The effects of strain and ploidy on the
physiological responses of rainbow trout
(*Oncorhynchus mykiss*) to pH 9.5 exposure

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Abstract

British Columbia (BC) has a well-established lake-stocking program that relies on hatchery-reared rainbow trout (*Oncorhynchus mykiss*) from multiple wild and domesticated strains. These strains are stocked into BC lakes as diploids and triploids and, in general, high mortality rates are common after lake stocking due to environmental conditions. Of particular concern is that some lakes in BC are approaching a pH of 9.5, and it is not known if strain and ploidy affects the physiological responses of trout to high pH exposure. The goal of this thesis is to understand the effects of pH exposure, in both soft and hard water, on wild and domestic strains of trout as diploids and triploids. In soft water, high pH exposure resulted in more than 40% loss of equilibrium in the wild strains of trout while the Fraser Valley domesticated strain had fewer than 10% of individuals lose equilibrium overall. There were no clear differences between ploidies in loss of equilibrium. High pH exposure caused significant increases in plasma and tissue ammonia, with no differences between strains or ploidies in ammonia accumulation. In the brain, glutamine increased in response to high pH exposure and glutamate decreased suggesting a protective mechanism of glutamine production in high pH. Plasma lactate accumulated in all groups, suggesting an increase in anaerobic metabolism as a result of high pH exposure. There were no physiological differences between high pH exposure in hard and soft water among the strains tested. However, triploid rainbow trout suffered a greater loss of equilibrium than diploid trout, occurring in conjunction with a significant elevation of brain ammonia in triploid rainbow trout when compared to diploid trout in high pH water. Overall, the

results of this thesis demonstrate an effect of strain on high pH tolerance in trout, but the differences in tolerance appear to not be explained by differences in ion regulation and ammonia balance.

Preface

Chapters 2 and 3 of this thesis are co-authored by William A. Thompson, Tammy Rodela, and Jeffrey G. Richards. I conducted the research in both chapters under the supervision of Dr. Jeffrey G. Richards, with the exception of the measurements of plasma cortisol and brain composition percentages in Chapter 3, which were collected by Tammy Rodela (UBC) and an undergraduate volunteer, Amir Pourghardiri (UBC), respectively. I wrote all 4 chapters of this thesis and I received editorial feedback from Drs. Jeffrey G. Richards, Patricia Schulte, and Colin J. Brauner.

All procedures involving animals were performed using protocols approved by the Animal Care Committee, certificate A13-0024.

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For my grandmother

[1] General introduction

General overview

Given the rainbow trout's importance to sport fishing in British Columbia, experimentally addressing their abilities and limitations will be of great financial benefit to the province. In this thesis I assess the physiological response of diploid and triploid strains of rainbow trout to a high pH environment. I focus primarily on physiological changes related to ammonia balance, ionoregulation, and markers of stress.

1.1 Rainbow trout

Rainbow trout (*Oncorhynchus mykiss*) are members of the family *Salmonidae* and genus *Oncorhynchus* and are closely related to Pacific Salmon (Benhke, 2010). Rainbow trout are not to be confused with the anadromous form of *Oncorhynchus mykiss*, the Steelhead trout, as the rainbow trout possess a different life history in which all life stages live in a freshwater environment (McCusker et al. 2000). The body of work presented in this thesis solely focuses on the physiology and biochemistry of rainbow trout. The rainbow trout is native to the areas around the Pacific Ocean, extending from the coastline of California to Eastern Russia (McCusker et al. 2000). Geographical distributions across a variety of organisms have led to the theory that in the Pleistocene era, a path was formed from the Columbia River that allowed the radiation of the rainbow trout to previously unpopulated areas in California (Benhke, 1979). Rainbow trout have been

introduced to a wide range of locales including Europe, Asia, Australia, Africa, South America, and are prominent on the Western and Eastern coasts of North America (MacCrimmon, 1972; Benhke, 1979). Rainbow trout prefer temperatures ranging from 13°C to 18°C, and can tolerate variations of water quality, and other environmental variables (Hardy, 2002). Their range of tolerance of varied environments further advanced their propagation around the world (Keeley et al. 2005; Keeley et al. 2007). They are a major food source across the globe and as a result many countries have developed rainbow trout hatcheries to meet this demand (MacCrimmon, 1971; Benhke, 1979).

1.2 Aquaculture

The successful growth of fish in hatcheries allowed the development of lake stocking programs to supplement a growing recreational fishing industry. The first report of hatchery-reared rainbow trout was in the 1870's by the California Acclimatization Society (Benhke, 1979). The success of the hatchery allowed the introduction of rainbow trout to new regions throughout the United States and the world (MacCrimmon, 1972). Rainbow trout currently comprise nearly half of the 8 million fish stocked in BC, and the system that handles stocking began to develop in the late 1880's (GSGislason and Associates Ltd, 2009). At that time, BC had 17 government run hatcheries primarily culturing sockeye salmon (Robson, 2004). These hatcheries discontinued salmon production by 1941 and shifted their focus to managing freshwater species through a provincial effort coordinated by the Ministry of Fisheries to supervise all aquaculture sites (Robson, 2004). Today, the

culturing of freshwater fish is largely managed by a non-profit organization, the Freshwater Fishery Society of British Columbia (FFSBC), who play an integral role in maintaining a provincial fish stocking program that supports sport fishing (Robson, 2004).

1.3 Provincial fish stocking program

The sport fishing industry in BC generates over 400 million dollars in revenue from approximately 300 thousand anglers per year (Clarke, 2012). To meet this incredible demand from tourism for this activity, the FFSBC supplements high traffic lakes with fish raised in local hatcheries. Between 2004 and 2009 nearly 8 million fish were stocked annually into 837 lakes across BC, and of these stocked fish nearly 4 million were rainbow trout (GSGislason and Associates Ltd, 2009).

To accommodate the variety of environment conditions present in BC lakes, the FFSBC stock lakes using a variety of wild and domesticated strains. A strain is defined as a population of rainbow trout that is geographically isolated and has adapted to its local environment. It is believed that many of the adaptations are genetically based and therefore heritable (FFSBC, 2004). As a result, the FFSBC attempts to match appropriate strains to specific environments to optimize fish survival (Clarke, 2012). There are six strains that are commonly stocked in BC including four wild strains in this thesis (Blackwater, Tzenzaicut, Pennask, and Carp Lake), and one domesticated strain (Fraser Valley). Wild strains are kept in a number of broodstock lakes across BC that are maintained by the FFSBC in order to limit the number of fish removed from the original wild populations (FFSBC, 2004).

These wild strains show differences in growth rates and display diverse preferences in diet and within lake habitat use. The Blackwater strain originates from Blackwater River, and is a fast growing, piscivore commonly stocked in lakes with large shallow shoal areas with a non-salmonid fish community (FFSBC, 2004). The Tzenzaicut strain originates from Tzenzaicut Lake, and is a piscivore as well, preferring colder, pelagic regions (FFSBC, 2004). The Pennask strain originates from Pennask Lake, and is a slow growing insectivore, preferring pelagic regions (FFSBC, 2004). The Carp Lake strain is a recent addition to the lake stocking program, and originates from Carp Lake, BC (Clarke, 2012). The Fraser Valley strain is the only domesticated strain that is currently stocked in BC. Its genetic roots originate from the McCleary strain of rainbow trout from the trout lodge hatchery in Washington, USA, and it is considered to have a fast growth rate (FFSBC, 2004).

A serious concern with stocking of hatchery-reared fish in a natural environment is the gene flow of domesticated genes into the indigenous gene pool. The combination of reversed sex rainbow trout in conjunction with a shock technique to induce triploidy creates a sterile triploid female population to be introduced for sport fishing. Triploidy is a condition that occurs naturally in some vertebrates with cells containing a third set of chromosomes as opposed to the normal two. Triploidy is typically lethal in mammals (Beatty, 1970), with few species capable of forming individuals that can reach an adult age with triploid cells (Jones et al. 1969; Dunn et al. 1970; Fechheimer et al. 1983; Lamborot and Vasquez, 1998). In rainbow trout the rate of spontaneous triploidy generation is small (Thorgaard et al. 1982), yet triploid rainbow trout are typically able to survive to

adults (Cuellar and Uyeno, 1972; Thorgaard and Gall, 1979). It is hypothesized that the natural mechanism for spontaneous triploidy in rainbow trout is the fertilization of an unreduced oocyte combined with the suppression of the second meiotic division (Cuellar and Uyeno, 1972).

Artificially, triploidy has been induced by suppressing the second meiotic division through a number of different means including pressure shock and temperature. The prevention of the second meiotic division occurs immediately after fertilization of the egg by a sperm, caused by applying a shock to prevent the extrusion of the second polar body from the fertilized egg. The effectiveness of the shock in different species is highly dependent on the time of exposure and the type of shock being used (Gillet et al. 2001; Komen and Thorgaard, 2007; Filep et al. 2009). Chemical induction by cytostatic chemicals has shown some success in Atlantic salmon for the induction of triploidy, but has also resulted in the production of tetraploid individuals (Allen and Stanley, 1979). When chemicals such as cytochalasin B have been used to induce triploidy in rainbow trout, high mortality rates have occurred (Refstie, 1981; Pawson, 2003). More successful methodology has since been found in exposure to high-heat or high-pressure environments to suppress the second meiotic division. Triploidy rates of 77-100% have been reported in Brown trout by applying different temperatures (26°C-32°C) to recently fertilized eggs for a variable time course. In rainbow trout, Lincoln and Scott (1983) saw varying success with different strains receiving the same heat shock. However, heat shock may induce the purging of the sperm from the egg therefore eliminating the induction of triploidy (Thorgaard et al. 1995). The most consistent technique

used in aquaculture to induce triploidy in rainbow trout has been the implementation of a hydrostatic pressure shock to recently fertilized eggs. The pressure can range from 7,000 to 12,000psi (Chourrout, 1984; Luo and Purdom, 1984; Lincoln, 1989; Yesaki et al. 1996) but produces near 100% effectiveness in triploidy induction. Although this process does cause some mortality its effectiveness makes it the current mechanism of choice. At the FFSBC the treatment is strain specific and can be either a heat shock or a hydrostatic pressure shock (FFSBC, 2004).

Stocking only female fish in lakes is another important measure to prevent gene flow to natural lake populations of rainbow trout. The generation of an all-female population starts with the production of sex reversed female rainbow trout. Reversed sex rainbow trout are normal fry females that have been introduced to a testosterone-based chemical, such as 17α -methyltestosterone, that causes the cessation of ovary development and the production of normal testes resulting in a modified male phenotype (Kuzminksi and Dobosz, 2010). As the rainbow trout are females, they are of an XX genetic background and subsequently will only be capable of passing on an X chromosome to their offspring, viably producing a sterile triploid all female population.

The combination of the reversed sex rainbow trout in conjunction with the shock technique creates a sterile female population to be introduced for sport fishing. The benefits of sterility appear to be dependent on the sex of the fish made sterile. For females, sterility is believed to divert energy that would normally be used for gonadal development towards somatic growth (FFSBC, 2004). However,

sterile triploid male rainbow trout still produce reproductive hormones, which are believed to reduce flesh quality due to the onset of secondary sexual characteristics (FFSBC, 2004).

In the wild, stocked triploid fish have been shown to have variable success in terms of survival. Studies have reported reduced triploid returns (Blanc et al. 1992; Simon et al. 1993; Dillon et al. 2000), while others report no difference between ploidies (Guo et al. 1990; Wagner et al. 2006). In BC, gillnetting assessments have reported reduced returns of triploid fish after stocking, and the exact cause of this increased mortality is unknown. There appears to be no real difference in growth, haematological, and biochemical parameters between ploidies when exposed to stress (Galbearth et al. 1994; Sadler et al. 2000), however, Scott (2012), reported reduced time to loss of equilibrium of triploid rainbow trout in hypoxia. No work has been done to explore the physiological response of triploid fish to high pH water.

1.4 The environmental parameters of stocked lakes in British Columbia

In addition to stocking BC's lakes, the FFSBC regularly records water parameters of various lakes across the province. The society has reported increasing water pH in many lakes. Records have shown that lakes, such as Green Lake, have increased to a pH of almost 9.5, and are expected to continue to increase to a pH of 10.0 in the coming decade.

The pH of an aqueous environment is a measure of hydrogen concentration and is calculated by:

$$\text{pH} = -\log_{10}[\text{Hydrogen}]$$

and can vary due to a variety of physical and chemical factors. In the natural environment, water pH can be greatly influenced by photosynthesis and respiration of aquatic plants (Jones et al. 1998; Hansen, 2002), human eutrophication (Jones et al. 1998), and basic bedrock geology of the aquatic environments (Sutcliffe and Carrick, 1973; Krueger and Water, 1983). In BC, bedrock geology releases high levels of carbonates resulting in lakes with high pH. The pH tolerance of hatchery-reared trout in BC has seen limited investigation despite the great range in lake water pH that these fish may be introduced to.

1.5 Morphological and physiological responses to elevated ambient pH

Growth and survival are reduced upon exposure to high pH water. Studies on carp (*Cyprinus carpio* L.) have reported a decrease in growth rate at the larval stage in high pH to almost a third of that seen in more neutral waters (Korwin-Kossakowski, 1992). Mortality studies have reported relative tolerances of trout, with the upper limits of brook trout survival occurring at a pH of 11.0 (Daye and Garside, 1975). Jordan and Lloyd (1964) reported up to 50% mortality in rainbow trout at a pH of 9.8 after 24h, and Daye and Garside (1975) noted histological damage of the skin at a pH of 10.

High water pH impacts gill function, which ultimately leads to mortality. Daye and Garside (1976) noted superficial damage to the gill and operculum after brook trout were exposed to an ambient pH of 9.0, far below the levels reported for mortality (Daye and Garside, 1975; Daye and Garside, 1976). Damage to the gill was

primarily attributed to degradation of the connective tissue between epithelial cells and the hypertrophy of mucous secreting cells and their subsequent release of excess mucous (Daye and Garside, 1976). Moreover, high pH significantly affects the tight junction proteins in the gill. Tight junctions are multifunctional apical proteins comprised of claudins, that act as junctions of cells that are present at the apical side of the gill membrane, and that are directly responsible for regulating paracellular solute movement and water flux in epithelial cells (Schneeberger and Lynch, 2004; Sandbichler et al. 2011). Low water pH causes enhanced permeability of the gill through the disruption of tight junctions (Tang and Goodenough, 2003) and the same may be true of high pH.

1.5.1. Ionoregulation in high pH

Under normal conditions, freshwater fish have to actively maintain the ionic composition of their body fluids, due to their hypo-osmotic environment (Evans et al. 1999). The exchange of electrolytes in the rainbow trout is tightly controlled by the chloride cells, or mitochondria rich cells, and to a lesser extent the pavement cells of the gill in adult fish (Avella et al. 1987; Perry and Laurent, 1989; Perry, 1997; Evans et al. 2005). Sodium levels within the body are maintained by an electrochemical gradient with a basolateral sodium-potassium ATPase (NKA), and with exchange of sodium and protons across the apical membrane (NHE) (Evans, 2011). An apical bicarbonate exchanger transports chloride, and chloride channels carry chloride ions across the basolateral membrane into the blood (Evans, 2011).

The gill is the major ion regulatory organ in fish and it is susceptible to damage during high pH exposure, and various studies have reported severe ion loss of sodium (Na^+) and chloride (Cl^-) and large histological changes in rainbow trout exposed to high pH water (Wilkie and Wood, 1991; Yesaki and Iwama, 1992; Wilkie and Wood, 1995; Laurent et al. 2000). Sodium and chloride are the dominant ions in plasma and consequently are primary determinants of osmolality (Wood and Randall, 1973). Both of these ions play a large role in acid/base balance (McDonald and Wood, 1981; Goss and Wood, 1990; Perry et al. 1992; Goss et al. 1998), particularly in exchange for their acidic and basic equivalents, H^+ and HCO_3^- (Eddy, 1976; Goss et al. 1998). Exposure to high pH water in rainbow trout results in an immediate loss of sodium to the environment, presumably due to gill damage (Wilkie et al. 1996; McGeer and Eddy, 1998; Laurent et al. 2000), despite a maintained unidirectional sodium uptake rate. High pH water is associated with increases in chloride cell surface area and their surface area to volume ratio, increases in the amount of surface area available for pavement cells, and a reduction of the lamellar thickness of cells at the gill (Laurent et al. 2000). Furthermore, increased secretion of mucus from epithelial cells under high pH conditions, as reported by Daye and Garside (1976), may provide a charged microenvironment, which can be expected to act as a trap for cations and anions such as chloride and sodium. Mucus is comprised of water, ions, and cationic glycoproteins that are secreted in high stress situations by hypertrophy of mucosal cells (Handy, 1989).

1.5.2 Nitrogen balance in high pH

Ammonia (referred to as the sum of NH_3 and NH_4^+) is a by-product of amino acid catabolism and is either readily excreted across the gills, converted to another molecule for reuse, or converted to urea for release from the body (Wilkie, 2002; Randall, 2011). Rainbow trout typically excrete most of their wastes as ammonia across the gill (Wilson et al. 1994). At a normal physiological pH (7.4 to 7.8), approximately 95% of the ammonia exists as NH_4^+ . In vertebrates, if NH_3 accumulates within the body it may lead to seizures, convulsions, and even death through disruption of the central nervous system (Randall and Tsui, 2002; Monfort et al. 2002; Randall, 2011). The toxicity of ammonia is directly related to the concentration of the un-ionized form (NH_3) in the body (Smart, 1978). The proportion of NH_3 compared to the ionized ammonium ion (NH_4^+) is directly determined by the pKa (~ 9.3) of ammonia, which is influenced by both temperature and pH (Emerson et al. 1975; Smart, 1978). Ammonia is typically excreted across the gill as NH_3 by simple diffusion down its concentration gradient through rhesus glycoproteins (Nawata et al. 2007; Hung et al. 2008). The gill creates an acidic microenvironment by pumping H^+ across the apical gill creating a boundary layer or “pocket” for NH_3 to interact with H^+ and to be converted to NH_4^+ , keeping NH_3 levels low and promoting further ammonia diffusion (Randall and Wright, 1989; Wilson et al. 1994). Sodium can also be exchanged for H^+ by the NHE to maintain this acidic “pocket” for ammonia conversion (Wright and Wood, 2009; Wright and Wood, 2012).

Environmental NH_3 increases in high pH water impairing ammonia excretion, and leading to an elevation in whole body ammonia (Wilkie and Wood, 1994; Wilkie et al. 1996; Wilkie, 1997). The retention of ammonia within the body increases the amount of unionized ammonia within the tissues, and it can interact with free H^+ to raise the intracellular pH of the tissues that can, in turn, affect cellular mechanisms (Randall, 2011). Studies have demonstrated that white muscle tissue increases its ammonia content 2-fold when exposed to a prolonged alkaline environment (Wilkie and Wood, 1994). Under conditions where excretion is inhibited, ammonia can be shuttled to metabolite production. Sanderson et al. (2010) reported that rainbow trout could convert ammonia to less harmful metabolites such as glutamate and glutamine in their high external ammonia exposure. The production of glutamine is expected to be a protective response to ammonia accumulation in the brain, which is sensitive to ammonia (Sanderson et al. 2010; Randall, 2011)

Ammonia can also be sequestered as another metabolite, urea, through the ornithine-urea cycle (OUC; Huggins et al. 1969; Depeche et al. 1979; Anderson, 2001). Lake Magadi tilapia (*Oreochromis alcalicus grahami*) live in an extremely alkaline environment (pH >10.0) and have adapted their metabolism to synthesize and excrete their nitrogen wastes as urea (Wood et al. 1989). While the early life stages of rainbow trout are able to synthesize urea due to the expression of OUC enzymes (Wright et al. 1995; Pilley and Wright, 2000), these same enzymes are not highly expressed in adult stages and urea is not readily used by adult rainbow trout to sequester and excrete ammonia from the body (Wright, 1995; Wilkie et al. 1996; McGeer and Eddy, 1998).

1.5.3 Blood parameters in high pH

Disturbances to haematology, changes in the hormone cortisol, and the production of lactate often indicate organismal stress. A change in the osmolarity of the plasma affects hematocrit, and this can adversely affect the viscosity of the blood (Leray et al. 1981). Hematocrit can be highly variable in blood due to stress (Wells and Weber, 1991) and measurements of hematocrit have been used as a proxy for stress in humans (Ring et al. 2008), and may potentially act as a measure of the stress response to high pH.

Upon exposure to a stressful event, fish respond with rapid increases in the production of lactate and the stress hormone cortisol. Cortisol promotes energy metabolism in organisms (DiBattista et al. 2005) prompting the release of ammonia as a by-product of protein catabolism (Leach and Taylor, 1982; Mortimore and Poso, 1987; Van Der Boon et al. 1991). Cortisol significantly upregulates the amount of chloride cells at the gill (Laurent and Perry, 1990; Laurent et al. 1994) and can produce rapid changes in the activity of Na^+/K^+ ATPase expression (Shrimpton et al. 1994; Shrimpton and McCormick, 1999; Seidelin et al. 1999). The presence of high cortisol levels in the plasma is thought of as an indicator of stress (Weil et al. 2001; Jentoft et al. 2005); lower cortisol levels in the plasma may be representative of an organism that is more tolerant of high pH. Lactate is produced during anaerobic metabolism (Huckabee 1958; Skinner and McLellan, 1980; Milligan and Girard, 1993; Juel, 1997), increasing in response to stress (Turner et al. 1983). In

high pH experiments, lactate significantly increases immediately after exposure and returns to control levels after a few days (Wilkie et al. 1996; Wilson et al. 1998)

1.5.4 Hard water and high pH

Lakes that are near a pH of 9.5 often have hardness levels (measured as concentration of CaCO_3) above 500mg/L, which are considerably higher than their rearing conditions at the hatchery (Intermediate hardness of 70-180mg/L). Dissolved calcium in the environment assists in maintenance of the gill, presumably by tightening leaky junctions through direct binding to the gill (Hunn, 1985; Perry and Wood, 1985). The “shoring” up of leaky channels is confirmed in other studies where the efflux of ions is often reduced upon the addition of calcium to the ambient environment (Eddy, 1975; Hunn, 1985), which also increased activity of the H^+ ATPase at the gill (Lin and Randall, 1995). If increased hardness assists in reducing the normal efflux of ions across the gill, then the natural environment may play a role in maintaining ionic levels in a high pH environment. However, the reduced flux across the gill may prevent the formation of the acidic boundary layer, which could abolish ammonia trapping and the re-establishment of ammonia excretion after the ammonia load. The buffer capacity of hard water may also prove to be detrimental, as exposure to water buffered with glycine resulted in rainbow trout losing a significant amount of ions (McGeer and Eddy, 1998). Whether or not calcium and hardness in general will provide an advantage to our fish remains to be seen.

1.6 Objectives

The goal of this thesis was to explore the physiological responses to high pH exposure in four strains of diploid and triploid rainbow trout. Each of the strains used in this study had varied genetic origins and one strain may have exhibited an advantageous physiological response to high pH water. Given the prevalence of triploidy in stocking lakes in British Columbia, assessing the physiological change of triploid trout compared to diploid trout is essential for appropriate stocking efforts. Therefore, for my first objective, I explored: *the effects of strain and ploidy on the physiological responses of trout to pH 9.5 water*

For the second objective, I was aware that high pH environments are complex, and that lake settings can affect the response of an organism with its environment. Therefore, for my second objective, I investigated: *the effects of water hardness on the physiological response to high pH water of various strains and ploidies of rainbow trout*

[2] The physiological effects of high pH (9.5) exposure on various strains of diploid and triploid Rainbow trout (Oncorhynchus mykiss)

2.1 Introduction

Increases in water pH are known to have profound physiological effects on fish. In rainbow trout (*Oncorhynchus mykiss*), exposure to pH above 9.5 can impair ionoregulation and result in an accumulation of ammonia in blood and tissues, which if uncorrected, can result in death. Disturbances in ionoregulation occur because exposure to high pH causes gill damage (Daye and Garside, 1976; Wilkie and Wood, 1994), which increases mucous production, tight junction loosening, and degradation of the secondary lamellae (Daye and Garside, 1976; Heisler, 1989; Tang and Goodenough, 2003). These changes in gill structure lead to increased gill permeability and increased ion efflux across the gill (Wilkie and Wood, 1994; Wilkie et al. 1997). In response to the high-pH induced increase in gill permeability, rainbow trout up-regulate gill chloride cell density (Wilkie and Wood, 1994; Laurent et al. 2000) in an attempt to balance ions, but these mechanisms are not entirely effective as high pH has been shown to directly inhibit ion influx (Wilkie et al. 1999).

Trout exposed to pH 9.5 also accumulate endogenously produced ammonia in their blood and tissues, increasing more than 3-fold in plasma (Wilkie and Wood, 1991; Wilkie and Wood, 1994) due to a pH-dependent decrease in ammonia efflux across the gills (Wright and Wood, 1985). At physiological pH, the ammonium ion (NH_4^+) is the predominant form of ammonia in the blood and tissues (Ip et al. 2001; Randall and Tsui, 2002); when water pH is above the pK_a for $\text{NH}_4^+/\text{NH}_3$ ($\text{pK}_a=9.3$),

NH_4^+ shifts towards NH_3 (Cameron and Heisler, 1983). This alters the concentration gradient that normally allows the passive diffusion of ammonia, causing an ammonia accumulation in the blood and tissues (Wilkie and Wood, 1994; Wilkie et al. 1996; Wilkie et al. 1997). Ammonia is lethal at high concentrations (Randall and Tsui, 2002; Monfort et al. 2002; Ip and Chew, 2010; Randall, 2011), causing over activation of NMDA receptors (Xu and Tao, 2004; Ip and Chew, 2010). These physiological effects combined with ion loss are thought to be the primary mechanisms associated with mortality in fish exposed to high environmental pH (Yesaki and Iwama, 1992; McGeer and Eddy, 1998).

Over the past several decades there has been a gradual increase in pH of many freshwater lakes across British Columbia (BC) such that pH is presently approaching 9.5 and is expected to exceed 10 in the coming decade. The primary cause of the increasing pH is unknown, but bedrock geology and low flushing rates are thought to be the primary reason (Krueger and Water, 1983; Jones et al. 1998). BC has maintained an active lake-stocking program for decades with more than 4-million rainbow trout (*Oncorhynchus mykiss*) stocked annually into 837 lakes. BC's lake stocking program uses trout from at least 6 different strains of which roughly half are stocked as triploids, and generates more than \$400 million annually. Rising pH in many of these lakes, particularly popular fishing lakes such as Green Lake that has already reached pH ~9.3, puts continued stocking efforts in jeopardy. In order to continue a successful lake-stocking program in British Columbia it is essential to characterize the effects of high pH exposure on the various strains and ploidies of

trout used by the Freshwater Fishery Society of British Columbia (FFSBC), in hopes of identifying the most high pH tolerant trout strain for future stocking efforts.

The objective of this study was to compare the physiological responses of exposure to pH 9.5 among five strains of juvenile rainbow trout, four wild and one domesticated, as diploid and triploid, all of which are currently used by the FFSBC for stocking. In this study I exposed rainbow trout to pH 9.5 in soft water and sampled blood and tissues for up to 72h and analyzed aspects of ion regulation and ammonia balance. My goal was to determine whether there is an affect of strain or ploidy on the physiological effects of pH 9.5 exposure.

2.2 Materials and methods

2.2.1 Experimental animals

This study used four wild strains and one domesticated strain of rainbow trout. The fish were obtained from the Fraser Valley freshwater trout hatchery in Abbotsford, British Columbia. Wild strains were named after their site of origin, and included strains from Blackwater River (named Blackwater), Tzenzaicut Lake (named Tzenzaicut), Pennask Lake (named Pennask), and Carp Lake (named Carp lake). Each spring, FFSBC staff collected breeding pairs of each strain from their native environment and bred three families at the Fraser Valley trout hatchery. The progeny of each family were divided, and half were allowed to develop as diploids and the fertilized eggs of the other half underwent hydrostatic pressure shock to induce triploidy, creating full siblings that differed only by ploidy. Three families of

the Fraser Valley domesticated strain were also bred and diploid and triploid siblings were generated using the same strategy as used in the four wild strains. Strains used each year were based on availability. Fish were reared at the Fraser Valley trout hatchery until December of each year when fish were tagged with visible implant elastomere tags to distinguish between strains, ploidies, and families. In January, the wild strains were transferred to the University of British Columbia (UBC) and were housed in a common garden flow through system separated by strain. The Fraser Valley domesticated strain grows faster than the wild strains, therefore they were bred later and tagged in March of each year, transferred to the UBC in early April, and subsequently housed in the same system. At the UBC, the fish were reared for 6 months in flow through de-chlorinated Vancouver city tap water (pH ~6.7, hardness as $[\text{CaCO}_3]$ <17.9mg/L) and were held at 10°C-15°C in 2011, and 8°C-12°C in 2012. Hardness was measured with a colourmetric assay (API General hardness test kit). Fish were fed twice daily with 2% bodyweight of Bitovita fry food. All holding and experimental conditions were conducted according to the guidelines of the Canadian Council on Animal Care.

2.2.2 Experimental protocol

To examine the effects of pH 9.5 exposure on diploid and triploid trout strains, two studies were conducted, with minor differences in protocol and the number of strains used. In 2011, I exposed diploid and triploid Blackwater, Tzenzaicut, Pennask and Fraser Valley trout to pH 9.5 for 48h. All trout were fasted for 24h in their stock tank before 12 size-matched individuals of each strain and

ploidy were transferred to a randomly chosen compartment of a 160L exposure tank. Equal numbers of each family (4 individuals/family) were included in the compartment. Trout were allowed to recover from the transfer under flow-through conditions for 24h. Water was circulated from the tank to a header tank using two submersible pumps and this water circulation facilitated mixing within the exposure system. After the 24h recovery period, two fish of each group were sampled for plasma, muscle, brain, and gill and this sampling served as the control. Fish were removed by netting and transferred into a bucket containing 250mg/L benzocaine. Once the fish showed loss of equilibrium (defined as an inability to maintain dorso-ventral orientation) and ceased ventilating, they were removed from the anaesthetic and sampled. Whole blood was drawn into heparinized capillary tubes after caudal severance. Capillary tubes were centrifuged at 9200g to separate plasma from red blood cells. Plasma was then removed and placed into a micro-centrifuge tube, frozen in liquid nitrogen, and stored at -80°C. I pipetted 5µL of whole blood from the exposed caudal vein and mixed in 125µL of propidium iodide solution (125µL Citrate acid dextrose (2.088% trisodium citrate, 2.45% glucose, 0.693% citric acid of total volume), 0.025mg propidium iodide, 5µL IGEPAC detergent, and 0.00425mg ribonuclease A) and used it for ploidy verification (details below). An additional 10µL of whole blood was taken from the caudal vein and mixed with 2mL of Drabkin's reagent (Sigma #D5941) for the spectrophotometric determination of hemoglobin concentration. Following blood sampling, a 1cm segment of caudal muscle was excised, brain and the entire gill basket were excised and all tissues were immediately frozen in liquid N₂ and stored at -80°C. Following control

sampling, pH was adjusted from a pH of 6.7 to 9.5 over 6h with the addition of 0.2M NaOH directly to the header tank. Once pH 9.5 was achieved it was maintained using a pH electrode (Cole-Parmer) connected to an alpha-560 Eutech instruments pH regulator. This controlled a peristaltic pump that delivered 0.2M NaOH when measured pH deviated from the set point by 0.01 pH units. The regulator was calibrated every 12h and I verified water pH using a Fisher Scientific hand-held pH meter to ensure that pH remained at 9.5. Dissolved oxygen and water ammonia were measured every hour by a handheld-oxygen probe (Oakton Acorn Series DO6) and colourmetric assay (API Ammonia test kit), to ensure oxygen levels remained above 90% air saturation and ammonia levels remained below 0.25mg/L. Water changes were performed immediately after sampling, with pH 9.5 water at 6, 12, 24, and 36h. Two more fish from each compartment were sampled at 6, 12, 24, and 48h using the same protocol as outlined above. Fish were assessed for loss of equilibrium (LOE) throughout the study. LOE was established when the fish lost the ability to maintain normal dorso-ventral orientation and did not correct orientation when gently prodded with a fish net. If upon prodding, the fish could not re-establish equilibrium, it was removed and the time was recorded. No fish that lost equilibrium were sampled for tissue analysis. This experiment was replicated four times, using diploid Blackwater of 15.49 ± 1.1 g (mean $\pm$ SEM; n=29), triploid Blackwater of 15.62 ± 1.28 g (n=25), diploid Tzenzaicut of 24.48 ± 1.72 g (n=26), triploid Tzenzaicut of 19.25 ± 1.09 g (n=22), diploid Pennask of 16.72 ± 2.89 g (n=26), triploid Pennask of 10.97 ± 1.19 g (n=26), diploid Fraser Valley of 20.02 ± 0.79 g (n=40), and triploid Fraser Valley $18.05 \pm .65$ g (n=40) for a total of 96 fish per

replicate. Two-way ANOVA and post-hoc analysis of main effects showed a significant difference between strains ($p < 0.0001$), but no significant difference in size between ploidies of a strain. This study was carried out in June of 2011.

I repeated the above experiment in 2012 with minor modifications. This study was repeated in order to collect more tissue samples for analysis and because a new trout strain, the Carp Lake strain, had become available. This experiment consisted of 3 replicates, with diploid Blackwater of $6.49 \pm 0.51\text{g}$ ($n=36$), triploid Blackwater of $5.92 \pm 0.41\text{g}$ ($n=36$), diploid Tzenzaicut of $4.91 \pm 0.26\text{g}$ ($n=36$), triploid Tzenzaicut of $4.41 \pm 0.12\text{g}$ ($n=35$), diploid Pennask of $3.78 \pm 0.2\text{g}$ ($n=35$), triploid Pennask of $3.47 \pm 0.19\text{g}$ ($n=34$), diploid Fraser Valley $5.31 \pm 0.32\text{g}$ ($n=36$), triploid Fraser Valley of $4.49 \pm 0.13\text{g}$ ($n=36$), diploid Carp Lake of $5.37 \pm 0.37\text{g}$ ($n=36$), and triploid Carp lake of $3.99 \pm 0.18\text{g}$ ($n=36$). Two-way ANOVA and post-hoc analysis of main effects showed a significant difference between strains ($p < 0.0001$), but no significant difference in size between ploidies of a strain. A 160L tank was divided into 10 separate compartments with a total of 15 fish of each strain and ploidy in each compartment. Three fish were removed for sampling at 0 (control), 12, 24, and 72h in the 2012 study using the protocol described above. Water changes were performed at 6h, 12h, 18h, 24h, 36h, 48h, and 60h with pH 9.5 water.

2.2.3 Analytical techniques

In 2011, plasma hemoglobin, hematocrit, mean cellular hemoglobin concentration, ammonia, urea, and white muscle ammonia was measured. In 2012, plasma sodium, chloride, lactate, brain ammonia, brain glutamate, and brain

glutamine was measured. Capillary tubes were centrifuged at 9200*g* to separate plasma and red blood cells and hematocrit was determined as the percent of total blood occupied by red blood cells. Hemoglobin measurements were divided by hematocrit values to give MCHC content. Separated plasma was removed from the capillary tubes, and placed into a micro-centrifuge tube and frozen in liquid nitrogen. Ploidy was confirmed in all fish using the protocol established by Arumuganathan and Earle (1991). Briefly, whole blood samples stored in the propidium iodide solution were analyzed for cell size using a FACSCalibur bench flow cytometer (BD Biosciences, California, USA). Triploidy was confirmed to be 94% effective in 2011 and 96% effective in 2012. Hemoglobin concentration was obtained spectrophotometrically by the methods described by Blaxhall and Daisley (1973).

Plasma was used for the analysis of total ammonia, urea, chloride, sodium, and lactate. Metabolite plasma samples were diluted in 8% perchloric acid and the homogenate was centrifuged at 18000*g* for 10 minutes. The supernatant from the centrifuged sample was removed and neutralized in 2M KHCO₃, and centrifuged again at 18000*g* for 10 minutes. The supernatant from the neutralized plasma sample was removed for analysis. Ammonia values were obtained with a commercial kit (SIGMA Cat#A0100), using glutamate dehydrogenase and the extinction coefficient of NADH at 340nm of 6,220L/mol•cm. Plasma urea values were obtained by using the protocol of Rahmatullah and Boyde (1980). For sodium and chloride measurements, frozen plasma was thawed and 5µL of plasma was diluted 200-fold in dH₂O. The diluted plasma sample was aliquoted into two

separate tubes: (1) the aliquot was diluted an additional 6-fold and run through a flame photometer (Varian AA240FS) (2) the aliquot of the 200-fold diluted plasma sample was mixed with 13mM mercuric thiocyanate and 0.5M ferric nitrate and was immediately read at 480nm by spectrophotometer for the determination of plasma chloride. Plasma lactate was measured using the methods outlined in Bergmeyer (1983).

White muscle was used for the analysis of total ammonia, and brain tissue was used to obtain total ammonia, glutamine, and glutamate values. Brain and white muscle ammonia were measured using the same protocol used for determination of plasma ammonia. For the determination of white muscle ammonia, briefly, white muscle tissue was isolated and ground under liquid nitrogen, pre-weighed, sonicated with 8% perchloric acid using a micro ultrasonic cell disrupter (Kontes), and neutralized with 2m KHCO_3 . The neutralized sample was centrifuged at 18000g for 10 min, and the supernatant was removed. Brain samples were weighed, sonicated with the same protocol as white muscle tissue, and neutralized. 5M TRIS was used to neutralize brain samples instead of KHCO_3 to help buffer the neutralization reaction. Brain glutamate and glutamine concentrations were determined using a modifying version of the protocol described in Lund (1970). Neutralized homogenates were aliquoted into two separate tubes. One aliquot was subjected to an additional hydrolysis reaction, being mixed with 0.5M acetate buffer and 2mM glutaminase solution and incubated at 38°C for 1 h to convert all glutamine in the homogenate to glutamate. Both aliquots were then mixed with 0.5M Tris/Hydrazine buffer, 30mM NAD^+ solution, 100mM ADP solution, and

glutamate dehydrogenase (1200kU/L) and incubated at room temperature for 40 minutes prior to being read on the spectrophotometer at 340nm. Both samples were read against a standard curve for glutamate, and taking the total glutamate from the hydrolysis reaction and subtracting the glutamate from the aliquot that did not undergo a hydrolysis reaction, I calculated glutamine. I assumed that glutamine converts to glutamate stoichiometrically.

2.2.4 Statistical analysis

All data are reported as mean values $\pm$ SEM. Family was collapsed in the statistical analysis to focus on the effects of time/treatment, strain, and ploidy. Three-way analysis of variance (ANOVA) was performed on all data sets with time, strain, and ploidy as fixed variables. When interaction terms were significant, a two-way ANOVA was performed followed by a Tukey post-hoc analysis, with data sets collapsed by strain or ploidy where appropriate. LOE data was analyzed using a Kruskal-Wallis chi-squared test. All data was analyzed using R version 2.15.1.

2.3 Results

2.3.1 Loss of Equilibrium 2011 study

In 2011, a Kruskal-Wallis chi-squared test revealed a significant effect of strain on LOE ($p < 0.0001$). Trout began losing equilibrium as pH reached 9.5 at 6h, with the Pennask triploid trout showing the greatest loss of equilibrium (Figure 2.1). By 12h exposure to pH 9.5, all diploid and triploid wild strains had a greater

loss of equilibrium than the Fraser Valley domestic strain, which had no individuals suffering a LOE. By 24h, pH 9.5 exposure caused more triploids to lose equilibrium than the diploids within the same strain. The Fraser Valley domestic strain only began to show loss of equilibrium at 24h, with triploid Fraser Valley domestic trout showing a greater loss of equilibrium compared with their diploid counterparts. Even at 48h exposure to pH 9.5, greater than 70% of the Fraser Valley domestic trout remained, whereas no wild strains were available to sample.

2.3.2 Haematology

Three-way ANOVA revealed a significant effect of time ($p=0.033$) and strain ($p=0.0002$) on blood hemoglobin concentration with no significant effect of ploidy ($p=0.302$) and no significant interaction between time and strain ($p=0.459$), time and ploidy ($p=0.372$), strain and ploidy ($p=0.537$), and time, strain, and ploidy ($p=0.627$) (Figure 2.2A). Three-way ANOVA determined a significant effect of time ($p<0.0001$), strain ($p<0.0001$), and ploidy ($p<0.0001$) for hematocrit (%) following exposure to pH 9.5 and there was no significant interaction between time and strain ($p=0.075$), time and ploidy ($p=0.836$), strain and ploidy ($p=0.713$), and time, strain, and ploidy ($p=0.673$) (Figure 2.2B). A post-hoc analysis showed a significant decrease in all time points compared with controls, a significant increase of hematocrit in diploid trout, and that the Blackwater strain was significantly decreased from the Tzenzaicut ($p=0.0005$) and Pennask ($p=0.0001$) strains. There was no significant effect of time ($p=0.0502$), strain ($p=0.144$), or ploidy ($p=0.098$) on mean cellular hemoglobin concentration (MCHC). There were also no significant

interaction between time and strain ($p=0.253$), time and ploidy ($p=0.503$), strain and ploidy ($p=0.222$), and time, strain, and ploidy ($p=0.88$) for MCHC (Figure 2.2C).

2.3.3 Nitrogen balance

There was a significant effect of time ($p<0.0001$), but no effect of strain ($p=0.891$) or ploidy ($p=0.477$) on plasma ammonia (Figure 2.3A). There were no significant interactions between time and strain ($p=0.829$), time and ploidy ($p=0.984$), strain and ploidy ($p=0.906$), and time, strain, and ploidy ($p=0.741$). Plasma ammonia increased significantly by 6h exposure to pH 9.5 compared with controls, but by 12h exposure to pH 9.5, plasma ammonia decreased and was no longer significantly elevated relative to controls.

Three-way ANOVA revealed a significant effect of time ($p=0.004$), but no effect of strain ($p=0.262$) or ploidy ($p=0.897$) on plasma urea (Figure 2.3B). There was also no significant interaction between time and strain ($p=0.812$), time and ploidy ($p=0.503$), strain and ploidy ($p=0.909$), and time, strain, and ploidy ($p=0.903$) between main effects for plasma urea. Post-hoc analysis of the time effect indicated that plasma urea was significantly lower at 48h exposure compared with control values ($p=0.001$; Figure 2.3B).

Three-way ANOVA revealed a significant effect of time ($p<0.0001$), and no significant effect of strain ($p=0.3443$) and ploidy ($p=0.788$) on white muscle ammonia concentration (Figure 2.3C). There were no significant interactions between time and strain ($p=0.189$), time and ploidy ($p=0.645$), strain and ploidy ($p=0.275$), and time, strain, and ploidy ($p=0.802$) between main effects for white

muscle ammonia. White muscle ammonia increased 3-fold over control values after 6h exposure to pH 9.5 and remained at this elevated level for the duration of the experiment.

2.3.4 Loss of equilibrium 2012 study

In 2012, a Kruskal-Wallis chi-squared test revealed a significant effect of strain on LOE ($p=0.04$). Fewer trout showed a LOE in response to pH 9.5 exposure in 2012 (Figure 2.4) than in 2011 (Figure 2.1). Similar to the 2011 study, the Fraser Valley strain had the least number of individuals showing limited LOE throughout the exposure with only 1 individual of the Fraser Valley triploid group losing equilibrium. Among the wild strains of trout, loss of equilibrium was noted in all strains by 24h and at 72h the greatest total loss of equilibrium occurred in the Carp Lake diploid and Pennask triploid trout, with the least in the Fraser Valley diploid strain.

2.3.5 Brain metabolites

There was a significant effect of time ($p<0.0001$) and strain ($p=0.001$), but no effect of ploidy ($p=0.241$) or significant interaction between time and strain ($p=0.113$), time and ploidy ($p=0.806$), strain and ploidy ($p=0.762$), and time, strain, and ploidy ($p=0.598$) on brain ammonia. All trout exposed to pH 9.5 had significantly higher brain ammonia than controls ($p<0.00001$) (Figure 2.5A). Within strains, tukey post-hoc analysis showed that the Blackwater individuals had significantly lower brain ammonia concentrations than the Fraser Valley ($p=0.003$)

and Pennask ($p=0.002$) strains. There was a significant effect of time ($p<0.0001$) but no significant effect of ploidy, strain or interactions on brain glutamate. Fish exposed to elevated pH had significantly lower brain glutamate compared to the control values ($p<0.0001$)(Figure 2.5B). All strains, except for the Tzenzaicut strain, showed a significant decrease in brain glutamate at 24h, and concentrations remained at this level for the duration of the exposure. The analysis reveals a significant effect of time ($p<0.0001$) and strain ($p<0.0001$) on brain glutamine. A post-hoc test revealed that pH exposed fish had significantly higher brain glutamine at all time points compared to the control group; the 12h time point increased tissue glutamine 2.5 fold at 12h, and continued to increase to up to 3-fold higher than control values at 24h (Figure 2.5C). Among strains, there was a significant difference between the Pennask, and Blackwater ($p=0.007$), and Carp Lake ($p=0.001$) strains, with the Pennask strain having significantly higher brain glutamine.

2.3.6 Plasma ions

There was a significant effect of time ($p=0.003$) and strain ($p=0.008$), but no significant effect of ploidy ($p=0.459$) or interaction between time and ploidy ($p=0.331$), strain and ploidy ($p=0.409$) for plasma sodium. There was a significant interaction between strain and time in this experiment ($p=0.007$), with a post-hoc analysis revealing that plasma sodium was significantly higher following high pH exposure at the 12h ($p=0.007$) and 72h ($p=0.011$) compared with control fish (Figure 2.6A). At the strain level, Fraser valley domestics had plasma sodium levels

that were significant lower than Blackwater ($p=0.028$) and Tzenzaicut ($p=0.011$) strains.

There was a significant effect of time ($p<0.0001$) on plasma chloride, however, there was no significant of strain ($p=0.449$) and ploidy ($p=0.455$) or interaction between time and strain ($p=0.829$), time and ploidy ($p=0.785$), strain and ploidy ($p=0.81$), and time, strain, and ploidy ($p=0.441$). A post-hoc analysis revealed that the 24h time point was significantly decreased from all other time points (Figure 2.6B).

2.3.7 Plasma lactate

There was a significant effect of time ($p<0.0001$) and strain ($p<0.0001$) on plasma lactate and no significant effect of ploidy ($p=0.274$). There was a significant interaction between strain and ploidy ($p=0.02$), and no significant interaction between time and strain ($p=0.503$), time and ploidy ($p=0.372$), and time, strain, and ploidy ($p=0.463$) on plasma lactate. All plasma lactate values were significantly higher following high pH exposure compared to control individuals, with the 12 and 24h time points showing a 2-fold increase and 1.5-fold increase at 72h (Figure 2.6C). Pennask trout had significantly higher plasma lactate than the Carp Lake and Fraser Valley groups ($p=0.001$). The Pennask triploid group was significantly different from the diploid Blackwater group ($p=0.027$), the Tzenzaicut diploids ($p=0.004$), the Carp Lake diploids ($p=0.026$), the Carp Lake triploids ($p=0.0001$), the Fraser Valley diploids ($p=0.003$), and the Fraser Valley triploids ($p=0.0005$) (Figure 2.6C).

2.4 Discussion

The most striking outcome of the present study was that the Fraser Valley domesticated strain of rainbow trout were more tolerant of pH 9.5 exposure than the four wild strains used in this study, and this was observed over two consecutive years of analysis (2011 Figure 2.1 and 2012 Figure 2.4). This is consistent with previous work comparing the responses of various trout strains to an environmental challenge, where Scott (2012) demonstrated that the Fraser Valley domesticated trout strains were more tolerant of low oxygen stress compared with wild strains. This suggests that, in general, domestication may improve how animals perform under stressful conditions. Indeed, it has been shown that domesticated strains of trout and salmon handle stress far better than their wild strains, and this better stress tolerance has been associated with a blunted cortisol response (Woodward and Strange 1987; Fevolden et al. 1991).

There was considerable variation in the time to loss of equilibrium at pH 9.5 in the wild strains. In 2011, no wild strain was capable of surviving past 24h of the high pH exposure, however, in 2012, all strains survived to 72h. The reason for the year-to-year variation in high pH tolerance is unknown, but may be associated with differences in size. The wild strains used in 2012 were significantly smaller than the trout used in 2011 (see section 2.3.2 of the methods section), however unpublished FFBC results suggest that larger fish should be advantageous in high pH. The role of size in high pH should be explored in future work.

Although the present study suggests a large effect of strain and year on high pH tolerance, all of the trout used in the present study appear to be far more

sensitive to a high pH exposure compared with other studies (Wilkie and Wood, 1991; Wilkie et al. 1996; Wilson et al. 1998). Wilkie and Wood (1991) reported that free-swimming rainbow trout survived up to 5 weeks of exposure to pH 9.5 water. There are several possible explanations for lower survival in this study compared with previously published studies. For example, the increased sensitivity in my fish may be due to the greater magnitude of change in pH from 6.7 to 9.5, whereas most other studies only change pH from a control value of 8.0 to 9.5 (Wilkie and Wood, 1991; Wilkie et al. 1996; Laurent et al. 2000). Murray and Ziebell (1984) increased pH to 9.5 from 7.2 over 6h and reported 50% mortality, but improved survival by gradually increasing pH to 9.8 over 5 days demonstrating the benefit of a gradual pH increase. However, experiments by Wilson et al. (1998) and Yesaki and Iwama (1992), were conducted in similar conditions to this study, and only the latter reported losses in their soft water high pH (10.1) condition. The underlying cause of sensitivity may be again based in size of the fish, as both previous studies used adult rainbow trout (approximately 200 and 400g respectively) compared to the juvenile fish used in this study. Other studies have shown an effect of size on metabolite storage in rainbow trout (Ferguson et al. 1993). Larger fish may be able to regulate a physiological disturbance better, as has been shown in larger brook trout that maintain lower blood osmolarity after transfer to seawater, than smaller trout (McCormick and Naiman, 1984). Water hardness varies between experiments as well, with many studies conducted in water of intermediate to high levels of hardness ($[\text{CaCO}_3] > 140\text{mg/L}$) (Wilkie and Wood, 1991; Wilkie et al. 1996), in comparison to the soft water used at the UBC ($[\text{CaCO}_3] < 17.9\text{mg/L}$).

Despite the significant effects of strain on LOE at pH 9.5, there were no apparent differences in the physiological responses of the different strains to high pH exposure. This may, in part, be due to the fact that I sampled surviving fish for physiological analysis, which may preclude me from defining the physiological basis for differences in loss of equilibrium between strains. In addition, it is possible that other physiological or biochemical factors, besides the ones measured here, may differ between the strains and account for the differences in high pH tolerance.

High pH exposure is well known to affect ammonia excretion and ionregulation in trout (Wilkie and Wood, 1994; Randall and Tsui, 2002). In particular, it has been shown previously that ammonia excretion is greatly reduced upon exposure to a high pH environment (Wilkie and Wood, 1994; Wilkie and Wood, 1995; McGeer and Eddy, 1998; Wilson et al. 1998) due to the difficulty of maintaining the blood to water ammonia excretion gradient facilitated by the acidification of the gill boundary layer (Weihrauch et al. 2009; Wright and Wood, 2009). Ammonia accumulation in tissues, like the brain, has been proposed to cause the production of reactive oxygen species that ultimately lead to death (Norenberg, 2004). The results from the present study show a 2-fold increase in plasma ammonia after 6h exposure to pH 9.5 (Figure 2.3A). This was accompanied by a 2-fold increase in brain ammonia (Figure 2.5A). Sanderson et al. (2010) showed two-fold higher brain ammonia than values presented in the rainbow trout in the present study, which suggests that ammonia could accumulate to higher levels in the brains before resulting in toxicity. As ammonia can readily be incorporated into citric-acid cycle metabolites such as α -ketoglutarate and oxaloacetate, I expected to

see an increase in the production of glutamine to deter the amount of ammonia present in the body (Randall, 2011). This is supported by the 3-fold increase in brain glutamine levels and the reduced levels of brain glutamate upon the onset of high pH (Figure 2.5B and 2.5C). It also could be expected that ammonia could be accumulated in other, ammonia insensitive cellular compartments (i.e. muscle) in order to keep plasma levels low and minimize accumulation in the brain or other more ammonia sensitive tissues. Wilkie and Wood (1995) showed that trout white muscle had a large capacity to store ammonia because it comprises up to 65% of the body mass (Stevens, 1968). The data collected in the present study on white muscle ammonia shows an accumulation of ammonia that is rapid and to a concentration that is 7.5 times greater than observed in the blood (assuming 0.8ml water/g of tissue for muscle), demonstrating the capacity of the white muscle to store ammonia during an accumulation.

Exposure to high pH had a small, but significant effect on plasma sodium and chloride concentrations. Greater plasma ion loss following elevated pH exposure was expected as a consequence of the weakening of the tight junctions at the gill and general hypertrophy that occurs in fish, like the Brook trout, exposed to high pH (Gaye and Darside, 1976; Laurent et al. 2000). The present data shows a significant decrease in plasma chloride at 24h (Figure 2.6B). The subsequent removal of HCO_3^- may account for the recovery of chloride by 72h (Figure 2.6B), via the $\text{Cl}^-/\text{HCO}_3^-$ exchanger at the gills. This is speculative, as I did not measure HCO_3^- , however soft water high pH studies have shown increases in HCO_3^- as a result of high pH exposure (Heming and Blumhagen, 1988; Yesaki and Iwama 1992, McGeer and

Eddy, 1998) suggesting the presence of HCO_3^- reserves for exchange. I expected sodium levels to drop as reported in other studies (Wilkie and Wood, 1993; Wilkie and Wood, 1995), but rather saw limited change in sodium much like the study by Wilkie et al. (1996). The additional 0.01M sodium from the NaOH used to adjust the pH may have assisted in maintaining plasma sodium values. The limited change in plasma sodium and chloride suggest that osmoregulation was generally maintained in the trout strains during exposure to pH 9.5, which is further supported by the relatively stable effect of pH 9.5 exposure on blood hemoglobin, hematocrit, and MCHC (Figure 2.2A; 2.2B; 2.2C). The results indicate a significant increase in lactate initially, followed by a return to control values by 72h. This transient increase is similar to the one reported by Wilson et al. (1998), where lactate remained significant elevated at 72h in high pH (9.5) exposed fish, but returned to control values by 96h. The increase in lactate reported in this study suggests that my fish use anaerobic metabolism upon exposure to high pH, although the cause of the increase in lactate is unknown.

There is no clear and consistent effect of ploidy on any of the responses of the trout to high pH exposure. The lack of an effect of ploidy is consistent with other studies that have seen similar results with no real difference in growth, hematological and biochemical parameters between ploidies when exposed to stress (Galbearth et al. 1994; Sadler et al. 2000). Gillnetting returns by the FFSBC and other studies have shown reduced triploid returns in the wild (Blanc et al. 1992; Simon et al. 1993; Dillon et al. 2000), however others report no difference between ploidies (Guo et al. 1990; Wagner et al. 2006). The data does not support triploid

sensitivity to high pH water, but an in-lake study would need to be run to confirm if ploidy adversely affects high pH tolerance.

2.5 Conclusion

The results of this study demonstrate that the Fraser Valley strain of trout is more tolerant of pH 9.5 compared with 4 wild strains; however, I did not discover a physiological explanation for this greater high pH tolerance. The Fraser Valley strain was visibly sessile throughout the exposure (personal observation), which may minimize energy expenditure and contribute to their greater high pH tolerance. Future studies should examine aspects of energy balance in trout exposed to high pH and determine if some form of metabolic rate suppression is responsible for the enhanced tolerance of the Fraser Valley trout. The natural environments of the wild strains used in this study are typically found in mountainous regions where water has a relatively high level of CaCO_3 . Measuring tolerance in a high pH environment more similar to a natural environment of the brood stocks may give a better representation of each strains actual level of tolerance in a high pH setting which is the focus of the next chapter.

Figures

Figure 2.1. LOE data from 2011 showing the Blackwater (circles), Tzenzaicut (downward triangles), Pennask (squares), Fraser Valley (diamonds) strains and their diploid (filled symbols) and triploid (open symbols) conditions exposed to high pH (9.5) water for 48h (n=4 for all data points). All groups have been staggered at their respective time points for clarity of viewing. P values from significant main effects are denoted on figure.

Figure 2.2. Hemoglobin concentration (A), hematocrit % (B), and Mean Cell Hemoglobin concentration in Blackwater (circles), Tzenzaicut (downward triangles), Pennask (squares), Fraser Valley (diamonds), Carp lake (upward triangles) strains and their diploid (filled symbols) and triploid (open symbols) conditions exposed to high pH (9.5) water for 48h (n=2-5 for all groups except Fraser Valley (n=8) at 24h mark). All groups have been staggered at their respective time points for clarity of viewing. P values from significant main effects are denoted on figure.

Figure 2.3. Total plasma ammonia (A), plasma urea (B), and white muscle ammonia (C) in Blackwater (circles), Tzenzaicut (downward triangles), Pennask (squares), Fraser Valley (diamonds), Carp lake (upward triangles) strains and their diploid (filled symbols) and triploid (open symbols) conditions exposed to high pH (9.5) water for 48h (n=2-5 for all groups except Fraser Valley (n=8) at 24h mark for A and C; n=1-2 for all groups except Fraser Valley (n=8) at 24h mark for B). All groups have been staggered at their respective time points for clarity of viewing. P values from significant main effects are denoted on figure.

Figure 2.4. LOE data from 2012 showing the Blackwater (circles), Tzenzaicut (downward triangles), Pennask (squares), Fraser Valley (diamonds), Carp lake (upward triangles) strains and their diploid (filled symbols) and triploid (open symbols) conditions exposed to high pH (9.5) water (n=3 for all data points). All groups have been staggered at their respective time points for clarity of viewing. P values from significant main effects are denoted on figure.

Figure 2.5. Total brain ammonia (A), brain glutamate (B), and brain glutamine (C) in Blackwater (circles), Tzenzaicut (downward triangles), Pennask (squares), Fraser Valley (diamonds), Carp lake (upward triangles) strains and their diploid (filled symbols) and triploid (open symbols) conditions exposed to high pH (9.5) water for 72h (n=5-8 for all groups except at 72h; n=3-8 for all groups at 72h). All groups have been staggered at their respective time points for clarity of viewing. P values from significant main effects are denoted on figure.

Figure 2.6. Plasma sodium (A), plasma chloride (B), and plasma lactate (C) in Blackwater (circles), Tzenzaicut (downward triangles), Pennask (squares), Fraser Valley (diamonds), Carp lake (upward triangles) strains and their diploid (filled symbols) and triploid (open symbols) conditions exposed to high pH (9.5) water for 72h (n=5-8 for all groups except at 72h; n=3-8 for all groups at 72h). All groups have been staggered at their respective time points for clarity of viewing. P values from significant main effects and interactions are denoted on figure.

[3] The physiological response of wild and domestic strains of diploid and triploid rainbow trout (Oncorhynchus mykiss) in hard and soft high pH (9.5) water

3.1 Introduction

British Columbia (BC) has a rich history of stocking lakes with rainbow trout (*Oncorhynchus mykiss*) to support recreational fishing. At least 6 different strains originating from lakes, rivers, and hatcheries are stocked across BC every year, resulting in the addition of over 4 million trout to freshwater lakes (GSGislason and Associates Ltd, 2009). To mitigate the genetic influence this addition would have on indigenous populations of trout, hatcheries commonly stock sterile triploid trout to prevent genetic mixing between stocked and native trout populations. However, numerous published reports have shown a reduced survival of triploid trout in natural settings compared with their diploid counterparts (Blanc et al. 1992; Simon et al. 1993; Dillon et al. 2000). The Freshwater Fishery Society of BC (FFSBC) is also facing the startling trend of increasing pH in freshwater lakes in BC, with lakes increasing to a pH near 9.5 as a result of their bedrock geology and low flushing rates (Krueger and Water, 1983; Jones et al. 1998). The FFSBC and other agencies have reported reduced returns and survival of stocked trout in high pH lakes (Yesaki and Tsumura, 1992; Mathias et al. 1996; Wagner et al. 1997), and a pH of 9.5 has been reported to result in severe physiological responses in rainbow trout used for stocking (Chapter 2).

Previous work demonstrated that there is an effect of strain on high pH tolerance in trout, with the Fraser Valley domestic strain being more tolerant of pH 9.5 exposures than four wild strains (Chapter 2). However, despite large differences in tolerance, there were no significant differences between domestic and wild strains in ammonia and ion balance (Chapter 2). My previous study was conducted using de-chlorinated Vancouver City tap water, which is considered to be soft ($[\text{CaCO}_3] < 17.9 \text{ mg/L}$) and does not reflect the water hardness seen in many of BC's lakes (Unpublished FFSBC data).

A high level of water hardness ($[\text{CaCO}_3] = 320 \text{ mg/L}$) enhances survival in rainbow trout exposed to high pH (Yesaki and Iwama, 1992). Hard water is expected to strengthen tight junctions at the gill, which results in less ion loss from the plasma to the freshwater environment (Hunn, 1985; Tang and Goodenough, 2003). Furthermore, increases in water hardness affects ammonia excretion in trout exposed to high pH. In soft water, high pH exposure results in an accumulation of ammonia in plasma and tissues due to an inhibition of ammonia efflux across the gills (Wilkie and Wood 1994; Wilkie et al. 1996; Wilkie, 1997), which is partially alleviated in hard water (Yesaki and Iwama, 1992; Iwama et al. 1997; Wilson et al. 1998).

The goals of this study were to compare the short-term physiological response of diploid and triploid trout from one domestic and three wild strains to pH 9.5 exposure in both soft water ($[\text{CaCO}_3] < 17.9 \text{ mg/L}$) and hard-water ($[\text{CaCO}_3] = 320 \text{ mg/L}$). To accomplish this, I measured the changes in plasma ions, cortisol, and levels of ammonia in the plasma, liver, and brain as well as brain water

content after 24h exposure. Fish were abruptly transferred from soft water control conditions (pH 6.7) to soft water controls, soft water at pH 9.5 and hard water at pH 9.5 and I recorded any loss of equilibrium (LOE) of trout in each condition.

3.2 Materials and methods

3.2.1 Experimental animals

Strains were obtained based on their availability, and in this study I used three wild strains and one domesticated strain of rainbow trout. For the wild strains, three breeding pairs were taken from their natural environment and crossed at the hatchery. Wild strains were obtained from Blackwater River (named Blackwater), Pennask Lake (named Pennask) and Carp Lake (named Carp lake). Half of the progeny from each pair were allowed to develop as diploid fish, while the remainder was exposed to a hydrostatic pressure shock during development to induce triploidy. This created full siblings from three families that differed only by ploidy. The domestic Fraser Valley strain (named Fraser Valley) was crossed several months after the wild strains, producing diploid and triploid full siblings by the same protocol described above. Fish were reared at the Fraser Valley fish hatchery until December of 2012, when the fish were tagged with visible implant elastomere tags to distinguish strain, ploidy, and family prior to transfer from Abbotsford to the University of British Columbia (UBC) in January of 2013. Fraser Valley domestics were tagged in March of 2013, and brought to the UBC in early April of the same year. At the UBC, all trout were held in a partially re-circulated common garden

system using de-chlorinated Vancouver city tap water (pH ~6.7; $[\text{CaCO}_3] < 17.9 \text{ mg/L}$). The fish were reared until June of 2013 at the UBC and fed twice daily Monday to Friday, and once daily on weekends for a total of 2% bodyweight of Bitovita 1.2mm fry food. All holding and experimental conditions were conducted until the guidelines of the Canadian Council on Animal Care.

3.2.2 Experimental protocol

Fish were fasted 24h before being sorted and size matched. For this experiment, diploid Blackwater trout were $9.68 \pm 0.16 \text{ g}$ ($n=24$)(Mean $\pm$ S.E.M), the triploid Blackwater were $9.56 \pm 0.19 \text{ g}$ ($n=22$), the diploid Pennask were $9.08 \pm 0.16 \text{ g}$ ($n=24$), the triploid Pennask were $8.21 \pm 0.16 \text{ g}$ ($n=24$), the diploid Carp lake were $10.09 \pm 0.3 \text{ g}$ ($n=24$), the triploid Carp lake were $9.58 \pm 0.48 \text{ g}$ ($n=21$), the diploid Fraser valley were $5.34 \pm 0.3 \text{ g}$ ($n=24$), and the triploid Fraser Valley strain weighed $5.28 \pm 0.18 \text{ g}$ ($n=24$). Two-way ANOVA revealed a significant difference between strains ($p < 0.0001$), but not between ploidies or treatments within a strain for size. Four trout from each strain and ploidy were placed into 4L opaque plastic jars that had 4 square sections of the sides replaced with mesh and was covered with transparent plastic to allow observations of specimens throughout the exposure. After fish were placed into each plastic container, they were put back into the stock tank. Polyethylene foam was used for flotation of each container. The fish were allowed to acclimate to the container for 24h, before being placed into a mobile 150L tank for transport to the experimental chamber, where they were moved into the experimental exposure system.

Three, blue 150L oval experimental tanks were set up at least 24h before exposures, consisting of a soft water control condition (pH=6.7; $[\text{CaCO}_3]<17.9\text{mg/L}$), a soft water high pH (pH=9.5; $[\text{CaCO}_3]<17.9\text{mg/L}$) condition, and a hard water high pH (pH 9.5; $[\text{CaCO}_3]=320\text{mg/L}$) condition. The soft water treatment used de-chlorinated Vancouver city tap water with the addition of 0.2M NaOH to maintain a pH of 9.5. Two pumps circulated water to a header tank where NaOH was added and mixed via heavy aeration before being returned to the exposure tank. The control tanks followed the same protocol without the addition of NaOH. The hard water condition consisted of 38.5g $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$, 3.2g CaCO_3 , and 34.6g $\text{MgCl}_2\cdot 6\text{H}_2\text{O}$ addition to the tank (150L) for a final hardness of 320mg/L as CaCO_3 . The pH of 9.5 was maintained by addition of 0.2M NaOH. NaOH addition for the alkaline soft/hard treatments was controlled by use of a Cole-Parmer electrode connected to an alpha-560 Eutech instruments pH regulator that electrically manipulated a peristaltic pump. Tanks were observed every hour and pH was maintained at 9.5. To ensure proper mixing in the hard water exposure, a total of four circulating pumps were placed in the bottom of the tank to create circular flow, and agitation was supplemented with heavy aeration. Hardness was measured with a colourmetric assay (API General hardness test kit). Each tank was covered with opaque plastic sheeting. This set up was identical for each condition.

Fish were exposed to each treatment for 24h. To sample the fish, a container with four fish was removed and fish were anesthetized by the addition of MS-222 (buffered with sodium bicarbonate) to a final concentration of 0.1g/L directly into the container. All 4 fish were removed and fork length, weight, and elastomere tag

position were recorded. The collection of blood and tissue samples was identical to the protocol described in the previous chapter (Chapter 2). Briefly, whole blood was drawn into heparinized capillary tubes after caudal severance, and an additional 5 μ L of whole blood was pipetted from the exposed caudal vein to be mixed with 125 μ L of propidium iodide solution for determination of ploidy. I then excised a posterior 1cm segment of caudal muscle, sliced open the cranial cavity to extract brain tissue, and dissected the entire gill basket out. Samples were wrapped in aluminum foil, flash frozen in liquid nitrogen, and stored at -80°C. Capillary tubes were centrifuged at 9200*g* to separate plasma from red blood cells. Plasma was removed and placed into a micro-centrifuge tube, frozen in liquid nitrogen, and stored at -80°C. This protocol was used for all 3 experimental conditions and groups. Fish were also assessed for LOE throughout the study. Any fish that lost equilibrium was prodded with a net, and if they could not re-establish normal dorso-ventral orientation, it was removed and the time of LOE was recorded.

3.2.3 Analytical techniques

Ploidy was confirmed following the protocol outlined in Arumuganathan and Earle (1991) by using a FACSCalibur bench flow cytometer (BD Biosciences, California, USA). The triploidy induction protocol was 98% efficient. Plasma was used for analysis of ammonia, cortisol, sodium, and chloride. Frozen plasma was thawed and 10 μ L was removed for the analysis of ammonia. For all plasma ammonia samples, perchloric acid (8%) was used to dilute the thawed plasma sample, and the sample was spun for 10 minutes at 18000*g*. The supernatant was

removed and neutralized using 2M KHCO_3 ; the neutralized sample was spun again to remove the supernatant. Ammonia values were obtained using commercial kits (Sigma; Cat#A0100), and use of the extinction coefficient of NADH at 340nm of 6,220L/mol•cm. For sodium and chloride measurements, frozen plasma was thawed and 5 μL of plasma was diluted 200-fold in dH_2O . The diluted plasma sample was aliquoted into two separate tubes: (1) the aliquot was diluted an additional 6-fold followed by being run through a flame photometer (Varian AA240FS) (2) the aliquot of 200-fold diluted plasma sample was mixed with 13mM mercuric thiocyanate and 0.5M ferric nitrate and was immediately read at 480nm by spectrophotometer for the determination of plasma chloride. Plasma cortisol concentration was measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit from Neogen Corporation (Lexington, KY). Due to the small size of fish used in this study only 10 μL of plasma was available for steroid analysis. As a result, the initial step of the extraction process was modified and scaled down to use only 10 μL of plasma dissolved in 100 μL of ethyl ether. The rest of the protocol was carried out according to the manufacturer's specifications.

Liver and brain tissue was assessed for total ammonia. Briefly, sections of liver were weighed in micro-centrifuge tubes and sonicated in 8% perchloric acid using a micro ultrasonic cell disrupter (Kontes). The liver suspension was centrifuged at 18000g for 10 minutes, and the supernatant was removed and neutralized in 2M KHCO_3 . The neutralized sample was centrifuged again for the removal of the final neutralized supernatant. Brain samples were handled identically to liver samples with the exception of the neutralizing solution. To assist

in buffering of the neutralization, 5M TRIS was used. To determine water content of the brain, portions of frozen brain tissue were allowed to thaw for 3 minutes before being placed on the scale for wet weights. After wet weights were recorded, samples were placed in an incubator at 55°C. Dry weights were obtained after 24h in the incubator, and the percent water and percent dry mass are presented here.

3.2.4 Statistical analysis

All data reported are mean values $\pm$ SEM. Family was collapsed in the statistical analysis to focus on the effects of treatment, strain, and ploidy. In order to test for a significant effect of ploidy on time to LOE, I collapsed treatment and strain and perform an independent 2-group Mann-Whitney-U test. A three-way analysis of variance (ANOVA) was used on all physiological and biochemical measurements and treatment, ploidy, and strain were used as the fixed variables. If an interaction between fixed variables was seen to be significant, I collapsed non-significant variables, and performed two-way ANOVA, one-way ANOVA, and Tukey-post hoc tests. In the case of significant three-way interactions, a main effect was isolated and a two-way ANOVA was performed using the remaining main effects. Data was analyzed using R version 2.15.1.

3.3 Results

3.3.1 Loss of equilibrium

A total of eight rainbow trout lost equilibrium in this study and they were all triploid (Figure 3.1). A Mann-Whitney-U test revealed a significant effect of ploidy on time to loss of equilibrium ($p=0.037$). The soft water high pH treatment resulted in two fish losing equilibrium, one Blackwater triploid and one Fraser Valley triploid. Half of the Carp Lake triploid fish lost equilibrium in the hard water high pH treatment.

3.3.2 Plasma ions

Three-way ANOVA revealed a significant effect of treatment ($p=0.0002$), but no effect of ploidy ($p=0.135$), strain ($p=0.155$), and no interaction between treatment and ploidy ($p=0.11$), treatment and strain ($p=0.972$), strain and ploidy ($p=0.645$), or strain, ploidy and treatment ($p=0.789$) on plasma sodium (Figure 3.2A). Compared with the soft water controls (pH 6.7), exposure to pH 9.5 resulted in a significant decrease in plasma sodium in both soft water ($p=0.0002$) and hard water ($p=0.0003$).

Three-way ANOVA yielded a significant effect of treatment ($p<0.0001$), and a strain effect ($p=0.047$), but no significant effect of ploidy ($p=0.145$) on plasma chloride (Figure 3.2B). There was a significant interaction between treatment and ploidy ($p=0.02$), and no significant interaction between treatment and strain ($p=0.07$), strain and ploidy ($p=0.476$), and strain, ploidy, and treatment ($p=0.437$). A

tukey post-hoc test showed that the control diploid group was significantly elevated from the soft water diploid ($p=0.03$), triploid ($p<0.0001$), and the hard water diploid ($p<0.0001$), and triploid ($p=0.001$) high pH groups. The control triploid group was significantly elevated from the soft water triploid ($p<0.0001$), and the hard water diploid ($p<0.0001$), and triploid ($p=0.025$) high pH groups.

3.3.3 Plasma cortisol

The three-way ANOVA revealed a significant effect of treatment ($p<0.0001$), and strain ($p=0.0005$), with no significant effect of ploidy ($p=0.917$) on plasma cortisol (Figure 3.3). There was a significant interaction between treatment with ploidy and strain ($p=0.034$), treatment and strain ($p=0.04$), and no significant interaction between treatment and ploidy ($p=0.788$), and strain and ploidy ($p=0.74$) with cortisol as well. Isolating the diploid groups, there is a significant increase in soft high pH Blackwater plasma cortisol compared to the control Blackwater group ($p=0.018$), Carp Lake group ($p=0.0006$), Fraser Valley group ($p=0.0007$), the soft water high pH Pennask ($p=0.025$), and the hard water high pH Fraser valley group ($p=0.02$). Isolating the triploid groups, a significant increase of plasma cortisol occurs in the hard water high pH Blackwater group compared to the control Blackwater ($p=0.01$), Carp Lake ($p=0.025$), and the hard water high pH Pennask group ($p=0.011$).

3.3.4 Ammonia and brain weights

Three-way ANOVA revealed a significant effect of high pH treatment ($p < 0.0001$), and no significant effect of ploidy ($p = 0.316$), and strain ($p = 0.086$) on plasma ammonia in the rainbow trout. There was no significant interaction between treatment and ploidy ($p = 0.666$), treatment and strain ($p = 0.448$), strain and ploidy ($p = 0.803$), and strain, ploidy and treatment ($p = 0.39$). All groups experienced a 4-fold increase in plasma ammonia in both the hard water and soft water treatment (Figure 3.4A).

Three-way ANOVA demonstrated that there was no significant effect of treatment ($p = 0.1459$), ploidy ($p = 0.399$), and strain ($p = 0.318$) on liver ammonia. There were no significant interactions between treatment and ploidy ($p = 0.839$), treatment and strain ($p = 0.95$), strain and ploidy ($p = 0.179$), and strain, ploidy, and treatment ($p = 0.788$) on liver ammonia. Liver ammonia was stable throughout all groups regardless of exposure treatment (Figure 3.4B).

A three-way ANOVA revealed a significant effect of treatment ($p < 0.0001$), ploidy ($p = 0.035$), and strain ($p = 0.013$) on brain ammonia. There were no significant interactions between treatment and ploidy ($p = 0.06$), treatment and strain ($p = 0.179$), strain and ploidy ($p = 0.36$), and strain, ploidy and treatment ($p = 0.954$). High pH produced a 2.5 fold increase in brain ammonia from control values (Figure 3.4C).

Brain dry weights and water content did not vary across conditions with a three way ANOVA showing no significant effect of treatment ($p = 0.776$), ploidy ($p = 0.21$), and strain ($p = 0.31$) (Figure 3.5A and 3.5B). There were no significant

interactions between treatment and ploidy ($p=0.11$), treatment and strain ($p=0.971$), strain and ploidy ($p=0.645$), and strain, ploidy, and treatment ($p=0.789$).

3.4 Discussion

The results of this study suggest that water hardness does not affect the physiological responses of trout to pH 9.5 exposures. A total of 8 trout showed LOE over the 24h of exposure to high pH water (Figure 3.1) and 6 of the 8 fish that lost equilibrium were exposed to pH 9.5 in hard water. These results are in contrast to those of Yesaki and Iwama (1992) who observed no deaths during 24h exposure to pH 10.1 in hard water ($[CaCO_3]= 320mg/L$), but had 28.5% mortality in the trout exposed to pH 10.1 in soft water. Also, Wilson et al. (1998) did not observe any mortality in trout exposed to pH 10.0 for 15 days in soft ($[CaCO_3]= 4mg/L$) or hard ($[CaCO_3]= 320mg/L$) water. Clearly there is large study-to-study variation in high pH tolerance in trout. Fish size may be one factor contributing to the variation in high pH tolerance among studies. Compared to this study, Yesaki and Iwama (1992) and Wilson et al. (1998) used trout weighing over 400g and 200g, respectively, while I used juvenile trout weighing no more than 12g. Weight has been shown to be an advantage as larger fish have been shown to have less stress-induced sodium loss as shown following injection of adrenaline (McDonald and Milligan, 1997), and have shown a greater capacity to adjust to seawater transfer (McCormick and Naiman, 1984). Smaller fish are expected to experience greater osmotic stress as a result of their larger gill surface area to total body mass (McDonald and Milligan, 1997).

Clearly, future studies should focus on the effects of body mass on high pH tolerance in trout.

Apart from increasing survival in high pH water, the addition of divalent ions to the water was expected to limit ion loss across the gill. In the study by Yesaki and Iwama (1992) exposure of trout to hard ($[\text{CaCO}_3]=320\text{mg/L}$) pH 10.1 water resulted in fewer ionic disturbances than in trout exposed to pH 10.1 in soft water ($[\text{CaCO}_3]=4\text{mg/L}$). Plasma sodium and chloride concentrations significantly decrease as a result of high pH exposure (Figure 3.2A and 3.2B), but there was no effect of water hardness on plasma ion concentrations. Remodeling of the gill to a soft water environment in fish has been shown to take numerous days (McDonald and Rogano, 1986; Greco et al. 1996), and 24h may not be sufficient time for the rainbow trout to interact with hard water as long-term exposure to hard water has been shown to limit ion loss to the environment (McDonald and Robinson, 1993; McDonald and Milligan, 1997).

Cortisol is a stress hormone that has been shown to increase the capacity for gill ion regulation by proliferating gill chloride cells (Laurent and Perry, 1990; Laurent et al. 1994), and increasing the activity of Na^+/K^+ ATPase in trout (Seidelin et al. 1999) and salmon (Shrimpton et al. 1994; Shrimpton and McCormick, 1999). Overall, a significant increase in plasma cortisol occurs as a result of high pH exposure, which correlates with the ionoregulatory challenge the trout experienced. The change in cortisol in this study is not consistent across treatments, with no decreased production of plasma cortisol as a result of exposure to hard high pH water (Figure 3.3). Yesaki and Iwama (1992) showed that there was a doubling of

plasma cortisol by 96h in their soft water high pH exposure compared to a stable level of cortisol in hard water high pH exposed fish. The continued increase in plasma cortisol was most likely exacerbated by the continuing decrease of plasma ions in their soft water trout (Yesaki and Iwama, 1992). However, their results showed no significant difference between treatments at the 24h time point, similar to the results presented here.

My study showed no differences between soft and hard water in terms of ammonia accumulation in high pH. Hard water has been shown to improve the excretion rate of ammonia across the gill (Yesaki and Iwama, 1992; Wilson et al. 1998), and was expected to limit ammonia accumulation in the plasma, liver, and brain tissue. Plasma ammonia, however, increases 3-fold after 24h in both high pH conditions (Figure 3.4A). This is consistent with other studies (Wilkie and Wood, 1991; Wilkie et al. 1994; Wilson et al. 1994; McGeer and Eddy, 1998), but does not demonstrate a benefit of hard water. Liver ammonia also did not change in any condition during the exposure (Figure 3.4B), which is in contrast to the result obtained by Wilson et al. (1998), who demonstrated a significant doubling of liver ammonia in soft water (pH 10). Wilson et al. (1998) reported that their rainbow trout were capable of maintaining control levels of brain ammonia throughout their high pH exposure. The 3-fold increase in brain ammonia I report in this study is consistent with the trend presented in the previous experiment (Chapter 2). The significant 3-fold increase in brain ammonia (Figure 3.4C) as a result of high pH exposure indirectly suggests that protective metabolites such as glutamine are being produced. An immediate increase in brain glutamine occurs in conjunction with an

increase in ammonia (Sanderson et al. 2010; Chapter 2), in an apparent attempt to protect the brain from a lethal ammonia load. The brain water content suggests that while the metabolite concentration rises the organism maintains the osmolarity of the brain (Figure 3.5A). Rainbow trout have been shown to accumulate water in response to a high level of external ammonia (Sidhu, 2012), and increased water content may have resulted in increased cranial pressure and mortality. However, water content of the brain does not increase with high pH exposure, suggesting that the brain does not swell in response to high pH water (Figure 3.5A).

There was a significant effect of ploidy on LOE during high pH exposure (both soft water and hard water combined; Figure 3.1). Across all measurements, only brain ammonia accumulation at high pH showed a ploidy effect with diploid trout having lower brain ammonia than triploid trout (Figure 3.4C), suggesting, albeit tentatively, a link between the increased LOE in triploid trout at high pH and brain ammonia accumulation. However, the levels of ammonia in the brain tissue of triploids are lower than the values obtained from my previous study (Chapter 2) and those obtained during exposure to high external ammonia (Sanderson et al. 2010), suggesting that despite the difference brain ammonia results are within physiologically manageable levels.

Comparing this study to my previous work shows that there is great year-to-year variation in high pH tolerance, as we did not observe a significant strain effect in the present study yet a significant ploidy effect is obtained that was absent in previous experiments (Chapter 2). The previous study demonstrated a pronounced sensitivity of rainbow trout to high pH water, with the domesticated Fraser Valley

strain outperforming wild strains in terms of duration to high pH exposure (Chapter 2). The Fraser Valley strain saw limited LOE in a multi-day high pH (9.5) exposure over consecutive years, while all wild strains began to lose equilibrium hours into each assessment (Chapter 2). Individual trout begin to lose equilibrium within 24h into this study, but the Fraser Valley strain does not exhibit the same tolerance I noted in previous years. Domestication has been shown to benefit trout in terms of limiting cortisol production in high stress environments (Woodward and Strange, 1987), and although I report a significant strain effect in my fish in regards to plasma cortisol, only the Blackwater strain is significantly elevated from the Fraser Valley domesticated strain in the soft water high pH treatment.

3.5 Conclusions

Hard water did not significantly reduce ammonia accumulation, the production of cortisol, or limit ion loss to the environment. Furthermore, hard water provided no benefit in terms of time to LOE amongst the rainbow trout used in this study. Given the immediate exposure of the trout to the environment, an adjustment period may be required to fully take advantage of the water chemistry. However, this is similar to what the trout would experience during lake stocking, suggesting that the immediate exposure may be lethal. A longer-term study would be required for the effect that hard water evokes on the survival of the trout, and is warranted, as many lakes in BC possess nearly double the hardness (as CaCO_3) used in this study. I note that there is a ploidy effect of high pH water in my trout, one that was not present in previous years. The outright cause of increased LOE in triploid fish is

unknown, but is unlikely to be a result of the physiological measurements made here.

Figures

Figure 3.1: Number of fish showing a loss of equilibrium over 24h exposure to soft water at pH 6.7 (control; black bars), soft water at pH 9.5 (soft pH 9.5; grey bars) and hard water at pH 9.5 (hard pH 9.5; open bars). Blackwater (Bw), Pennask (Pn), Carp Lake (CL), and Fraser Valley (FV) strains as both diploids (2n) and triploids (3n). P values from significant main effects are denoted on figure.

Figure 3.2: Plasma sodium (A) and chloride (B) in the Blackwater (Bw), Pennask (Pn), Carp Lake (CL), and Fraser Valley (FV) strains, as both diploid (2n) and triploid (3n), exposed for 24h to soft water at pH 6.7 (controls; black bars), soft water at pH 9.5 (grey bars), and hard water at pH 9.5 (open bars) (n=6-8 for all groups except for the CL 3n (n=4) and FV groups (n=1-4)). P values from significant main effects and interactions are denoted on figure.

Figure 3.3: Total plasma cortisol in the Blackwater (Bw), Pennask (Pn), Carp Lake (CL), and Fraser Valley (FV) strains, as both diploid (2n) and triploid (3n), exposed for 24h to soft water at pH 6.7 (controls; black bars), soft water at pH 9.5 (grey bars), and hard water at pH 9.5 (open bars) (n=6-8 for all groups except for the CL 3n (n=4) and FV groups (n=2-4)). P values from significant main effects and interactions are denoted on figure.

Figure 3.4: Total ammonia in the plasma (A), liver (B), and brain (C) in the Blackwater (Bw), Pennask (Pn), Carp Lake (CL), and Fraser Valley (FV) strains, as both diploid (2n) and triploid (3n), exposed for 24h to soft water at pH 6.7 (controls; black bars), soft water at pH 9.5 (grey bars), and hard water at pH 9.5 (open bars) (n=6-8 for all groups except for the CL 3n (n=4)). P values from significant main effects and interactions are denoted on figure.

Figure 3.5: Percent composition of the brain in terms of water (A) and dry mass (B) in the Blackwater (Bw), Pennask (Pn), Carp Lake (CL), and Fraser Valley (FV) strains, as both diploid (2n) and triploid (3n), exposed for 24h to soft water at pH 6.7 (controls; black bars), soft water at pH 9.5 (grey bars), and hard water at pH 9.5 (open bars) (n=6-8 for all groups except for the CL 3n (n=4)).

[4] General Discussion

4.1 The effects of strain and ploidy on the physiological response of trout to pH 9.5 water

The results suggest that among all the trout strains examined in this study, the Fraser Valley domesticated strain is best suited for stocking to high pH lakes. In 2011 and 2012 the Fraser Valley domesticated strain showed significantly less LOE in pH 9.5 water (Figure 2.1; Figure 2.3) compared with the wild strains of trout investigated in this study. The most dramatic difference between strains occurred in my first experiment in 2011 (Figure 2.1), with more than 30% of wild strains losing equilibrium within the first 12h exposure to pH 9.5 and over 80% of wild strains losing equilibrium by 24h. Due to the variation in the results between years I cannot conclusively state whether ploidy affects high pH tolerance. In the 2013 study, all fish that lost equilibrium were triploid, with no diploid fish succumbing to high pH in the 24h experiment. However, the 2011 and 2012 studies showed no difference in loss of equilibrium between the ploidies.

Each year used a slightly different exposure protocol to increase pH to 9.5 (2011 6h gradual increase in pH; 2012 1h gradual increase in pH; 2013 immediate transfer), but the trout consistently exhibited sensitivity to high pH. My finding that high pH elicits an extreme response is in contrast to various other studies (Wilkie and Wood, 1991; Wilkie et al. 1996; Wilson et al. 1998), which instead show that rainbow trout are capable of prolonged survival in high pH. As discussed in chapter 2, various other factors play a role in determining tolerance in a high pH medium.

Very few conditions overlap across studies as many use larger organisms, performed their experiments in different seasons, had remarkably different rearing conditions, and used brood stocks of rainbow trout from very different environments (Wright and Wood, 1985; Yesaki and Iwama, 1992; Wilson et al. 1994; Wilkie et al. 1996; Wilson et al. 1998; Laurent et al. 2000; Wood and Nawata, 2011).

The exposure to a high pH environment caused the retention of ammonia in various tissues across the body, and changes in ion levels of the rainbow trout, but there were no physiological disturbances that were different between domestic and wild fish. All fish experienced increases in plasma ammonia (Figure 2.3A; Figure 3.4A), brain ammonia (Figure 2.5A; Figure 3.4C) brain glutamine (Figure 2.5C), and plasma lactate (Figure 2.6C), with decreases in brain glutamate (Figure 2.5B), plasma sodium (Figure 3.2A), and plasma chloride (Figure 2.6B; Figure 3.2A). All these changes are within physiological levels that have previously been reported. My values for ammonia accumulation in plasma and tissues are similar to those seen in the literature (Wilkie and Wood, 1994; Wilkie and Wood, 1995; Wilson et al. 1998; Wang et al. 2003; Zimmer et al. 2013), with glutamate, and glutamine levels well within those reported in high ammonia studies (Arillo et al. 1981; Sanderson et al. 2010). There is a significant increase in brain ammonia across triploids coinciding with the increased LOE of triploid fish in the 2013 study, but the values measured were lower than those collected in groups from the 2012 study and are half of what has been noted in high external ammonia studies (Sanderson et al. 2010). It should be noted that the ion levels presented in this study are below what

is commonly seen in literature (Wood and Randall, 1973; Wilkie et al. 1999). Given the remarkably soft water used in the rearing conditions ($<17.9\text{mg/L}$) a loss of plasma ions is not surprising as a result of ions leaking out across the gill to the hypo-osmotic environment (McDonald and Rogano, 1986). This could result in the subsequent establishment of a lower steady-state condition. The physiological measurements made in chapters 2 and 3 were chosen because previous studies had indicated that they were most affected by high pH exposures. The results suggest that the Fraser Valley strain is more tolerant of high pH, and that the wild strains are more sensitive to high pH water, but no physiological measurement yielded a clear difference between domestic and wild fish. As I only sampled surviving fish, my measurements cannot elucidate the underlying cause of LOE in high pH.

4.2 The effects of water hardness on the physiological response to high pH water of various strains and ploidies of rainbow trout

Exposure to hard water did not change the physiological response of any strain or ploidy to high pH water. All groups experienced significant increases in plasma and brain ammonia, and significant decreases in plasma sodium and chloride. The most notable outcome of the hard water exposure was the significant effect of ploidy on high pH, as presented in the LOE data. The only trout to lose equilibrium throughout the high pH exposure were triploid, and this corresponded to a significant accumulation of brain ammonia. Originally I assumed that hard water exposed fish would exhibit an advantage over soft water exposed fish during high pH exposure because of the previous work done by Yesaki and Iwama (1992).

However, the results presented here suggest that all physiological changes are a result of high pH exposure rather than hard water.

4.3 Recommendations and future research

Despite the results suggesting that the Fraser Valley strain show greater pH tolerance, I do not believe they would be an effective strain to stock in the wild. While the Fraser Valley have proven to be rather successful in lab trials (Scott, 2012; Chapter 2) the translation to high performance in the wild setting has been disappointing. As mentioned earlier, domestication has been shown to be advantageous in exposures to abiotic stressors in trout (Woodward and Strange, 1987; Lepage et al. 2001; Scott, 2012), theorized to be due to a reduced cortisol response post-stress (Woodward and Strange, 1987; Pottinger and Carrick, 1999). Domestication selects for growth rate and size and often performs poorly in the wild (Brauhn and Kincaid, 1982; Huntingford, 2004; Araki et al. 2008) as a result of their increased aggression and risk taking behavior (Vincent, 1960; Johnsson and Abrahams, 1991; Biro et al. 2004). The domesticated strain performance in this study may be an artifact of generations in aquaculture, adjusting to confined quarters, multiple handlings, and sudden changes in water parameters. Crosses with the Fraser Valley strain may potentially enhance the capability of wild strains to environmental extremes, or reduce their survival by alteration of a phenotype that has been selected for by nature.

I would recommend the implementation of a process that selects individuals amongst the wild strains that exhibit greater tolerance of a high pH environment.

My LOE observations demonstrate that there are individuals that are capable of surviving for extended periods of time in high pH, and selecting for these organisms may yield offspring that are also high pH tolerant. However, this warrants future work in determining if pH tolerance is genetically heritable. Another facet of tolerance may be exposure time, or acclimation time. Pre-exposure to reasonably high pH (8.0 and above) may allow the organism time to adjust physiologically to a pH a magnitude higher in lake settings (Wilkie and Wood, 1994; Wilkie and Wood, 1995; Wilkie et al. 1996). The change from the holding conditions (pH 6.7) to the experimental pH (9.5) may have been the cause of fish losing equilibrium in my study.

Future studies should be geared towards studying the effects of long-term exposure to high pH. All the studies presented in this thesis are limited in duration, with the longest exposure lasting 72h. With lakes in BC expected to reach a pH of 9.5, exploring whether or not stocked strains are capable of adjusting to a high pH in the long-term is paramount. Lahontan cutthroat trout exposed to highly alkaline lake water (pH 9.4) for two years experienced a suite of changes to adjust long-term, with increases in chloride cell density, plasma ammonia, and internal pH (Wilkie et al. 1994). In the 2012 study, the number of fish losing equilibrium is limited after 24h, suggesting that there is a critical period of adjustment, and that there are individuals in these stocks capable of greater high pH tolerance. A potential experiment would be to run a larger group of trout in a high pH exposure over 6 months, with measurements directed to ammonia and ion balance. One could hypothesize that fish capable of surviving longer in a high pH environment would

have similar histological and physiological changes as those seen in Lahontan cutthroat trout.

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