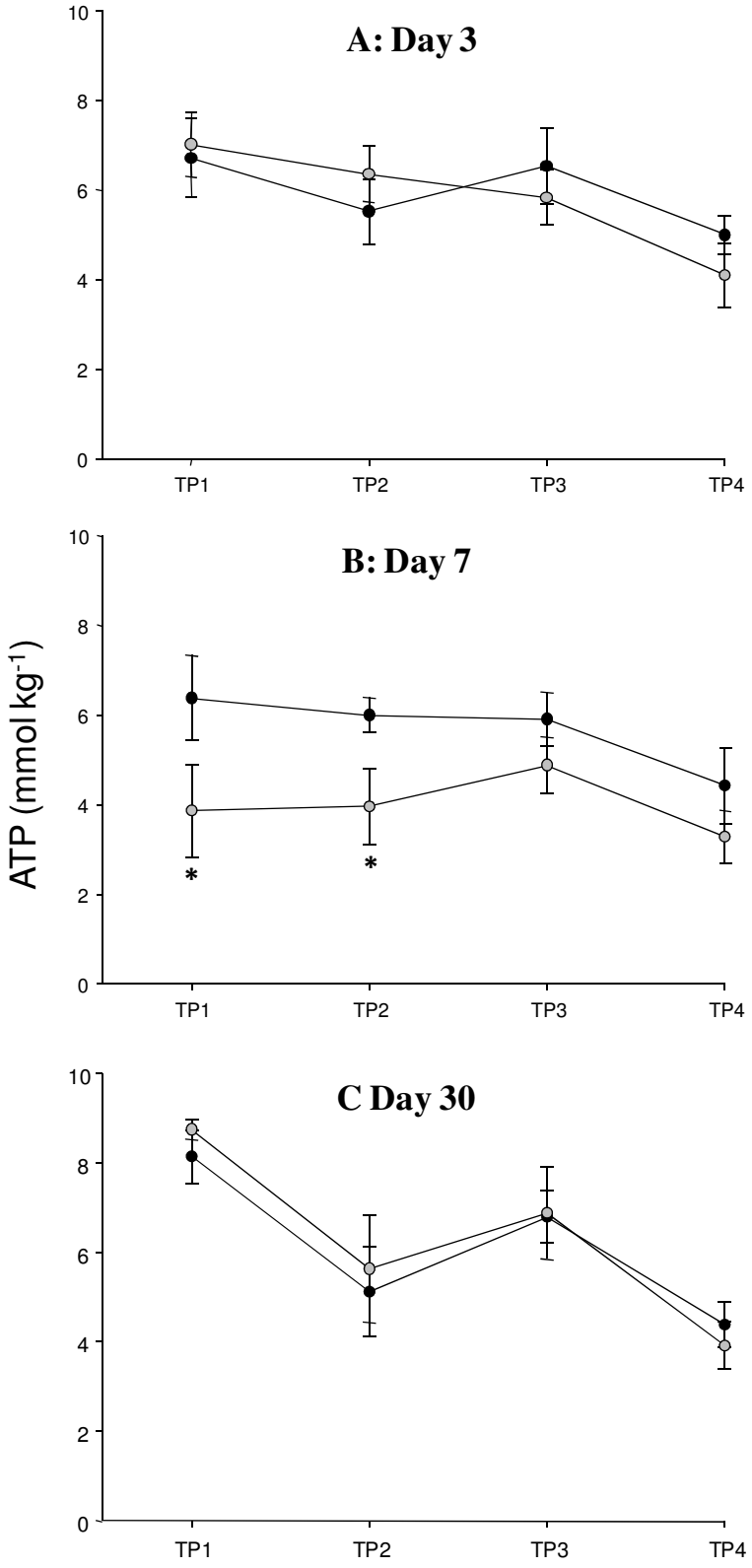


Figure 2.11

Figure 2.11 Muscle ATP levels in lake whitefish under both control (black points) and Cd exposed (18 nM Cd: grey points) conditions on days 3 (A), 7 (B) and 30 (C) of exposure. Represented on the x axis are the four time points described previously. Briefly, time point 1 represents unexercised fish, time point 2 represents fish sampled following the first swim. Time point 3 represents fish that have been sampled after the first swim and subsequent 30 min recovery period. And finally time point 4 is representative of fish sampled immediately after completing two successive swims. Values are mean ± 1 SEM, with n=6. A * represents a significant difference from controls at that time point ($P<0.05$).

Chapter 3

The effect of chronic sublethal waterborne cadmium exposure and recovery time on repeated swim performance in rainbow trout (Oncorhynchus mykiss)

3.1 Abstract

Swimming ability is frequently used to characterize the integrated metabolic costs associated with exposure to contaminants, including Cd. Repeat swimming challenges provide a more sensitive indicator of effects by identifying impairment that was not observed in a singular swim challenge alone. Sublethal Cd exposure has previously been shown not to affect swimming performance in individual swim challenges; however we have recently shown that exposure to 18 nM Cd (2 µg Cd L^{-1}) resulted in a reduction in performance in subsequent swim trials (See Chapter 2). For example, performance in the second swim trial, following an initial swim and 30 min recovery period, resulted in reductions of sustained swimming capacity (U_{crit}) of 31% and 38% in Cd exposed brown trout (*Salmo trutta*) and lake whitefish (*Coregonus clupeaformis*) respectively. Drawing on this, recovery periods of varying duration were used in order to determine the role of the rest period in the recovery of swimming ability and metabolic parameters. This study employs two consecutive swimming challenges, separated by a recovery period of varying duration (0.5, 1.5 and 6 h) in order to investigate the effects of 6 d Cd (18 nM Cd, ~85 mg $CaCO_3$) exposure on repeated swimming ability in juvenile rainbow trout (*Oncorhynchus mykiss*). Blood, organ and muscle samples were taken before and after each of the swim trials and rest periods, and tissue Cd burden, plasma ion concentrations, metabolic fluctuations and stress response were measured. Following a 30 minute recovery period, Cd exposed fish had a decreased U_{crit} of 3.0 ± 0.3 bl s^{-1}, compared with 4.4 ± 0.3 bl s^{-1} in controls, in subsequent challenges. However, with a 1.5 hour recovery period Cd exposed fish performed significantly better in subsequent swimming challenges (5.2 ± 0.2 bl s^{-1}) than did controls (4.3 ± 0.2 bl s^{-1}), with no differences

observed between treatments after 6 hours of recovery. Metabolites and metabolic fuel sources, utilized to power muscular contraction and physical activity, such as glycogen and ATP were directly linked to the changes in swim performance at different recovery times. The increased swimming capacity of Cd exposed fish following a 1.5 h recovery was correlated with greater restoration rate of glycogen and ATP and increased removal of lactate compared to controls. The results establish linkages between exposure, bioaccumulation and the physiological disruption induced by Cd to provide an improved understanding of the mechanisms underlying impairment of whole animal performance.

3.2 Introduction

Cadmium, unlike other metals such as zinc and copper is not required for biological processes and thus has no dietary requirement (McGeer *et al*., 2011). Elevated Cd concentrations are associated with its natural occurrence as well as anthropogenic activities such as mining and smelting. There are no Cd mines but rather the production of Cd is closely linked with the mining of other metals such as zinc, lead and copper (Panagapko, 2007). The majority of Cd produced (~83%) is utilized in the production of batteries, with much of the remaining applications including use as a stabilizer and in pigments (WHO, 1992; Panagapko, 2007, ATSDR, 2008).

The mechanism of acute Cd toxicity is related to an inhibition of Ca uptake. Cd^{2+} acts as a Ca^{2+} analogue, causing an antagonistic relationship between the two ions (Verbost *et al*., 1988; Pratap *et al*., 1989; Playle *et al*., 1993; Wicklund-Glynn *et al*., 1994; Hollis *et al*., 2000; Niyogi and Wood, 2004; Niyogi *et al*., 2008). At the apical membrane Cd^{2+} ions are thought to pass through lanthanum-sensitive voltage-independent epithelial Ca^{2+} channels (ECaC) in the mitochondria rich chloride cells of the gill (Verbost *et al*., 1989; Wicklund-Glyann *et al*., 1994; Galvez *et al*., 2006). At the basolateral membrane, transport to the blood occurs by high-affinity Ca^{2+} pumps (Ca^{2+}-ATPase) and the Na^{+}/Ca^{+} exchanger (Verbost *et al*., 1989; Flick, 1990). The Ca^{2+}-ATPase associated with fish gills has been shown to have an affinity of at least 3 orders of magnitude greater for Cd^{2+} vs. Ca^{2+} (log K_{Cd2+} 7.5-8.6 vs log K_{Ca2+} 3.7-5; Verbost *et al*., 1988, Playle *et al* 1993a, b; Niyogi *et al*., 2004, 2008, McGeer *et al*., 2011).

Increased concentrations of free, ionic Cd in the water will result in Cd^{2+} rather than Ca^{2+} being translocated into the blood, leading to a potentially fatal reduction in

whole organism Ca, known as hypocalcaemia (Verbost *et al*., 1989). Numerous studies have shown disruption of Ca homeostasis as a result of Cd exposure in tilapia (*Oreochromis mossambicus*: Pratap *et al*., 1989; Chang *et al*., 1997), tambaqui (*Colossoma macropomum*: Matsuo *et al*., 2005) and rainbow trout (Giles, 1984; Reid and McDonald, 1988; McGeer *et al*., 2000a; Chowdhury *et al*., 2004). Sublethal exposure to Cd has been linked to a variety of physiological and whole organism disruptions (reviewed McGeer *et al*., 2011), however, studies on its effects on swimming performance are somewhat equivocal.

Swimming is easily observed and is a highly relevant measure for assessing organism health, stress and/or impairment (Beitinger and McCauley, 1990). Swimming ability has implications for important processes such as feeding, attracting mates, avoidance of unfavourable conditions, reproductive behaviour and migration (Plaut, 2001). There are three methods of swimming (sustained, prolonged and burst; Beamish 1978) and each represents at different duration, intensity and the type of metabolic fuel utilized. Prolonged swimming is the method studied most frequently (Beitinger and McCauley, 1990). The characterization of the capacity for prolonged swimming involves tests where fish swim against water velocities for consistent durations that are incremented at regular intervals until exhaustion occurs. The critical swimming speed (U_{crit}, Brett, 1964) is the calculated endpoint for the test and it provides a measure of maximum sustained swimming capacity (Beitinger and McCauley, 1990; Farrell, 2008).

U_{crit} swimming challenges can also be used to demonstrate the integrated additional metabolic costs associated with contaminant exposure (Little and Finger, 1990). As such, U_{crit} has been used to characterise the effects of metal exposure on the

swimming performance of salmonid species. For example reduced U_{crit} values associated with chronic exposure have been documented for brown trout (*Salmo trutta*: Beaumont *et al*., 1995) and rainbow trout (McGeer *et al*., 2000a) exposed to Cu as well as rainbow trout exposed to Ni (Pane *et al*., 2004). Unlike these metals, however, chronic sublethal Cd exposure does not to affect sustained swimming performance in rainbow trout (Hollis *et al*., 1999, McGeer *et al*., 2000a). Month long exposure to 27 nM Cd in moderately hard water did not result in reductions in U_{crit} in spite of significant tissue burdens and obvious physiological disruption (e.g. ionoregulation; Hollis *et al*., 1999; McGeer *et al*., 2000a). Therefore, U_{crit} swim performance testing may provide useful measures of the metabolic costs associated with chronic contaminant exposure but there are exceptions.

The studies that have used swimming challenges to evaluate the effects of chronic Cd exposure have only used a single swim U_{crit}. A more sensitive and ecologically relevant measure of the potential impacts of chronic exposure can be provided through measures of performance in repeat swimming challenges. Jain *et al*. (1998) found that the use of a repeat swimming trial identified reduced performance in dehydroabietic acid (DHA) exposed fish that was not identified in a single swimming challenge alone. This theory was tested in Chapter 2, where a reduction in swimming performance in the second swimming trial, following a 0.5 hour recovery, was observed in brown trout and lake whitefish (*Coregonus clupeaformis*) exposed to 18 nM Cd but not in controls.

The extent and frequency to which a fish can undergo repeated bouts of high intensity exercise is determined by the time required for recovery, particularly the time necessary to restore energy reserves such as glycogen, and to clear accumulated lactate; a process which can take in excess of 12 h to complete (Milligan, 1996). However, many

fish species, including salmonids, can undergo successful bouts of swimming following much shorter recovery periods, indicating that a full metabolic recovery is not required to sustain high performance exercise (Hochachka, 1961).

An important component of performance in the second swim in a repeat swim challenge is the recovery period. Various studies have shown that to fully restore swim capacity in salmonids (i.e. second U_{crit} is not significantly less than first) 40 min to several hours may be required (Randall *et al*., 1987; Brauner *et al*., 1994; Farrell *et al*., 1998; Jain *et al*., 1998; Jain and Farrell, 2003; MacNutt *et al*., 2004). Brauner *et al*. (1994) observed a full recovery of swimming performance in juvenile coho salmon (*Oncorhynchus kisutch*) given a 2 h recovery period. Randall *et al*. (1987) observed that chinook salmon (*Oncorhynchus tshawytscha*) given a 60 minute recovery period performed equally well in a second U_{crit} challenge, while a 45 minute recovery period was sufficient for the restoration of swimming performance in three successive swim challenges in mature sockeye salmon (*Oncorhynchus nerka*: Farrell *et al*., 1998). Recovery durations similar to those observed for pacific salmon were also observed in trout species. Jain *et al*. (1998) found that full recovery of swimming ability following exhaustive exercise was restored in hatchery reared rainbow trout following a 70 minute recovery. Even shorter recovery periods of 40 (Jain and Farrell, 2003) and 45 minutes (MacNutt *et al*., 2004) were sufficient to restore swimming ability in a second swim trial with mature rainbow trout.

While swimming ability in salmonids can typically be restored in under two hours, full metabolic recovery can take much longer, sometimes in excess of 12 h (Milligan, 1996). An individual bout of exhaustive exercise can deplete glycogen stores

within the white muscle by as much as 90% (Wang *et al*., 1994b; Milligan, 1996). Following this, the two key fuel sources available to trout muscle are lactate accumulated as a result of glycolysis and remaining lipid-based fuel stores. Milligan (1996) reviews recovery following exhaustive exercise in rainbow trout, with particular emphasis on the recovery of anaerobic fuel sources, metabolites and accumulated waste products. In mammals glycogen is recovered through a system known as the Cori cycle which shuttles lactate that has accumulated in the muscle to the liver where it is converted to the glucose required to replenish glycogen stores (Moyes and Schulte, 2008).

In fish, the role of the Cori cycle is under debate. Rather than being transported to the liver, the majority of lactate (80-85%) is retained within the white muscle as a substrate for glycolysis (Milligan and Wood, 1986; Pagnotta and Milligan, 1991). The clearance of lactate corresponds with the rate of recovery of glycogen (Schulte *et al*., 1992; Milligan and Girard, 1993). The clearance of lactate corresponds with the restoration of glycogen with both taking approximately 8 h to complete (Milligan, 1996). Following exhaustive exercise, a portion of the accumulated lactate (15-20%) is transferred to the blood space (Turner *et al*., 1983; Milligan and Wood, 1986; Pagnotta and Milligan, 1991). Lactate appears in the blood more slowly, generally reaching peak levels 2 h after exhaustion and returning to resting levels after approximately 8 h. A significant portion of blood borne lactate has been observed to have a primarily oxidative fate; being utilized by red and cardiac muscle and other aerobic tissues. Some however, is re-synthesized to glycogen to supply the white muscle (Milligan and Girard, 1993).

Much of the energy required for initial muscular contraction is supplied by the hydrolysis of phosphocreatine (PCr). The decline in PCr as well as ATP as a result of

exhaustive exercise is quite variable, with reductions from 40% (Wang *et al*., 1994b) to as great as 90% (Milligan and Wood, 1986; Dobson and Hochachka, 1987; Schulte *et al*., 1992) have been reported. ATP has been shown to recover much faster than glycogen with concentrations typically being returned to pre-exercise conditions approximately 2 h post-exercise (Milligan, 1996).

The present study draws upon and the research presented in Chapter 2 in association with previous research conducted on metabolic recovery in trout. While it is known that chronic Cd exposure will not result in significant reduction in single swim U_{crit} values in we anticipate that it will result in reduced performance in a second swim challenge following a brief recovery period (0.5 h) as was observed in lake whitefish and brown trout in Chapter 2. If Cd exposure does reduce performance in the second swim then this would indicate that the metabolic effects of Cd result in impairment of either the recovery processes that restores fuel sources or the mobilization of additional fuel sources (or both). To evaluate these possibilities our experimental approach was to compare different durations of the recovery period, ranging from 0.5 to 6 h under the hypothesis that if the second swim impairment is due to recovery processes then longer recovery times should result in improved performance. If the effect of Cd is related to mobilization of additional fuel sources (e.g. lipids) then we would anticipate that longer recovery times would not dramatically improve performance in the second swim. Comparing different recovery times will also establish a time course of recovery of metabolic parameters and any effects of Cd.

The goals of this study were two fold; to determine if Cd exposure impaired sustained swimming ability following an initial swim challenge in rainbow trout and then

to understand the effect of varying the recovery duration between swims in relation to both critical swimming speed and metabolic recovery. Juvenile rainbow trout were selected for this study as they have well characterised responses to Cd exposure, including single swim tests, but the effect on repeat swimming has not been studied. As well, this species provides a good model organism for studying swim performance (Milligan, 2003). A waterborne Cd exposure concentration of 18 nM (2 ug L^{-1}) was chosen to provide a sublethal physiological disruption and acclimation response with no sustained swimming impairment during the initial swim (Hollis *et al*., 1999; McGeer *et al*., 2000a). Recent research with brown trout and lake whitefish (Chapter 2) showed that similar Cd exposure concentrations resulted in impaired swim capacity in the second swim (after 0.5 h recovery) and this was particularly evident after six days of exposure. Therefore exposures to rainbow trout in this study were for six days and recovery times between swims of 0.5, 1.5 and 6 h were compared.

3.3 Materials and Methods

3.3.1 Fish Culture

Juvenile rainbow trout were purchased from Rainbow Springs Trout Hatchery (Thamesford, ON). Fish were transported to the aquatics facility at Wilfrid Laurier University where they were held in 200 L polyethylene tanks with flowing fresh water for at least 1 month prior to experimentation. Fish were monitored daily and fed commercial fish food (Skretting, Moore Clarke Canada, Vancouver) at 2% of body weight daily. Culturing and experimentation was conducted in accordance with the Canadian Council

on Animal Care as reviewed and approved by the Wilfrid Laurier University Animal Care Committee.

3.3.2 Sublethal Cd Exposure

Exposures were in duplicate in 30 L tanks with a control group (no added Cd) and an exposure group (18 nM Cd) for 6 d. Rainbow trout (mass 46.5g ± 1.4g; fork length=15.4 cm ± 0.2 cm; maximum depth=3.4 cm ± 0.05 cm; maximum width=1.7 cm ±0.02 cm (mean ± SEM, n = 128) were transferred from the holding tanks and acclimated to the exposure conditions (without Cd) for a week before being non-selectively distributed to exposure tanks (Fig. A2). Exposure water was a combination of reverse osmosis processed water and well water, mixed to give a hardness of 84.2 mg $CaCO_3$ (524 ± 3.4 µM Ca, 206 ± 1.4 µM Na, 318 ± 2.1 µM Mg; mean ± SEM, n=160) with a mean pH of 7.2 ± 0.02, ambient temperature of 10.1 ± 0.2 ^{o}C and conductivity of 185 ± 2.3 µS (n=30). Fish were fed a daily ration of 2% body weight, with the exception of the day prior to swim testing. Cd exposures involved metering (QG6 pump, Fluid Metering Inc., Oyster Bay NY) a concentrated $CdCl_2$ solution into a mixing head tank before delivery to fish tanks and to initiate the exposure a sufficient volume of concentrated metal solution was added to the head tank and fish tanks to bring them to 18 nM Cd instantly. Header and fish tanks were supplied with continuous aeration to ensure mixing. Water samples (10 mL) were collected every 3 days and acidified to 1% with 16 N HNO_3 (trace metals grade; Fisher Scientific, Mississauga ON) and subsequently measured for total Cd concentration.

3.3.3 Swim Performance Testing

Fish were not fed for the 24 hours prior to the commencement of each swimming test to ensure a post absorptive state. Swim performance was evaluated after 6 days of exposure. Fish were non-selectively netted from the exposure tanks and transferred into a 30 L swim flume (D30 Loligo Systems; Tjele, Denmark) with a swimming chamber of 55x14x14 cm. The flow through the swim chamber flume was calibrated relative to the speed of the motor with a flow meter (HFA, Hüntzsch; Waiblingen, Germany) so that flow could be adjusted on a body length per second (bl s^{-1}) basis.

Prior to testing repeat swimming capacity in exposed (or control) fish a group of unexposed controls were given a single sustained swimming challenge to determine the U_{crit} value of control fish. Trout underwent a standard, single, exhaustive U_{crit} swimming challenge following the protocol of Jain *et al.* (1997). This produced a mean U_{crit} value of 4.5 ± 0.15 bl s^{-1}, allowing for the determination of swimming speeds based on a percentage of the full critical swimming speed.

The repeated swim test protocol was adapted from Jain and Farrell (2003) and is presented as a schematic diagram, in Figure 3.1. Briefly, in pairs, fish were measured for fork length and placed in the holding chamber of the swim flume, where they were given a 30 minute low velocity moving water (0.5 bl s^{-1}) acclimation period. Following this water velocity was increased to 1 bl s^{-1} and was then rapidly ramped to 3 bl s^{-1} representing approximately 65% of the final U_{crit} value of control fish, over a 10 minute period. At the completion of this velocity ramping, water velocity was maintained at 3 bl s^{-1} for 30 minutes. Following this, water velocity was then increased to 3.75 bl s^{-1} where the fish swam at this velocity for an additional 30 min (this velocity, represented

approximately 85% of the typical U_{crit} of control fish). Following the completion of this stage fish were given either a 0.5, 1.5 or 6 hour recovery period at a water velocity of 0.5 bl s^{-1}. Following the recovery period fish were once again subjected to the ten minute velocity ramping stage, until a swimming velocity of 3.0 bl s^{-1} was attained. Following this, water velocity was increased by 0.75 bl s^{-1} every 30 min until exhaustion. Exhaustion was defined as the time when a fish could no longer hold its position in the swim chamber and was swept against the rear grid despite being replaced within the chamber twice. The duration of the 30 min interval and water velocity at exhaustion was noted for determination of U_{crit}.

3.3.4 Sample Collection and Storage

To asses metabolic effects over the course of a repeat swim challenge, fish were sampled at four distinct time points; Before exercise (sampled directly from the exposure tanks; TP 1) at the end of the first swim (TP 2), at the end of the recovery period (TP 3), and after the exhaustive second swim (TP 4). Fish were euthanized with an overdose of tricaine methanesulfonate (MS222: 0.6 g L^{-1}; buffered with $NaHCO_3$), blotted dry with paper towel, weighed and measured for fork length, maximum depth and maximum width. Blood was collected via caudal puncture using heparinised syringes and was transferred to 1.5 ml centrifuge tubes containing 10 µl of heparinised saline (Li salt, Sigma Alderich Canada, Oakville ON). Whole blood samples were then centrifuged (Spectrafuge 16M, Labnet International, Edison NJ) at 13,000 rpm for 4 min. Plasma was collected, placed in liquid nitrogen, and subsequently stored at -80^{o}C. White muscle samples were removed from the right side of the fish from a region between the pectoral and dorsal fins. Muscle samples were immediately frozen in liquid nitrogen and later

stored at -80°C. Liver and head kidney samples were also removed, frozen in liquid nitrogen and stored at -80°C for metabolic analysis. Gill samples were removed and rinsed thoroughly in deionized water and blotted dry. Finally, additional samples of white muscle, liver and kidney were collected and, along with gills, stored at -40°C prior to characterization of Cd burden.

3.3.5 Analytical Techniques

3.3.5a Tissue Metal Bioaccumulation

Tissue metal bioaccumulation was measured in the gill, liver, kidney and white muscle following a protocol adapted from Janes and Playle (1995). Briefly, tissues were sub-sampled and amounts of approximately 100 mg were digested with 5:1 (volume: weight) 1N trace metals grade nitric acid (Fisher Scientific Canada, Ottawa ON) and heated to 80°C for 3 h. Digested samples were re-suspended by vortexing (30 s), left to settle for a minimum of 12 h, and centrifuged at 10 000g for 2 min before the supernatant was appropriately diluted in 1% nitric acid and analysed by graphite furnace atomic absorption spectrophotometry (SpectraAA, Varian Inc., Palo Alto, CA). Reference standards (TM 26.3 and TM 28.3) certified by the National Water Research Institute (Environment Canada) were used to verify analyzed values.

3.3.5b Plasma Ion Composition

Plasma ion compositions (Ca and Na) were analysed by flame atomic absorption spectrophotometry. Plasma was diluted 50 x for calcium and 1000 x for sodium with Milipore deionised water.

3.3.5c Metabolic Analysis

Unless noted, all enzymes and reagents were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Sample processing for metabolic determination of glycogen, ATP and PCR was adapted from Wilkie *et al.* (1997, 2001) following the protocol of Wang *et al*. (1994a). Briefly, a mortar and pestle was used to grind 100 mg (approx.) of each muscle sample to a fine powder in liquid nitrogen, followed by deproteination in 4 volumes of 8% perchloric acid (PCA) containing 1mM ethylenediaminetetraacetic (EDTA). Samples were left to deproteinate on ice for 10 min at which time a 100 µL sub-sample (sub-sample one) of this tissue and PCA slurry was removed, neutralized with 3M K_2CO_3, frozen in liquid nitrogen and stored at -80^{o}C for subsequent glycogen characterization. The remaining slurry (sub-sample two) was then centrifuged at 4^{o}C for 2 minutes at 10 000 x g. The supernatant was drawn off, weighed to the nearest 0.001g, and neutralized with 0.5 volumes of 2M KOH cocktail (0.4M imidazole and 0.4M KCl). The solution was vortexed and then centrifuged as before. The remaining supernatant was drawn off and stored at -80^{o}C for subsequent analysis for lactate and ATP.

Determination of muscle glycogen was done by adding one part 2M acetate buffer (sodium acetate, pH 4.5) to one part of the tissue + PCA slurry (sub-sample 1) and then incubating at 37^{o}C for 2 h with 40 units (U) of amyloglucosidase to convert glycogen to glucose. After this 25 µl of 70% PCA was added to terminate the reaction and approximately 75 µl of 3M K_2CO_3 was subsequently added to neutralize the sample. An additional sub sample was collected prior to the digestion with amyloglucosidase and was

flash frozen immediately in liquid nitrogen without undergoing the incubation steps. This sub-sample was used to determine free glucose. All samples were stored at -80 ^{o}C.

Glycogen concentrations were determined by adding 500 µL of a glucose cocktail solution comprised of 0.25 M triethylamin hydrochloride (TEA-HCl) containing Mg, NAD, ATP and glucose-6-phosphate dehydrogenase to the 20 µL of tissue homogenate. Concentrations were measured spectrophotometrically (Pharmacia LKB-Novaspec II) at 340 nm, following the addition of hexokinase (5 units) to the cocktail and measuring the appearance of NADH in the solution due to the conversion of glucose to glucose-6-phosphate. Both free glucose and total glucose were determined via this method and free glucose was subtracted from the total glucose concentration in the muscle tissue in order to yield white muscle glycogen concentrations which were expressed as glycosyl units on a tissue weight adjusted basis.

Sub-sample two was analysed enzymatically for lactate following the method of Bergmeyer (1983). In the presence of saturating NAD, lactate is converted to pyruvate via lactate dehydrogenase (LDH) and the change in absorbance at 340 nm due to conversion of NAD to NADH is used to quantify the initial lactate concentration. Here 10 µL of subsample two was added to a NAD solution (0.2 M hydrazine buffer and NAD) followed by the addition of the enzyme lactate dehydrogenase (5 units) which allowed for the determination of white muscle lactate based on the conversion of lactate to pyruvate resulting in the generation of NADH.

Sub-sample two was also used for the analysis of white muscle ATP. The basis of the ATP assay (Bergmeyer, 1983) uses the hexokinase catalyzed reduction of ATP and glucose to glucose-6-phosohate (G6P) and ADP (reaction 1). This G6P then acts as a

substrate, when combined with NAD to produce 6-phosphogluconate, H^+ and NADH in the presence of glucose-6-phosphate-dehydrogenase (G6P-dehase; reaction 2). The NADH produced is directly proportional (1:1 basis) to the original concentration of ATP in sub-sample 2. ADP produced in the first reaction reacts with creatine phosphate, in the presence of creatine kinase to regenerate ATP (reaction 3). By measuring the amount of ATP formed in this third reaction the levels of PCr in the sample can be determined using the previous two reactions.

$$\text{ATP} + \text{glucose} \xrightarrow{\text{Hexokinase}} \text{Glucose-6-phosphate} + \text{ADP} \qquad \text{(reaction 1)}$$

$$\text{Glucose-6-phosphate} + \text{NAD} \xrightarrow{\text{G-G-P dehase}} \text{6-phosphogluconate} + \text{NADH} + H^+ \qquad \text{(reaction 2)}$$

$$\text{Creatine phosphate} + \text{ADP} \xrightarrow{\text{Creatine Kinase}} \text{Creatine} + \text{ATP} \qquad \text{(reaction 3)}$$

3.3.5d Cortisol Analysis

Plasma cortisol was assessed using a cortisol enzyme-linked immunosorbent assay (ELISA) kit (402710, Neogen Corporation, Lexington, KY). The ELISA operates on the basis of competition between cortisol present in the sample and enzyme conjugate for a limited number of binding sites within each well of an antibody coated 96 well microplate. Cortisol was present in the plasma at sufficiently high concentrations and the optional extraction step (given in test protocol) was not required. Rather, plasma samples required dilution with the extraction buffer included in the kit. After the addition of sample/standard and enzyme conjugate to each well an incubation period of one hour was used so as to allow time for competition for binding sites. Following this the plates wells were cleaned three times with the supplied wash buffer so as to remove unbound

material. Finally, substrate was added to each well and was given an incubation time of 30 min so as to maximize enzyme conjugate binding (and hence colouration). Plates were measured at 650 nm on a plate spectrophotometer, with colour development being inversely proportional to the amount of cortisol present in the sample. Samples were measured in duplicate and the protocol provided with the test kit was followed with no deviations.

3.3.6 Calculations and Statistical Analysis

Tissue accumulation is expressed as Cd (nmol g^{-1} tissue wet weight), while plasma ions are shown as the element concentration in mmol L^{-1} of plasma. Tissue metabolite concentrations are also expressed on a tissue weight basis, as mmol kg^{-1} wet weight while plasma metabolites are shown as mmol L^{-1}. Plasma cortisol is expressed in ng mL^{-1}. The critical swimming speed at exhaustion was calculated in cm s^{-1} for each fish using the following formula:

$$Ucrit = Ui + \left(\frac{Ti}{Tii} \times Uii\right)$$

Where U_i is the highest velocity maintained for the whole interval (cm s^{-1}), U_{ii} is the velocity increment (cm s^{-1}), T_i is the time spent at fatigue velocity (min), and T_{ii} is the interval length (min: Brett, 1964). To normalize the data the individual U_{crit} values were divided by the fork length of the fish in order to produce a U_{crit} value in bl s^{-1}.

All data are presented as the mean ± 1 SEM and statistical analysis was performed using SigmaPlot (version 11.0, SPSS Inc., Chicago, IL). Comparisons between control and Cd exposed fish following 6 days of exposure were analyzed through individual T-Tests (ex. plasma ions, tissue specific Cd accumulation, and swim performance). Comparisons of multiple groups were computed by two-way ANOVA followed by the

Student-Newman-Keuls post-hoc test when required (ex. metabolites, cortisol). These methods were used for all pairwise comparisons of means among the different treatment groups. The limit of $P<0.05$ was used to indicate significance. Recovery curves for glycogen and ATP recovery and lactate clearance were computed by determining the change in concentration between TP 2 and TP3 for different recovery durations (0.5, 1,5 and 6 h) and expressing this as a percentage of the change that occurred between TP 1 and TP 2. Curve fitting parameters were used to compare the rate data (recovery rates for glycogen and ATP and for lactate the clearance rate) for Cd exposed fish and controls.

3.4 Results

3.4.1 Mortality and Plasma Ion Composition

Exposure to 18 nM Cd resulted in 25% mortality and all of these occurred within the first four d of exposure (Fig 3.2). Controls experienced 5% mortality (3 of 64 fish). Plasma ion concentrations, measured after 6 days of exposure, showed no significant difference between Cd exposed and control fish in either plasma Ca or Na concentrations (Fig. 3.3).

3.4.2 Tissue Metal Accumulation

After 6 days of exposure the gills, liver and kidney had all accumulated significant concentrations of Cd compared to unexposed controls (Fig. 3.4). The gill was the primary site of accumulation, with concentrations increasing 25 times above that of controls. Increases in Cd content of the liver and kidney were 3 and 4 times higher than their respective controls. Accumulation was not significant in white muscle.

3.4.3 U_{crit} Swimming Ability

In a single swim challenge, the mean U_{crit} value for unexposed trout was 4.5 ± 0.15 bl s^{-1}. This allowed for the water velocity during the ramping stage (65% U_{crit}) to be calculated to 3.0 bl s^{-1}. The target swim speed for the first swimming challenge (85% U_{crit}) was calculated at 3.75 bl s^{-1}. U_{crit} values determined in the second swim of the repeat swim trial demonstrated that swim performance varied with recovery time for Cd exposed trout but not controls (Fig. 3.5). Unexposed controls had consistent U_{crit} values of 4.4 ± 0.3, 4.3 ± 0.2 and 4.4 ± 0.2 bl s^{-1} (n=8 for each) for 0.5, 1.5 and 6 h of recovery and these were not significantly different from the single swim mean U_{crit} value. Given a 0.5 h recovery Cd exposed fish showed a significant reduction in swim performance (U_{crit} of 3.0 ± 0.3 bl s^{-1}, n=8) however, when the recovery period was increased to 1.5 h the mean U_{crit} was 5.2 ± 0.2 bl s^{-1} (n=8), a significant increase compared with controls and the single swim challenge (Fig 3.5). After 6 h of recovery there was no significant difference in secondary swim performance between control and Cd exposed fish with the U_{crit} for the latter being 4.6 ± 0.2 bl s^{-1} (n=8, Fig 3.5).

3.4.4 Metabolic Analysis

3.4.4a White Muscle Glycogen and Lactate

Prior to exercise (TP 1) both control and Cd exposed fish had similar white muscle glycogen concentrations of 11.2 ± 2.0 mM kg^{-1} and 12.1 ± 1.6 mM kg^{-1} (mean ± SEM, n=8 for each) respectively (Fig 3.6). Following the first swimming challenge, both control and Cd exposed fish showed a significant reduction in white muscle glycogen stores between time points 1 and 2. After the 0.5 hour recovery period there was no recovery of white muscle glycogen stores in either treatment group. When the recovery

period was increased to 1.5 hours a significant recovery of 42% of white muscle glycogen was observed in fish exposed to 18 nM Cd. In control fish however, there was only a 7% increase in white muscle glycogen concentrations, which was not significantly different from glycogen levels at the end of the first swim. Finally, after a 6 hour recovery there were no significant difference between treatments with controls showing a 57% recovery and Cd exposed fish a 51% recovery of depleted white muscle glycogen stores.

Parallel to the reduction and recovery pattern for white muscle glycogen during the first swim and subsequent recovery periods there was an inverse pattern of lactate accumulation and clearance in the white muscle. Lactate levels were lowest prior to exercise (TP 1). Control fish had a mean lactate concentration of 9.4 ± 1.7 mmol kg^{-1}, while fish exposed to 18 nM Cd had a significantly higher resting lactate concentration at 15.6 ± 1.3 mmol kg^{-1} (Fig. 3.7). Both treatment groups showed significant increases in lactate concentrations as a result of the first swimming challenge (TP 2). In controls the lactate concentrations rose to 15.3 ± 1.6 mmol kg^{-1} (a 39% increase, n=8), while Cd exposed fish had an average muscle lactate concentration of 22.0 ± 1.2 mmol kg^{-1} (a 29% increase n=8).

After 0.5 hours of recovery lactate levels in control fish remained elevated with very little clearance occurring as the mean concentration actually increased slightly from 15.3 ± 1.6 mmol kg^{-1} to 16.6 ± 1.6 mmol kg^{-1} (n=8). Cd exposed fish however, showed a rapid recovery. With a recovery period of only 0.5 h, white muscle lactate levels were significantly decreased from 22.0 ± 1.2 mmol kg^{-1} to 15.8 mmol kg^{-1}, representing a nearly 100% recovery and a return to pre-exercise levels. Following a 1.5 hour recovery,

white muscle lactate was still elevated in control fish, with minor reductions occurring only after 6 h of recovery was given. Reductions of muscle lactate were much more rapid in trout exposed to 18 nM Cd compared to controls. Lactate concentrations declined rapidly, by 1.5 h dropping to 8.7 ± 1.7 mmol kg^{-1} (n=8), which was significantly lower than levels before the first swim. Concentrations remained low through to the 6 h of recovery (Fig. 3.7B). After the second swim challenge, lactate levels in Cd exposed fish were greatly increased and were significantly greater than in controls, which showed only minor increases.

3.4.4b Liver Glycogen and Lactate

Prior to exercise liver glycogen levels were significantly lower in Cd exposed fish (57.6 ± 8.5 mmol kg^{-1}: Fig. 3.8) relative to controls (91.9 ± 9.2 mmol kg^{-1}, n=8). As in white muscle, liver glycogen levels declined significantly as a result of the first swimming challenge and there was no recovery of liver glycogen stores following a 0.5 hour recovery period in either treatment. After 1.5 hours of recovery, fish exposed to Cd showed significantly greater recovery (68.1 ± 10.1 mmol kg^{-1}) compared with controls which showed no recovery of glycogen stores from levels at the end of the first swim (40.6 ±7.2 mmol kg^{-1}). After 6 hours of recovery there was partial restoration of liver glycogen stores in controls (58.7 ± 7.2 mmol kg^{-1}), however, recovery in Cd exposed fish was still significantly greater than controls (102.8 ± 14.3 mmol kg^{-1}).

Heptic lactate levels in control and Cd exposed fish were similar prior to exercise. As observed in the white muscle, the initial swim challenge resulted in increased lactate concentrations in the liver. These increases were much more prominent in Cd exposed fish (56% increase from mean resting values) compared to unexposed controls (27%

increase: Fig. 3.10). Cd exposed fish cleared the lactate that accumulated in the liver very quickly, a 0.5 h recovery period was sufficient to reach pre-exposure levels while a longer recovery time was required in controls (Fig 3.9).

3.4.4c Plasma Glucose

Plasma glucose remained constant in control fish over the course of the repeat swim challenge, regardless of recovery duration (Fig. 3.10). Following a 0.5 h recovery period, plasma glucose concentrations were similar to those observed in controls, and similar to concentrations observed prior to exercise. After 1.5 h of recovery the plasma glucose levels of Cd exposed fish were significantly decreased (8.54 ± 0.7 mmol L^{-1}) relative to controls (18.3 ± 0.4 mmol L^{-1}). The plasma glucose of Cd exposed fish was still depressed after 6 h of recovery (11.7 ± 1.7 mmol L^{-1}).

3.4.4d Plasma Lactate

Lactate levels in the plasma (Fig. 3.11) were significantly lower than those observed in the white muscle or the liver. Circulating lactate concentrations in control fish were significantly higher (0.6 ± 0.06 mmol L^{-1}) than in fish exposed to 18 nM Cd (0.3 ± 0.07 mmol L^{-1}, n=8). Both exposure and control fish showed significant increases in circulating lactate as a result of the initial swim challenge. Lactate levels in the plasma were returned to pre-exercise conditions in control fish after 6 h of recovery. After 6 h of recovery the plasma lactate of Cd exposed fish remained elevated at concentrations roughly double those observed prior to exercise.

3.4.4e White Muscle ATP

Prior to exercise, the Cd exposed fish had significantly lower ATP levels (5.0 ± 0.6 mmol kg^{-1}) in the white muscle compared to controls (6.8 ± 0.4 mmol kg^{-1}). ATP stores in the white muscle were depleted to 3.4 ± 0.7 mmol kg^{-1} (50%) in controls and to 2.3 ± 0.5 mmol kg^{-1} (46%) in d exposed fish following the first swim challenge (Fig. 3.12). Following a 0.5 h recovery period there was a significantly increase in white muscle ATP in Cd exposed fish (5.7 ± 0.6 mmol kg^{-1}) relative to controls (3.6 ± 0.9 mmol kg^{-1}: Fig. 3.10A), with significant improvement in ATP stores not observed in controls until after the 1.5 hour mark, at which time Cd exposed fish still showed significantly higher levels, with levels returning to those observed prior to exercise. Pre-exercise ATP levels were re-established in both treatment groups after a 6 hour recovery.

3.4.4f Liver ATP

ATP levels in the liver were quite low, compared to those in the white muscle. ATP supply did not change within the liver of control fish, with concentrations remaining constant throughout rest, exercise and recovery (Fig. 3.13). Fish exposed to Cd exhibited a significant decrease in ATP stores as a result of the first swim challenge; however, concentrations were restored to pre-exercise levels after only 0.5 h of recovery, and did not change following the second swim challenge.

3.4.5 Plasma Cortisol

Plasma cortisol concentrations were lowest prior to exercise, and increased significantly during the first swim challenge in both control and Cd exposed fish. Cd exposure resulted in significantly lower cortisol concentrations compared to controls (Fig 3.14). Recovery for up to 6 h resulted in few changes in plasma cortisol, with only

controls showing a reduction, however concentrations were still roughly double those observed prior to swimming. The second swim challenge did not resulted in any further significant changes.

3.5 Discussion

3.5.1. Mortality and Plasma Ion Concentrations

In Cd exposed fish, all mortalities occurred within the first four days of exposure (Fig.3.2). Cd exposure did not result in alterations in plasma Ca or Na after 6 d (Fig.3.3). Ionoregulatory disruption is considered the primary cause of acute toxicity in fish exposed to metals (Wood, 2001). Specific to Cd exposure, acute toxicity has been associated with Ca inhibition. Upon exposure, Cd accumulates within the PNA^+ MR of the branchial epithelium, causing an inhibition of Ca^{2+}-ATPase transporters, inhibiting Ca^{2+} transport (Galvez *et al*., 2006). Very low concentrations of waterborne Cd can inhibit Ca^{2+} transport as Ca^{2+}-ATPase present on the basolateral membrane has an extremely high affinity for Cd (I_{50}=3nmol^{-1}: Verbost *et al*. 1988). Disturbances to Na^+ homeostasis associated with Cd exposure are typically less prominent and have a shorter duration than disturbances to Ca^{2+} homeostasis (McGeer *et al*., 2000a).

In the present study, Cd exposures were 6 days in duration; this corresponds with the initial shock phase of the damage-repair model proposed by McDonald and Wood (1993). This phase is associated with initial physical damage as a result of metal exposure. More specifically, this phase is characterized by accumulation of metal at the gills which results in homeostatic disruptions, including alterations to ionoregulation

(McDonald and Wood, 1993). Typically, the initial shock phase lasts several days to one week before transitioning into the second, or recovery, stage.

Following 6 days of exposure mean plasma Ca levels of rainbow trout did not differ from controls. The acute effects of Cd exposure on plasma Ca have been well characterised. Pratap *et al*. (1989) observed that 2 days of exposure to 89 nM Cd resulted in a 40% reduction in plasma Ca^{2+} in tilapia (*Oreochromis mossambicus*) in low Ca water (0.2 mM) and a 32% reduction in high Ca water (0.8 mM). These reductions were lessened by the 4^{th} day of exposure, where a 31% reduction was observed in low calcium water and a 20% reduction in higher calcium water. These reductions were no longer observed after 14 days of exposure (Pratap *et al*., 1989). In rainbow trout, a 44% reduction in plasma Ca was observed after 3 days of exposure to 89 nM Cd by Chowdhury *et al*. (2004). When Cd exposures were extended to 6 days, Pelgrom *et al*. (1995) observed varying effects to plasma Ca in tilapia. Here, fish exposed to the highest Cd concentration (623 nM Cd) had plasma Ca concentrations approximately half those of control fish. Moderate exposure (311 nM Cd) did not cause an alteration in plasma Ca relative to controls, while fish exposed to the lowest dose (178 nM Cd) actually showed a significant increase in plasma Ca after 6 days of exposure. Disruptions of plasma Ca homeostasis are typically transient and eliminated during chronic exposure. Hollis *et al*. (2000) observed that plasma Ca of juvenile rainbow trout was not reduced following a 30 day exposure to 1nM Cd in soft water.

Disturbances to plasma Na concentrations as a result of Cd exposure are typically less prominent, shorter in duration and more difficult to predict than alterations to Ca homeostasis (McGeer *et al*., 2000a). In the present study, plasma Na concentrations were

not significantly affected by 6 d of exposure to 18 nM Cd. Several other studies have reported no alterations to plasma Na concentrations as a result of Cd exposure (Pratap *et al*., 1989; Verbost *et al*., 1989; Baldisserotto, 2004). Chowdhury *et al*., (2004) observed a significant increase in plasma Na as a result of 2 days exposure to (89 nM Cd) in juvenile rainbow trout, while Pelgrom *et al*. (1995) observed a significant reduction in plasma Na in tilapia exposed to 623 nM Cd for 6 days. The primary focus of the experimental design was based on developing an understanding of swimming impairment. To clearly establish the ionoregulatory impact and subsequent response of trout to Cd, multiple samplings over the course of a longer exposure would be required.

3.5.2. Tissue Specific Cd accumulation

After 6 d of exposure to 18 nM Cd rainbow trout accumulated significant concentrations of Cd in the liver, kidney and gills (Fig.3.4) with the greatest concentrations in the latter. This is expected in waterborne exposures and Hollis *et al*. (1999, 2000) identified gills as the site of the greatest Cd accumulation in juvenile rainbow trout compared to other tissues. McGeer *et al*. (2000b), using rainbow trout exposed to 27 nM Cd, observed significant accumulation in the gills that stabilized after 5 d of exposure. Other tissues, particularly the kidney and liver showed slower rates of accumulation but longer times to saturation and by d 65 of exposure both had surpassed the concentration in the gill. Szebedinsky *et al*. (2001) similarly observed that following 30 d of exposure to 18 nM Cd the kidney was the primary site of Cd accumulation in rainbow trout, with gills having the next highest concentration. Therefore the d 6 tissue concentrations in this study are consistent with the pattern of rapid uptake of Cd into gills with slower partitioning from gills to the blood and then accumulation to internal tissues (Wicklund Glynn, 1991).

There was no significant accumulation of Cd in white muscle. This is similar to the study of de Conto Cinier *et al*. (1999) where levels in white muscle of carp (*Cyprinus carpio*) were not significantly different from controls until more than 3 months of Cd exposure had elapsed. The differences in metal accumulation among internal tissues may reflect the differences in partitioning (i.e. perfusion rates) as well as the mechanisms of uptake and elimination, which are not well understood for Cd.

3.5.3 Repeat Swimming Ability

Control fish performed equally as well in the second swim challenge as they had in a single U_{crit} swim trial alone, regardless of the recovery duration. However, in Cd exposed fish the effect of the different recovery duration significantly affected performance in the second U_{crit} swim trial (Fig. 3.5). As anticipated from the results of chapter 2, a 0.5 h recovery period resulted in a significantly reduced U_{crit} value in Cd exposed fish in the second swim. When recovery duration was extended to 1.5 h the Cd exposed fish had a significantly higher U_{crit} value than controls and following a 6 h recovery both control and Cd exposed fish performed equally.

The effect of Cd exposure on the performance in a single swim challenge has been shown not to impact U_{crit} swimming ability of rainbow trout in the lab (Hollis *et al*., 1999; McGeer *et al*., 2000a; Cunningham and McGeer, 2009) as well as swimming stamina (Hollis *et al*., 1999). In the field, Rajotte and Couture (2002) observed equal U_{crit} swim performance in perch (*Perca flavescens*) from across a gradient of Cd contaminated lakes.

While the impacts of Cd exposure on single U_{crit} performance have been characterized the effect of Cd exposure on other types of swimming, and for a variety of

swimming challenges has received limited attention. To the best of our knowledge, with the exception of the data presented in Chapter 2 the effect of Cd exposure on recovery and the ability to undergo successive swim challenges has not been evaluated. Although it has received limited attention, the use of repeated swim tests has proved to act as a more sensitive indicator of physiological impairment than a single swim challenge alone. Jain and Farrell (2003) reported a significant decrease in U_{crit2} in warm water (17 ^{o}C) acclimated rainbow trout compared with controls (5 ^{o}C) following a 40 minute recovery period; a difference that was not observed following the first swim trial. Jain *et al*. (1998) observed sockeye salmon (*Oncorhynchus nerka*) exposed to dehydroabietic acid (DHA) performed equally as well as controls in an initial U_{crit} challenge. However, in a second swim trial, following a 40 minute recovery period exposed fish had a reduced U_{crit}, as measured by the recovery ratio relative to controls. The recovery ratio is the ratio of the second and first U_{crit} values. Ratios less than one represent a decrease in performance in the second swim. The calculation of a recovery ratio requires that both swim trials be exhaustive (Jain *et al*., 1998). In the present study, the initial swim was selected to reproduce more naturally occurring conditions and thus was not fully exhaustive but rather to 85% of a single U_{crit} and thus the recovery ratio cannot be calculated. However, alterations in swimming ability are clearly observable through significant differences in U_{crit} performance in the second swim challenge when compared with controls. It is also notable that all fish successfully completed the first swim, suggesting some consistencies with previous studies demonstrating no impact on single swim challenges.

3.5.4 Metabolic Effects

3.5.4.1. Initial metabolic effects

White muscle glycogen concentrations were highest prior to exposure and there were no significant differences between exposed and control fish at TP1. These represented the highest concentrations observed over the course of the repeat swimming protocol (Fig.3.6). There were no differences between control and rainbow trout exposed to 18 nM Cd. Lowe-Jinde and Niimi (1984) found no alterations to white muscle glycogen in rainbow trout exposure to 36 nM Cd but at 320 nM Cd, concentrations were reduced to half that of controls after 3 days of exposure. Similarly, Cicik and Engin (2005) observed a 24% reduction in white muscle glycogen concentrations compared with controls in common carp exposed to 9 µM Cd.

While no reduction in glycogen stores was observed in the white muscle, in the liver glycogen concentrations were significantly reduced in Cd exposed fish compared with controls (Fig. 3.8). A similar reduction was observed by Cicik and Engin (2005) in common carp. Here, Cd reductions in the liver were as great as 29% at doses up to 9 µM Cd. Lowe-Jinde and Niimi (1984) also observed a reduction in liver glycogen to 1/3 that of control rainbow trout as a result of exposure to 320 nM Cd. However, exposure to 36 nM Cd did not affect muscle or liver glycogen (Lowe-Jinde and Niimi, 1984). The observed decrease in liver glycogen may signal that stored glycogen is being utilized to provide energy required for acclimation, while stores within the white muscle remain unaffected to maintain performance in high intensity exercise, such as may be required for predator avoidance or prey capture. Reductions in resting liver glycogen concentrations may be as a result of increased stimulation of enzymes required for

glycogenolysis. Soengas *et al*. (1996) observed an increase in circulating glucose which was associated with increased glycogenolysis in the liver as a result of Cd exposure. This corresponded with increased activity of glycogen phosphorylase (GPase *a*) and decreased activity of glycogen synthase (GSase *a*) in Cd exposed fish (Soengas *et al*., 1996). The ratio of GSase reduction to GPase activation was consistent with glycogen depletion.

Observations of reduced liver glycogen were accompanied by increases in plasma glucose (Soengas *et al*., 1996) but this was not observed in the present study where plasma glucose levels were slightly, but significantly reduced in Cd exposed fish (Fig. 3.10). This is in contradiction to Cicik and Engin (2005) who observed a dose dependent increase in serum glucose as a result of Cd exposure. Plasma glucose concentrations can fluctuate diurnally and also in relation to feeding status and in tank activity, which may explain this variation in results.

Lactate concentrations in the white muscle were increased in Cd exposed fish relative to controls while lactate concentrations in the liver were not affected by Cd exposure (Fig.3.7). In the plasma a significant decrease in lactate concentrations was observed in Cd exposed fish relative to controls. This is contrary to Tort and Torres (1988) where no variation between control and Cd exposed dogfish (*Scyliorhinus canicula*) was observed. A study by De Smet and Blust (2001) also identified that Cd exposure did not affect resting concentrations of muscle and plasma lactate in common carp (*Cypinus carpio*) exposed to up to 178 nM Cd.

While common in mammals, the shuttling of accumulated lactate between the white muscle and the liver, via the blood stream is less well defined. The majority of lactate is maintained within the tissue to fuel in situ glycogen re-synthesis. Particularly

within the plasma, metabolite levels can vary significantly as a result of feeding status and in tank activity, which could explain the observed elevation of lactate in control fish. ATP stores in the white muscle (Fig.3.12) of resting Cd exposed fish were significantly reduced while ATP stores in the liver (Fig. 3.13) were observed to be slightly, but significantly increased in resting, Cd exposed fish. A similar reduction in ATP was observed in brown trout in the previous chapter.

3.5.4.2. Effects of sub-maximal swimming

The initial swim challenge to 85% U_{crit} was non-exhaustive; however this was sufficient to require recruitment of both aerobic and anaerobic metabolic pathways in order to supply energy demands. Richards *et al*. (2002b) described 90% U_{crit} as an unsustainable level of swimming and associated it with significant recruitment of anaerobic metabolism and subsequent depletion of white muscle metabolic fuel stores. Reductions in glycogen stores and increases in lactate indicate that oxidative phosphorylation of carbohydrates (glycogen) is occurring (Richards *et al*., 2002b). Following the initial swim challenge to 85% U_{crit} both white muscle glycogen (Fig. 3.6) and liver glycogen (Fig. 3.8) concentrations decreased significantly with no significant differences between treatments. Accompanying decreases in glycogen was significant accumulation of lactate in both the muscle (Fig. 3.7) and the liver (Fig. 3.9).

A study on sub-maximal swimming in rainbow trout by Burgetz *et al*. (1998) evaluated metabolic parameters following swimming to 70, 80, and 100% U_{crit}. Non-exhaustive swimming to 80% U_{crit} resulted in a significant increase in white muscle lactate. The effect of swimming to 80% U_{crit} was similar to that observed in fully exhausted fish (100% U_{crit}). Burgetz *et al*. (1998) also inferred a corresponding reduction

in muscle glycogen based on a ratio (2 mol lactate: 1 mol glycosyl unit) described by Moyes *et al*. (1990) and Arthur *et al*. (1992), as a result of swimming at both speeds. Such alterations, as well as those observed in the present study indicate that high intensity but sub-maximal swimming (85% U_{crit}) cannot be supported aerobically and requires the recruitment of substrate level phosphorylation. A significant increase in white muscle lactate as a result of exhaustive exercise in rainbow trout was similarly observed by Kieffer *et al*. (1994). The increase observed in the present study (10 nmol kg^{-1}) is less than that reported in Kieffer *et al*. (1994; 30 nmol kg^{-1}) however, measurements in the latter case were on exhausted fish while in this study they were not. A significant increase in circulating lactate was also observed (Fig. 3.11) while plasma glucose was unaffected. The alterations in metabolic parameters observed in the present study indicate that although sub-maximal, swimming to 85% U_{crit} required significant recruitment of anaerobic metabolic processes though the activation of substrate level phosphorylation.

In the white muscle, both control and Cd exposed fish utilized a significant amount of stored ATP (Fig. 3.12), while ATP decreases in the liver were only significant in Cd exposed fish (Fig. 3.13). Burgetz *et al*. (1998) similarly observed depletions in ATP (as well as phosphocreatine) in white muscle as a result of swimming at 80 and 100% U_{crit}. Kieffer *et al*. (1994) observed up to a 4.3 mmol kg^{-1} decrease in muscle ATP following exhaustive exercise in rainbow trout. In conjunction with the observed reductions in muscle and liver glycogen stores, and associated accumulation of lactate, the utilization of stored ATP was anticipated. At low swimming speeds ATP demands are typically low, and can be met by oxidative phosphorylation, however, as demand increases, even with recruitment of anaerobically derived ATP energy demands may not

be met, requiring that stored ATP be utilized in order to sustain high intensity activity (Kieffer, 2000).

3.5.4.3. Metabolic Recovery and its Relation to Repeat Swim Performance

Metabolic recovery following high intensity exercise in fish is a lengthy process dependent upon multiple factors including duration and intensity of exercise as well as species specific differences. Full metabolic recovery can take in excess of 12 hours to complete (Milligan, 1996; Kieffer, 2000). In salmonids, ATP and PCr stores are generally replenished relatively rapidly following exhaustion, with concentrations matching those observed prior to exercise within the first one to two hours post exercise (Wood, 1991; Wang *et al*., 1994b: Kieffer, 1995; reviewed by Milligan 1996). The replenishment of metabolic fuel sources, such as glycogen, and the associated removal of lactate can be a much longer process. Varying recovery times have been reported, however the majority describe 6 to 12 hours as the minimum period of time required for recovery of these parameters in salmonids (Wendt and Saunders, 1973; Milligan and Wood, 1986; Schwalme and Mackay, 1991; Kieffer *et al*., 1994; Wang *et al*., 1994b; Wilkie *et al*., 1997; Milligan, 2003).

ATP in the white muscle of control fish remained depressed at 0.5 and 1.5 h following the first swim and was fully restored after 6h (Fig. 3.12). Although not significantly increased after 1.5 hours a trend towards recovery seemed evident. In Cd exposed fish, recovery of ATP stores occurred much more rapidly. In fish exposed to 18 nM Cd the concentrations of ATP in white muscle were restored to pre-exercise concentrations after only 0.5 h of recovery. Liver ATP stores in control fish were not altered by the initial swim, however, Cd exposed fish exhibited a significant reduction in

liver ATP as a result of exercise. ATP concentrations in Cd exposed fish were returned to pre-exercise levels after 1.5 h of recovery (Fig. 3.13).

As a result of exercise, white muscle glycogen stores can be decreased as much as 90% with accompanying increases in lactate (Wang *et al*., 1994b). Some of the lactate produced will be transferred into the blood stream, where peak levels are typically observed 2 hours following exercise and cleared after 8 h (Milligan, 1996). The majority of the lactate produced however, is retained within the white muscle for *in situ* glycogen synthesis, as such reductions in lactate are accompanied by corresponding increases in glycogen stores. In the present study, elevated lactate levels as a result of exercise were slow to clear from white muscle, with only partial recovery observed after 6 h in control fish (Fig. 3.7). Elevations in liver lactate were less prominent, but did not show any reduction over the 6 h recovery period in control fish (Fig. 3.9). Farrell *et al.* (1998) observed that muscle lactate levels in normoxic sockeye salmon were significantly elevated as a result of an initial U_{crit} swim challenge. During the subsequent two U_{crit} challenges, separated by 45 min recovery periods lactate levels remained elevated (Farrell *et al*., 1998). This may indicate that 45 minutes was not a sufficiently long recovery period for the clearance of muscle lactate. Based on the review of Milligan (1996) a recovery period of 6 hours (as we had in this study), corresponds with partial, but not complete recovery of lactate in unexposed (control) trout. As a result of exposure to 18 nM Cd the clearance of lactate in the white muscle and the liver occurred much more rapidly than was observed in control fish. In the white muscle lactate levels were significantly reduced 1.5 h following exercise, while lactate levels declined in the liver after only 0.5 h. In the plasma, lactate levels declined quickly in Cd exposed fish, with

significant decreases observed after 1.5 h, while after 6 h only partial recovery was observed in control fish.

Reductions in lactate typically correspond with the reestablishment of glycogen stores. In the present study, the restoration of glycogen occurred in a similar fashion to the clearance of lactate. In the white muscle, glycogen levels were only significantly restored after 6 h of recovery. After 6 h of recovery muscle glycogen levels had reached ~ 65% of pre-exercise levels. A similar result was observed by Scarabello *et al*. (1991) who noted that whole body glycogen levels fell to 13% of resting levels in juvenile rainbow trout as a result of exhaustive exercise and then returned to 65% of resting levels after 4 hours. Whole body glycogen then remained at 65% of pre-exercise values over the course of a 24 h recovery period (Scarabello *et al*., 1991). Milligan (2003) also observed a slow recovery of muscle glycogen with ~50% recovery observed after 4 h in trout. In Cd exposed fish the reestablishment of glycogen stores occurred much more rapidly than was observed in controls. In both the white muscle and the liver no recovery was observed after 0.5 h, however by 1.5 h there was a significant increase in muscle glycogen, while controls did not show significant recovery until at least 6 h post exercise.

When given a 0.5 h recovery Cd exposed fish had a significantly lower U_{crit} than did control fish. This reduction in swim performance does not appear to be as a result of alterations to anaerobic metabolism. After 0.5 h ATP stores were increased in Cd exposed fish but not in controls, while other metabolic parameters such as glycogen or lactate remained unchanged. The observed reduction in swimming ability by Cd exposed fish may be as a result of a number of altered physiological parameters associated with Cd exposure. For example, it is possible that aerobic recovery may be impacted by Cd

exposure. As the initial stages of each swimming trial utilize predominantly aerobic metabolism, if Cd exposure limits recovery of substrates (lipid derived) then oxidative phosphorylation cannot be employed for as long. This will then cause an early recruitment of substrate level phosphorylation, which in turn could cause the observed reduction in secondary swim performance.

Interestingly, when the recovery period was extended to 1.5 h trout exposed to 18 nM Cd performed significantly better in the second swim challenge than did controls. This augmented swimming ability may be as a result of a faster recovery rate in Cd exposed fish. After 1.5 h of recovery a significant replenishment of ATP and glycogen in both the muscle and liver were observed, along with significant reductions in lactate in both tissues. This is compared with controls in which recovery of these parameters was primarily not observed until after 6 h and was still often incomplete. The increased recovery of metabolic fuel sources, such as glycogen allows for an increase in substrate required to power high intensity exercise. This in turn, may correlate to increased swim performance in Cd exposed fish. Following a 6 h recovery period, the majority of metabolic parameters measured in controls are similar to in Cd exposed fish. As a result, no variations in secondary swim performance were observed between treatments

3.5.5. Plasma Cortisol

Stressor induced elevations in plasma corticosteroid levels is an evolutionarily conserved response though to be essential to the restablishment of homeostasis (Sapolsky *et al*., 2000). Prior to exercise, plasma cortisol levels were relatively low, falling within the standard range of resting plasma cortisol levels for trout (10-50 ng mL^{-1}: Milligan, 1996). As a result of the first swim challenge cortisol levels increased significantly in

both treatments, however, the observed increase was much greater in control fish (Fig. 3.14). The increase in circulating cortisol in Cd exposed fish was only approximately 60% of that observed in controls. Following exercise, cortisol levels can be up to five times higher than at rest, with levels remaining elevated for more than 12 h post exercise (Milligan, 1996; Kieffer, 2000; Milligan, 2003). This was observed in the present study as plasma cortisol remained elevated in both treatments after 0.5 and 1.5 h of recovery. After 6 h, cortisol levels showed a significant decrease in controls, however, concentrations were still double those observed in resting fish. The plasma cortisol concentrations of Cd exposed fish were not altered after 6 h of recovery.

At rest and prior to swimming, little difference in plasma cortisol concentrations was observed. A similar result was obtained by Ricard *et al*. (1998) who reported no alterations in the plasma cortisol levels of juvenile rainbow trout exposed to 41 nM Cd over a 30 day period. Sandhu and Vijayan (2011) also observed that Cd exposure did not affect basal cortisol secretions by the head kidney of rainbow trout. However, Pratap and Wendelaar Bonga (1990) observed significant increases in plasma cortisol in tilapia exposed to 89 nM Cd. Here, plasma cortisol levels in Cd exposed fish increased to nearly 11 x those observed in controls.

In the present study, plasma cortisol levels were significantly increased as a result of the first swim challenge. However, the increase observed in Cd exposed fish was smaller than in control fish, with cortisol concentrations only reaching 60% of that observed in controls. Similarly, Scott *et al*. (2003) observed inhibition of the cortisol response in juvenile rainbow trout exposed to 18 nM Cd when subjected to an alarm cue. *In vivo*, Lacroix and Hontella (2004) found that Cd exposure eliminated cortisol secretion

through the inhibition of adrenocorticotropic hormone (ACTH) in the interrenal cells of the kidney. More specifically, Cd disrupts the signalling pathway of cortisol synthesis at a step prior to the formation of pregnenolone (Lacroix and Hontella, 2004). The effect of Cd exposure was also studied on an *in vitro* model by Sandhu and Vijayan (2011) who observed a dose related suppression of ACTH-mediated cortisol production in the head kidney of rainbow trout. This suppression of the cortisol response correlated with significant reductions of melanocortin 2 receptor (MC2R), steroidogenic acute regulatory protein (StAR) and cytochrome P450 side chain cleavage enzyme (P450scc) RNA levels. Such inhibition indicates that Cd exposure disrupts expression of genes critical for the synthesis of corticosteroid hormones, particularly that MC2R, the required gene for the ACTH induced first step of corticosteroidogenis is the target of Cd (Sandhu and Vijayan, 2011).

Cortisol is a hormone which has been shown to increase the metabolic rate of fish species (Chan and Woo, 1978). Increases in circulating cortisol have been associated with the mobilization of lipids for gluconeogenisis from amino acids (Vijayan *et al*., 1996). Following exhaustive exercise elevations in plasma cortisol appear to inhibit glycogenesis, as there is no net glycogen synthesis in the muscle during periods of elevated circulating cortisol (Pagnotta *et al*., 1994). When the characteristic increase in plasma cortisol following high intensity exercise is inhibited, muscle glycogen stores are replenished more quickly than in the presence of cortisol (Pagnotta *et al*., 1994).

Cortisol has also been implicated in the clearance of lactate following high intensity exercise. When a standard cortisol response occurs, complete clearance of lactate from the white muscle generally takes in excess of 8 hours to complete. However,

Pagnotta *et al*. (1994) found that when the cortisol response was inhibited using metyrapone (2-methyl-1, 2-di-3-pyridyl-1-propanone) lactate did not accumulate in a characteristic manner. Fish with an inhibited cortisol response accumulated less than half of the lactate observed in controls. Clearance of lactate was also impacted, with fish with an inhibited cortisol response recovering much faster than controls. Here, lactate took in excess of 8 hours to be fully cleared from the white muscle of control fish; while in the absence of cortisol full lactate clearance occurred after only 2 hours (Pagnotta *et al*., 1994). This indicates that increasing plasma cortisol concentrations can influence metabolic recovery, and actually produce an inhibitory effect (Milligan, 1996; Milligan, 2003).

Exposure to 18 nM Cd resulted in a significant decrease in circulating cortisol in juvenile rainbow trout. Also observed was an increased metabolic recovery rate in Cd exposed fish. The more rapid recovery from exercise observed in Cd exposed fish could be associated with inhibition of the cortisol response. As the cortisol response has been implicated in regulating the rate at which metabolic recovery occurs. When the cortisol response is eliminated, the restoration of metabolic fuel sources and elimination of muscle lactate occurred in less than one quarter of the time that was required for controls (Pagnotta *et al*., 1994). In the present study, the cortisol response was significantly reduced relative to controls and this may be associated with the observed increase in recover rate of metabolic parameters, which may correlate to increased swimming ability following a initial swim challenge and 1.5 h recovery.

3.6 Conclusions

The initial objective of this research project was to establish the acute effects of 6 d of exposure to 18 nM Cd. The dose of 18 nM Cd was selected to be sublethal but still cause significant homeostatic disruptions. This was apparent by the 25% mortality in Cd exposed fish. No alterations to plasma ion (Ca and Na) concentrations were observed, however, ions were only evaluated after 6 days of exposure. Full analysis of the ionoregulatory effect of Cd exposure would require much more extensive sampling. Cadmium was observed to accumulate significantly within the liver, kidney and gills, with the latter accumulating significantly more Cd than the other tissues.

The second objective was to determine the effect of acute Cd exposure on the repeat swimming ability of juvenile rainbow trout when given a short (0.5 h), intermediate (1.5 h) and extended (6 h) recovery period. Thirdly, we aimed to assess fluctuations in anaerobic metabolic properties and effects to the stress response as a result of exercise as well as how this was affected by Cd exposure. As anticipated, based on the results of the Chapter 2, when given a short time to recover (0.5 h) Cd exposed trout had a significantly reduced U_{crit} relative to controls. This reduction is swimming ability could not, however be explained by effects to anaerobic metabolism, as after the 0.5 h recovery period, little recovery of any of the properties studied (glycogen, lactate, ATP, cortisol) was observed in either treatment. After 1.5 h of recovery the swimming ability of Cd exposed fish was actually better than that of unexposed controls. The increased swimming ability of Cd exposed fish corresponded to more rapid recovery of metabolic fuel sources and clearance of waste products. Following the extended recovery period (6 h) swimming performance did not differ between treatments. Metabolically, after 6 h

significant recovery was observed in controls. In many cases the recovery of control fish was then equal to that of Cd exposed fish.

In both control and Cd exposed fish, swimming resulted in a significant increase in plasma cortisol, however, the increase observed in Cd exposed fish was 40% less than that observed in controls. This reduced cortisol response may partially account for the more rapid recovery of metabolic parameters observed in Cd exposed fish as cortisol has previously been shown to restrict the recovery rate of such metabolic fuel sources (Lacroix and Honetlla, 2004).

Alterations to swim performance and recovery ability can have implications not only for the individual but at a population level as well. Reduced recovery ability can influence foraging ability and migration, resulting in reductions to organism health and reproductive fitness. Additionally, reductions in stress response can alter preditor-prey interactions, altering population dynamics. As a result, although Cd exposure does allow for increased recovery rate following a moderate recovery period of 1.5 h, its' presence in an aquatic ecosystem can result in a host of negative effects to fish species.

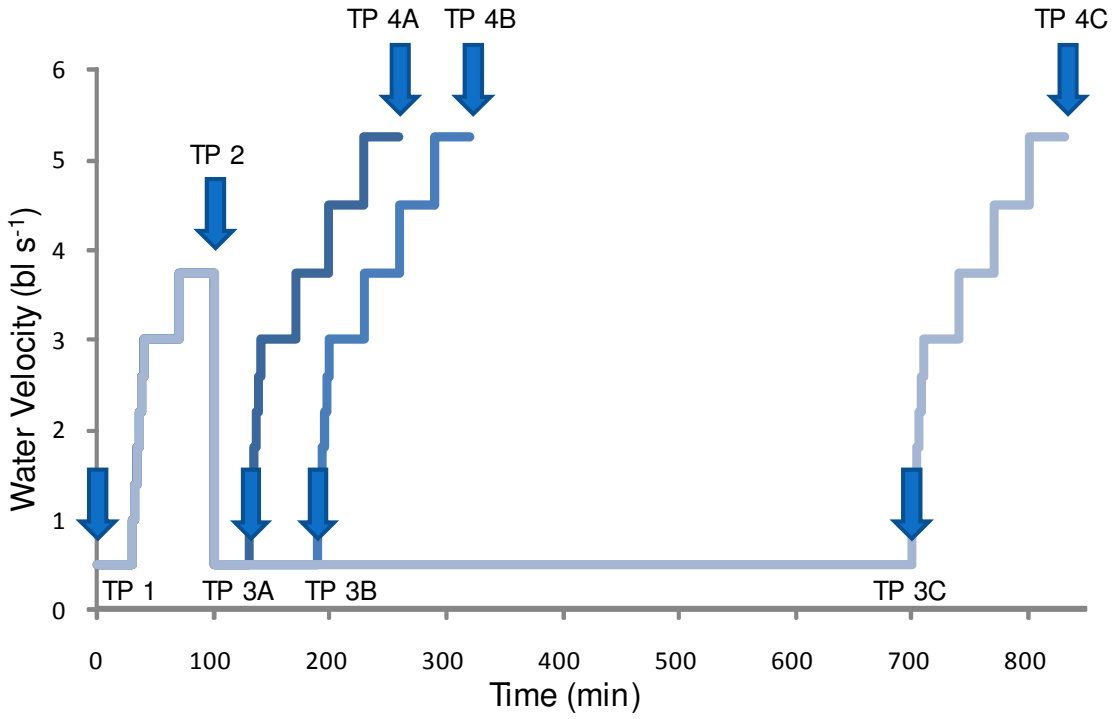

TP 4A
TP 4B
TP 4C
TP 2
TP 1
TP 3A
TP 3B
TP 3C
Water Velocity (bl s-1)
Time (min)
0
1
2
3
4
5
6
0
100
200
300
400
500
600
700
800

Figure 3.1

Figure 3.1. The velocity of water (bl s^{-1}) in the swim flume over the course of a repeat swimming challenge. Initially, water velocity is set at 0.5 bl s^{-1} to allow for a 30 minute acclimation. Following this is a 10 minute velocity ramping step adapted from Jain *et al*. (1997) where water velocity is increased to 3 bl s^{-1}, or approximately 65% of the U_{crit} of control fish. Fish then swim, in half hour increments with velocity increases of 0.75 bl s^{-1}, to a velocity of 3.75 bl s^{-1}, representing approximately 85% of the U_{crit} value of control fish. Following this, fish were given a 0.5, 1.5 or 6 hour rest period at 0.5 bl s^{-1}. Following the rest period fish underwent another swim challenge. Water velocity increased as in the first challenge, and continued until exhaustion. The arrows represent the four sampling time points. Briefly, time point 1 (TP 1) represents unexercised fish, time point 2 (TP 2) represents fish sampled following the first swim. Time point 3 (TP 3A-C) represents fish that have been sampled after the recovery period. And finally time point 4 TP 4A-C) is representative of fish sampled immediately after completing two successive swims.

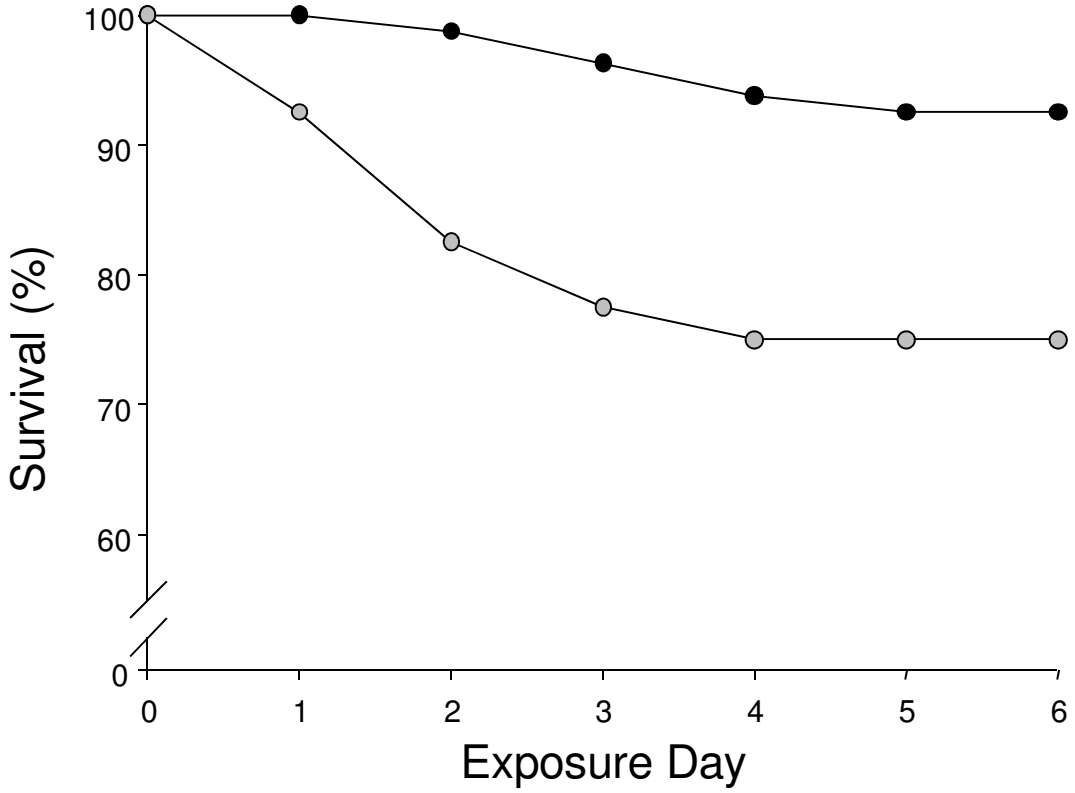


Figure 3.2

Figure 3.2. Survival (%) of rainbow trout exposed to 18 nM waterborne Cd for 6 d (grey symbols). A group of unexposed (control) trout are also shown (black symbols).

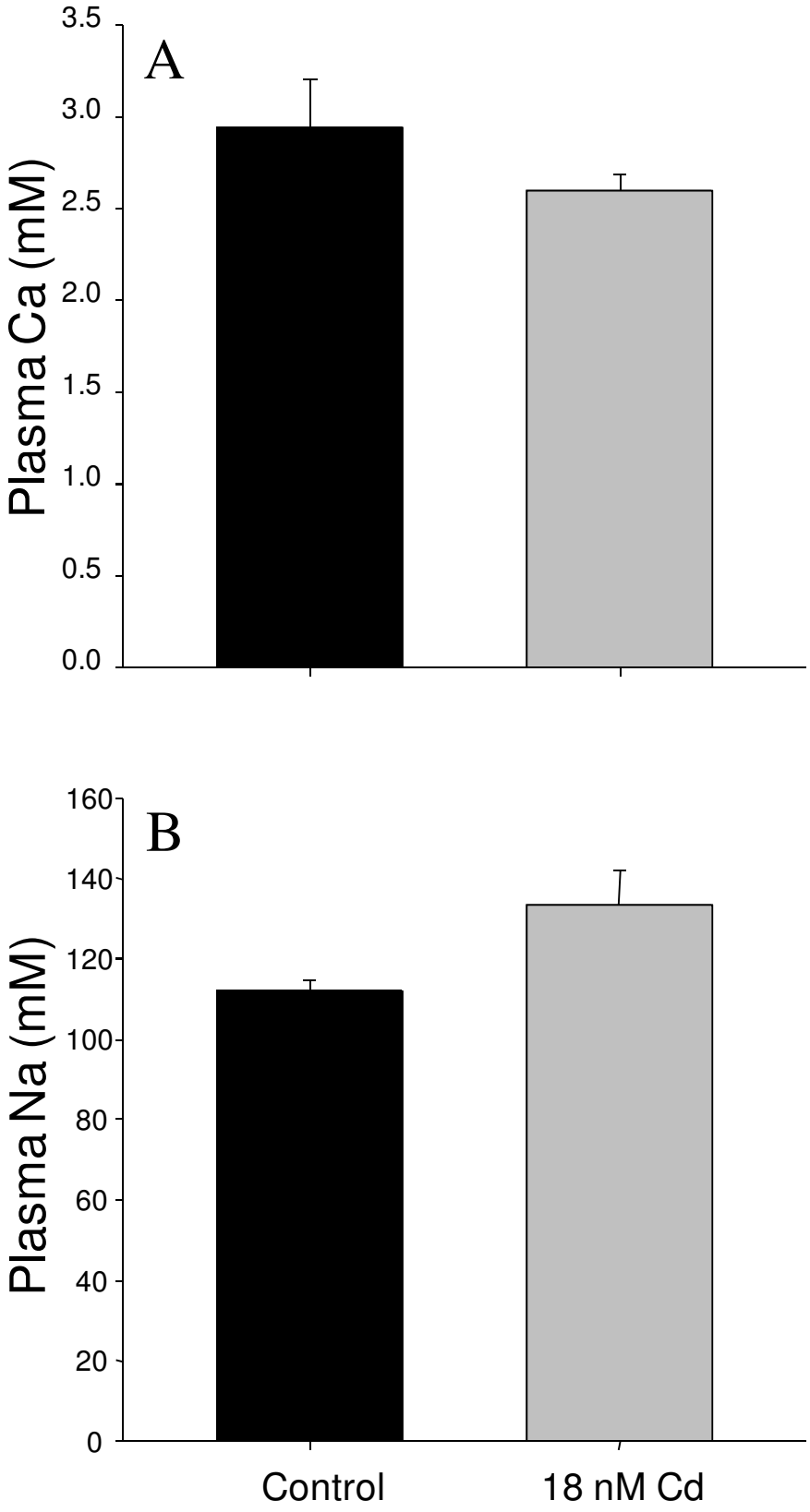

A
Plasma Ca (mM)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
B
Plasma Na (mM)
160
140
120
100
80
60
40
20
0
Control
18 nM Cd

Figure 3.3

Figure 3.3. Mean (± 1 SEM) plasma Ca (A) and Na (B) concentrations in rainbow trout after 6 days of exposure to 18 nM waterborne Cd (grey bars). Ion concentrations in a group of unexposed (controls) fish are also shown (black bars). For each mean, n=8 and * indicates a significant difference ($P<0.05$) between control and exposed fish.

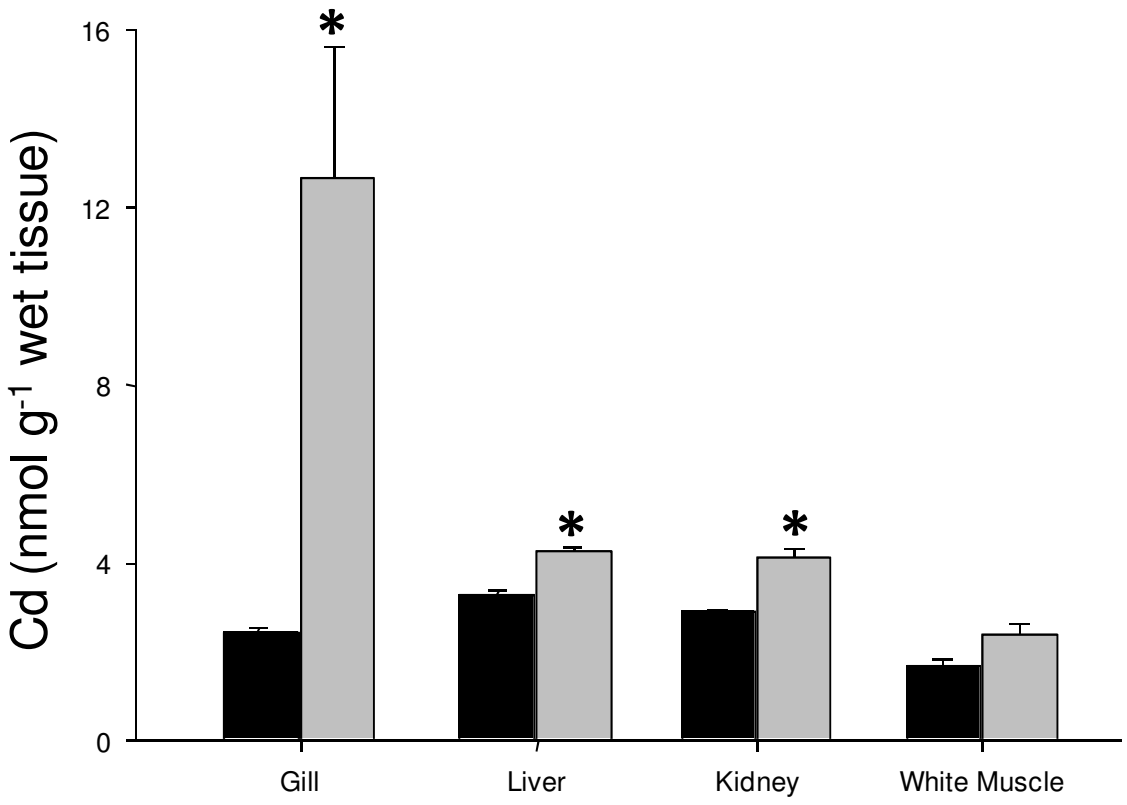

Cd (nmol g-1 wet tissue)
16
12
8
4
0
*
*
*
Gill
Liver
Kidney
White Muscle

Figure 3.4

Figure 3.4. Mean (± 1 SEM) concentration of Cd accumulation in tissues of rainbow trout after 6 days of exposure to 18 nM waterborne Cd (grey bars). Concentrations in a group of unexposed (controls) fish are also shown (black bars). For each mean, n=8 and * indicates a significant difference ($P<0.05$) between control and exposed fish.

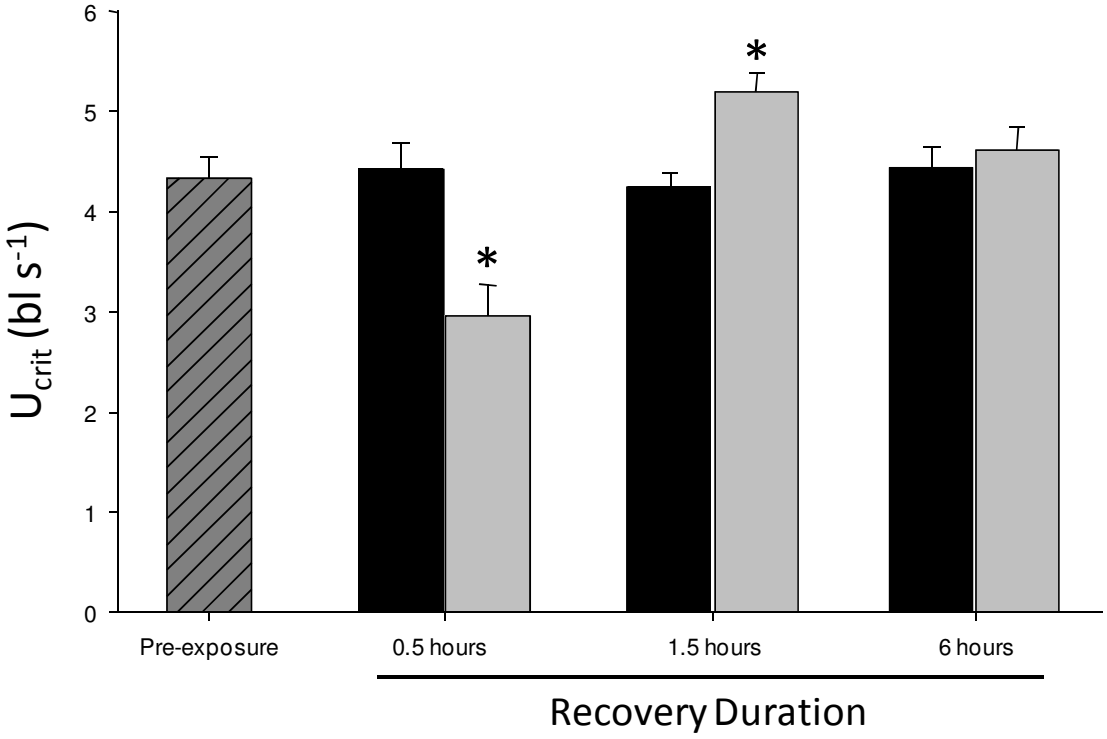
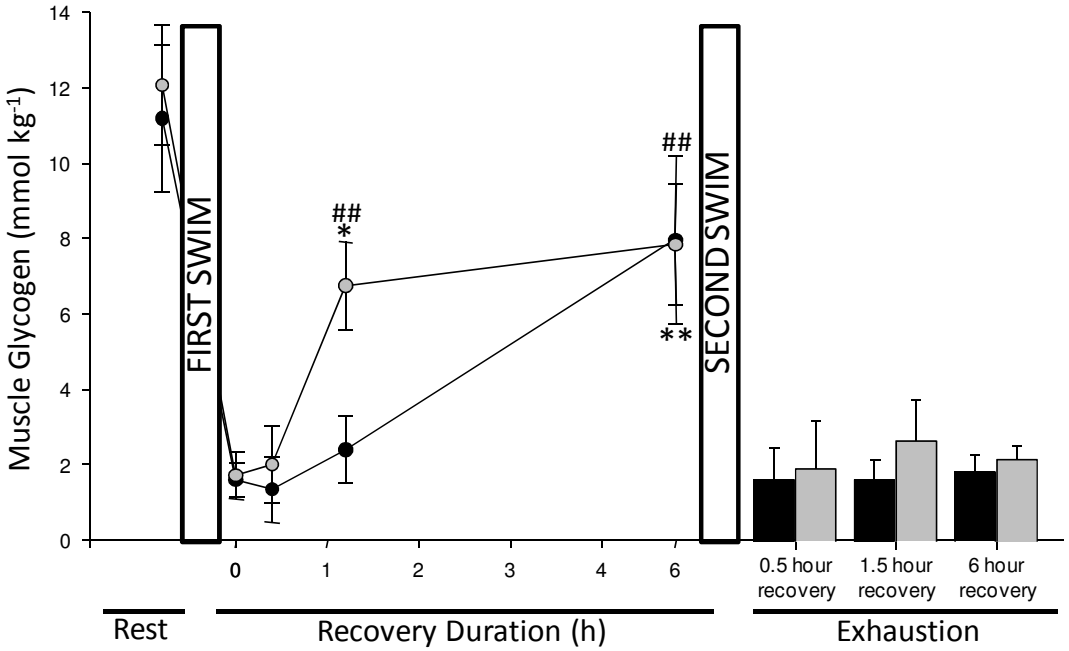

6
5
4
3
2
1
0
U_{crit} (bl s^{-1})
*
*
Pre-exposure
0.5 hours
1.5 hours
6 hours
Recovery Duration

Figure 3.5

Figure 3.5. Mean (± 1 SEM) U_{crit} swim performance of control rainbow trout in a single swim challenge (hatched bar). Remaining bars represent U_{crit} swim performance following an initial swim challenge to 85% U_{crit} in rainbow trout after 6 days of exposure to 18 nM waterborne Cd (grey bars). Swim performance in a group of unexposed (control) fish are also shown (black bars). Recovery periods between each swim trial of 0.5, 1.5 and 6 h are indicated on the x axis. For each mean n=8 and a * indicates a significant difference ($P<0.05$) between control and exposed fish.


Muscle Glycogen (mmol kg-1)
FIRST SWIM
SECOND SWIM
##
*
##
**
0
1
2
3
4
6
0.5 hour recovery
1.5 hour recovery
6 hour recovery
Rest
Recovery Duration (h)
Exhaustion

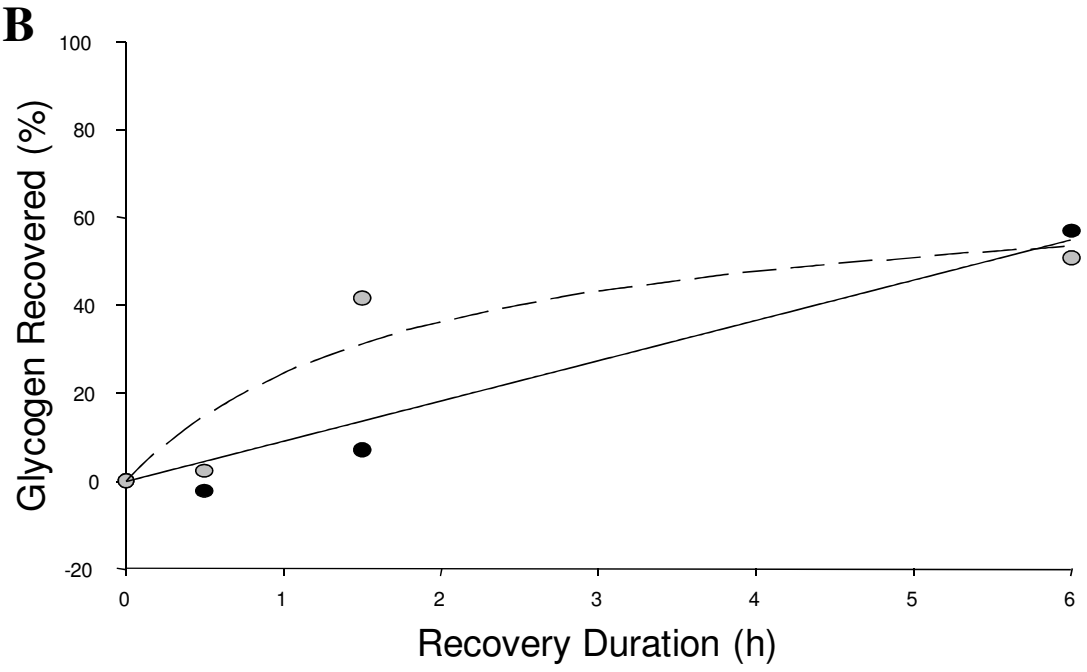

B
Glycogen Recovered (%)
Recovery Duration (h)

Figure 3.6

Figure 3.6. **A:** Mean (± 1 SEM) white muscle glycogen stores in rainbow trout exposed to 18 nM Cd for 6 days (grey symbols). White muscle glycogen stores are shown for unexposed (control) fish (black symbols). The first open bar represents the first swim challenge to 85% U_{crit} and separate pre-exercise concentrations from those following the initial swim challenge. The centre portion represents the pattern of recovery over the 6 h rest period. Finally, the second swim bar separates recovery points from concentrations at exhaustion following the second swim. For each mean n=8 and a * indicates a significant difference ($P<0.05$) between control and exposed fish. A ** indicates a significant difference ($P<0.05$) from 0 h of recovery in controls while a # # indicates a significant difference ($P<0.05$) from 0 h in Cd exposed trout (Two-way ANOVA, Student-Newman-Keuls).

B: The percent recovery of white muscle glycogen stores over time in rainbow trout exposed to 18 nM Cd (grey circles and hatched lines). Percent recovery over time is also shown for a group of unexposed (control) rainbow trout (black circles and lines).

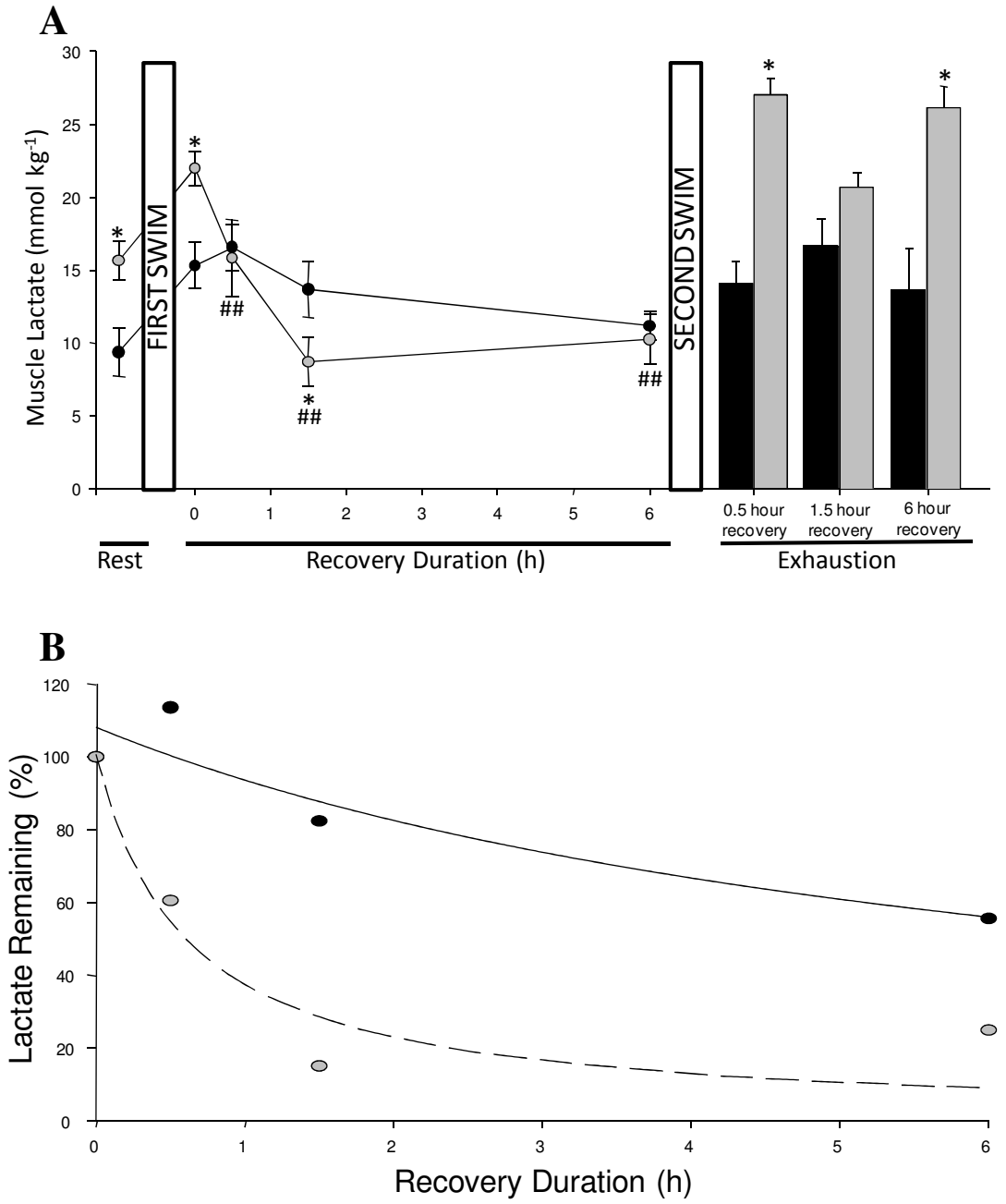

A
Muscle Lactate (mmol kg-1)
FIRST SWIM
SECOND SWIM
0.5 hour recovery
1.5 hour recovery
6 hour recovery
Rest
Recovery Duration (h)
Exhaustion
B
Lactate Remaining (%)
Recovery Duration (h)

Figure 3.7

Figure 3.7 **A:** Mean (± 1 SEM) white muscle lactate accumulation in rainbow trout exposed to 18 nM Cd for 6 days (grey symbols). White muscle lactate concentrations are shown for unexposed (control) fish (black symbols). The first open bar represents the first swim challenge to 85% U_{crit} and separate pre-exercise concentrations from those following the initial swim challenge. The centre portion represents the pattern of recovery over the 6 h rest period. Finally, the second swim bar separates recovery points from concentrations at exhaustion following the second swim. For each mean n=8 and a * indicates a significant difference ($P<0.05$) between control and exposed fish. A ** indicates a significant difference ($P<0.05$) from 0 h of recovery in controls while a # # indicates a significant difference ($P<0.05$) from 0 h in Cd exposed trout (Two-way ANOVA, Student-Newman-Keuls).

B: The percent clearance of white muscle lactate over time in rainbow trout exposed to 18 nM Cd (grey circles and hatched lines). Percent recovery over time is also shown for a group of unexposed (control) rainbow trout (black circles and lines).

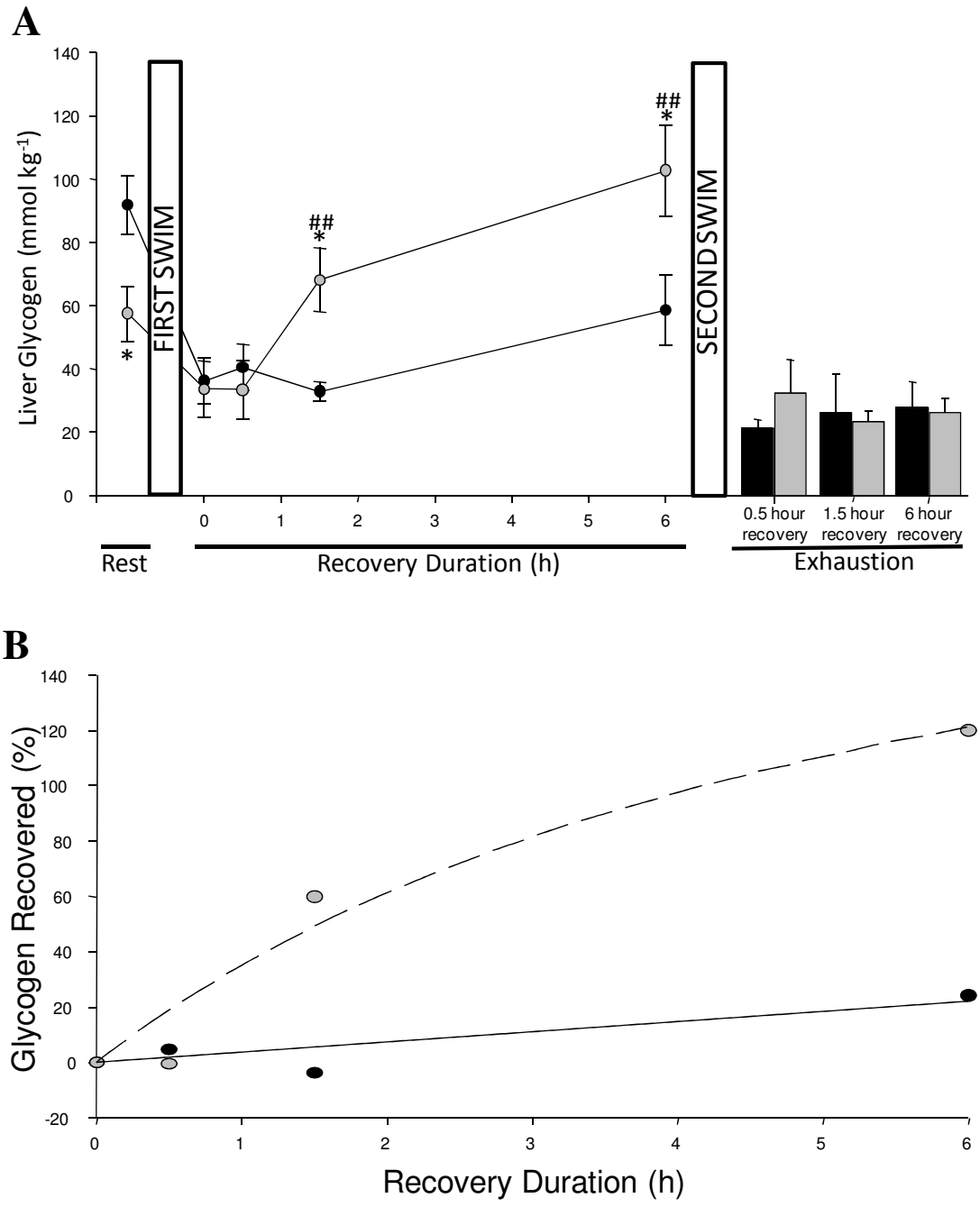

A
Liver Glycogen (mmol kg-1)
140
120
100
80
60
40
20
0
FIRST SWIM
SECOND SWIM
##
*
##
*
*
0
1
2
3
4
5
6
0.5 hour recovery
1.5 hour recovery
6 hour recovery
Rest
Recovery Duration (h)
Exhaustion
B
Glycogen Recovered (%)
140
120
100
80
60
40
20
0
-20
0
1
2
3
4
5
6
Recovery Duration (h)

Figure 3.8

Figure 3.8 **A:** Mean (± 1 SEM) liver glycogen stores in rainbow trout exposed to 18 nM Cd for 6 days (grey symbols). Liver glycogen stores are shown for unexposed (control) fish (black symbols). The first open bar represents the first swim challenge to 85% U_{crit} and separate pre-exercise concentrations from those following the initial swim challenge. The centre portion represents the pattern of recovery over the 6 h rest period. Finally, the second swim bar separates recovery points from concentrations at exhaustion following the second swim. For each mean n=8 and a * indicates a significant difference ($P<0.05$) between control and exposed fish. A ** indicates a significant difference ($P<0.05$) from 0 h of recovery in controls while a # # indicates a significant difference ($P<0.05$) from 0 h in Cd exposed trout (Two-way ANOVA, Student-Newman-Keuls).

B: The percent recovery of liver glycogen stores over time in rainbow trout exposed to 18 nM Cd (grey circles and hatched lines). Percent recovery over time is also shown for a group of unexposed (control) rainbow trout (black circles and lines).

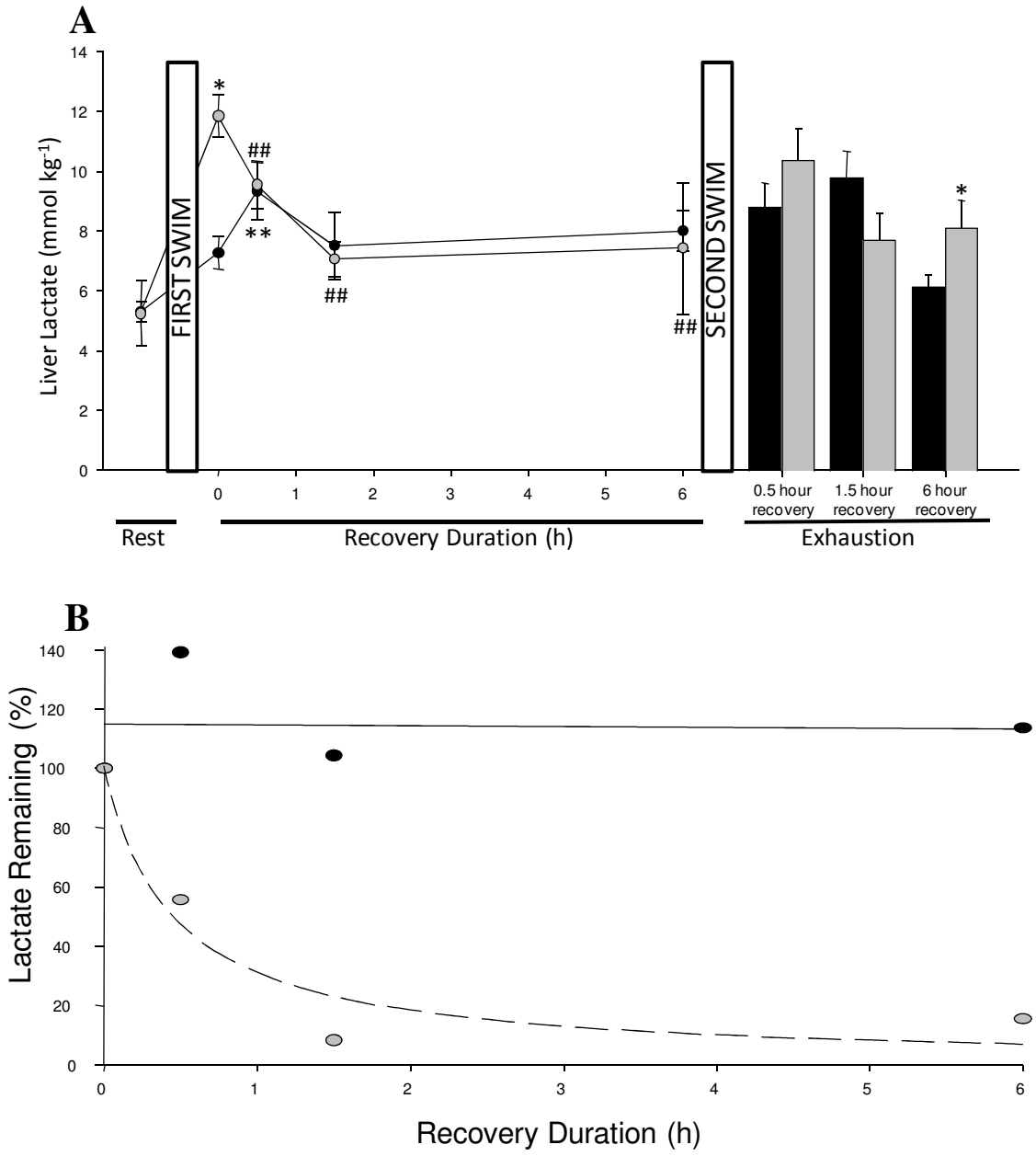

A
Liver Lactate (mmol kg-1)
FIRST SWIM
SECOND SWIM
0.5 hour recovery
1.5 hour recovery
6 hour recovery
Rest
Recovery Duration (h)
Exhaustion
B
Lactate Remaining (%)
Recovery Duration (h)

Figure 3.9

Figure 3.9. **A:** Mean (± 1 SEM) liver lactate accumulation in rainbow trout exposed to 18 nM Cd for 6 days (grey symbols). Liver lactate concentrations are shown for unexposed (control) fish (black symbols). The first open bar represents the first swim challenge to 85% U_{crit} and separate pre-exercise concentrations from those following the initial swim challenge. The centre portion represents the pattern of recovery over the 6 h rest period. Finally, the second swim bar separates recovery points from concentrations at exhaustion following the second swim. For each mean n=8 and a * indicates a significant difference ($P<0.05$) between control and exposed fish. A ** indicates a significant difference ($P<0.05$) from 0 h of recovery in controls while a # # indicates a significant difference ($P<0.05$) from 0 h in Cd exposed trout (Two-way ANOVA, Student-Newman-Keuls).

B: The percent clearance of liver lactate over time in rainbow trout exposed to 18 nM Cd (grey circles and hatched lines). Percent recovery over time is also shown for a group of unexposed (control) rainbow trout (black circles and lines).

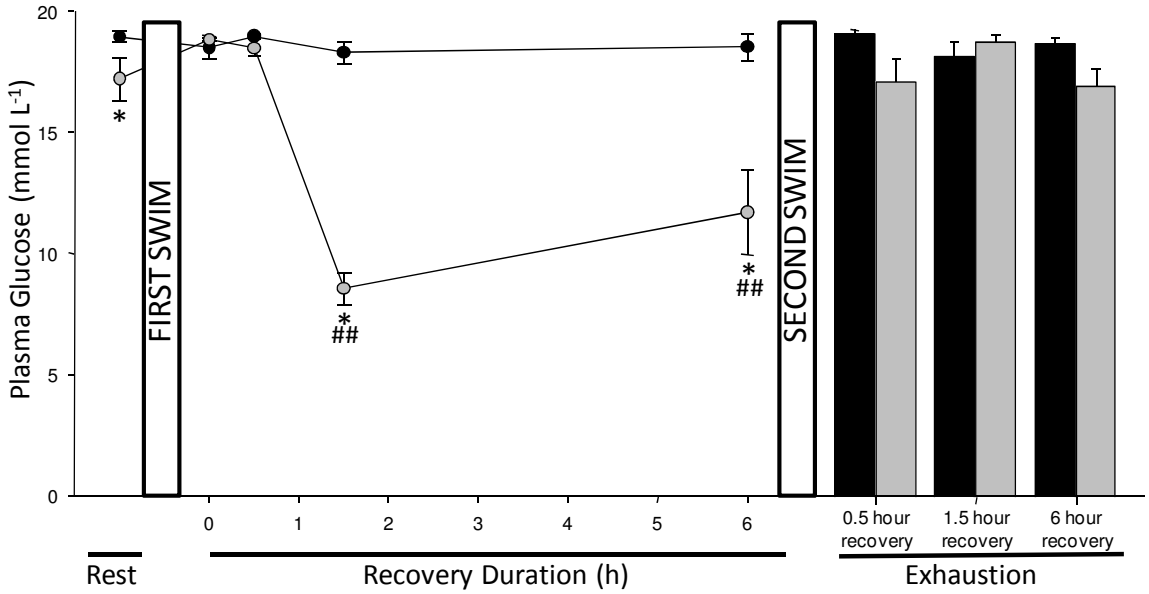

Plasma Glucose (mmol L-1)
20
15
10
5
0
FIRST SWIM
SECOND SWIM
*
*
##
*
##
0
1
2
3
4
5
6
0.5 hour recovery
1.5 hour recovery
6 hour recovery
Rest
Recovery Duration (h)
Exhaustion

Figure 3.10

Figure 3.10. Mean (± 1 SEM) plasma glucose in rainbow trout exposed to 18 nM Cd for 6 days (grey symbols). Plasma glucose concentrations are shown for unexposed (control) fish (black symbols). The first open bar represents the first swim challenge to 85% U_{crit} and separate pre-exercise concentrations from those following the initial swim challenge. The centre portion represents the pattern of recovery over the 6 h rest period. Finally, the second swim bar separates recovery points from concentrations at exhaustion following the second swim. For each mean n=8 and a * indicates a significant difference ($P<0.05$) between control and exposed fish. A ** indicates a significant difference ($P<0.05$) from 0 h of recovery in controls while a # # indicates a significant difference ($P<0.05$) from 0 h in Cd exposed trout (Two-way ANOVA, Student-Newman-Keuls).

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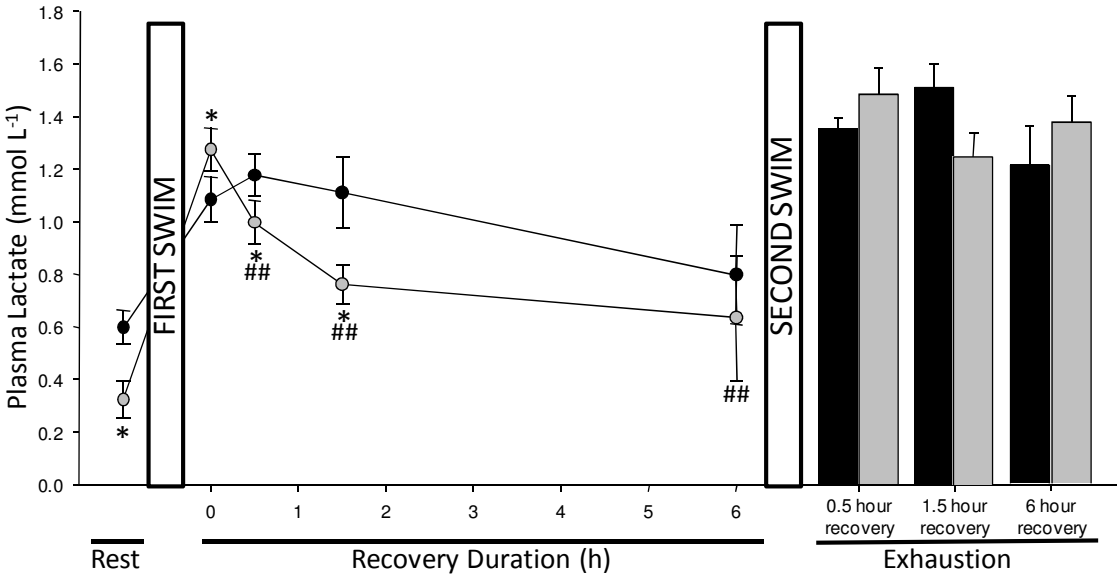
Plasma Lactate (mmol L-1)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
FIRST SWIM
SECOND SWIM
*
*
*
##
*
##
##
0
1
2
3
4
5
6
0.5 hour recovery
1.5 hour recovery
6 hour recovery
Rest
Recovery Duration (h)
Exhaustion

Figure 3.11

Figure 3.11 Mean (± 1 SEM) plasma lactate concentrations in rainbow trout exposed to 18 nM Cd for 6 days (grey symbols). Plasma lactate concentrations are shown for unexposed (control) fish (black symbols). The first open bar represents the first swim challenge to 85% U_{crit} and separate pre-exercise concentrations from those following the initial swim challenge. The centre portion represents the pattern of recovery over the 6 h rest period. Finally, the second swim bar separates recovery points from concentrations at exhaustion following the second swim. For each mean n=8 and a * indicates a significant difference ($P<0.05$) between control and exposed fish. A ** indicates a significant difference ($P<0.05$) from 0 h of recovery in controls while a # # indicates a significant difference ($P<0.05$) from 0 h in Cd exposed trout (Two-way ANOVA, Student-Newman-Keuls).

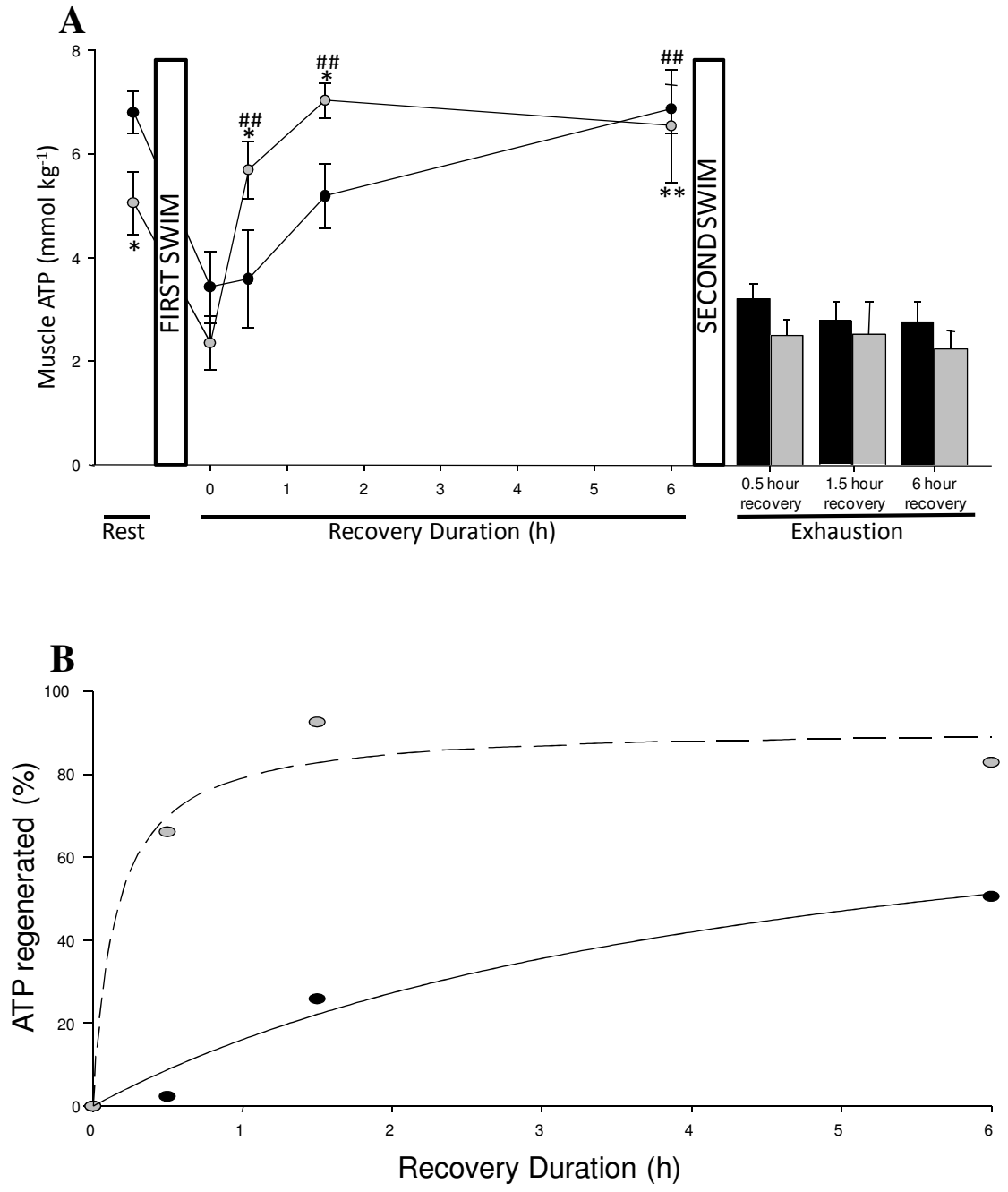
A
Muscle ATP (mmol kg-1)
FIRST SWIM
SECOND SWIM
0.5 hour recovery
1.5 hour recovery
6 hour recovery
Rest
Recovery Duration (h)
Exhaustion
B
ATP regenerated (%)
Recovery Duration (h)

Figure 3.12

Figure 3.12. A: Mean (± 1 SEM) white muscle ATP stores in rainbow trout exposed to 18 nM Cd for 6 days (grey symbols). White muscle ATP concentrations are shown for unexposed (control) fish (black symbols). The first open bar represents the first swim challenge to 85% U_{crit} and separate pre-exercise concentrations from those following the initial swim challenge. The centre portion represents the pattern of recovery over the 6 h rest period. Finally, the second swim bar separates recovery points from concentrations at exhaustion following the second swim. For each mean n=8 and a * indicates a significant difference ($P<0.05$) between control and exposed fish. A ** indicates a significant difference ($P<0.05$) from 0 h of recovery in controls while a # # indicates a significant difference ($P<0.05$) from 0 h in Cd exposed trout (Two-way ANOVA, Student-Newman-Keuls).

B: The percent recovery of white muscle ATP stores over time in rainbow trout exposed to 18 nM Cd (grey circles and hatched lines). Percent recovery over time is also shown for a group of unexposed (control) rainbow trout (black circles and lines).

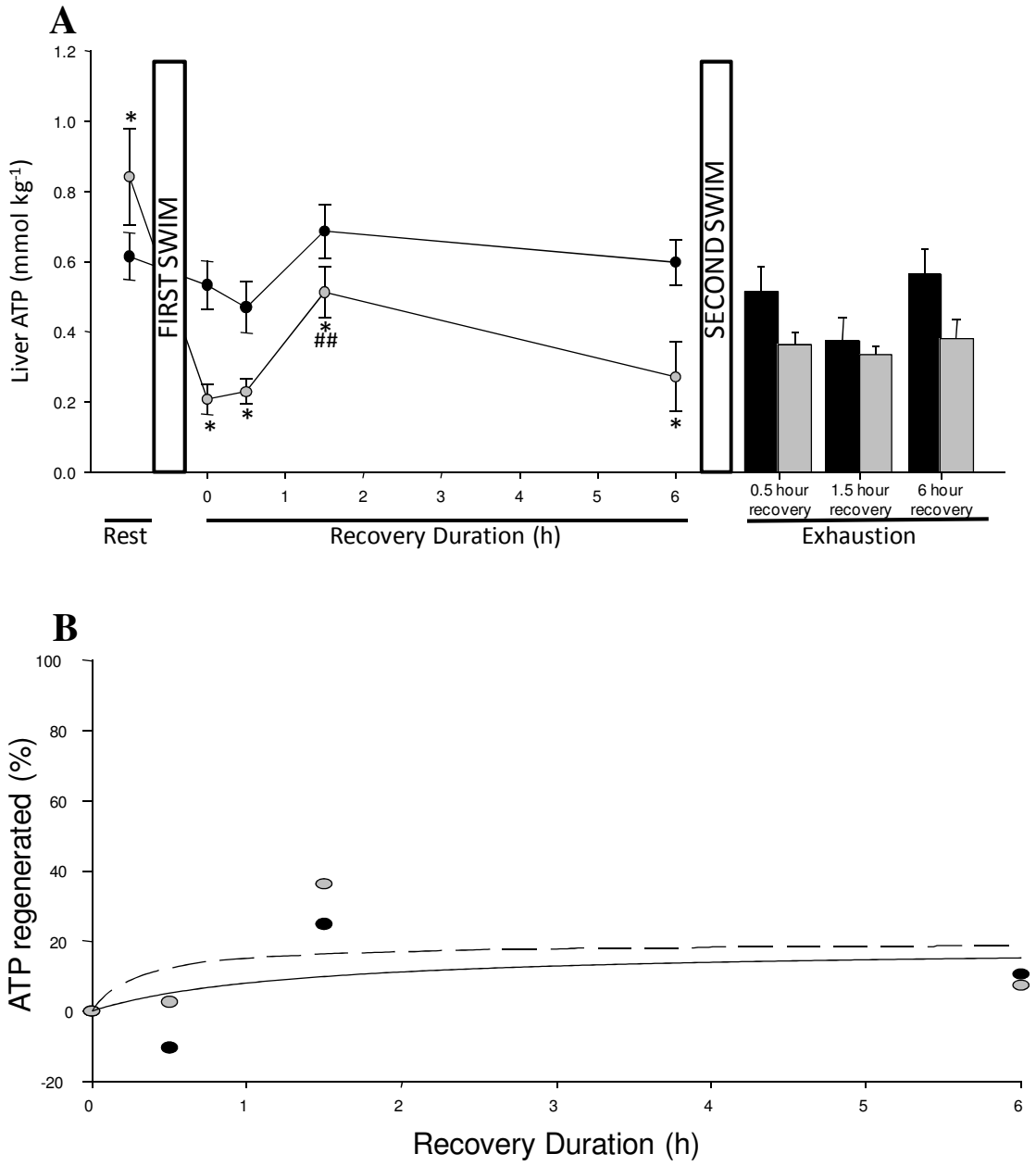
A
Liver ATP (mmol kg-1)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
FIRST SWIM
SECOND SWIM
*
*
*
*
##
*
0
1
2
3
4
5
6
0.5 hour recovery
1.5 hour recovery
6 hour recovery
Rest
Recovery Duration (h)
Exhaustion
B
ATP regenerated (%)
100
80
60
40
20
0
-20
0
1
2
3
4
5
6
Recovery Duration (h)

Figure 3.13

Figure 3.13. **A:** Mean (± 1 SEM) liver ATP stores in rainbow trout exposed to 18 nM Cd for 6 days (grey symbols). Liver ATP concentrations are shown for unexposed (control) fish (black symbols). The first open bar represents the first swim challenge to 85% U_{crit} and separate pre-exercise concentrations from those following the initial swim challenge. The centre portion represents the pattern of recovery over the 6 h rest period. Finally, the second swim bar separates recovery points from concentrations at exhaustion following the second swim. For each mean n=8 and a * indicates a significant difference ($P<0.05$) between control and exposed fish. A ** indicates a significant difference ($P<0.05$) from 0 h of recovery in controls while a # # indicates a significant difference ($P<0.05$) from 0 h in Cd exposed trout (Two-way ANOVA, Student-Newman-Keuls).

B: The percent recovery of liver ATP stores over time in rainbow trout exposed to 18 nM Cd (grey circles and hatched lines). Percent recovery over time is also shown for a group of unexposed (control) rainbow trout (black circles and lines).

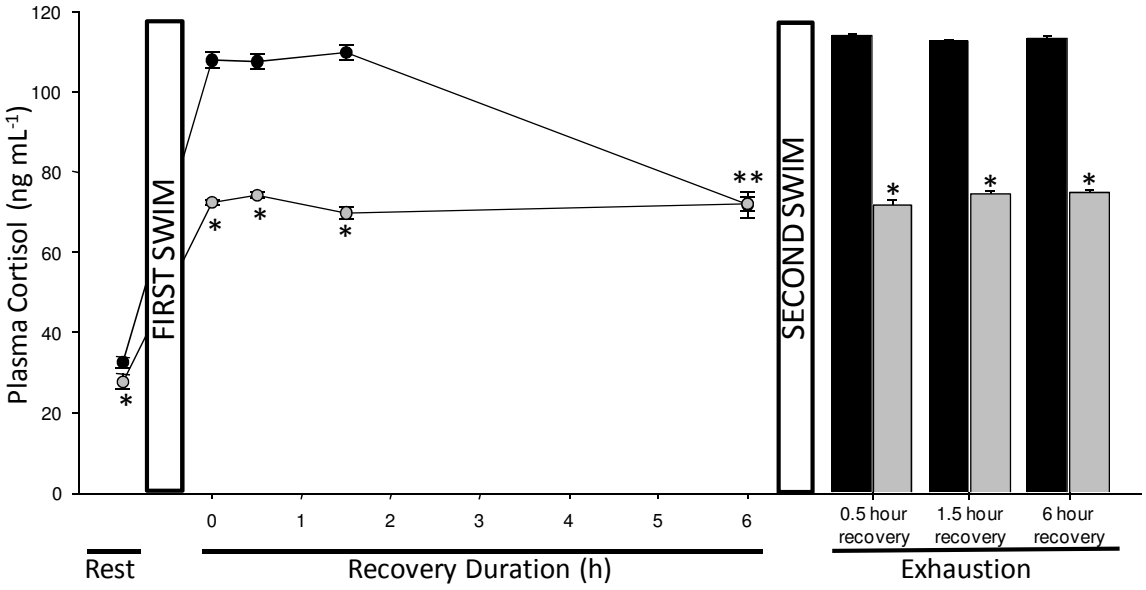
Plasma Cortisol (ng mL-1)
120
100
80
60
40
20
0
FIRST SWIM
SECOND SWIM
0
1
2
3
4
5
6
0.5 hour recovery
1.5 hour recovery
6 hour recovery
Rest
Recovery Duration (h)
Exhaustion

Figure 3.14

Figure 3.14. A: Mean (± 1 SEM) plasma cortisol concentrations in rainbow trout exposed to 18 nM Cd for 6 days (grey symbols). Plasma cortisol concentrations are shown for unexposed (control) fish (black symbols). The first open bar represents the first swim challenge to 85% U_{crit} and separate pre-exercise concentrations from those following the initial swim challenge. The centre portion represents the pattern of recovery over the 6 h rest period. Finally, the second swim bar separates recovery points from concentrations at exhaustion following the second swim. For each mean n=8 and a * indicates a significant difference ($P<0.05$) between control and exposed fish. A ** indicates a significant difference ($P<0.05$) from 0 h of recovery in controls while a # # indicates a significant difference ($P<0.05$) from 0 h in Cd exposed trout (Two-way ANOVA, Student-Newman-Keuls).

Chapter 4

General Discussion and Integration

4.1 General Discussion- Integrated model of the effects of Cd

The research presented in Chapters 2 and 3 examines the relationship between repeat swim performance, metabolic expenditure and recovery processes, and the stress response. Theses parameters were evaluated to establish a time course of recovery both in terms of swim performance and the restoration of depleted metabolic fuel stores in unexposed rainbow trout and then to compare this to responses in trout exposed to 18 nM Cd to identify the effect of sub-lethal, waterborne, Cd exposure on whole organism performance. Measures of repeat swimming ability were performed in brown trout, rainbow trout and lake whitefish so as to identify any differences that may exist between species.

The effect of sub-lethal waterborne Cd exposure on the critical swimming speed of salmonids has previously been described. Typically, Cd exposure at sub-lethal concentrations does not result in alterations to the critical swimming speed (Hollis *et al*., 1999; McGeer *et al*., 2000a) and swimming stamina (Hollis *et al*., 1999). This absence of an effect of Cd on U_{crit} swimming has been shown for individual swim challenges only. However, the effect of Cd exposure on other evaluations of swim performance, such as repeat swimming ability, have not been examined. Similarly, the recovery of metabolic parameters (i.e. re-establishment of energy stores and fuel sources and the clearance of metabolic waste products) following high intensity exercise has been well documented in unexposed (control) fish, however, there is limited research on how these parameters may be impacted by chronic sublethal exposure to contaminants.

The aim of this research project has been to further characterize the effects of sub lethal waterborne Cd exposure on whole organism performance through measures of

tissue specific metal accumulation, plasma ionic disturbances, metabolic expenditure and recovery, and repeat swim performance. More specifically, the objectives of this research project were to a) characterise the effect of Cd exposure on sustained, repeat swimming ability and to examine the role of the rest period in the recovery process. Secondly, we have aimed to b) identify the anaerobic metabolic and stress response related effects of repeat swimming and to characterize the effects of Cd exposure on these parameters. Finally, this research project has aimed to c) complement measures of both acute and chronic Cd toxicity through evaluation of basic parameters such as mortality, tissue specific Cd accumulation and alterations in plasma ion composition (Ca and Na).

The first objective of this thesis, which was to determine the effect of Cd exposure on repeat swimming ability of three salmonid species, was achieved through the performance of acute (6 d) and chronic (30 d) Cd exposures. Chronically, it was observed that performance in the second swim trial was reduced on days 1, 6 and 14 in brown trout (Fig.2.5A) and day 3, 7 and 30 in lake whitefish (Fig.2.5B). In brown trout the reduction was most pronounced at day 6 while the greatest decrease in swim performance in lake whitefish was observed on day 7. Subsequent acute Cd exposure to rainbow trout similarly identified a reduction in secondary swim performance in rainbow trout given a 0.5 h recovery period.

When metabolic parameters were examined it was evident that the initial swim challenge to 85% U_{crit} resulted in significant depletion of glycogen (Figs. 2.6 and 2.7) and ATP stores (Figs. 2.10 and 2.11) and an accumulation of lactate within the white muscle (Figs. 2.8 and 2.9) in both control and Cd exposed fish. This indicates that, although not exhaustive, the metabolic demands to support high intensity activity (85% U_{crit}) could not

be met through oxidative phosphorylation alone and required the recruitment of substrate level phosphorylation. Similar metabolic debts were incurred in both control and Cd exposed fish, indicating that substrate level phosphorylation played an equal role in metabolism regardless of treatment, and thus the initial recruitment of anaerobic metabolic pathways is not affected by Cd exposure. It was also observed that little recovery occurred between time points 2 and 3, indicating that a 0.5 h recovery period was not sufficient to allow for significant metabolic recovery. This was anticipated as full metabolic recovery can take in excess of 8 h to complete in salmonids. All three species tested showed limited variation in expenditure and recovery of anaerobic metabolic fuel sources (or accumulation and removal of waste products) while still demonstrating a reduction in secondary swim performance as a result of Cd exposure.

The similar metabolic response, despite the reduction in swimming ability allows for the conclusion that, when given a short (0.5 h) recovery period decreases to secondary swim performance in Cd exposed fish is not as a result of alterations to anaerobic metabolism. The initial portion of each swim challenge would have required limited recruitment of anaerobic metabolism, and would have been supported predominantly aerobically through oxidative phosphorylation. As such, although significant recovery of anaerobic metabolic parameters was not observed after a 0.5 h recovery, these factors would continue to be recovered during swimming at lower intensities. After 0.5 h of recovery metabolic recovery was not observed in either treatment, but reductions in secondary swim performance were observed in Cd exposed fish only. This variation in the recovery of swimming ability could be related to recovery of aerobic metabolic properties. It is possible that if oxidative phosphorylation, or one of the precursor

pathways (ie. fatty acid β oxidation, aerobic glycolysis, TCA) or recovery processes (lipogenesis) was impacted by Cd exposure, that recovery of aerobic metabolic properties may be impacted (Fig. 4.1). This would limit the potential for aerobic metabolic activity and would require premature recruitment of anaerobic metabolism thus exhausting anaerobic metabolic fuel sources available for substrate level phosphorylation faster in the second swim. This may ultimately account for the decreased swimming capacity by Cd exposed fish following a 0.5 h recovery period.

The effects of Cd exposure on the repeat swimming ability of rainbow trout given a moderate recovery duration of 1.5 h was also evaluated. Unlike following the short 0.5 h recovery period, Cd exposed trout given a 1.5 h recovery period performed significantly better in the second swim trial than did control fish. The increased performance of Cd exposed fish after the 1.5 h recovery period correlated an increased rate of recovery of several metabolic parameters. For the most part, recovery of ATP (Figs. 3.12 and 3.13) and glycogen (Figs. 3.6 and 3.8) in the white muscle and liver and removal of lactate from the white muscle, liver and plasma (Figs. 3.7, 3.9 and 3.11 respectively) all occurred more rapidly in Cd exposed fish with significant recovery observed much sooner than in control fish. When oxygen is limited, NADH donates a proton to pyruvate, producting lactate and NAD^+ which is required for the continuation of glycolysis. This conversion to lactate, and subsequent alteration back to pyruvate may be impacted by Cd exposure, causing the conversion to occur at a greater rate (Fig. 4.1). The goal of identifying the metabolic effects associated with Cd exposure was met, and illustrated that Cd exposure has an accelatory effect on the recovery of metabolic fuel stores and clearance of associated waste products.

Also associated with exposure to 18 nM Cd to rainbow trout was a decrease in the cortisol response (Fig. 3.14). While elevations in plasma cortisol were observed in both treatments, the elevation observed in Cd exposed fish was 40% less than that observed in control fish. Elevations in plasma cortisol following exercise have been linked with an inhibition of the recovery of muscle glycogen stores and removal of muscle lactate (Lacroix and Hontella, 2004). Therefore, it is possible that the reduced mobilization of cortisol in Cd exposed fish facilitated an increased rate of metabolic recovery and this resulted in an improved swimming ability after 1.5 h of recovery.

Mortalities, plasma ion disruption and elevated tissue metal concentrations were all observed as a result of Cd exposure, and may also have helped to account for the alterations in swimming ability observed in Cd exposed fish. Exposure to 18 nM Cd resulted in 25% mortality in rainbow trout (Fig. 3.2) and 7% mortality in brown trout while no mortalities were observed in lake whitefish. In both rainbow and brown trout all mortalities were observed during the first 4 days of exposure. The pattern of mortality may have been correlated with an initial period of ionoregulatory disruption. Brown trout showed significant reductions in plasma Ca^{2+} (Fig. 2.2A) after only one day of exposure. In lake whitefish (Fig. 2.2C) a significant decrease was not observed until day 7. No significant disruption of plasma Ca^{2+} was measured in rainbow trout (Fig. 3.3A) but measurements were only performed on d 6 of exposure. The most significant decreases in swimming ability were observed after approximately one week of exposure, which also generally correlated with significant disruptions to plasma ion status. This indicates that disruptions to plasma ions may negatively affect swimming performance.

There was also significant accumulation of Cd in the gills, liver and kidney of all three species. It was hypothesized that the liver would chronically accumulate the most Cd, however it was observed that following 30 d of exposure the gill had accumulated the most Cd (brown trout: Fig. 2.3A, and lake whitefish: Fig. 2.4A). Acute exposure resulted in similar accumulation in both the liver and kidney. However, following chronic exposure lake whitefish accumulated higher concentrations of Cd in the liver than brown trout (Fig. 2.4B and 2.3B respectively). This may play a role in the variation in recovery of repeat swim performance in brown trout (recovered) and whitefish (impacted) after 30 d of Cd exposure, as this may signify an increased capacity for detoxification in brown trout compared with lake whitefish as a result of variations in accumulation between these species as lake whitefish had accumulated more Cd in the liver than brown trout (Figs. 2.4B and 2.3B respectively). Typically, the kidney represents the site of greatest Cd accumulation following chronic exposure as it is the primary site for the sequestration of metalothionine bound, and thus biologically unavailable, Cd. Increased presence of Cd in the liver may indicate that less of the Cd present in the organism has been bound to metallothionine, and thus is causing increased physiological damage, such as decreased recovery ability.

4.2 Integration

Cadmium is present in the environment at elevated concentrations particularly as a result of anthropogenic sources such as mining and smelting of ores, industrial activity and fuel combustion (McGeer *et al*., 2011). Cadmium is a non-essential element with no dietary requirement. At elevated levels, Cd can have significant and potentially lethal

effects on aquatic organisms (Hollis *et al*., 2000). Salmonids, including trout species, are amongst the most sensitive teleosts to waterborne Cd exposure. Effects of Cd exposure include ionoregulatory disturbances (Verbost *et al*., 1988 and 1989), oxidative damage (Livingstone, 2001) and decreased growth (Hansen *et al*., 2002). Cd exposure has previously been shown not to reduce the critical swimming speed of trout in a single swim challenge (Hollis *et al*., 1999; McGeer *et al*., 2000a; Cunningham and McGeer, 2009).

The ability to swim is central to the health and survival of fish species. As such, swim performance is one of the most observable and critical measures of the effect of various stressors on fish health and performance (Beitinger and McCauley, 1990). Swimming ability is important for a variety of behavioural responses such as prey capture and predator avoidance, avoidance of unfavourable environmental conditions and migration (Beaumont *et al*., 1995; Drucker, 1996; Plaut, 2001).

Associated with swimming performance is recovery ability or the time required for the restoration of un-impacted swimming. Impairment of recovery ability can have significant ecological impacts as fish with longer recovery periods are more susceptible to predation and will perform poorly in foraging and migration (Farrell *et al*., 1998). The ability to recover from high intensity exercise and to perform well in subsequent swimming challenges has been shown to be a more sensitive indicator of impairment than the use of a singular swim challenge alone (Jain *et al*. 2003). The use of a repeat swim test allows for a more in depth examination of the effects of a contaminant on whole organism performance. A repeated swim challenge is able to test for the parameters evaluated by a singular U_{crit} challenge, as well as the ability to recover. This allows for

other physiological processes to be tested then would be employed in a single swim challenge alone. This ultimately allows for a greater potential to detect altered performance as a result of contaminant exposure.

In the present study, exposure to sub-lethal concentrations of Cd was shown to affect swimming ability. When recovery periods were very short (0.5 h) Cd exposed fish did not perform as well as did controls. A reduced capacity to swim following high intensity exercise could result in adverse effects to organism health if foraging is impacted as well as to reproductive success if migration is impacted. Following an intermediate recovery period of (1.5 h), Cd exposure resulted in increased recovery ability, both of swimming ability and of metabolic fuel sources. While improvements to repeat swimming ability may confer an advantage to migrating fish, Cd exposure was also associated with decreased stress response (plasma cortisol) which plays an important role in the avoidance of predators and unfavourable conditions, both factors with can be detrimental to fish health. Altered predator-prey interactions and migratory behaviour can have significant ramifications not only to individuals, but at a population level as well. If reproductive success is impacted this can shift population dynamics which can in turn incur significant ecological stress.

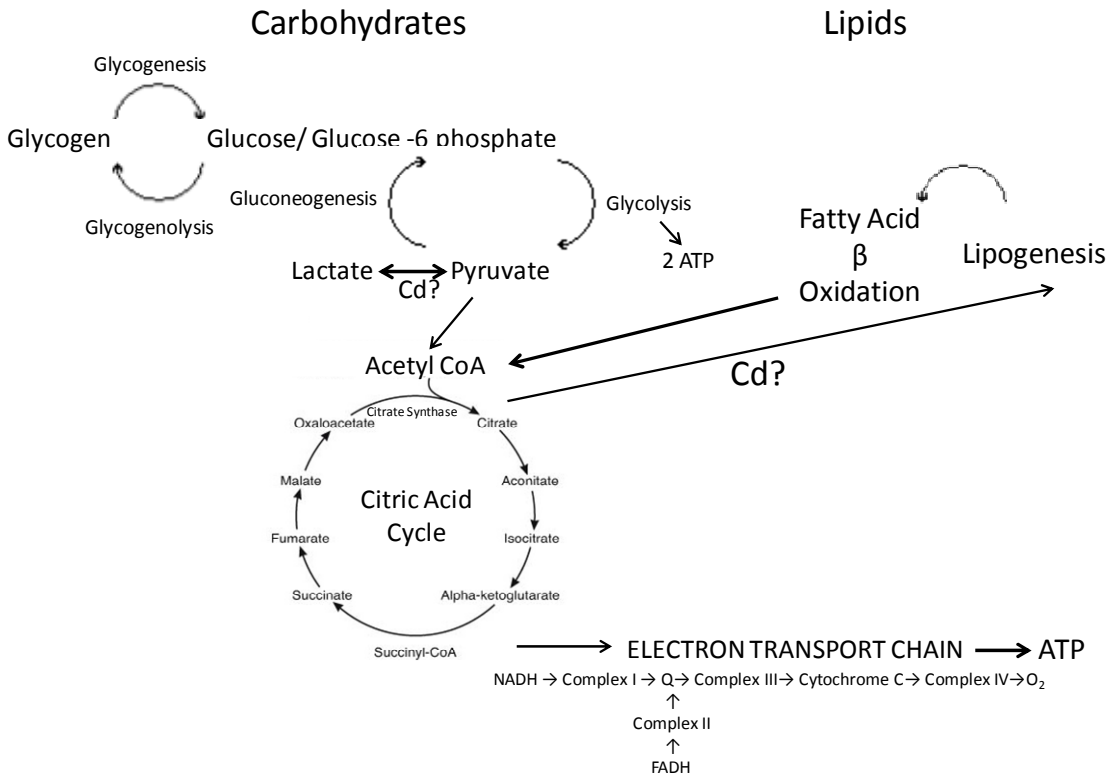
Carbohydrates
Lipids
Glycogenesis
Glycogen
Glucose/ Glucose -6 phosphate
Glycogenolysis
Gluconeogenesis
Glycolysis
2 ATP
Lactate
Pyruvate
Cd?
Fatty Acid
β
Oxidation
Lipogenesis
Acetyl CoA
Cd?
Citrate Synthase
Oxaloacetate
Citrate
Malate
Aconitate
Citric Acid
Cycle
Fumarate
Isocitrate
Succinate
Alpha-ketoglutarate
Succinyl-CoA
ELECTRON TRANSPORT CHAIN
ATP
NADH → Complex I → Q→ Complex III→ Cytochrome C→ Complex IV→O_2
Complex II
FADH

Figure 4.1

Figure 4.1. Representation of aerobic and anaerobic metabolic pathways utilized to support repeat swimming, and where it may be potentially impacted by sub-lethal waterborne Cd exposure.

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Appendices

Appendix A: Summary

Mortality:

Exposure to 18 nM Cd resulted in 25% mortality in rainbow trout with 5% mortality observed in controls (Fig. 3.2). In brown trout 7% mortality was observed in Cd exposed fish with no mortalities in controls. Control and Cd exposed lake whitefish both had 100% survival. All mortalities occurred within the first 4 days of exposure.

Plasma Ion Concentrations:

Plasma Ca showed a characteristic pattern of damage and repair. In brown trout (Fig. 2.2A), a significant reduction in plasma Ca was observed in Cd exposed fish, relative to controls, on all days evaluated, with the exception of day 6 where the plasma Ca of controls was decreased from other days. In lake whitefish (Fig. 2.2C) a significant reduction in plasma Ca of Cd exposed fish was not observed until day 7, but was restored by day 30. In rainbow trout, plasma Ca was not significantly different from controls (Fig. 3.3A).

Alterations to plasma Na were less predictable. In brown trout, exposure to 18 nM Cd resulted in a significant increase in plasma Na after 1 day of exposure. However, by days 14 and 30 a significant reduction in plasma Na was observed in Cd exposed fish relative to controls (Fig. 2.2B). In lake whitefish, a significant decrease in plasma Na was observed on days 3 and 7 of Cd exposure, with differences between control and Cd exposed fish being eliminated by the 30^{th} day of exposure (Fig. 2.2D). In rainbow trout a plasma Na levels did not vary significantly from controls as a result of Cd exposure (Fig 3.3B).

Tissue Specific Cd Accumulation:

Cd accumulation was greatest both acutely (rainbow trout: Fig. 3.4) and chronically (brown trout: Fig. 2.3, and lake whitefish: Fig. 2.4) was the greatest in the gill. Acutely, the liver and kidney accumulated Cd at a similar rate. Chronically, lake whitefish accumulated nearly twice the amount of Cd in the liver as did brown trout. Cd concentrations in the liver were also greater in lake whitefish and in brown trout after 30 days of exposure. The white muscle in all three species did not significantly accumulate cadmium.

Repeat Swimming Performance:

Performance in a second swim trial was affected by exposure to Cd. In brown trout (Fig. 2.5A), performance in a secondary swim challenge following a 30 minute recovery period was reduced in Cd exposed fish on days 1, 6 and 14. Swimming was evaluated on day 30 but it was not significantly different from controls. In a similar experiment, Cd exposure reduced the secondary swimming ability of lake white fish (Fig.2.5B) acutely on days 3 and 7 as well as chronically on day 30.

Rainbow trout (Fig. 3.5) exposed to 18 nM Cd for 6 days similarly showed a reduced Ucrit in the second swim trial when given a 0.5 h recovery period. When the recovery period was extended to 1.5 h, Cd exposed fish performed significantly better than did controls in the second swim challenge. Both treatments performed equally after 6 h of recovery.

Glycogen:

In all three species tested glycogen levels were highest prior to exercise (TP 1). No differences in resting glycogen concentrations in the white muscle were observed

between control and Cd exposed lake whitefish (Fig. 2.7) and rainbow trout (Fig. 3.6). Brown trout showed transient increases and reductions in resting glycogen concentrations varying between test days (Fig. 2.6). A significant reduction in the resting hepatic glycogen supply was observed in rainbow trout after 6 d of exposure. Swimming to 85% U_{crit} resulted in a significant decrease in glycogen concentrations in all tissues and all species studied. In brown trout and lake whitefish little recovery of muscle glycogen was observed after 0.5 h of recovery (TP 3) in either treatment. As in brown trout and lake whitefish, rainbow trout did not show a significant recovery of glycogen stores following a 0.5 h recovery for either treatment. When the recovery period was extended, fish exposed to 18 nM Cd recovered both white muscle and liver glycogen stores much more rapidly than did controls.

Lactate:

Lactate dynamics were effectively the inverse of those observed for glycogen in brown trout (Fig. 2.8), lake whitefish (Fig. 2.9) and rainbow trout (Fig. 3.7 and 3.9). Prior to exercise (TP 1) lactate levels were lowest. In rainbow trout, lactate concentrations in the white muscle were significantly higher than those of controls at TP 1, however in the liver, no such increase was observed. White muscle lactate concentrations increased significantly as a result of the initial swim trial (TP 2), with increases being approximately equal between control and Cd exposed fish. In the liver of rainbow trout, the increase in lactate concentration was significantly greater in Cd exposed fish than in controls. As with glycogen, little recovery occurred after 0.5 h of recovery. However, when the recovery period was extended in rainbow trout a much more rapid removal of

muscle and liver lactate is observed. Lactate levels were elevated to levels observed following the first swim at the completion of the second swim challenge.

ATP:

The depletion and recovery of white muscle ATP in brown trout (Fig. 2.10) and lake whitefish (Fig. 2.11) showed little difference between control and Cd exposed fish. A notable exception is at day 6 in brown trout and 7 in lake whitefish were there exists a sizable reduction in the resting white muscle ATP stores of Cd exposed fish. This decrease was not significant in brown trout but was in lake whitefish. This reduction was also observed in the white muscle of rainbow trout (Fig. 3.12), evaluated after 6 d of exposure to 18 nM Cd.

The recovery of white muscle ATP occurred more rapidly than the recovery of glycogen or clearance of lactate. Recovery again occurred more quickly in Cd exposed fish, with full recovery of muscle ATP stores observed after only 0.5 h of recovery. A similar recovery was not observed until more than 1.5 h in controls. In rainbow trout, liver ATP (Fig.) levels were relatively low and their pattern of depletion and restoration was altered from that observed in the white muscle with significant reductions as a result of exercise only observed in Cd exposed fish. Such reductions were rapidly restored following 0.5 h of recovery.

Cortisol:

In rainbow trout, plasma cortisol (Fig. 3.14) was lowest prior to exercise (TP 1). Following the initial swim challenge a significant increase in plasma cortisol was observed for both control and Cd exposed fish, however the increase was significantly greater in control fish. No reductions in plasma cortisol were observed during the rest

period until after 6 h of recovery, where a significant reduction was observed in control fish, however, concentrations were still significantly elevated compared with resting fish. Cortisol levels remained elevated following the second swim trial.

Appendix B: Exposure Systems

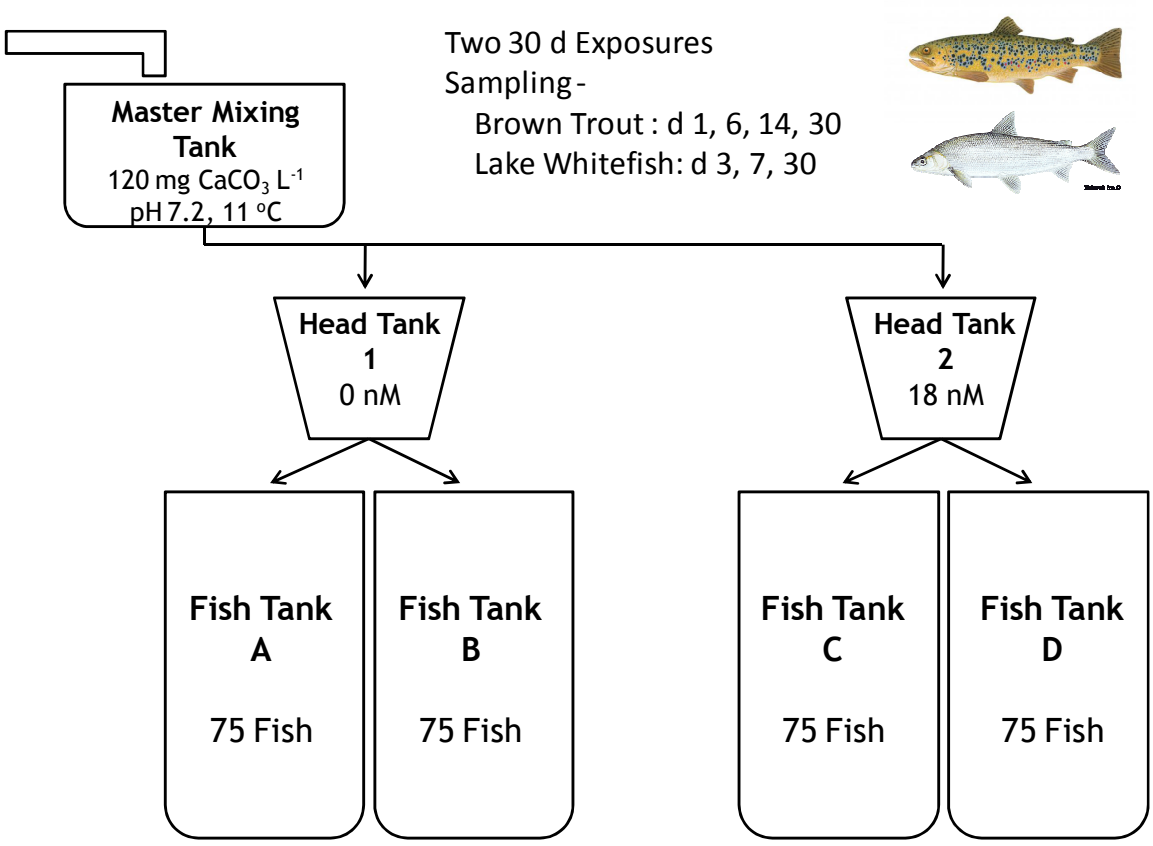


Figure: A1

The distribution of water through the exposure system used in Chapter 2 for brown trout and lake white fish. Exposure water was a mixture of reverse osmosis and dechlorinated city tap water for brown trout and reverse osmosis and well water for lake whitefish. Water was first added to a master mixing tank to allow for mixing. Water was then distributed to the head tanks, where Cd was metered in. Finally, from the head tanks water was delivered, in duplicate to the fish tanks.

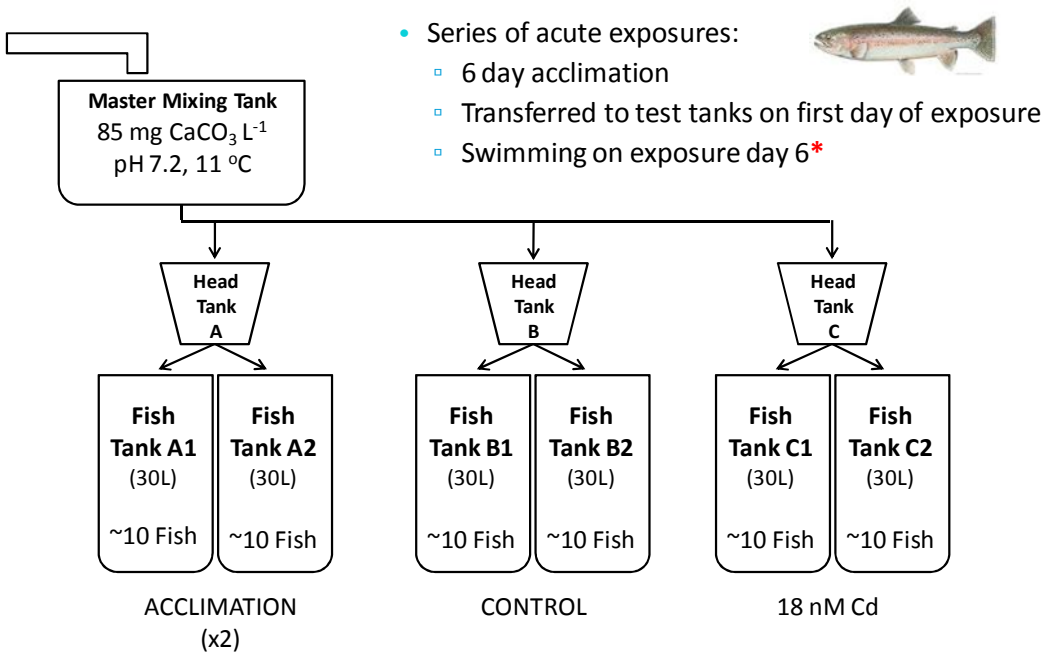


Figure: A2

The distribution of water through the exposure system utilized in Chapter 3 for rainbow trout. Exposure water was a mixture of reverse osmosis and dechlorinated city tap water for. Water was first added to a master mixing tank to allow for mixing. Water was then distributed to the head tanks, where Cd was metered in. Finally, from the head tanks water was delivered, in duplicate to the fish tanks. There were two sets of acclimation tanks (4 tanks total), as well as two tanks for duplicate control and Cd exposures.

Appendix C: Protocols for Analysis of Anaerobic Metabolism

1. Tissue Metabolite Extraction

Reagents Required:

1. 8% PCA Solution

    - Dilute 70% PCA to 8% with epure

    - Add 1 mM EDTA

    Refrigerate, store up to 3 months

2. 3M K_2CO_3

    - Make with 50 mL epure

    - 20.73 g K_2CO_3

    Refrigerate, store up to 3 months

3. 2 M KOH Cocktail

    - 100 mL epure

    - 2M KOH (11.2222 g KOH)

    - 0.4M Imidazol (2.7235 g)

    - 0.4M KCl (2.9822 g)

Extraction Protocol

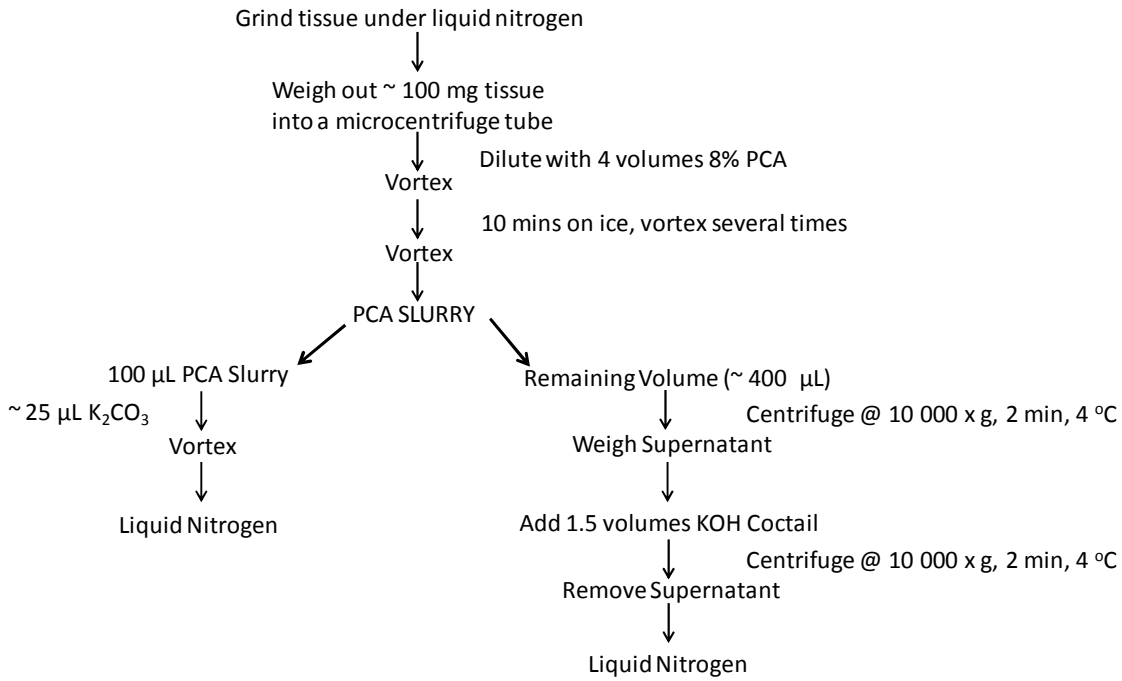
Grind tissue under liquid nitrogen
- Weigh approximately 100 mg tissue into a microcentrifuge tube
- Add 4 x tissue weight 8% PCA
- Leave to deproteinate on ice for 10 min, vortex several times

After 10 minutes
- Remove 100 µL into a microcentrifuge tube (subsample 1)
- Neutralize with ~ 25 µL K_2CO_3
- Place in liquid nitrogen and store at -80 ^{o}C for further analysis of glycogen

Remaining volume (~400 µL) centrifuge @ 10 000 x g (2 mins, 4 ^{o}C)
- Draw off and weigh supernatant

- Dilute with 0.5 volumes KOH cocktail
- Centrifuge as before
- Draw off supernatant into another microcentrifuge tube
- Place in liquid nitrogen and store at -80 ^{o}C for further analysis of lactate and ATP/PCr (subsample 2)

Schematic of Tissue Grinding Protocol:



2. Tissue Glycogen/Glucose Assay

Part 1: Glycogen Digestion

Solutions Required:

Amyloglucosidase: 40 units/ sample (U)

2M sodium acetate (pH to 4.5 with glacial acetic acid)

3M K_2CO_3

Digestion Protocol:

Following the tissue extraction protocol the microcentrifuge tube designated for glycogen/glucose analysis contains 100 µL PCA slurry + ~20 µL 3M K_2CO_3 (~120 µL total: subsample 1)

To this add 1 part sodium acetate (~120 µL)

Vortex

Remove 50 µL of slurry and neutralize with ~5 µL K_2CO_3

Place in liquid nitrogen for subsequent analysis of background glucose concentrations

To the remaining sample add a 20 µL aliquot of 40 U amyloglucosidase

Vortex

Place in water bath or heating block at 37 ^{o}C for 2 h

After 2 h terminate the reaction with 25 µL 70% PCA

Vortex

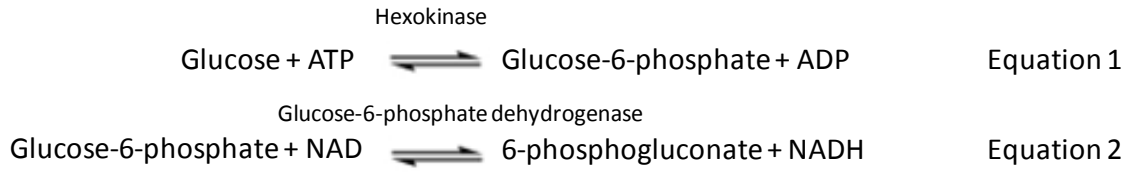
Neutralize with ~80 µL K_2CO_3

This will react violently, add in 10 µL aliquots upto ~40 µL, if the reaction slows can add larger volumes at a time. Add until neutralized

Place in liquid nitrogen for subsequent analysis of liberated glucose concentrations

Part 2: Glucose Assay

Principle of the Assay:



The enzyme catalyzed conversion of glucose to glucose-6-phosphate, followed by the conversion of glucose-6-phosphate to 6-phosphogluconate in the presence of saturating NAD causes the generation of NADH (with a high specific absorbance at 340 nm) in a 1:1 ratio.

Reagents Required:

TEA-HCl (make in advance)

To a 500 mL volumetric flask add:

- 200 mL epure
- 250 nM TEA-HCl

pH to 7.5-7.6 with NaOH as required

Bring volume up to 500 mL with epure

Refrigerate, good for 3 months

Glucose Cocktail (make day of)

To 25 mL TEA-HCl add:

- 0.11g $MgCl_2 \cdot 6H_2O$ (0.02M)
- 0.025g NAD
- 0.025g ATP
- 2.4 units/mL glucose-6-phosphate dehydrogenase

Hexokinase (make day of)

Make with TEA-HCl

- 5 µL/sample and 5 U/sample

Glucose Stock (0.1M)

To 10 mL epure add 0.1802g D-glucose

Glucose Standards:

[Glucose] mM	Volume 0.1M Glucose Stock (µL)	Volume TEA-HCl (µL)
0	0	1000
0.25	2.5	997.5
0.5	5	995
1.0	10	990
1.5	15	985
2.0	20	980
4.0	40	960
6.0	60	940
8.0	80	920
10.0	100	900

The Assay:

Performed using a NOVA Spec (1 mL cuvettes)

To each cuvette add:

- 20 µL sample/standard
- 500 µL glucose cocktail

Wait 5 min

Read $ABS_{initial}$ @ 340 nm

Add 5 µL hexokinase

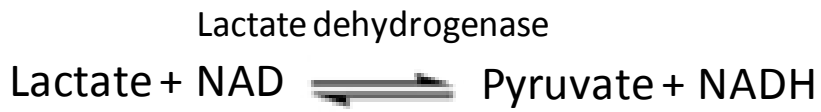
Wait 20 min

Read ABS_{final} @ 340 nm

[G] α ΔABS (ABS_F-ABS_I)

3. Tissue Lactate Assay

Principle of the Assay:



Reagents Required:

Hydrazine Buffer (0.2 M: make in advance)

To a 500 mL volumetric flask add:

- 400 mL epure
- 13 g hydrazine sulphate
  * dissolve on spin plate*
- pH to 9.5 with 10 M KOH (take ~25-30 mL)
- top up to 500 mL with epure

NAD Solution (make day of)

Based on 100 mL hydrazine buffer add:

- 0.033g NAD

LDH Solution (make day of)

- 5 µL/sample and 5 U/sample

Lactate Stock and Standards (make day of)

Lactate Stock 4.44 M

- Make with sodium-L-lactate (FW: 112.03 g/mol)

Make 50 mL of 100 mM stock

112.06g/mol x 0.1mol/L x 0.050 L

=0.5603g sodium-L-lactate

*account for the mass of Na

112.06 g/mol/1 g = 89.07g/mol/xg

=0.7948 g sodium-L-lactate to 50 mL hydrazine buffer

Note: 112.06/89.07 = 1.2581g of sodium-L-lactate has 1/0 g lactate

Dilute the 100 mM stock 10 x

Lactate Standards:

[Lactate] mM	10 mM Lactate Stock (µL)	Hydrazine Buffer (µL)
0	0	1000
0.125	12.5	9875
0.25	25	975
0.5	50	950
1.0	100	900
2.0	200	800
5.0	500	500
10.0	1000	0

The Assay:

In a 96 well microplate add:

- 10 µL sample/standard
- 200 µL NAD solution

Wait 5 mins

Read ABSinitial @ 340 nM

Add: 5 µL of LDH solution (5 U/sample)

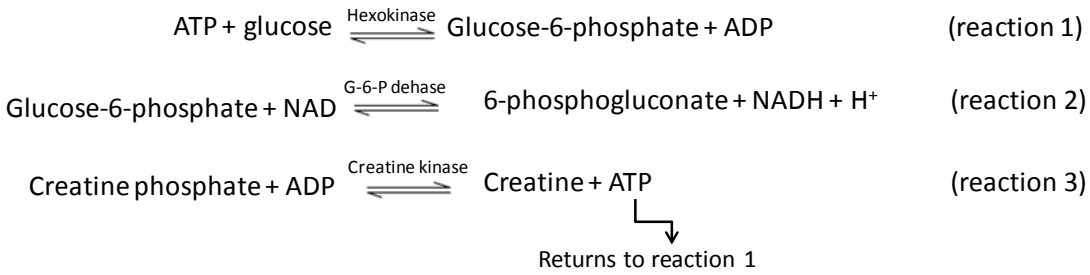
Wait 60-90 mins @ room temperature (or 30-45 mins @ 37 ^{o}C)

Read ABS final @ 340 nM

ΔABS=ABSfinal-ABSinitial

4. White Muscle ATP and Phosphocreatine (PCr) Assay

Principal of the Assay:



The increase in NADH, following the addition of hexokinase results in increased absorbance at 340nm (NAD → NADH). The NADH is directly proportional (1:1) to the original concentration of ATP in the solution analyzed. By measuring the amount of ATP formed in reaction 3, using reactions 1 and 2, one can elucidate the levels of PCr in the sample solution.

Preparation of Reagents

1. Cocktail A. G-6-P Dehydrogenase Solution

   A) First prepare 500 ml 250 mM triethanolamine buffer (TEA)
      To 200 ml epure add
      - 250 mM TEA-HCl
      - pH with NaOH as required to a pH of 7.5-7.6
      - Add enough epure to yield a final volume of 500 ml

   B) To 300 ml TEA add:
      - 0.1063g of NAD
      - 0.187g of $MgCl_2 \cdot 6H_2O$
      - 0.054g of D-glucose
      - 1 unit/ml of G-6-P dehydrogenase (ie 300 units)

2. Cocktail B. Creatine Kinase/ADP Solution
   A) Prepare 6 ml of 0.25 M glycine buffer
      - Add 2.5 ml 0.6 M glycine buffer solution
      - Add 3.5 ml epure

   B) To 2 ml of the 0.25 M Glycine buffer add:
      - 0.025g of ADP

- 5 units/sample of Creatine Kinase

3. 4 units/sample and 5 µl/sample
    - Make with TEA

4. Preparation of 1.25 mM ATP and 4.0 mM PCr Stock/Standards

    To 50 ml TEA add:
    - The appropriate amount of ATP
    - The appropriate amount of PCr

    (Calculate mass of ATP and PCr based on chemical provider)

    ATP/PCr Standards:

% ATP/PCr	[ATP] mM	[PCr] mM	Stock to add (ml)	TEA-HCl to add (ml)
0	0	0	0	1.0
10	0.125	0.4	0.1	0.9
20	0.25	0.8	0.2	0.8
40	0.5	1.6	0.4	0.6
60	0.75	2.4	0.6	0.4
80	1.0	3.2	0.8	0.2
100	1.25	4.0	1.0	0

Assay Protocol:

- To a 96 well plate add:

    - 20 µl sample/standard
    - 200 µl Cocktail A

-Wait ~ 5 minutes to read ABS 1 @ 340 nm

-Add 5 µl Hexokinase to each well

-When the absorbance of the highest ATP standard stabilizes (15-30 min) record ABS 2

-Add 5 µl Cocktail B to each well

-When the absorbance of the highest PCr standard stabilizes (60-90 min) record ABS 3