A GENETIC BASIS OF ADAPTATION TO HIGH pH IN RAINBOW TROUT

by

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Abstract

Exposure to high environmental pH is physiologically stressful for fish. In British Columbia, this has led to low survivorship among Rainbow Trout stocked into alkaline lakes. Early studies have shown promising results for stocking the progeny of brood stock collected in high pH lakes into similar alkaline environments. Here I follow up by characterizing the high pH tolerance of fish with parents collected from an alkaline lake, Stump Lake. I also look at the effects of acclimation and rearing fish at pH 8.8 on subsequent pH 9.5 tolerance. I found that this population had a short time to loss of equilibrium, with only 10% of fish remaining after a 3 day exposure to pH 9.5. Acclimation resulted in significant improvements to tolerance and rearing resulted in almost none of the fish losing equilibrium over a 3 day exposure. A genome wide association study on non-acclimated and acclimated individuals did not show any significant genetic marker associations with high pH tolerance. However this analysis did identify some potential SNPs associated with genes involved in acid-base regulation, muscle function, neural signaling, and DNA transcription in the non-acclimated fish. The pH 8.8 acclimated fish only showed association with genes involved in neural signaling and DNA transcription. These data suggest that acclimation may remove limitations associated with some of these other processes. Overall the Stump Lake population does not appear to have genetic adaptations that improve tolerance to high pH exposure, but can improve tolerance through acclimation to moderately high pH.

Preface

Chapter 2 of this thesis is co-authored by Tara L. McBryan, Sara Northrup, and Patricia M. Schulte. Data on LOE_{pH} was collected by Sara Northrup and data analysis and write up were performed by Tara McBryan.

Chapter 3 of this thesis is co-authored by Tara L. McBryan, Timothy M. Healy and Patricia M. Schulte. All laboratory procedures were performed by Tara L. McBryan. Genome by sequencing and SNP calling was performed by the University of Cornell Genomic Diversity Facility. Timothy Healy performed paralog and missing data filtering. Tara McBryan ran all statistical analysis and completed the write up.

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[1] General Introduction

1.1 Freshwater Fisheries

Recreational fisheries have significant economic, social and ecological impacts around the World. In Canada, the recreational fishing industry contributed $8.3 billion in 2010 to the national economy (DFO 2010). This revenue is particularly concentrated in small remote communities in British Columbia and Ontario and is an important economic driver in these areas. Revenue consists of recreational sporting goods and fishing-trip-associated costs.

Fishing plays a positive role in the lives of many Canadians and is thought to reduce stress and bring families together in the outdoors. In a 2005 Survey of Recreational fishing in British Columbia (Gislason 2009) anglers reported on average that relaxation, companionship and family bonding was more important than actually catching fish. Fishing is an activity which is enjoyed across demographics that provides access to recreation and food (DFO 2010, Gislason 2009).

Protecting recreational fisheries has spill-over benefits into the conservation of aquatic ecosystems at large. Historic over-exploitation of many fisheries has led to declining numbers resulting in the implementation of management programs to rebuild stocks, set quotas, and require anglers to buy fishing licenses. Money generated from fishing licenses is, in part, directed into stock assessments and research into maintaining fisheries. Creel surveys allow anglers to contribute directly to stock assessment data and promote an interaction between scientists and anglers. Anglers also extensively volunteer within fish and game clubs and community based fish hatcheries (Gislason 2009).

Within British Columbia, various species of Pacific salmon are the mainstay of the recreational fishery. In tidal waters Chinook and Coho Salmon are the most targeted species, whereas in freshwater Rainbow Trout is the most popular target of the recreational fishery (DFO 2010). Management of anadromous salmon is handled at the federal level within the Department of Fisheries and Oceans and anadromous Steelhead and freshwater trout are managed at the provincial level. In 2005, $141 million was generated from anglers targeting Rainbow Trout in British Columbia (Gislason 2009).

1.2 Rainbow Trout

Rainbow Trout are part of the family salmonidae, subfamily Salmoninae; which includes the trout, char, and Pacific Salmon (Nelson *et al*. 2004). Rainbow Trout and cutthroat trout were long thought to be part of the genus Salmo (associating them more closely with Atlantic Salmon); however, mitochondrial and genomic DNA evidence has placed them in the genus Oncorhynchus with the pacific salmon (Stearley and Smith 1993, Devlin 1993). The species under study in this thesis, *Oncorhynchus mykiss*, is further divided into two ecotypes: Rainbow Trout referring to the freshwater residents and Steelhead Trout referring to anadromous fish. Hecht *et al.* (2013) have detected genetic variation between these groups that may account for the Steelhead Trout's propensity to migrate seaward. Hybridization between Rainbow and Cutthroat Trout (*Oncoryhnchus clarkii*) occurs across British Columbia (Rubidge and Taylor 2005, Rubidge *et al.* 2001). In this thesis I use a population of Rainbow Trout that has an entirely freshwater life history and has no evidence of cross species hybridization.

Rainbow Trout play an important role in freshwater ecosystems throughout their life cycle. Due to different life history phases, Rainbow Trout occur at many trophic levels in aquatic food webs. After yoke-sac absorption Rainbow Trout begin feeding on drift insect larva, and may convert to a diet of larger insects, molluscs, tadpoles or smaller fish depending on availability of prey and the population of Rainbow Trout (McPhail 2007). Although Rainbow Trout have the capacity to spawn several times (iterparous), a secondary spawning event depends on the population and environmental factors (Hootten *et al.* 1987).

Rainbow Trout can be found in both lakes and river systems and are native to Western North America and Northeastern Siberia (McPhail 2007). Rainbow Trout thrive in waters between 13-23°C and have many traits which make them good candidates for aquaculture (Hardy 2002). This has led to these fish being introduced to freshwater systems around the world for recreational and commercial intent (McPhail 2007, Quinn 2005). Within British Columbia, it is unclear which lakes are native habitat and which lakes are inhabited due to extensive and often-times unrecorded stockings of Rainbow Trout over the last 100 years to meet recreational fishing needs (McPhail 2007).

1.3 Freshwater Fisheries Society of British Columbia

The Freshwater Fisheries Society of B.C. (FFSBC) is a private non-profit organization. Their mandate is to enhance and conserve BC's freshwater fisheries for public benefit. Working in partnership with government, industry and anglers, their goal is to improve fishing in BC through the enhancement and conservation of BC's freshwater fish resources. The FFSBC stocks hatchery reared trout, char, and sturgeon

into more than 800 lakes, rivers and streams around the province annually. Each year, FFSBC stocks approximately 8 million fish with 5 million of these fish being Rainbow Trout. These Rainbow Trout are comprised of different strains, which meet a variety of environmental, catchability and ecological requirements.

Currently, the main strains in production within the FFSBC are the Fraser Valley Domestic, Blackwater River, and Pennask Lake strains. The Fraser Valley domestic strain has been in production at various hatcheries since the 1940s. This strain is usually reared as sterile triploids to catchable size (250g) in the hatchery and then released into urban lakes. The Blackwater and Pennask strains are from wild populations which are stocked into brood stock lakes to facilitate large scale production. These strains are suitable for stocking into different types of aquatic environments. For example, the Blackwater River strain can be stocked into lakes with competition which has allowed this strain to reach large sizes on a diet of shiners within Courtney Lake, Merritt BC. Whereas the Pennask Lake strain is not as resilient to competition, but performs well in cold-water lakes, for example it reaches large sizes at high elevation within Vinson Lake, Merritt BC. The combined use of all of these strains has allowed the FFSBC to successfully stock lakes around the province used for recreational angling. Many of these lakes have been experiencing increases in pH, with a quarter having pH which exceeds an average of 8.5. Reports from the late 1980's began to suggest that angler days on alkaline lakes in British Columbia were falling (Tredger 1990).

1.4 High pH Lakes in British Columbia

Alkaline lakes exist around the world (Grant and Jones 2000). The causes of the high pH in these lakes can be attributed to biotic and abiotic factors. On a biotic level, carbon fixation by aquatic plants and algae reduces the amount of carbonic acid present in the lake, therefore, effectively raising pH (Hansen 2002). This natural process can be intensified by agricultural runoff and sewage seepage, causing eutrophication. For example, in Slapton Ley (UK), this process has left parts of the lake with pH= 10.5 (Scott *et al.* 2005). Food processing and cement manufacturing also directly increase lake pH through NaOH and $Ca(OH)_2$ emissions (Grant and Jones 2000).

The interactions between the bedrock surrounding the lake and water movement in a lake can also cause high alkalinity. In order for the bedrock to cause high pH, the rocks surrounding these lakes must be high in Na^+ but low in Ca^{2+} and Mg^{2+} (e.g. trachyte lava surrounding Lake Magadi, Kenya, Africa) (Grant and Jones 2000). This leads to Na^+ being the dominant ion binding to CO_3^-, instead of Ca^{2+} or Mg^{2+}, which normally causes the formation of neutral precipitates such as calcite, magnesite or dolomite (Grant and Jones 2000). Instead, the formation of Na_2CO_3 (soda brine aka. soda ash) results in increasing pH as it remains water soluble (Horikoshi and Grant 1998). In order for this brine to concentrate enough in the lake to appreciably increase pH, inflowing water needs to be exceeded by evaporation (Grant and Jones 2000). A number of small lakes in the interior of BC meet this criteria, for example, Stump Lake, Green Lake, and Pigeon Lake (Strahler and Archibold 2008).

1.5 Physiology of Aquatic High pH Exposure

Fish in alkaline lakes are required to constantly lower blood pH while maintaining ion balance and ammonia excretion against environmental gradients. In fish, one of the most direct results of high environmental pH is increases in blood pH upon initial exposure (Yesaki and Iwama 1992, Wilkie and Wood 1991, Wilkie *et al.* 1996, Wilkie and Wood 1995). As part of the mechanism to bring plasma pH back down to physiological levels, the gills exchange Na^+ in the blood for H^+ in the water and Cl^- in the water for HCO_3^- in the blood which results in perturbances in blood osmolarity (Wilkie and Wood 1996, Yesaki and Iwama 1992). A second important consequence of exposure to high environmental pH is an increase in plasma ammonia. Although the majority of ammonia excretion does not rely directly on the transport of acid and bases, its passive diffusion across the gills becomes unfavorable due to the chemical parameters of high environmental pH (*see* Wilkie and Wood 1996 *for review*). In order to survive high pH, a fish must be able to reduce ammonia load and maintain blood pH and ionic composition.

Within hours of exposure to environmental high pH (>9.5) plasma pH dramatically increases (Yesaki and Iwama 1992, Wilkie and Wood 1991, Wilkie *et al*. 1996, Wilkie and Wood 1995). This is caused by a right shift in the $CO_2 \leftrightarrow HCO_3 + CO_3$ reaction in the aquatic environment which creates a CO_2 deficiency in the water surrounding the gill. This in turn establishes a "CO_2 vacuum" and draws CO_2 out from the blood (Johansen *et al.* 1975). This loss of CO_2 results in increases in the pH of the blood and is characterized as a respiratory alkalosis (Yesaki and Iwama 1992, Wilkie and Wood 1991, Wilkie *et al.* 1996, Wilkie and Wood 1995). In addition, the alkaline environment usually has many negatively charged ions (OH^- and HCO_3^-), which create an

electrical gradient causing H^+ to leave the blood and HCO_3^- to enter the blood (Wilkie *et al*. 1994). Just as in acidic environments, fish are able to offset the respiratory pH imbalance by metabolic compensation. An activation of anaerobic metabolism in white muscle facilitates a metabolic acidosis, which appears to occur to counter the respiratory alkalosis (Wilkie and Wood 1995, Thompson *et al.* 2015). Across most species studied it appears that these shifts result in a full or significant recovery of plasma pH; however, this approach is quite energetically costly and it has been hypothesized that in the long term, acid-base regulation at the gills may play a more significant role (Wilkie and Wood 1996).

At the freshwater gill, acid-base regulation is intimately tied to ion regulation (see Gilmour and Perry 2009 for review). Sodium and chloride are taken into the blood in exchange for acid and base, respectively. Under low pH, a fishes capacity to regulate blood pH back to physiological norms is limited by available Cl^- in the blood by which to exchange with HCO_3^- (Kwong *et al*. 2014). By this same logic, Na^+ should become a limiting factor by which to bring H^+ into the blood under high pH exposure, or perhaps Cl^- levels would build in an effort to extract HCO_3^-. Ionic disturbances do occur under high pH exposure. These disturbances are partially corrected during chronic exposure because some species remodel their gills to increase chloride cell (sites of HCO_3^-/Cl^- exchange) fractional gill surface (Wilkie and Wood 1994). Some studies suggest that sodium levels may be kept from dropping too greatly due to the presence of a Na^+/NH_4^+ on the gills which plays a small part in ammonia excretion; however, other studies have suggested this exchange is insignificant (Yesaki and Iwama 1992, Wright and Wood 1985, Wilkie and Wood 1996).

While most fish have the physiological mechanisms by which to regulate blood pH and ion levels, establishing favorable ammonia efflux in a high pH environment is more challenging. Ammonia is continuously being produced by protein catabolism in the liver (Wilkie 2002). High levels of ammonia (NH_3) is toxic to animals as it binds to NMDA receptors in the brain causing an over-excitation which leads to convulsions, seizures, and ultimately death (Randall and Tsui 2002, Wilkie *et al*. 2011). In order to keep ammonia levels low in the blood birds convert it into uric acid and mammals convert it to urea to avoid toxicity, but the production of these compounds is energetically expensive (Nelson and Cox 2008). These processes are not generally necessary in fish, in part because living in an aquatic environment where waste can continually be released into the environment via the high surface area of the gills has made it possible for fish to be ammonotelic and avoid the costs of nitrogenous waste processing (Kardong 2009).

Our most current understanding of ammonia excretion in fish is that most ammonia (in the form of NH_3) passively diffuses out through rhesus proteins on the gill (Wright and Wood 2009). In near neutral waters, the gill of a freshwater fish augments this process by pumping protons, or producing them via carbonic anhydrase to create an "acid trap" in the boundary layer water of the gill (Wilkie *et al.* 1994). In this microenvironment NH_3 is efficiently protonated and NH_4^+ can then diffuse away into the surrounding environment (Weihrauch *et al.* 2009). High environmental pH breaks down this acid trap mechanism, and once pH exceeds 9.5, the pKa of ammonia, passive diffusion no longer occurs. Over time ammonia build up results in mortality unless the fish can mitigate ammonia's toxic effects.

1.6 Variation in High pH Tolerance between Species of Fish

Fish living in high pH need to compensate for blood pH, ionic, and ammonia disturbances. Species living in these lakes have adaptations which allow them to maintain homeostasis. Here I will briefly discuss the two most heavily researched alkaline adapted fish; the Lake Magadi Tilapia and Lahontan Cutthroat Trout in comparison to the non-alkaline adapted Rainbow Trout.

The Lake Magadi Tilapia, of Kenya, is the only fish species which can be found in this pH 10 lake. These fish have an adaptation which lowers HCO_3^- permeability in gill and allows electrogenic HCO_3^- extrusion (Wood et al. 2012) but also are adapted to handle high plasma pH (Johansen *et al*. 1975; Wood *et al*. 1994). Na^+ and Cl^- levels remain stable as Lake Magadi has high salinity and therefore ions passively diffuse in through the gut as this fish drinks water (Wood *et al*. 2002). This species avoids the difficulties associated with ammonia excretion by converting ammonia to the far less toxic compound, urea, which can then be transported out through the gills via the UT-A transporter (Wood *et al.* 2002 and Walsh *et al.* 2001). Although most teleost have the genes coding the proteins necessary for this biochemical process, most fish do not express all of the enzymes in adult life, therefore suggesting this is an adaptation to life by Lake Magadi Tilapia in this high pH lake (Wilkie and Wood 1996).

Pyramid Lake, Nevada, USA, maintains a pH 9.4 yet has a thriving trophy Lahontan Cutthroat Trout Fishery (Galat *et al.* 1985). This subspecies appears to have slightly elevated blood pH compared to fish held in pH 8.4, however it has a very high chloride cell fractional surface area (CC FSA) on the gill to enhance acid-base regulation

(Wilkie *et al*. 1994). This gill modification is thought to aid in maintaining ionic composition of the blood, but it is also thought that the high salinity of the lake replenishes ions lost to acid-base regulation (Wilkie and Wood 1996). This species generates only slightly higher amounts of urea than non-alkaline fish, and the mechanism by which it handles nitrogenous waste is either via reducing ammonia production or via an alternative storage route (ie. creatine synthesis) which has not yet been assessed (Wilkie and Wood 1996).

Rainbow Trout generally do not persist well in alkaline environments, such as Pyramid Lake (Galat 1985). Reported high pH tolerance of this species greatly differ across studies. For example, Wilkie and Wood (1991) demonstrated that Rainbow Trout can survive for several weeks at pH 9.5 whereas at the same pH Thompson *et al*. (2015) observed fish losing equilibrium within hours. Even in the study reporting higher tolerance, high pH exposure made the fish more likely to die from other stressors (Wilkie and Wood 1991). Exposure to high pH induces considerable metabolic alkalosis, which does not fully recover over several days (Wilkie and Wood 1991; Yesaki and Iwama 1992). Ionoregulation appears to be modestly affected; as Na^+ and Cl^- slightly drop (5%), which is likely to be physiologically stressful but not lethal (Wilkie and Wood 1991, Wilkie *et al.* 1996). Plasma chloride may be regulated to some extent via up-regulation of CC FSA (Wilkie and Wood 1994). In the short term these fish do not appear to be able to counteract nitrogenous waste build up. In both short term and chronic exposure to high pH, blood ammonia levels remain elevated with no appreciable synthesis of urea (Wilkie and Wood 1991). In order to mitigate high plasma ammonia levels fish begin to sequester ammonia in white muscle (Wilkie *et al.* 1996, Thompson *et al.* 2015). Fish also attempt

to protect the brain from ammonia binding to and over-exciting NMDA receptors on neurons by binding ammonia to glutamate therefore increasing brain glutamine content (Thompson *et al.* 2015, Wilkie *et al*. 2011, Kosenko *et al*. 2003). There is some evidence to suggest that hard water reduces the stress of high pH (Yesaki and Iwama 1992); however, more recent studies have suggested that hard water does not affect tolerance to high pH (Thompson *et al*. 2016).

1.7 Variation in High pH Tolerance within Rainbow Trout

Over 20 years ago the Province of British Columbia funded an alkaline lakes enhancement project with the overall goal of enhancing Rainbow Trout populations in alkaline lakes (Yesaki and Tsumura 1991, Toth and Tsumura 1992, Toth and Tsumura 1993, Godin *et al.* 1994, Mathias *et al.* 1995). This project ran for 5 consecutive years and focused mainly on in-lake survival and compared a number of strains from both alkaline and non-alkaline brood stock. This study was broken down into long-term mark re-capture studies using gill-netting in experimental lakes, and short-term survivorship within net-pens in lakes. This study also began to explore the effects of acclimation and physiological parameters on high pH tolerance. Here I will focus on the findings of these extensive studies which pertain to this thesis.

One of the first objectives of this project was to identify good candidate alkaline lakes from which to draw brood stock (Yesaki and Tsumura 1991). The key idea was that fish from alkaline lakes may have adaptations not present in Rainbow Trout from circumneutral waters which would make them more tolerant when stocked into lakes with similar waters. Both Stump Lake (Merritt BC) and Green Lake (100 Mile House BC)

appeared to be good candidate lakes with an average pH of 9.2 in 1991. Both lakes had declining survivorship over the years proceeding this experiment so it was expected fish remaining in these lakes might have heritable pH tolerance. In addition Stump Lake had once been a popular recreational fishery lake and there was interest in revitalizing it.

A long-term mark recapture study in Experimental Lake 5567 (pH 9.2) was revealing in that it followed the progeny from the 1991 brood stock of Green Lake, Stump Lake, and Pennask Lake for the entire duration of the study. Brood stock were also collected in 1992 and 1993 from each lake for replication. The results of 4 years of high-pH lake exposure suggested that the fish derived from Stump Lake and Green Lake had significantly better survivorship than the Pennask strain. The results of the 1992 and 1993 brood stock did not show as clearly that survivorship was better, but did show that the alkaline strains had greater growth. In 1992 a second experimental lake was added, Till Lake (Williams Lake, BC; pH 9.2). Although catches were low, experiments in this lake provided evidence that Stump Lake was the most tolerant strain. In 1994 short-term net pen studies were carried out to compare mortality across the three strains in four alkaline test lakes. In two of these lakes, the progeny of Stump Lake appeared to be the most tolerant fish; however, in the other two lakes there were no strain differences suggesting that in-lake survivorship may depend on more than just high pH. By 1995, brood stock capture in Green Lake had collapsed therefore leaving Stump Lake as the only candidate from which to create an alkaline strain.

This project also looked at the effects of gradual exposure to high pH on tolerance to high pH (Yesaki and Tsumura 1991, Toth and Tsumura 1992). In the first year of this study the researchers did this by slowly moving fish (over the course of 7 hours) down a

channel which gradually transitioned from pH 8.02 to pH 9.3 in Green Lake and compared survival over 24 hours in a net pen with fish directly stocked into a net pen in the lake. They found 1% mortality and 10% mortality respectively (Yesaki and Tsumura 1991). The second year repeated this exposure over a week with similar results to the first year. They also looked at bringing the water in the transport truck slowly up to lake pH during transport and found that this too increased survivorship (Toth and Tsumura 1992).

In addition to field work, there was a small laboratory study performed during the second year of the study. This study simply compared the Na^+ and Cl^- levels of the blood across strains which were exposed to pH 9.3. This work suggested that Stump Lake and Green Lake fish had enhanced ability to regulate ions under high pH exposure (Toth and Tsumura 1992).

Laboratory tolerance assays investigating survivorship have also provided evidence that pH tolerance varies between strains of Rainbow Trout. Thompson *et al.* (2015) compared survivorship under laboratory conditions and examined physiological mechanisms involved in high pH tolerance to assess differences between the Fraser Valley Domestic, Blackwater, Tzenzaicut, Carp Lake, and Pennask strains. It is important to note that none of the wild strains used in these experiments were the progeny of parents living in high pH lakes. They compared time to loss of equilibrium as a proxy for tolerance among individuals of these strains across 2 days in the first year of the experiment and 3 days in the second year. In the first year and second year of their study the Fraser Valley Domestic strain demonstrated the highest tolerance compared to the wild strains. Across strains there was considerable ammonia build up in plasma, brain

and white muscle. Ammonia build up in the brain appeared to be mitigated by conversion of glutamate to glutamine.

Collectively, the alkaline lakes enhancement project and the work of Thompson *et al.* (2015) demonstrated that pH tolerance is highly strain-dependent under field and laboratory conditions. Early work also demonstrated how acclimation increases survivorship in high pH. My thesis will build on this work by assessing an alkaline population (Stump Lake) in the laboratory and looking at how acclimation and rearing under moderately high pH (8.8) will influence high-pH tolerance.

1.8 Overview of Genome Wide Association Studies

Despite many studies addressing inter and intra-species variation in high pH tolerance there have been no studies to assess genetic variation among individuals of varying pH tolerance. Until recently genotyping large numbers of individuals, especially from a non-model species, would have been financially difficult, but with the development of high through-put next generation sequencing (NGS) technology the price has dropped to a point where hundreds of individuals can be genotyped for a study. Genome by sequencing (GBS) has allows multiple samples to be pooled and then after sequencing to be sorted based on sequence barcodes which are unique to individuals (Elshire *et al.* 2011). The emergence of bioinformatic pipelines to analyze large amounts of data using relatively low computing power has made it possible for researchers to scan the genome of multiple individuals (Glaubitz *et al*. 2014). These genome scans can be compared based on a particular trait of interest for a genetic association, in a design known as a Genome-wide Association Study (GWAS).

Early sequencing approaches, such as Sanger's dideoxy sequencing (aka. Chain terminator sequencing) method, have very low sequencing throughput (96 bases at a time), whereas a NGS platform such as Illumina allows 150 million bases at a time by using bridge amplification on flow cells (Hutchison 2007). NGS is currently used for whole-genome sequencing whereby an entire genome is sequenced and assembled or for reduced representation sequencing where a subset of the genome is sequenced based on location to restriction enzyme sites (Davey *et al*. 2011). The reduced representation technique has been further improved by the addition of reversible terminator chemistry and barcodes so that numerous individuals can be simultaneously genotyped in flow cells (Bentley *et al*. 2008, Davy *et al*. 2011). This method can be modified for a lower coverage approach, whereby each site is not covered in every individual ie. Genotyping by sequencing (GBS) and more sites across samples can be discovered at a fraction of the cost (Davey *et al.* 2011).

Simplification of bioinformatics pipelines, and steps to reduce computational power, have been instrumental in reducing analysis time and giving biologists across fields the ability to use genetic data (Glaubitz *et al.* 2014). These pipelines can be used in species without reference genomes, but requires that a reference map be assembled around each marker. However, due to the low coverage of the GBS approach it is generally recommended for species with a reference genome (Davey *et al.* 2011). In part due to low coverage, filtering parameters are a very important aspect of processing GBS data. The TASSEL-GBS pipeline created by Glaubitz *et al.* (2014), first analyzes sequence reads and sorts them into counts. Any site with fewer than 3 reads is discarded. These sites are then mapped onto the reference genome and SNPs are called based on

variation among individuals. Each individual's genotype is assigned by sorting the data further based on their unique barcode read. Data can be further filtered to remove individuals or sites with high levels of missing data or to remove low frequency SNPs.

These SNPs can then be used in a GWAS, whereby each SNP can then be assessed to see if having one allele or the other is associated with a particular phenotype. These studies use general linear models (GLM) and mixed linear models (MLM) to allow the comparison of continuous traits across each SNP. GLMs include a principle component analysis to account for population structure, whereas MLMs also include a kinship matrix to account for the hidden effects of relatedness. These studies generally run tens of thousands of statistical comparisons and therefore require that false discovery rate be accounted for.

1.9 Genome Wide Association Studies in Salmonids

Following the development of high throughput sequencing technology with alignment pipelines, salmon biologists across disciplines were able to conduct GWAS to address the genetic basis for aquacultural, ecological and physiological traits. Since the Atlantic Salmon genome and the Rainbow Trout genome have been published, a number of studies have been able to identify genes which may be associated with various traits of interest. The current version of the Atlantic Salmon genome has been assembled and annotated whereas the current version of the Rainbow Trout genome has more than half of its sequences assembled on small scaffolds (Lien *et al*. 2016, Berthelot *et al*. 2014). In the current state of the Rainbow Trout genome it is not easy to resolve paralogs and this has led to issues in sequence alignment and certainty of SNP calling. Earlier studies with

Rainbow Trout have been cautious in making genetic arguments from markers associated with their traits of interest. Recently McKinney *et al.* (2016) developed a protocol to further improve accuracy of SNP calling in organisms (ie. the Rainbow Trout), with genomic duplication.

GWAS have become popular in aquaculture research as these data can inform marker-assisted breeding programs. Most of the applied aquaculture studies have focused on fillet production and disease resistance. This has allowed researchers to find that fillet production in Rainbow Trout is associated with having certain alleles of genes associated with myogenic precursor cell proliferation (Gonzalez-Pena *et al*. 2016). In Atlantic Salmon, GWAS is providing a genetic context for premature sexual development, growth and fillet fat content and firmness (Gutierrez *et al.* 2015, Sodeland *et al.* 2013). Other studies have sought to improve salmon health by identifying the genetic basis for resistance to bacterial cold water disease (BCWD), infectious hematopoieticnecrosis virus (IHV), Salmon Rickettsial Syndrome (SRS), and sea lice (Campbell et al. 2014, Correa et al. 2015, Liu et al. 2015, Tsai *et al*. 2016).

On an ecological level, GWAS have given scientist insight into genes affecting migration in salmonids. Hecht *et al.* (2013) and Hale *et al*. (2013) have identified several markers which differentiate Steelhead from Rainbow Trout populations. Researchers have also identified genes associated with Atlantic Salmon age of return from sea to gain insight into tradeoffs different populations make to enhance survivorship and reproduction (Johnston *et al.* 2014). Studying factors affecting salmon survivorship in the ocean is quite challenging; Bourret *et al.* (2014) have used GWAS to identify the genes

underlying sea mortality. Overall GWAS has provided answers to questions biologists have not historically been able to test.

GWAS may also be instrumental in understanding the genes involved in tolerance to abiotic stressors. Only one study thus far has conducted a GWAS to assess the genetic component of thermal tolerance in salmonids. Narum *et al*. (2013) investigated the genetic association of high thermal tolerance in Red Band Trout from montane and desert populations. They found a number of genes, such as heat shock protein coding genes, which grant the desert population higher thermal tolerance. These markers may be used on other populations to predict if they will have the genetic material to tolerate climate change. This type of study could be used on any abiotic stressor to identify markers which are associated with tolerance. GWAS may also prove useful in identifying which genes are associated with acclimation to an abiotic stressor.

1.10 Thesis Objectives

This thesis has four main objectives 1) to characterize high pH (9.5) tolerance in Rainbow Trout that are the progeny of parents from an alkaline lake (Stump Lake); 2) to compare whether acclimation or rearing fish under moderately high pH (8.8) improves tolerance to high pH; 3) to identify if there are genetic associations to high pH tolerance; 4) to characterize differences in genetic association to high pH tolerance between acclimated and non-acclimated fish. Overall these objectives will provide information to the FFSBC to improve stocking success in alkaline lakes.

[2] Characterization of Variation in pH 9.5 Tolerance in Rainbow Trout (*Oncorhynchus mykiss*) and the Effects of Acclimation and Rearing at pH 8.8

2.1 Introduction

High environmental pH is physiological stressful for most species of fish as it results in perturbations in blood pH, ionic composition and increases in ammonia. Initial exposure to pH higher than the blood (pH~ 7.8) is reflected by increases in blood pH; however, through respiratory and metabolic compensation, fish are to a great extent able to regulate their blood pH back close to initial physiological levels (Heisler 1984, Wilkie and Wood 1991). Branchial pH compensation involves exchanging ions for acid and base equivalents, which result in lowered Na^+ and Cl^- levels in the blood (Gilmour and Perry 2009, Wilkie and Wood 1991). However, these ionic reductions are not thought to be lethal (Wilkie and Wood 1996). The aspect of environmental high pH tolerance, which is thought to cause mortality in fish, is that it imposes limitations on branchial ammonia excretion (Wright and Wood 1985, Wilkie and Wood 1991, Thompson *et al*. 2015). Passive diffusion of ammonia across the gill ceases at pH ≥ 9.5 as the acid trapping mechanism that promotes favourable efflux fails (Wright and Wood 1985). As ammonia builds up within the fish it over stimulates NMDA receptors in the brain which result in seizures, convulsions and eventually death (Wilkie *et al*. 2011, Randall and Tsui 2002).

Some species of fish are extremely tolerant of high pH and have unique adaptations to deal with the physiological challenges of exposure. For example, the Lake Magadi Tilapia uses ureogenosis to prevent high levels of ammonia building up in the blood when external pH is high (*see* Walsh and Smith 2001 *for review*). Similarly, the Lahontan Cutthroat Trout (*Oncorhynchus clarkii henshawi*) thrives in Pyramid Lake at

pH 9.4 by increasing urea excretion, but it is thought that their success is ultimately limited by their ability to reduce nitrogenous waste production (Wright *et al*. 1993, Wilkie *et al*. 1994). In contrast, Rainbow Trout do not appear to have the plasticity for long-term survival in high pH environments and attempts to stock them into Pyramid Lake have failed (Galat *et al.* 1985). Unlike high pH tolerant species, Rainbow Trout do not show appreciable synthesis of urea and instead ammonia builds up in the blood at pH above 9.5 (Wilkie and Wood 1991, Yesaki and Iwama 1992, Wilkie and Wood 1995). Increased levels of blood ammonia can be tolerated at least up to 5 weeks in pH 9.5, but they result in the fish becoming more susceptible to death by other causes (Wilkie and Wood 1991).

In British Columbia, increasing alkalinity in a large number of lakes has created challenges for stocking Rainbow Trout. Some stocking attempts result in considerable mortality shortly after introducing fish into the alkaline lakes, whereas some fish can survive and naturalize to these same conditions. The Freshwater Fisheries Society of British Columbia (FFSBC) has selected a number of Rainbow Trout strains from natural source populations which are specialized for different lake requirements, (ie. catchability, ability to persist with other fish, over wintering capabilities), but does not currently have a strain in production for high pH lakes. This has led the FFSBC to investigate the mechanisms that allow fish to tolerate high pH and consider developing a new strain. Thompson *et al.* (2015) characterized high pH tolerance of the current FFSBC strains and found that they were even less tolerant of high pH than Rainbow Trout used in prior studies, which demonstrates the potential need for a new strain (Yesaki and Iwama 1992, Wilkie and Wood 1991, Wright and Wood 1985). In the early 1990's the alkaline lakes

enhancement project was initiated. In this project researchers experimentally stocked the progeny of fish from highly alkaline lakes (Stump Lake, Meritt, BC and Green Lake, 100 Mile House, BC) and compared their survivorship alongside an FFSBC strain of Rainbow Trout in high pH and circumneutral pH lakes (Yesaki and Tsumura 1991, Toth and Tsumura 1992, 1993, Godin *et al*. 1994, Mathias *et al*. 1995). The majority of their studies suggested that Stump Lake fish can survive and grow well in comparison to FFSBC strains. In this study we characterize the high pH tolerance of the progeny of Stump Lake fish in controlled laboratory conditions.

Fish are often stocked as juveniles and need to be able to survive and grow up to catchable size in high pH; however, there has been a lack of long-term laboratory studies focusing on the effects of high pH on fish. High pH exposure (9.5+) has been maintained in the lab from a few days (Wilkie and Wood 1991,1995, Yesaki and Iwama 1992, Thompson *et al*. 2015) up to 28 days (Wilkie *et al.* 1996) but, little is known about long-term effects. From the work of Wilkie and Wood (1991) high pH exposure initially causes plasma pH to increase; however, physiological adjustments allow pH to recover within 8hrs. With longer exposure, fish begin storing ammonia in white muscle, and they remodel their gills to enhance HCO_3^-/Cl^- exchange to counteract blood alkalosis (Laurent *et al.* 2000, Wilkie and Wood 1994, Wilkie *et al.* 1996). A few studies have looked at the effects of moderately high pH acclimation from a few hours to a week prior to high pH exposure as a mechanism to improve survivorship (Yesaki and Tsumura 1992, Toth and Tsumura 1993, Witschi and Ziebell 1979). These studies demonstrated improvements in high pH tolerance relative to fish held at neutral pH. High pH tolerance may be further improved by exposing fish to moderately high pH levels during development. However,

no studies have previously examined the effects of high pH rearing in the lab. Here I am interested in how a month of exposure to moderately high pH or rearing a fish under these conditions influences tolerance to high pH.

The objectives of this thesis chapter are 1) to characterize the tolerance of Rainbow Trout progeny from Stump Lake brood stock to high pH (9.5); 2) to establish if acclimation or rearing under moderately high pH (8.8) has an impact on tolerance to pH 9.5.

2.2 Materials and Methods

2.2.1 Experimental Animals

Adult Rainbow Trout were collected from Stump Lake, Merritt, British Columbia (GPS: 50.364088, -120.368242) in the spring of 2014. Ten males and ten females were batch spawned at the FFSBC Fraser Valley Trout Hatchery (FVTH), Abbotsford, British Columbia. Offspring produced in these mating crosses were randomly divided into two groups to be reared under different pH conditions: 1) near neutral well water (pH~ 7.2), which represents standard hatchery conditions, and 2) moderately high pH (8.8), which represents a typical pH for Stump Lake (data retrieved from the Freshwater Fisheries Society of BC Small Lakes Database). Eggs were reared in heath trays supplied with water at their respective pH treatment and upon developing to the alevin stage the fish were transferred to 200L oval tanks at the same pH levels. Each tank was fitted with a modified air-lift system made up of an elbow shaped piece of PVC pipe with an air stone suspended down the pipe deep into the tank. The bubbles coming off the air stone pulled tank water upwards and then mixed it at the bend in the pipe with fresh water delivered from the head tank. Incoming water flow was verified and adjusted on a weekly basis and increased with fish growth.

The FVTH uses onsite wells as a source of groundwater, which is aerated in large head tanks. The FVTH uses a recirculating aquaculture system for general production; however, to ensure biosecurity a flow through system is used for research production. For the pH 8.8 treatment 50% NaOH was added to a head tank such that when the water entered the culture environment, a pH of 8.8 was established in the heath trays or fish culture tanks. Maintenance of this pH was ensured by daily monitoring of culture tank pH

and adjustments to the pH set point of the head tank. Addition of NaOH into the head tank was performed using an American Marine Inc. Pinpoint pH controller with a pH sensor connected to a 1.1mL/min dosing pump. Because the system was designed to operate under flow-through conditions, it was not necessary to have an acid pump to compensate in the event of too much NaOH being dosed as it would quickly be flushed through the system.

Measurements of pH, oxygen levels and temperature were also recorded daily. Nitrogenous wastes were measured on a monthly basis and consistently remained low. Fish were fed at a level to ensure growth to pre-set size goals while ensuring healthy development. For this experiment, Stump Lake fish were reared to 3.8+/- 0.1 g. All holding and experimentation met the standards of the Canadian Council on Animal Care and University of British Columbia Animal Care Committee; permit: A14-0103.

In addition to the experiment described above to test the effects of developmental pH, an experiment to test the effects of shorter-term acclimation was also performed. For this experiment, a subset of the control (pH 7.2) fish were transferred into separate tanks for pH 8.8 acclimation. These tanks initially were supplied with control well water and then slowly adjusted over a week up to pH 8.8 and then were held at this level for a month prior to tolerance assays.

2.2.2 Determination of Time to Loss of Equilibrium in High pH

Time to loss of equilibrium at pH 9.5 (LOE_{pH}) was used as a measure of high pH tolerance. This pH was selected as it is similar to the pK' of ammonia and therefore it was predicted to impede branchial ammonia excretion in the fish (Cameron and Heisler

1983), and cause high ammonia in the blood with neurotoxic effects likely to result in loss of equilibrium. In addition, this pH has been used in prior studies of pH tolerance in the FFSBC Rainbow Trout strains (Cameron and Heisler 1983, Thompson *et al.* 2015). This pH is low enough though that it allows a distinction in time between high and low tolerance individuals. Prior to the transfer into assay tanks, fish from the pH 8.8 reared, pH 8.8 acclimated, and control treatments were distinctly fin-clipped so that the 3 treatments could be assessed in a common garden design.

The assay tanks were brought up to pH 9.5 and then fish were added and monitored for LOE_{pH} every hour. LOE_{pH} was defined by the fish being completely horizontally flipped over and incapable of locomotion. At the end of each hour, any fish that lost equilibrium were euthanized in a bucket with a lethal dose of MS222 and had their treatment group (identified by fin clip), weight, length and time to LOE_{pH} recorded. Another fin sample was collected and preserved in ethanol for later DNA extraction. The experiment was terminated at 72 hours and any fish remaining were euthanized and sampled; their LOE_{pH} was recorded as 72 hours.

2.2.3 Statistical Analysis

All statistical analysis was performed using R (v3.3.1). I analyzed the LOE_{pH} data using a Kaplan- Meier Survivorship analysis. This type of statistical approach is appropriate for analyzing data which has individuals that did not exhibit LOE_{pH} before the experimental endpoint (Rich *et al.* 2010). The Kaplan-Meier survivorship model was tested with a Wilcoxon test to establish any significant differences between each treatments survivorship curve. Differences among the curves were resolved through pair-

wise comparisons with a Bonferroni correction. This model has power to detect differences among treatment groups, but does not allow for numeric covariates to be included. Separate regression analyses were carried out to establish if weight, length or condition factor had an effect on LOE_{pH} as well as to see if there were differences in these measurements between treatments.

2.3 Results

Previous exposure to high pH significantly improved LOE_{pH} relative to the control ($P=3.01 \times 10^{-11}$). Interestingly only 10% of the control fish remained at the end of the experiment, whereas 93% of the pH 8.8 reared fish and 50% of the pH 8.8 acclimated fish remained at 72hrs (Figure 2.1). Posthoc tests revealed that all treatments significantly differed from one another ($P<0.005$). Weight did not differ between treatments ($P=0.72$) (Figure 2.2.A). However, the pH 8.8 reared fish were significantly longer than the control ($P= 0.02$) (Figure 2.2B). Regression analysis revealed that neither weight nor length was significantly associated with LOE_{pH} across treatments ($P>0.25$) (Figure 2.3 and 2.4).

2.4 Discussion

The most surprising results of this study was how few fish derived from a high pH lake (Stump Lake) were able to tolerate pH 9.5 for 72 hrs when they were reared under neutral pH conditions in the hatchery (*see Fig. 2.1*). However, exposing these fish to moderately high pH (8.8) for 1 month resulted in significant improvements in tolerance with 50% of the fish remaining at 72hrs and rearing these fish under moderately high pH (8.8) from fertilization resulted in 93% of the fish surviving high pH (9.5) exposure over

72 hrs. These results collectively show that phenotypic plasticity plays an important role in high-pH tolerance in this strain.

I hypothesized that fish from Stump Lake would have genetic adaptations that grant them tolerance to pH 9.5. My results did not support this hypothesis, as only 10% of the progeny from Stump Lake fish did not exhibit LOE_{pH} at 72hrs. This poor tolerance is further exemplified when I compare my results to those of fish from non-alkaline lakes. Thompson *et al*. (2015) characterized the high pH tolerance of 5 strains of Rainbow Trout from non-alkaline lakes. In the first year of their study they compared LOE_{pH} over 48hrs of exposure to pH 9.5 and found approximately 80% of the Fraser Valley Domestic remained in contrast with 0% in the wild strains (Pennask, Tzenziacut, and Blackwater) remained at the end of the experiment. In the second year of their study they found near 100%, 65%, 80%, 60%, and 60% remained at 72hrs for the Fraser Valley, Pennask, Tzenzaicut, Blackwater and Carp Lake strains, respectively. My results show approximately 25% and 15% of the Stump Lake population remained at 48hrs and 72hrs, respectively. Across both years this comparison suggests that the Fraser Valley Domestic strain was more tolerant than Stump Lake fish; however, the year-to-year variation in Thompson *et al.* (2015)'s study makes it difficult to resolve whether or not the non-alkaline wild strains are more or less tolerant than the Stump Lake strain. In addition it is difficult to directly compare my results to those collected by Thompson *et al.* (2015) because they used larger fish and their experimental design differed from mine in that in their study t=0 was measured from a neutral tank that was then gradually elevated to pH 9.5 over 6 hours, effectively allowing fish to acclimate.

Throughout the alkaline lakes enhancement project most of the findings suggested that Stump Lake fish had superior performance in alkaline lakes compared to the Pennask strain based on measurements of recapture success and growth (Yesaki and Tsumura 1991, Toth and Tsumura 1992, 1993, Godin *et al.* 1994, Mathias *et al.* 1995). However, this success was highly dependent on the broodyear from which the progeny were generated. The study followed the recapture of Pennask fish and the progeny of Stump Lake from brood stock collected in 1991, 1992, 1993 over 3, 2, and 1 years respectively, in an alkaline experimental lake (Lake 5567, pH 9.2). The Stump Lake progeny from 1991 and 1992 had higher recovery rates and growth rates than the Pennask strain. The experiment was replicated in Till Lake (pH 9.2) using the same strains over two years with similar findings. More consistent with my results, the 1993 brood stock progeny from Stump Lake demonstrated similar survivorship to the Pennask strain, only exceeding them in growth. The researchers attributed these differences among brood stock years in part to changes in pH levels, as the pH in Stump Lake varied between 8.8 to 9.3 within this 5 year window.

In the years since the alkaline lakes enhancement project assessment, Stump Lake has been stocked for angling with various Rainbow Trout strains by the FFSBC and presumably, pH has continued to cycle. Without the selective pressures of high pH it is possible that other strains have naturalized to this lake and therefore the genetic composition of the fish I used in my study may be notably different than 20 years prior.

Although, on average, Stump Lake fish did not show markedly superior performance at pH 9.5 relative to fish from other strains, I observed substantial inter-individual variation in tolerance within the Stump Lake population. This may suggest that

the fish that remained at 72hrs exposure to pH 9.5 were genetically distinct from those with LOE_{pH} under 20hrs.

Despite the poor performance of Stump Lake Fish reared under neutral pH in this experiment, my results demonstrate that prior exposure to pH 8.8 dramatically increases tolerance to pH 9.5. One month of acclimation resulted in ~50% of fish remaining until the end of the experiment whereas rearing fish from fertilization resulted in 93% fish remaining. It is unclear if rearing acts as a long-term acclimation period or if developmental plasticity results in these improvements. It is also difficult to compare my results with other studies, because to my knowledge prior to this study no one has tested the effects of longer term (1 month) acclimation to moderately high pH. Previous studies have only looked at short term gradual exposures to high pH, these results also demonstrated improvements in tolerance. For example, Murray and Ziebell (1984) show that small incremental increases in pH over 5 days result in higher survivorship than increases over 6 hours. Further, in the field Yesaki and Tsumura (1992) and Toth and Tsumura (1993) demonstrated that gradually increasing pH in transport water to that of the lake or slowly moving fish through channels with pH gradients into the lake promoted significantly higher survivorship than moving the fish directly from hatchery water into the lake.

Across studies Rainbow Trout appear to employ physiological adjustments to reduce the stress of high pH within hours of initial exposure. These adjustments include metabolically stabilizing the blood pH, remodeling of the gills to increase surface area for base excretion, handling ammonia build up through shunting it into other reactions ie. glutamine/ glutamate, urea synthesis and storing ammonia in white muscle (Wilkie and

Wood 1991,Thompson *et al.* 2015, Wilkie *et al.* 1996, Wilkie and Wood 1994). Acclimation to moderately high pH may prime these mechanisms prior to exposure to higher pH and therefore allow less tolerant individuals to persist. It would also seem reasonable to increase ammonia excretion, perhaps via increasing expression of a type of branchial ammonia metabolon (hypothesized by Wright and Wood 2009) which could use active transport to expel ammonia. A concurrent study is underway to investigate the physiological changes made during exposure to moderately high pH.

In conclusion, I demonstrate that there is large variation in high pH tolerance among the Stump Lake population and this group has the phenotypic plasticity to have markedly better survivorship in high pH environments with prior exposure to moderately high pH. Based on these results it appears that at this time Stump Lake would not be a good candidate population to use as brood stock for a high pH strain without artificial selection of the most tolerant individuals. Exposing fish to moderately high pH in the hatchery prior to stocking is an alternative approach worth exploring in hatchery production.

2.5 Figures

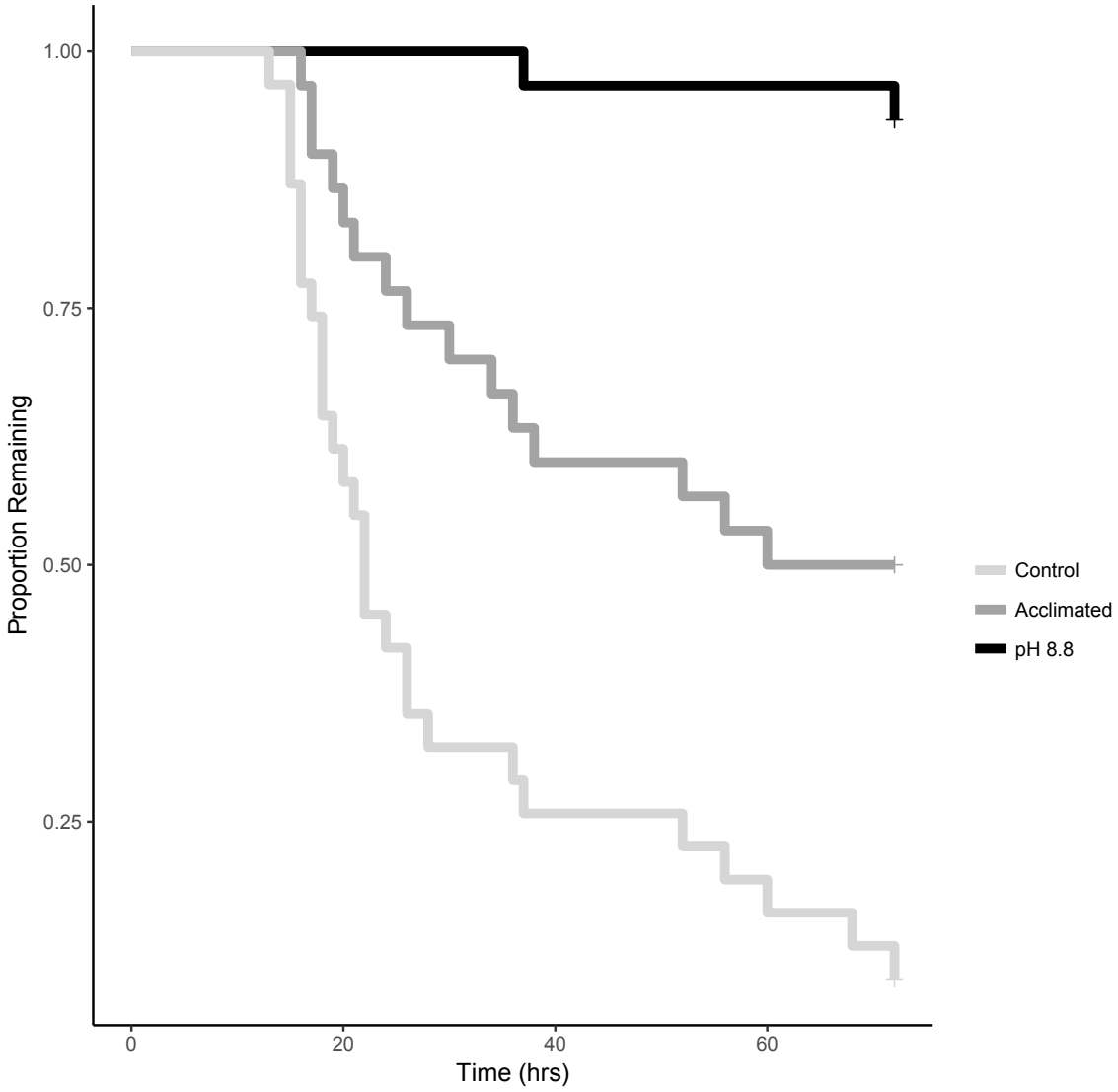

Figure 2.1. Proportion of fish remaining over time (hrs) of Rainbow Trout exposed to high pH 9.5. Fish that exhibited LOE were removed each hour. Control fish were reared at pH 7.2, Acclimated fish were reared at 7.2 and then transferred into pH 8.8 for 1 month, pH 8.8 fish were reared at high pH. All survivorship curves are significantly different (P=3.01e-11).

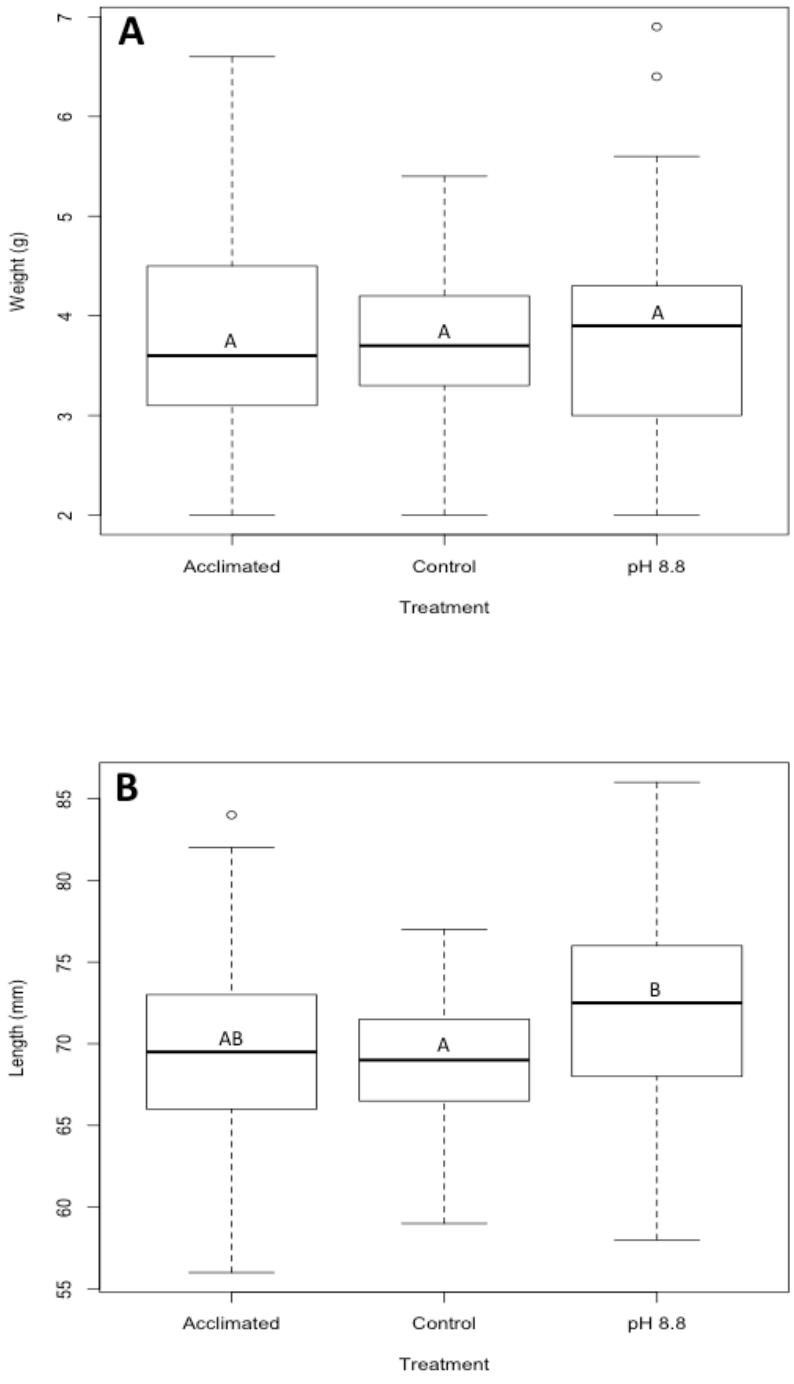

Figure 2.2. Median and 25% and 75% quantiles of A) weight (g) B) length (mm) compared of three treatments. Control fish were reared at pH 7.2, acclimated fish were reared at 7.2 and then transferred into pH 8.8 for 1 month, pH 8.8 fish were reared at high pH. Groups with different letters are significantly different at $P<0.05$.

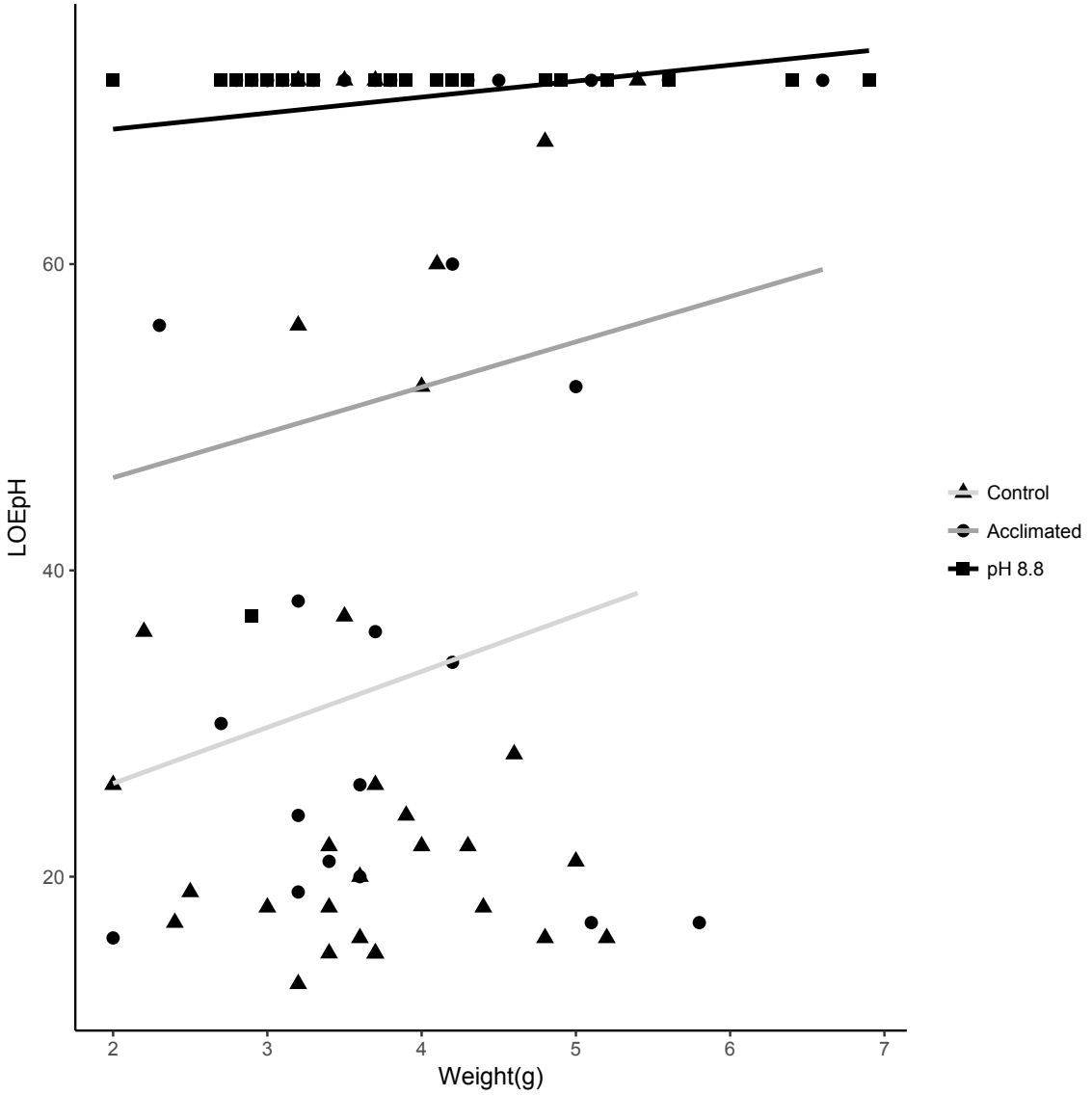

Figure 2.3. LOE_{pH} as a function of weight (g) in each treatment group. Control fish were reared at pH 7.2, Acclimated fish were reared at 7.2 and then transferred into pH 8.8 for 1 month, pH 8.8 fish were reared at high pH. Lines represent regression analysis of each group ($P>0.05$ for all).

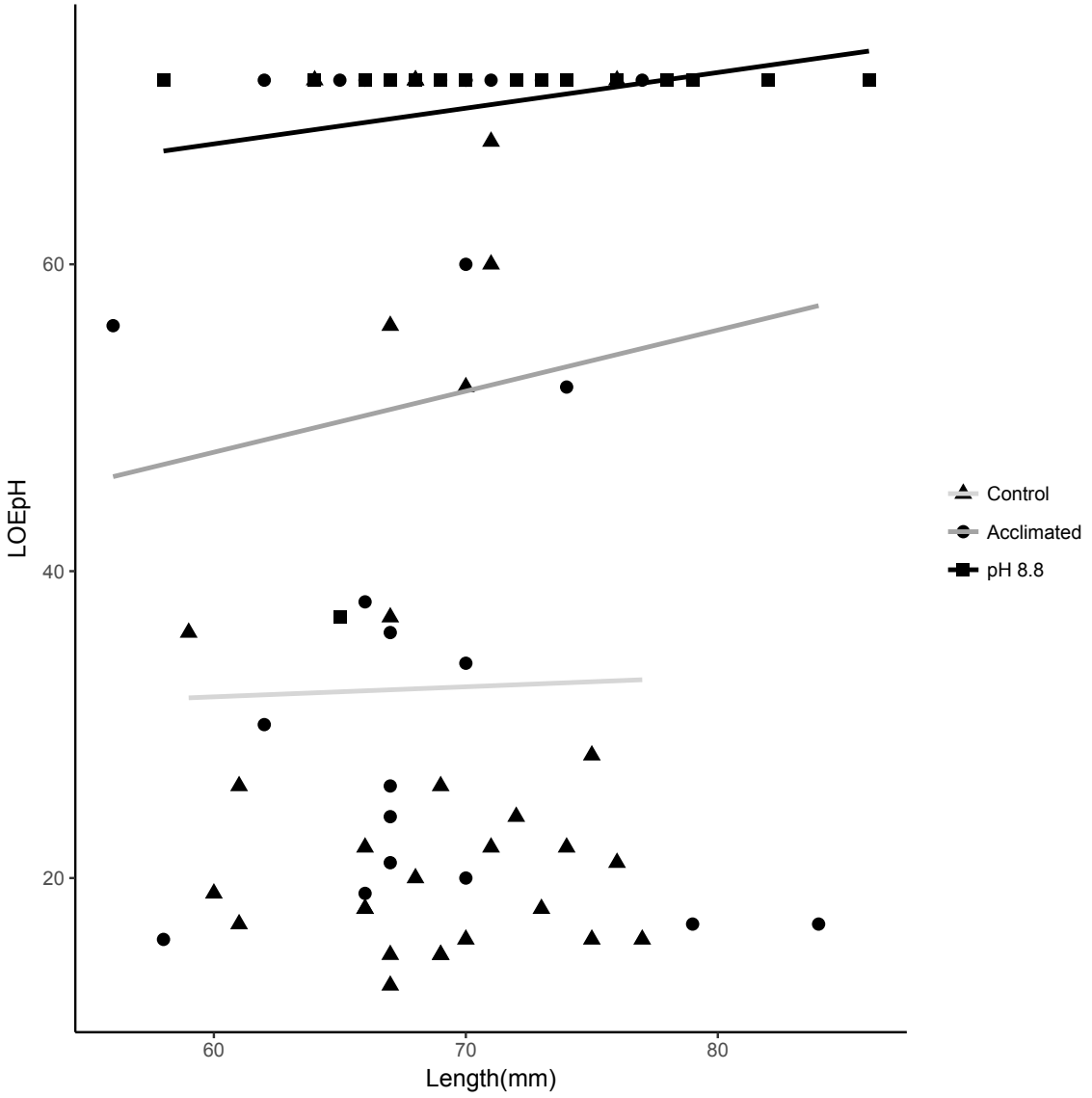

Figure 2.4. LOE_{pH} as a function of length (mm) for each treatment group. Control fish were reared at pH 7.2, acclimated fish were reared at 7.2 and then transferred into pH 8.8 for 1 month, pH 8.8 fish were reared at high pH. Lines represent regression analysis of each group ($P>0.05$ for all).

[3] The Genetic Basis of high pH Tolerance of Acclimated and Non-acclimated Rainbow Trout (*Oncorhynchus mykiss*) from an Alkaline Lake

3.1 Introduction

In recent years, biologists across disciplines have gained unprecedented access to genetic information as a result of advances in sequencing technology. This revolution is underway in large part due to the fact that it is possible to use restriction enzyme digestion followed by the ligation of molecular barcodes coupled with next generation sequencing to genotype large numbers of individuals economically (Elshire *et al.* 2011). These technological advances have made it possible to associate phenotypic variation with genetic variation across the genomes of multiple individuals through Genome Wide Association Studies (GWAS). These studies have the power of simultaneously questioning, with the use of linear models, if any one of the tens of thousands of single nucleotide polymorphisms (SNPs) in a given population is correlated with the trait of interest across multiple individuals. GWAS have been instrumental in identifying genetic association with disease and complex traits in humans (for review see Welter *et al.* 2014). In agriculture, GWAS have been used to identify SNPs for marker assisted selection for desirable traits in plants such as Barley and Maize (Elshire *et al.* 2011).

Recently GWAS have been applied to identify the genetic basis of ecological traits, disease resistance, growth and abiotic stress tolerance in salmonids. These studies have, for the first time, given ecologists insight into the genes involved in differentiation between Steelhead and Rainbow Trout populations and have shed light on genetic factors affecting at sea mortality and age of freshwater return in Atlantic salmon (Hecht *et al.* 2013, Hale *et al.* 2013, Johnston *et al.* 2014, Bourret *et al.* 2014). GWAS have also been important in identifying disease resistant genotypes to use for marker assisted selection to

improve survivorship in salmon aquaculture (Campbell *et al.* 2014, Liu *et al.* 2015, Correa *et al.* 2015). There has also been a great deal of interest in finding genetic association with growth and fillet quality in aquaculture (Gonzalez-Pena *et al.* 2016, Gutierrez *et al.* 2015, Sodeland *et al.* 2013). A few studies have even used GWAS to identify genes association with tolerance to abiotic stressors in salmonids including Narum *et al.* (2013) who used GWAS to identify SNPs associated with tolerance to thermal stress.

GWAS may serve as a strong conservation tool for identifying individuals that are resilient in changing environments. For example, these studies may be helpful for identifying genes in Rainbow Trout that convey tolerance to high pH in the alkaline lakes of British Columbia. For over 20 years, fisheries biologists have been investigating tolerance and stocking protocols to improve survivorship using various strains of Rainbow Trout that are part of the BC stocking program (Yesaki and Tsumura 1991, Toth and Tsumura 1992, 1993, Godin *et al.* 1994, Mathias *et al*. 1995, Thompson *et al*. 2015). Early work revealed that fish from Stump Lake, an alkaline lake of average pH 9.2, have greater survivorship than other non-alkaline strains in long-term lake survivorship studies (Mathias *et al.* 1995). Since that study the pH has cycled lower within Stump Lake and diploid Pennask and Blackwater fish have been stocked. This has likely resulted in high genetic diversity amongst this population with only some individuals with ancestors that were exposed to high pH having adaptations to tolerate high pH. A GWAS has the power to resolve genetic diversity within this mixed population for traits associated with survival in high pH. Any genes identified through

such a GWAS could then be selected for using marker-selected breeding to create a new high pH resistant strain.

In environments with fluctuating pH, having genes associated with phenotypic plasticity would likely improve survival (Lande 2009). Early work (Yesaki and Tsumura 1991, Toth and Tsumura 1992, Murray and Ziebell 1984) has suggested that even short periods of acclimation to moderately high pH result in improved high pH tolerance, but the genetic basis for this phenotypic plasticity is unknown. In this study I compare the results of GWAS studies addressing high pH tolerance performed on fish acclimated for 1 month to moderately high pH (8.8) to fish maintained at near neutral pH (7.2) until high pH (9.5) exposure. While this comparison does not allow the identification of a genetic basis for phenotypic plasticity, it allows me to determine whether the genes associated with high pH tolerance are similar or different under these two acclimation conditions.

The objectives of this thesis chapter are to use GWAS to 1) identify differences among Stump Lake individuals exhibiting different levels of tolerance to high pH; and 2) compare the significant markers between surviving control and acclimated individuals.

3.2 Materials and Methods

3.2.1 Experimental Design

I genotyped a total of 95 fish in this experiment from fin clips taken at the LOE_{pH} including: 1) the 20 parental fish collected from Stump Lake (Meritt BC) that were used to generate the fish phenotyped in this experiment, 2) 47 control fish which were reared under pH 7.2 hatchery conditions (from two independent experiments), and 3) 28 fish acclimated to pH 8.8 for 1 month prior to testing.

All of the acclimated fish and 24 control fish were collected from the experiment that was outlined in detail in the second chapter of this thesis; control and pH 8.8 acclimated fish were assayed for pH 9.5 tolerance with Stump Lake fish which had been reared at pH 8.8 from fertilization. All of these fish were reared under near neutral pH (7.2) in the hatchery up until acclimation or up until exposure to pH 9.5. Acclimated fish were transferred into separate culture tanks and brought up to pH 8.8 over one week and then held for one month at pH 8.8 prior to high pH (9.5) exposure.

Due to difficulties extracting high quality DNA from some of these samples I also used 23 control fish from a concurrent experiment. These fish were derived from a second experiment where control fish were exposed to high pH in a tank with other FFSBC strains. The fish in this experiment were reared in the same culture tanks as the fish used in the experiments from chapter two. These 23 samples were selected from this experiment in an alternating manner based on LOE_{pH}, to cover the distribution of this phenotype without biasing the results.

In both experiments, time to loss of equilibrium during exposure to pH 9.5 (LOE_{pH}) was recorded as a proxy for tolerance to high pH; each fish had its treatment, weight and length recorded and fins sampled for later DNA extraction. The control fish from these separate experiments did not statistically differ from one another in LOE_{pH}; however, I decided to analyze the control and acclimated sample separately due to differences in selection protocol and sample size. A summary of the phenotypes of the fish selected from both treatments can be found in the Appendix of this thesis.

3.2.2 DNA Preparation

Each fin clip was weighed and 10mg was used for DNA extraction. DNA extraction was completed using a Qiagen DNeasy Kit with replacement of vortexing steps with gentle inversions to prevent DNA shearing, extending the proteinase K digestion step overnight to ensure full digestion, and the addition of 5 uL of RNAse Cocktail (Thermo Fisher Scientific) to each sample prior to extraction. An aliquot of 10% of the samples was digested with the restriction enzyme HindIII for 3 hours at 37°C. Undigested and digested samples were visualized on an agarose gel using ethidium bromide. The purpose of this step was to ensure that the isolated DNA was intact and capable of effective digestion. Undigested DNA was quantified using a Quant-it Pico Green kit with a fluorometer and were diluted to 100ng/uL prior to submission to the Genomic Diversity Facility at Cornell University.

3.2.3 Genetic and Statistical Analysis

Samples were processed at the Genomic Diversity Facility, Cornell University using a genotyping by sequencing (GBS) approach as described in Elshire *et al.* (2011). Briefly, this procedure involved using HindIII restriction enzyme to cut DNA in each sample and then the ligation of an individual barcode to one end of the fragment and a common adaptor to the other before pooling all the samples. These samples were then amplified through PCR with primers that were complimentary to the adapters. These primers contained a region at the end opposite of DNA synthesis which was

complimentary to the oligonucleotides attached to surface of the flow cell. The samples were then sequenced on an Illumina HiSeq 2500 next generation sequencer (NGS). NGS uses reversible terminator bases with fluorophores attached to image DNA synthesis across all fragment clusters in real time (method described in Bentley *et al.* 2008).

Raw sequencing data were analyzed using the TASSEL- GBS "discovery" pipeline outlined in Glaubitz *et al.* (2014). Overall this approach takes raw sequencing data from NGS and filters out "good barcoded reads" (64 bp, with no out of phase "N" reads) and then sorts these reads into unique sequence "tags", which can then be mapped to a reference genome. For my study data was filtered so that each tag had at least 3 reads. SNPs were called based on the Rainbow Trout genome assembly available at https://www.genoscope.cns.fr/trout/ (Berthelot *et al*. 2014). Samples are also sorted based on barcode to give the genotype of individuals.

As a quality control measure we removed individuals with >60% missing data, which resulted in the removal of one individual in each treatment. Across the remaining individuals SNP sites with >80% missing data, SNPs with >2 alleles and sites with minor allele frequencies less than 2% were removed. Due to a recent genome duplication event, Rainbow Trout have many genetic paralogs across their genomes. Half of these paralogs have not shown appreciable pseudogenization and therefore persist as almost identical duplicate copies (Berthelot *et al.* 2014) and therefore can lead to errors in site mapping during the GBS pipeline resulting in false SNP calling. McKinney *et al.* (2016) developed a protocol for filtering out these errors using expected heterozygosity and deviations from expected uni-locus allele frequencies, analyzed using HDplots. The HDplot values we retained were $-7 \leq D \leq 7$ and $H \leq 0.6$ based on findings in Chinook

Salmon (*Onchorynchus tshawyscha*) which is a close relative of the Rainbow Trout (McKinney *et al.* 2016).

The resulting data were then uploaded into TASSEL (ver.5.2.35) with a trait file which contained weight and LOE_{pH} information for each fish. I ran a general linear model (GLM) which included a principle components analysis (using 5 components), DNA sequences and phenotypic data and a mixed linear model (MLM) which included the latter plus a kinship matrix. Each model was also analyzed for its fit to the data using a qqplot. The qq-plots in both treatments revealed that the GLM was a better fit than an MLM for these data sets. Thus the GLM results are presented here; MLM Manhattan plots and all qqplots are available in the appendix of this thesis. The raw p-values produced from these models were analyzed using FDR tests (Benjamini & Hochberg 1995) to correct for multiple testing at the genomic and chromosomal level using R (ver. 3.3.2). Due to the exploratory nature of this study, SNPs with raw P-values greater than 10^{-3} were also investigated. The identity of these SNPs was determined by using BLAST to compare the sequence of genes close to (within 100kbp) or encompassing that SNP against known genes in other species of fish (most commonly the Atlantic Salmon, *Salmo salar*).

3.3 Results

After the data were filtered, 37347 SNPs remained in the control group and 34864 SNPs remained in the acclimated group. At the genomic and chromosomal level there were no significant FDR corrected P-values in either control or acclimated fish. The results from the GLMs showed that most SNPs with raw $P<0.001$ were located on genetic

scaffolds in the current assembly of the Rainbow Trout genome (refer to Berthelot *et al*. 2014) (Figures 3.1 and 3.2). All of the SNPs that exceeded the raw (not FDR corrected) P-value threshold differed between the acclimated fish and the control fish (Tables 3.1 and 3.2). Genome BLAST results suggest within control fish one SNP was located close to the anion exchange protein gene (GSONMG00052646001) which is involved in HCO_3^- transport and 4 SNPs were in or close to genes involved in muscle function. In both treatments many of the SNPs were located within or close to genes which play roles in neuronal signaling and DNA expression regulation.

3.4 Discussion

Despite the fact that there was high inter-individual variation in high pH tolerance across fish from Stump Lake, no SNP that I detected in my GWAS was significantly associated with this variation. This was true under both control and acclimated treatments.

GWAS studies have well-documented limitations including multiple testing burden, poor detection of complex traits influenced by multiple loci, and large sample size requirements (Pe're *et al.* 2008, Korte and Farlow 2013). Each of these limitations may have hampered my ability to detect significant associations between genotype and phenotype. First, with respect to the multiple testing burden, FDR correction increases in stringency as the number of tests performed increases. Thus, in a GWAS study, the greater the number of markers examined, the more challenging it is to detect associations. The Stump Lake population of Rainbow Trout appears to have many more SNPs (~35000) than Rainbow Trout used in other GWAS such as Hale *et al.* (2013) ~7000

SNPs, Campbell *et al.* (2014) ~ 4500 SNPs, Hecht *et al.* (2014) ~3500 SNPs, and Liu *et al.* (2015) ~ 7800 SNPs. This made the power to detect genetic association low in this population. The second major limitation of GWAS is that these studies are poor at identifying SNPs which individually have small phenotypic effects, but may interact with many other SNPs to have large phenotypic effects (Korte and Farlow 2013). As discussed earlier in this thesis, the physiological response to high pH exposure employs many biochemical mechanisms in various parts of the body, which suggests that there may be many genes associated with this trait (i.e. polygenic influences). Finally, GWAS in other salmonids have used hundreds of individuals whereas in my study I only used 75 fish across both treatments. This in addition to high numbers of SNPs reduced the statistical power of my GWAS to detect associations. An additional issue to consider is the distribution of phenotypes within the population. In my analysis, rare allele variants were filtered out if they were below a frequency of 0.02. Thus, it is possible that filtering removed some of the underlying genetic association of high pH tolerance.

In order to explore some of the SNPs that may be associated with high pH tolerance, I set a threshold of raw $P < 0.001$ for analysis. Only one SNP was close to a gene coding for a protein directly involved in acid-base regulation, this was the HCO_3^- exchanger and was only seen in the control group (see Table 3.1). This protein is expressed in tissues throughout the body and at the gill is integral in maintaining blood homeostasis under high pH exposure. High environmental pH incurs a respiratory alkalosis whereby CO_2 passively moves out of the blood across the gill resulting in increased plasma pH (Johansen *et al*. 1975). The negative charge of the alkaline environment further increases plasma pH as it sets up an electrochemical gradient which

favors the entry of anions like HCO_3^- into the blood and the loss of H^+ from the blood (Wilkie and Wood 1996). In freshwater teleost acid and base movement is paired with Na^+ and Cl^- movement respectively, therefore highly alkaline environments result in ionic perturbation in the blood (Wilkie and Wood 1991). The HCO_3^-/Cl^- exchanger plays a role in excreting HCO_3^- from the blood and regaining Cl^- therefore bringing pH of the blood within physiological parameters and regulating blood ions throughout high pH exposure (Wilkie and Wood 1996). Perhaps this SNP results increased efficiency or increased expression of this transporter among tolerant individuals, or is linked to other genetic variation that has this effect.

The control group also had genes involved in muscle function which may contribute to maintaining blood homeostasis during high pH exposure. These genes consisted of Calpain-3, myotonin-protein kinase like, and IGFN-1. Like the gills, muscle also plays an important role in regulating blood pH and maintaining sub-lethal concentrations of ammonia in the blood (Wilkie and Wood 1991, Thompson *et al*. 2015). Within hours of exposure to high pH, the white muscle begins producing lactate to incur a metabolic acidosis to counter the respiratory alkalosis created by the alkaline environment (Wilkie 1994). This in concert with acid-base exchange at the gills results in a recovery in blood pH (Wilkie and Wood 1991). White muscle also appears to play a role in ammonia sequestration (Wilkie *et al*. 1996, Thompson *et al.* 2015). During environmental high pH exposure, passive ammonia excretion becomes unfavorable, and over a few hours of exposure ammonia begins building up in the plasma, which can eventually become lethal (Yesaki and Iwama 1992, Wilkie and Wood 1991, Wilkie *et al.*

1996, Thompson *et al*. 2015, Wilkie and Wood 1996). By storing ammonia in white muscle, fish can protect vital organs.

The reason these genes involved with acid-base regulation and muscle function are only associated with high pH tolerance in the control fish may be that acclimation facilitates improvements in these mechanisms by which they are no longer limiting to high pH tolerance across individuals. For example, during chronic exposure to high pH, Rainbow Trout remodel their gills to increase the fractional surface area covered by chloride cells, the site of HCO_3^-/Cl^- exchangers (Wilkie and Wood 1994, Laurent *et al*. 2000). Perhaps at high density, having variation in efficiency or expression levels of these exchangers does not further the fishes' ability to excrete HCO_3^-. This increase in chloride cells is also thought in large part to be a less energetically costly mechanism of decreasing blood pH than metabolic acidosis at the muscle (Wilkie and Wood 1996). Remodeling of the gills may reduce the role individual HCO_3^-/Cl^- exchangers and muscle acidosis play in tolerance, therefore resulting in insignificant association of these genes in within the acclimated group.

There were no SNPs associated with high pH tolerance in common between the control fish and acclimated fish (see Table 3.1 and 3.2). Despite this, in both treatments SNPs associated with high pH tolerance were close to or within genes coding for proteins involved in neural signaling and DNA expression. This suggests that even after acclimation to moderately high pH, high pH tolerance is still limited by having particular SNPs of these types of genes. It also suggests that the physiological stress incurred at pH 9.5 may be different than pH 8.8. At environmental pH of 8.8 passive ammonia efflux is still favorable across the gill, whereas 9.5 is the pKa of ammonia and therefore the NH_3 +

$H^+ \leftrightarrow NH_4^+$ reaction in the aquatic environment becomes left shifted and the chemical gradient for NH_3 movement across the gill becomes unfavorable (Wilkie and Wood 1991). As shown in chapter 2, even with acclimation, only 50% of the fish are able to tolerate high pH up to 4 days, so perhaps handling ammonia build up is limiting.

SNPs close to or within genes involved in the maintenance of the nervous system during high pH exposure appear to differ between low- and high-tolerant fish in each treatment. Ammonia build up in the blood is toxic because it overexcites neurons in the central nervous system (Wilkie *et al*. 2011, Randall and Tsui 2002). Defending the brain from ammonia by binding NH_3 to glutamate to produce glutamine (via glutamine synthetase) has already been demonstrated in Rainbow Trout under high pH exposure (Thompson *et al.* 2015). My results do not show an association of glutamine synthetase to high pH tolerance, but instead an association of intersection-1, neuroligin-2, and SEC13 genes in control fish and with neuroligin-1 and ERC-1 genes in the acclimated group. All of these genes are involved in neurotransmitter release at the synapse. Ammonia is thought to over activate NMDA receptors on neurons in the brain, leading to over excitation and excessive build up of Ca^{2+} in the post synaptic neurons which results in cell death (Randall and Tsui 2002, Wilkie *et al*. 2011). Perhaps individuals with higher pH tolerance have alleles of these genes involved in neurotransmitter release which reduce these toxic effects at the synapse.

Genes involved in regulation of DNA expression appear to influence high pH tolerance in both groups. This regulation was mainly mediated by DNA packing genes such as HMG-1, DNA topoisomerase-1, histone-lysine N-methyltransferase-2B in control fish and SMARCAL-1 and chromodomain-helicase-DNA-bind protein-6 in the

acclimated fish. It may be that tolerant fish in both treatments up-regulate expression of genes involved in combatting high pH which were not identified in my GWAS. A concurrent study is underway to address gene expression using RNA-Seq technology.

In conclusion, my GWAS failed to make any statistically significant genetic associations but it highlighted genes which may each have small phenotypic effects that when combined underlie high pH tolerance. Genes involved in acid base regulation, ion regulation, ammonia handling, and DNA transcription regulation are all associated with high pH tolerance among non-acclimated fish. Whereas only genes associated with ammonia handling and DNA transcription regulation were associated in acclimated fish. This suggests that acclimation to moderately high pH results in fish modifying their physiology to maintain acid-base and ionic composition when faced with high pH exposure, but does not prepare them for the issue of ammonia excretion faced at higher pH. Based on the results of this GWAS it appears that the genetic complexity of high pH tolerance may not make it a good candidate for marker- assisted selection.

3.5 Figures

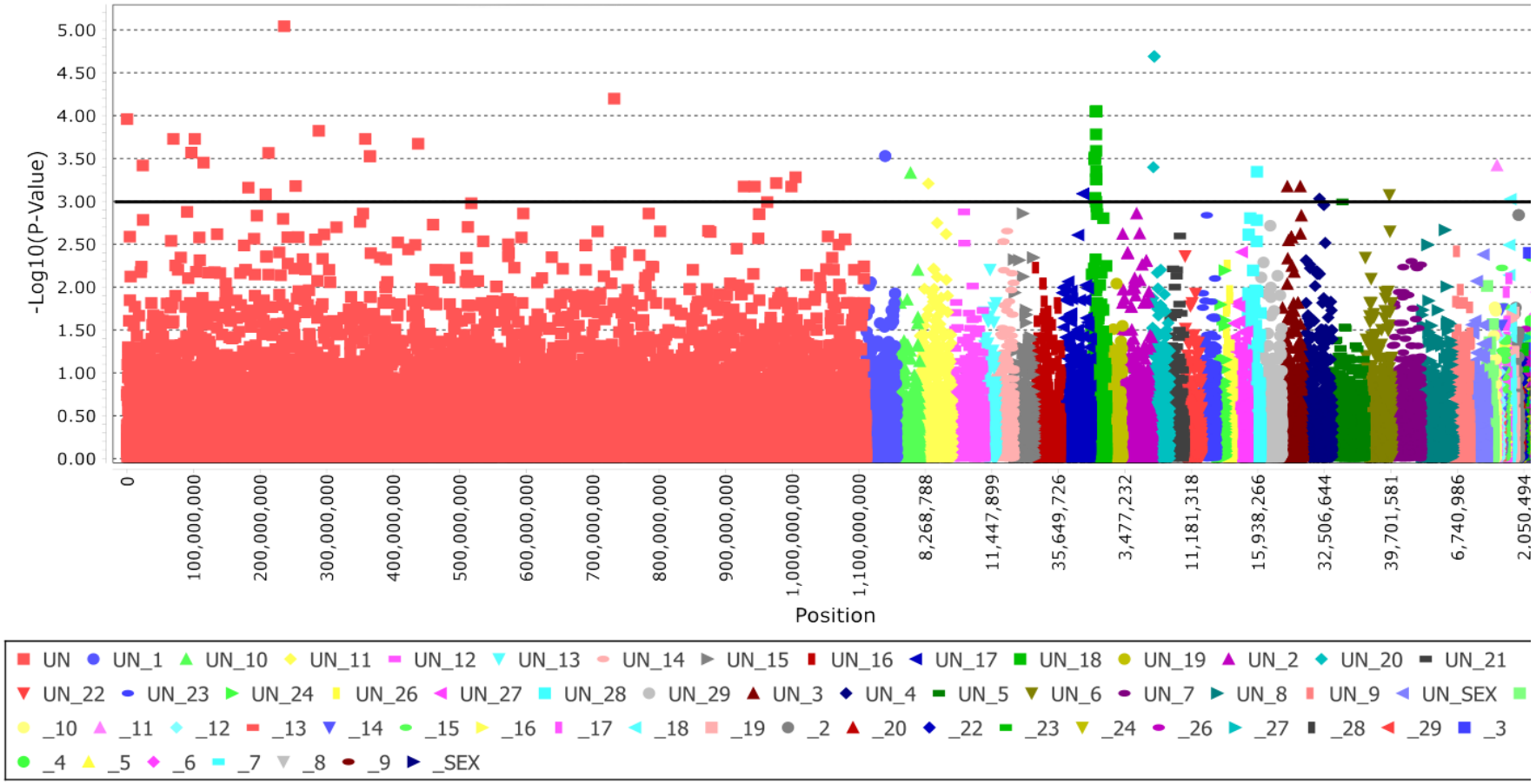

Figure 3.1 Plot of non-FDR corrected P-values for SNPs associated with tolerance to high pH (9.5) in fish reared at neutral pH (7.2) using a GLM in TASSEL (ver.5.2.35). The black solid horizontal line represents P=0.001; 37347 SNPs; n= 46.

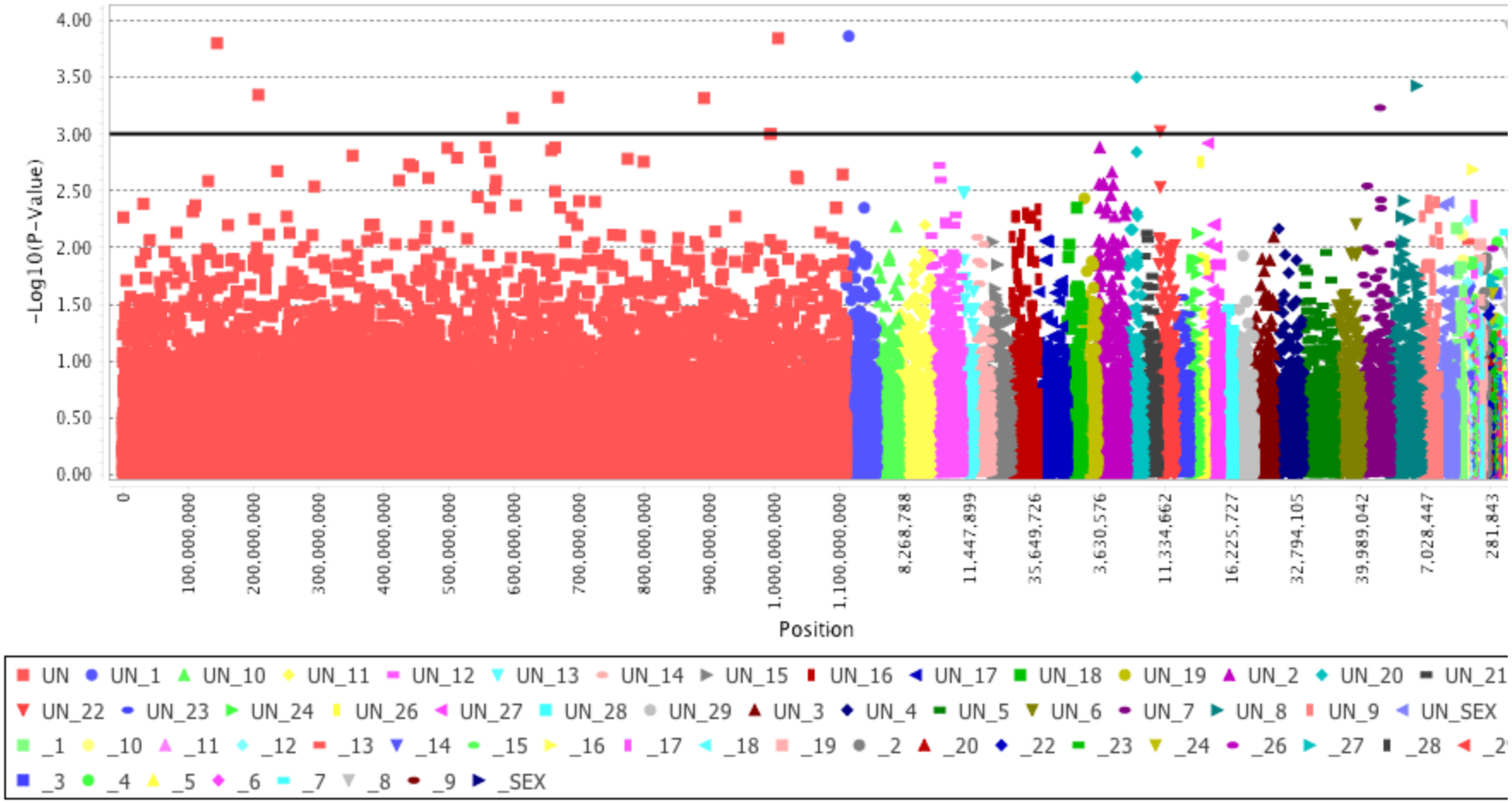

Figure 3.2 Plot of non-FDR corrected P-values for SNPs associated with tolerance to high pH (9.5) in fish reared at neutral pH (7.2) and then acclimated for 1 month at moderately high pH (8.8) prior to exposure using a GLM in TASSEL (ver.5.2.35). The black solid horizontal line represents P=0.001; 34864 SNPs; n= 27.

Position	Raw p-value	Gene	Distance to SNP(bp)	BLAST Sequence Match	Description
32928	1.10E-04	GSONMG00054473001	12997	*Salmo salar* Calpain-3 (can3)	Associated with titin in muscle
23654720	3.82E-04	GSONMG00050372001	96303	*Salmo salar* myotonin-protein kinase-like	Involved with calcium channels
69623141	1.87E-04	GSONMG00044331001	0	*Onchorynchus mykiss* HMG-1 gene	Involved in transcription and DNA organization
96653287	2.70E-04	GSONMG00045515001	0	*Salmo salar* DNA topoisomerase I mitochondrial	Breaks and rejoins DNA to releavee tension during transcription and replication
101837666	1.87E-04	GSONMG00041506001	26183	*Salmo salar* sphingomyelin synthase 2	Involved in making sphingomyelin, an important component of the golgi and cell membrane
114974358	3.54E-04	GSONMG00007226001	39377	*Salmo salar* par-3 family cell polarity regulator beta	May play a role in symmetric cell division
182422428	6.92E-04	GSONMG00044284001		*Salmo salar* Bardet-Biedl syndrome 4 protein homolog	Multiple functions
208446950	8.28E-04	GSONMG00044703001	62545	*Salmo salar* activating transcription factor 7-interacting protein 1	Multifunctional nuclear protein that associates with heterochromatin
212675461	2.73E-04	GSONMG00049684001	47851	*Salmo salar* methyltransferase	Methylates DNA
236117872	9.08E-06	GSONMG00029305001		*Salmo salar* intersectin-1	Vesicle transport perhaps in synaptic vesicle recycling
253448577	6.63E-04	GSONMG00054924001	67317	*Salmo salar* glycosylphosphatidylinositol anchor protein 2	May be involved in cell-cell interactions.
288217611	1.51E-04	GSONMG00035893001	26808	*Salmo salar* Golgi apparatus protein 1	Binds fibroblast growth factor to mediate neutrophil binding
358029318	1.87E-04	GSONMG00002462001	18294	*Salmo salar* A disintegrin and metalloproteinase with thrombospondin motifs 5	Cleaves aggrecan, a cartilage proteoglycan, and may be involved in its turnover.
365067727	2.98E-04	GSONMG00044635001	86903	*Salmo salar* neuroligin-2	May be involved in the formation and remodeling of central nervous
365067750	2.98E-04	SEE ABOVE	86926		
437669157	2.13E-04	GSONMG00034197001	8653	*Salmo salar* SEC13 homolog, nuclear pore and COPII coat complex component	Involved in vesicle biogenesis
732305648	6.35E-05	GSONMG00058390001	5872	*Salmo salar* calcium-binding mitochondrial carrier protein	
927266554	6.72E-04	GSONMG00057566001	30724	*Salmo salar* 2-oxoglutarate dehydrogenase, mitochondrial	This complex catalyzes the overall conversion of 2-oxoglutarate (alpha-ketoglutarate) to succinyl-CoA and CO(2)
943439907	6.72E-04	GSONMG00019088001	0	*Salmo salar* taste receptor type 1	G-protein coupled receptor involved in taste responses
975969470	6.13E-04	GSONMG00013975001			
998773630	6.72E-04	GSONMG00052646001	14026	*Salmo salar* anion exchange protein	Bicarbonate (HCO3-) transport mechanisms are the principal regulators of pH in animal cells.
1004919609	5.24E-04	GSONMG00014730001	30468	*Salmo salar* somatostatin receptor type 5	Receptor for somatostatin mediated by G proteins which inhibit adenylyl cyclase.
28975501	2.97E-04	GSONMG00010296001	78594	*Salmo salar* nuclear receptor coactivator 5	gene is a nuclear receptor coactivator that interacts with nuclear hormone receptors to enhance their transcriptional
28975502	2.97E-04	SEE ABOVE	78595		
20552008	4.67E-04	GSONMG00074639001	32809	*Salmo salar* ubiquitin carboxyl-terminal hydrolase 25	This enzyme is a thiol protease that hydrolyzes a peptide bond at the C-terminal glycine of ubiquitin. This gene is specifically expressed in the neurons and in cells of the diffuse neuroendocrine system.
12899033	6.20E-04	GSONMG00009056001	0	*Salmo salar* cadherin-6	Cadherins are membrane glycoproteins that mediate homophilic cell-cell adhesion and play critical roles in cell differentiation and morphogenesis.
30957555	8.15E-04	GSONMG00073110001	0	*Salmo salar* IGFN-1	paralog of TTN: This gene encodes a large abundant protein of striated muscle.
30957607	8.15E-04	NOT CLOSE TO ANY GENE			
5886230	3.06E-04	GSONMG00013694001	5237	*Salmo salar* ephrin type-A receptor 7	mediates developmental events, particularly in the nervous system.
5886244	3.23E-04	SEE ABOVE	5251		
5886246	3.23E-04	SEE ABOVE	5253		

Position	Raw p-value	Gene	Distance to SNP(bp)	BLAST Sequence Match	Description
6472845	9.18E-04	GSONMG00069689001	0	*Salmo salar* histone-lysine N-methyltransferase 2B	The protein encoded by this gene is a protein-lysine N-methyltransferase that can monomethylate Lys-20 of histone H4 to effect transcriptional repression of some genes.
6472846	9.18E-04	SEE ABOVE	0		
7854781	1.65E-04	GSONMG00046007001	4334	*Oncorhynchus mykiss* 40S ribosomal protein	
8001401	8.97E-05	GSONMG00080373001	30635	*Salmo salar* homocysteine-responsive endoplasmic reticulum-resident ubiquitin-like domain member 2 protein	May play a role in both unfolded protein responce and endoplasmic reticulum associated protein degradation
8001413	8.97E-05	SEE ABOVE	30623		
8001414	8.97E-05	SEE ABOVE	30622		
8001445	8.97E-05	SEE ABOVE	30591		
8001466	2.59E-04	SEE ABOVE	30570		
8153573	4.45E-04	GSONMG00080369001	0	*Oncorhynchus mykiss* eomesodermin-like protein a-2	encoded protein is a transcription factor which is crucial for embryonic development of mesoderm and the central nervous system in vertebrates.
8165619	5.54E-04	GSONMG00080369001	10690		
8165671	5.54E-04	SEE ABOVE	10742		
1638302	4.00E-04	NOT CLOSE TO ANY GENE			
2992893	2.04E-05	GSONMG00075031001	12425	*Salmo salar* serologically defined colon cancer antigen 8	May play a role in centrosome organization.
2992894	2.04E-05	SEE ABOVE			
2992944	2.04E-05	SEE ABOVE			
14550833	4.52E-04	GSONMG00042010001	0	*Oncorhynchus mykiss* caspase 8	Sequential activation of caspases plays a central role in the execution-phase of cell apoptosis.
7950518	6.72E-04	GSONMG00070236001	0	*Salmo salar* striated muscle preferentially expressed protein kinase-like	Isoform 3 may have a role in regulating the growth and differentiation of arterial smooth muscle cells
27453507	6.72E-04	NOT CLOSE TO ANY GEN-			
25065326	9.32E-04	GSONMG00066903001	53401	*Salmo salar* adhesion G protein-coupled receptor L2	This gene encodes a member of the G protein-coupled receptor family and regulates brain cortical patterning.
37343372	8.44E-04	NOT CLOSE TO ANY GENE			
1378485	3.82E-04	GSONMG00015115001	3512	*Salmo salar* insulin receptor substrate 2	This gene encodes the insulin receptor substrate 2, a cytoplasmic signaling molecule that mediates effects of insulin
2322115	9.45E-04	GSONMG00000890001	0	*Salmo salar* aryl hydrocarbon receptor 2 delta	Encodes a transcription factor involved in the regulation of biological responses to planar aromatic hydrocarbons.

Table 3.1 SNPs associated with high pH (9.5) tolerance for Stump Lake Rainbow Trout reared at pH 7.2, raw $P<10^{-3}$. SNPs close to or near genes involved in neural pathways, muscle function, DNA transcription and acid-base balance are highlighted in green, purple, orange and blue respectively.

Chromosome	Position	Raw p-value	Gene	Distance to SNP(bp)	BLAST Sequence Match	Description
UN	144683927	0.00015818	GSONMG00007743001	2367	*Salmo salar* transmembrane protein 97	plays a role in controlling cellular cholesterol
UN	207981123	0.00045118	GSONMG00001364001	13241	*Salmo salar* SMARCAL1	helicase that binds selectively to fork DNA and catalyzes the rewinding of the stably unwound DNA.
UN	598902034	0.00071919	GSONMG00060473001	4272	*Salmo salar* soluble lamin-associated protein of 75 kDa-like	found on the nuclear lamina, it's function is unknown.
UN	667786673	0.00047336	GSONMG00000905001	51421	*Salmo salar* chromodomain-helicase-DNA-binding protein 6	the encoded protein is thought to be a core member of one or more of these chromatin remodeling complexes
UN	892070616	0.00048195	GSONMG00039063001	0	Salmo salar protein NATD-1	unknown
UN	993844348	0.00099709	GSONMG00005086001	44834	*Salmo salar* neuroligin-1	may be involved in the formation and remodeling of central nervous system synapses
UN	993844371	0.00099709	SEE ABOVE	44857		
UN	1006003994	0.00014318	GSONMG00033526001	17175	*Salmo salar* ERC-1	May be involved in the organization of the cytomatrix at the nerve terminals active zone (CAZ) which regulates neurotransmitter release
UN_1	3478606	0.00013736	GSONMG00077752001	0	*Salmo salar* glycophorin-C-like	minor sialoglycoprotein in human erythrocyte membranes
UN_20	14972806	0.00031627	GSONMG00044001001	7384	*Salmo salar* PKMYT-1	Mediates phosphorylation of CDK1 predominantly on 'Thr-14'. Also involved in Golgi fragmentation.
UN_22	3433557	0.00094454	NOT CLOSE TO ANY GENE			
UN_7	27856739	0.00058724	NOT CLOSE TO ANY GENE			
UN_8	40602921	0.00037483	GSONMG00071170001	0	*Salmo salar* mitogen-activated protein kinase kinase kinase 5	Mitogen-activated protein kinase (MAPK) signaling cascades include MAPK or extracellular signal-regulated kinase (ERK), MAPK kinase (MKK or MEK), and MAPK
_8	5579743	0.00011316	NOT CLOSE TO ANY GENE			

Table 3.2 SNPs associated with high pH (9.5) tolerance for Stump Lake Rainbow Trout reared at pH 7.2 and then acclimated to pH 8.8 for 1 month, raw $P<10^{-3}$. SNPs close to or near genes involved in neural pathways, and DNA transcription are highlighted in green and orange respectively.

[4] General Discussion

4.1 Summary of Thesis Results

For the first objective of this thesis I characterized high pH tolerance of progeny of Rainbow Trout from Stump Lake, an alkaline lake. Approximately 90% of the fish reared under hatchery conditions at near neutral pH (~7.2) and then exposed to high pH (9.5) lost equilibrium within 3 days of exposure. These fish do not appear to have superior tolerance compared to values reported for fish from non-alkaline lakes. These results were in disagreement with field survival studies comparing Stump Lake fish to non-alkaline strains.

My second objective was to compare the effects acclimation and rearing fish under moderately high pH has on high pH tolerance. Both acclimation and rearing resulted in significant improvements in tolerance to high pH, with ~50% and ~90% of fish remaining at the end of a 3 day exposure to pH 9.5, respectively. This improvement was in agreement with the results of studies which showed that gradually exposing fish to high pH results in higher survivorship than direct exposure. This was the first study to look at long-term (1 month) acclimation to moderately high pH and effects of rearing on this trait. The physiological basis for these improvements is unknown.

My third objective was to perform a GWAS to assess the genetic basis of high pH tolerance among non-acclimated fish and my fourth objective was to compare these results with fish acclimated to moderately high pH. Across both treatments I did not find any statistically significant associations of genetic markers with high pH tolerance. This may have been because of the high numbers of SNPs I was comparing in my general linear models, the genetic complexity of high pH tolerance, or that my sample size was

not large enough. By applying an exploratory raw P-value threshold, I identified SNPs close to or within genes encoding proteins which regulate acid-base and ion levels in the blood within the non-acclimated fish and proteins which may be involved in protecting the central nervous system from ammonia toxicity within both treatments. These differences suggest that acclimation to moderately high pH improves acid-base and ionic regulation; however it does not prepare the fish for unfavorable ammonia excretion at higher pH.

4.2 Future Directions and Applications

As a whole, the progeny from Stump Lake do not appear to have adaptations for survival in high pH lakes, based on the results presented here. Individuals from this population that remained until the end of the high pH trial did not appear to have genetic markers that significantly differed from the less tolerant individuals. A complex trait such as high pH tolerance likely has too many markers associated with it to use for marker-assisted breeding. My GWAS did not have a large enough sample size to confirm this theory. Follow up GWAS on this trait should include a larger sample size. Based on my results I would recommend that the FFSBC does not develop this population as a strain for stocking in high pH lakes until further information is available.

Rearing and acclimating fish under moderately high pH has promising results for improving survivorship in high pH lakes. The only limitation of this approach is maintaining an alkaline water supply at a production level over the long term requires infrastructure as well as a higher degree of maintenance than neutral systems. Investigation into maintaining high pH within the recirculating aquaculture systems the

FFSBC uses in production will need to be conducted. It is therefore my suggestion that more work be done to look at how acclimation duration, and increasing the pH of the exposure effect tolerance under high pH.

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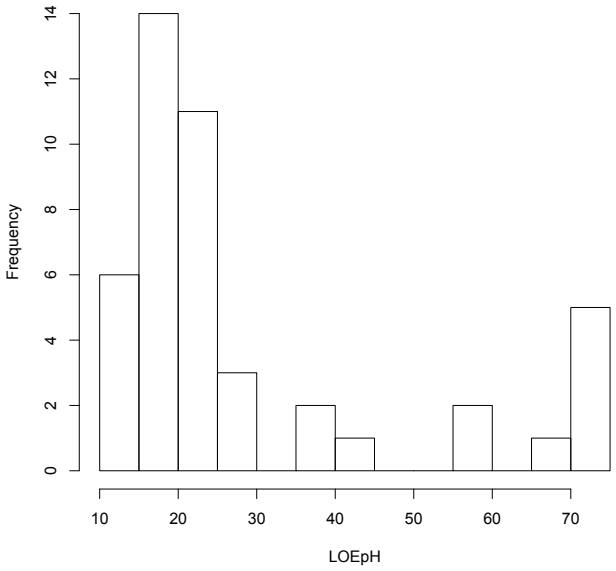
Appendix

A



B

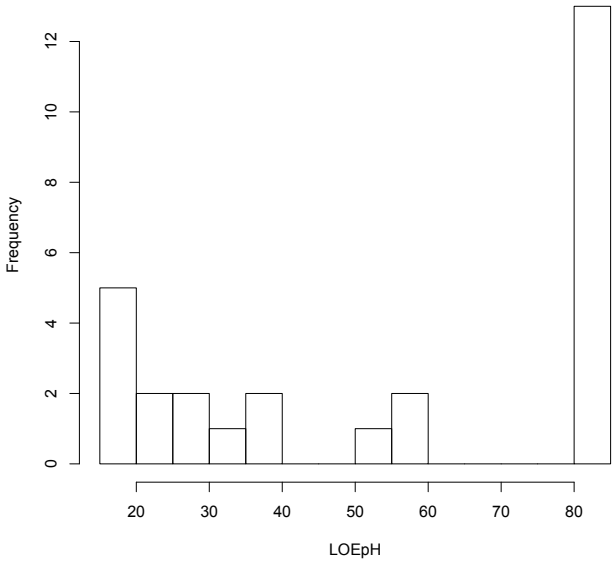


Figure A.1. Distribution of LOE_{pH} in Stump Lake Rainbow trout selected for the GWAS study. A) Fish reared under near neutral hatchery conditions and then exposed to pH 9.5 n=47 B) Fish reared under near neutral hatchery conditions and then acclimated to moderately high pH (8.8) one month prior to exposure to pH 9.5. n=28

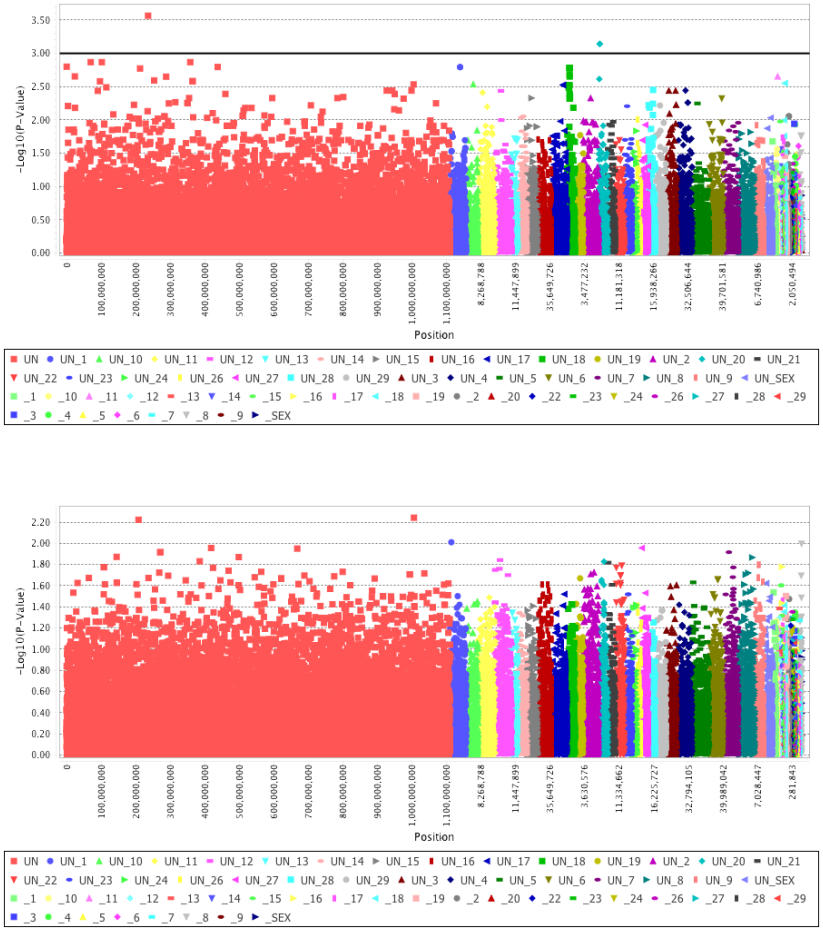


Figure A.2. Plot of non-FDR corrected P-values for SNPs associated with tolerance to high pH (9.5) using a mixed linear model (MLM) in TASSEL (ver.5.2.35) A) in fish reared at neutral pH (7.2) B) in fish reared at neutral pH (7.2) and then acclimated for 1 month at moderately high pH (8.8) prior to exposure to high pH (9.5). The black solid horizontal line represents P=0.001; 34864 SNPs; n= 27.

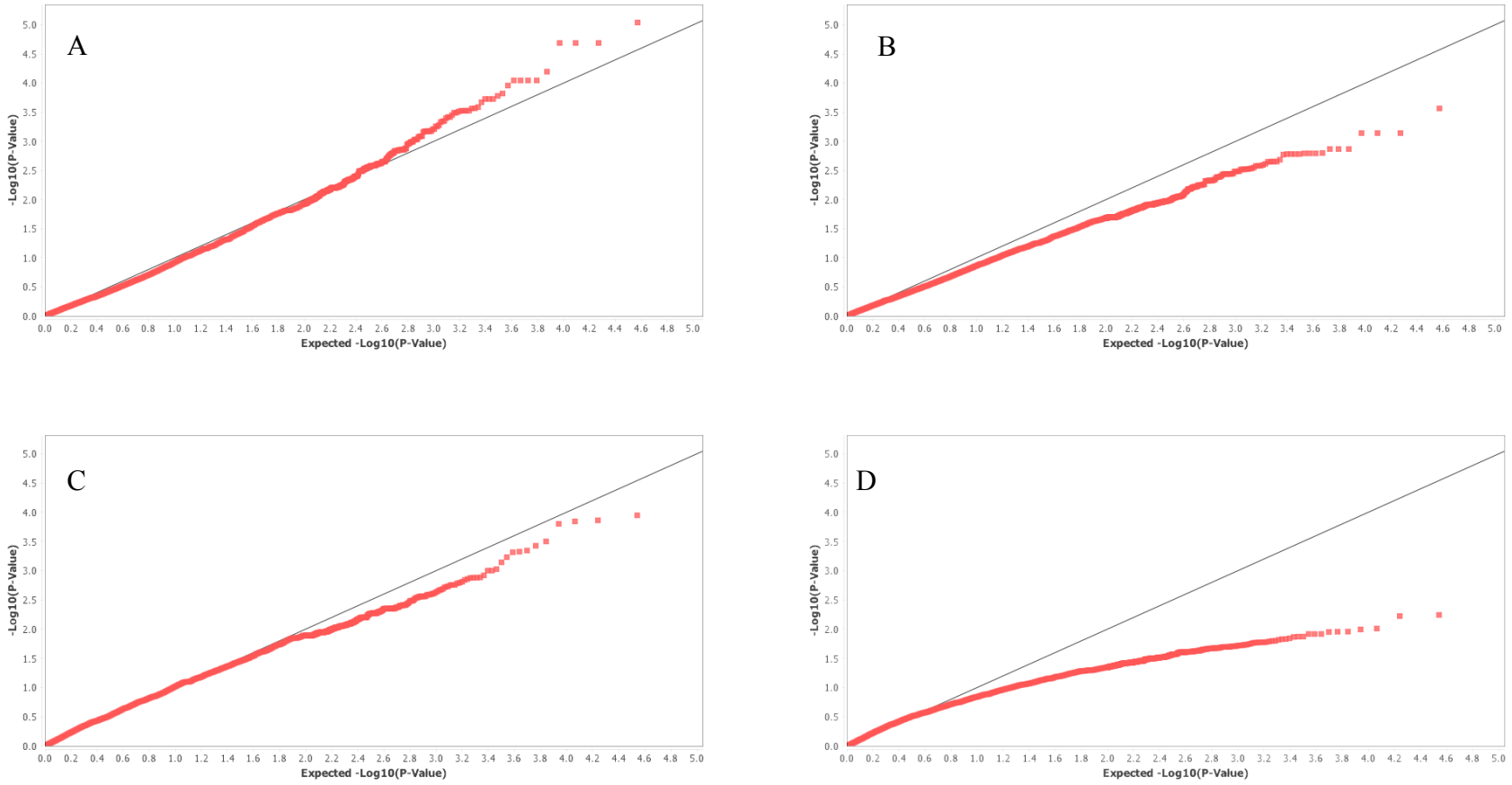


Figure A.3. Qqplots analyzing the fit of linear models analyzing the association of SNPS to high pH tolerance. The black line represents the 45-degree reference line deviations from this line suggest poor the fit of the linear model. a) Assesses the fit of a general linear model (GLM) and b) Assesses the fit of a mixed linear model (MLM) in fish held at neutral pH (7.2) up until high pH (9.5) exposure c) Assesses the fit of a general linear model (GLM) and d) Assesses the fit of a mixed linear model (MLM) in fish reared at neutral pH (7.2) and then acclimated for 1 month at moderately high pH (8.8) prior to exposure to high pH (9.5).