Freshwater Tolerance of Fourspine Stickleback

(*Apeltes quadracus*) Marine and Lake Populations

By
Alyssa Lynn Densmore

A Thesis Submitted to
Saint Mary's University, Halifax, Nova Scotia
in Partial Fulfillment of the Requirements for
the Degree of Bachelor of Science Honours (Biology)

May 2020, Halifax, Nova Scotia

Approved: Dr. Anne Dalziel
Supervisor

Approved: Dr. Timothy Frasier
Thesis Reader

Date: May 1st, 2020

Freshwater Tolerance of Fourspine Stickleback

(*Apeltes quadracus*) Marine and Lake Populations

By Alyssa L. Densmore

Abstract

Fishes have evolved freshwater tolerance multiple times, using different physiological mechanisms, and we have much to learn about how fish can adapt to freshwater. Sticklebacks in the family Gasterosteidae are becoming an important model system in which to study the evolution of freshwater tolerance. In this study I measured freshwater tolerance of a freshwater (lake) and a marine population of Fourspine Stickleback (*Apeltes quadracus*) to test overall tolerance in this species and determine if there is evidence of freshwater adaptation in the lake population. To assess tolerance, both populations acclimated to near isosmotic water (10 ppt) and were exposed to an acute freshwater (0 ppt) challenge and monitored over 48 days of freshwater acclimation. Survival, body water content, tissue ion content and standard metabolic rate were measured from fish from both populations during the study. We found that both the marine and lake populations were able to cope with freshwater transfer, as both had high survival rates and could maintain body-water homeostasis. My data suggests that ancestral Fourspine Stickleback that were landlocked at the end of the last ice age may have had higher freshwater tolerance than some other Gasterosteidae species, allowing them to easily colonize post-glacial lakes.

May 2020

Acknowledgements

I'd like to thank my amazing parents and brother for their enduring love and support throughout my undergraduate degree, I may not have survived the past five years without each of you. I would like to recognize the Dalziel-Weir Fish lab for the friendships and assistance that each member has provided me this past year. Specifically, I extend thanks to my roommate and fellow honours student Amanda Gove and Master's candidate Anna Bernhardsson for sparing her free time to assist in data collection. I would also like to express my deepest gratitude to my supervisor Dr. Anne Dalziel for going above and beyond her duties to assist me over the course of this thesis, allowing me to pursue my interests and find greater appreciation for the world of research. Most importantly, this project would not have been possible without my fish.

TABLE OF CONTENTS

LIST OF FIGURES

1. INTRODUCTION

1.1 How Animals Cope with Environmental Stressors

'Stress' is normally defined as the physiological response of an animal to an environmental factor which poses a threat to homeostasis (reviewed by Somero et al., 2017). New or unfamiliar abiotic and biotic factors can threaten an animal's well-being and act as stressors (Somero et al., 2017). These stressors must be accommodated by animals through beneficial phenotypic modifications if an organism is to survive (Somero et al., 2017). Stress can be combated by an individual through plastic responses within a lifetime (both acutely or with acclimation) or by populations through adaptation in response to persistent stressors over many generations leading to increased fitness (Piersma and Drent, 2003; Somero et al., 2017; Woods and Harrison, 2002).

Fluctuations in abiotic stressors can affect an individual animal's health. For instance, changes in salinity perturb ion and water homeostasis of aquatic species that exchange ions with their external environment. Salinity is characterized by the total concentration of dissociated inorganic salts into ions, including sodium (Na^+) and chloride (Cl^-) ions (Kültz et al. 2015; Moyes and Schulte, 2016). Animals maintain internal water and ion content though osmoregulatory systems (Moyes and Schulte, 2016). The integument and other epithelial tissues are key sites for water and ion transport; these tissues are equipped to counter excess ion and water gain or loss depending on the environment (Kültz et al. 2015; Moyes and Schulte, 2016).

Freshwater has a salinity of around 0 parts per thousand (ppt) and full-strength seawater has a salinity of approximately 30-35 ppt (Schultz and McCormick, 2013). Ionic components within water that contribute to salinity have a large influence on

electrochemical gradients between an animal and its environment. Animals placed in freshwater, a hypoosmotic environment, must fight against water influx and limit loss of ions to the surrounding environment (Moyes and Schulte, 2016). In saltwater, animals maintain osmotic balance through excreting excess ions to avoid internal accumulation of ions and fight to retain water (Moyes and Schulte, 2016). Changes associated with ion availability can modify biochemical processes and protein structure (Somero et al., 2017). In addition to directly altering cellular stability, water influx or efflux (in response to ion concentrations) can lead to cell swelling and rupture (respectively; Moyes and Schulte, 2016). Therefore, an inability to maintain iono-and osmo-regulatory homeostasis jeopardizes whole organism functioning and an inability to acclimate to changing salinity can lead to death.

1.2 How Teleost Fish Cope with Changes in Environmental Salinity

Many fish are stenohaline, or incapable of withstanding large salinity fluctuations, so are restricted to inhabiting either fresh or saltwater (Edwards and Marshall, 2013; Kültz, 2015). Indeed, only 3-5% of extant species are capable of living in both fresh and saltwater environments (McCormick et al., 2013). This small percentage of fish species are termed euryhaline and are able to thrive in varying salinities (Edwards and Marshall, 2013; Kültz, 2015).

As many fish species are incapable of altering osmoregulatory strategies, they are not able to transition between fresh and saltwater. Thus, water salinity often determines the distribution of fishes (McCormick et al., 2013; Kültz et al. 2015). In fish, osmotic and ionic regulation is a biological tug of war regardless of the external environment. In

freshwater, fish need to actively take up ions into the body against the concentration gradient and fight against the influx of water (Edwards and Marshall, 2013; Kültz, 2015). In seawater the opposite is true, fish must actively pass ions against the concentration gradient into their environment while tightly regulating internal water content. Ion and water regulation is normally accomplished in the gills, opercula, kidney and intestine through active transport mechanisms in adult fishes (Edwards and Marshall, 2013; Kültz, 2015).

The gills of a fish are the key site for both water and ion regulation. Ionocytes, or mitochondria rich cells, are specialist cells found in the epithelium of fish gills that house a number of ion transporters and are responsible for both ion uptake and secretion (Dymowska et al., 2012; Hwang et al., 2011). In particular, the Na^+/K^+-ATPase (NKA) is expressed on the basement membrane of ionocytes in both fresh and saltwater fish (Hiroi and McCormick, 2012; Hwang et al., 2011). Depending on the environmental salinity, transporters used by ionocytes differ in type, role and abundance (Dymowska et al., 2012; Hwang et al., 2011; Hiroi and McCormick, 2012). In seawater, the apical cystic fibrosis transmembrane regulator (CFTR) and basolateral Na-K-2Cl cotransporter (NKCC) allow excess Cl^- to leave the cell (Hwang et al., 2011); in addition to these proteins, tight junctions between ionocytes allow Na^+ excretion due to changes in the extra-cellular microenvironment (Hiroi and McCormick, 2012). However, the mechanisms leading to ion uptake in freshwater vary among species of fish and are not fully described for most taxa (Dymowska et al., 2012). Generally, a Na^+/Cl^- cotransporter (NCC), is used in combination with NKA for uptake of both Na+ and Cl- into the cell from the freshwater environment (Dymowska et al., 2012; Hiroi and McCormick, 2012; Hwang et al., 2011).

A fish's internal plasma osmolality (total concentration of ions; Kültz, 2015), ranges from approximately 260 mM kg^{-1}water in freshwater to 450 mM kg^{-1} water in seawater (reviewed by Marshall and Edwards, 2013). In a direct transfer from a higher salinity to pure freshwater the fish experiences a rapid decline of internal ion content which is reflected by a loss of ions in the plasma and tissues (Al-Jandal and Wilson, 2011; Scott et al., 2004). This ion loss can be in part combated by downregulation of salt secreting channels (e.g. NKCC and CFTR; limiting ion movement out of the body), and up-regulation of ion uptake channels, which vary among species (Dymowska et al., 2012; Hwang et al., 2011).

Maintaining osmotic balance in the face of changes in salinity can increase metabolic demands in teleost fishes (Ern et al., 2014). Metabolic costs that occur during exposure to salinity change reflect the need to remodel tissues to contain the specific cell types, ion transporters and associated enzymes needed to ion-regulate in each environment (Kültz et al. 2015; Tseng and Hwang, 2008). Therefore, differences in whole-animal metabolic rate can act as a proxy for the general amount of work the fish is putting into water and ion regulation during transitions to either freshwater or more saline environments (Ern et al., 2014; Tseng and Hwang, 2008).

1.3 The Gasterosteidae as a Model Clade in Which to Study Freshwater Adaptation

After the glacial retreat and lifting of the land after the last ice age, many fish species became isolated in landlocked lakes, which over time diluted to freshwater as seen today (Bell and Lee, 1999). Transitions to freshwater, as experienced by those fish isolated in post-glacial lakes, were likely difficult for the majority of fish species, as the physiological tactics to iono- and osmo-regulate differ greatly between fresh and

saltwater environments. Fish in the family Gasterosteidae, or stickleback, are one group

of ancestrally marine post-glacial fish that have invaded freshwater environments (shown

in Figure 1; Kawahara et al. 2009).

Many stickleback species, and populations, have independently invaded

freshwater (Schultz and McCormick, 2012). For example, populations of the Ninespine

(*Pungitius pungitius)* and Threespine (*Gasterosteus aculeatus*), have repeatedly, and

independently, evolved freshwater tolerance after moving from ancestral marine habitats

into freshwater at the end of the most recent ice age (Kawahara et al., 2009; Kültz 2015;

Lee and Bell, 1999). Notably, in Threespine Stickleback, salinity tolerance has been

shown to differ between isolated freshwater populations and ancestral-like euryhaline

populations (DeFaveri et al., 2011; Divino et al., 2016). Gibbons et al. (2016, 2017)

found that freshwater Threespine populations have evolved increased freshwater

tolerance compared to their putative marine ancestral populations. It has been shown that

various Threespine Stickleback populations have adapted to freshwater via increased

expression of genes encoding 'freshwater' ion transporters (Gibbons, 2017; McCairns and

Bernatchez, 2010; Shimada et al., 2011) indicating local adaptation in freshwater

populations through divergent expression of genes (DeFaveri et al., 2011, Shimada et al.,

2011, Gibbons et al., 2017). Such important genes include Na^+,K^+-ATPase a-subunit

(ATP1A1; Gibbons, 2017; McCairns and Bernatchez, 2010; Shimada et al., 2011) and the

Na^+–Cl^- co- transporter (Shimada et al., 2011).

The Brook Stickleback (*Culea inconstans*) is found almost exclusively in

freshwater of low salinity brackish waters (e.g. 10 ppt), but has been found in brackish

waters as well (A. Dalziel, personal communication; Gilhen and Claridge, 1985).

Blackspotted Stickleback (*Gasterosteus wheatlandi)* are primarily found in high salinity

areas, at the edge of tidal flux or salt marsh, and are considered a marine and brackish-

water species (Kawahara et al., 2009; Worgan and Fitzgerald, 1981). There are few

documented instances of freshwater Blackspotted populations apart from in

Newfoundland, Canada (van Vliet, 1970). Fourspine Sticklebacks (*Apeltes quadracus*)

are also considered to be primarily marine, but have been found in lakes across their

native range (Blouw and Hagen, 1984; Nelson, 1968). However, no studies have yet

looked at freshwater tolerance in the Fourspine Stickleback, or tested if adaptation to

freshwater has occurred in lake populations (but see Audet et al. 1985, 1986 for studies of

salinity preference).
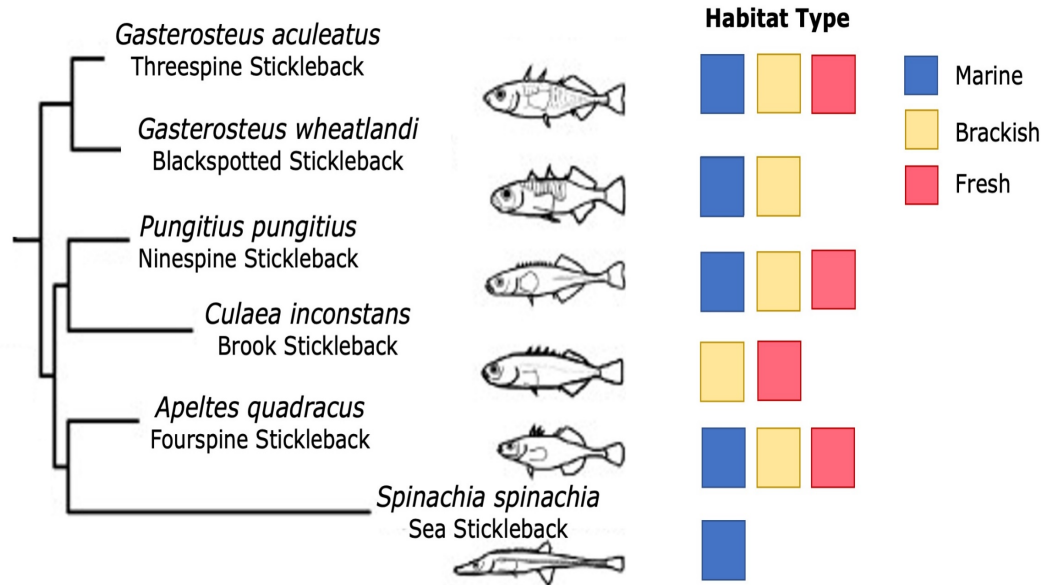
Figure 1. Representative Phylogeny of the Family Gasterosteidae. Colour coding shows which species contain populations living in marine water (blue), brackish water (yellow) and freshwater (red). The Brook Stickleback (*Culea inconstans*) has freshwater populations and some brackish water populations (A. Dalziel, personal communication; Gilhen and Claridge, 1985). Populations of Sea Stickleback (*Spinachia spinachia*) are exclusively marine while Blackspotted Stickleback (*Gasterosteus wheatlandi*) have populations residing in both marine and brackish water (Kawahara et al., 2009; Worgan and Fitzgerald, 1981). Three species, Threespine Stickleback (*Gasterosteus aculeatus*), Fourspine Stickleback (*Apeltes quadracus*) and Ninespine Stickleback (*Pungitius pungitius*) have populations in marine, brackish and freshwater (Kawahara et al., 2009). Freshwater adaptation has occurred in many Threespine and Ninespine Stickleback freshwater populations (see Gibbons et al., 2016; Kawahara et al., 2009; Worgan and Fitzgerald, 1981; Shimada et al., 2011) after divergence from their marine ancestor, but the freshwater tolerance of Fourspine Stickleback has not yet been studied. Figure adapted from Kawahara et al. (2009).

An important question in evolutionary biology is how often similar mechanisms underlie local adaptation to the same environmental factors (Losos et al., 2011; Rosenblum et al. 2014). Convergence describes the process by which a similar phenotype evolves among different species and often occurs as a result of similar selective pressures (Rosenblum et al., 2014). Parallelism describes a situation when similar molecular mechanisms underlie these convergent phenotypes (Rosenblum et al., 2014). Euryhaline capabilities, and freshwater tolerance, are believed to represent convergent evolution, where different mechanisms have evolved in many different species to accommodate exposure to freshwater (Dymowska et al., 2011; Kültz, 2015, Schultz and McCormick, 2013).

While research using Threespine Sticklebacks as model organisms to study freshwater adaptation is extensive, little is known about if, and how, populations of closely related species, including the Fourspine Stickleback (*Apeltes quadracus*), have evolved to cope with freshwater (Blouw and Hagen, 1981). If local adaptation to freshwater has occurred in Fourspine Stickleback, then the mechanisms contributing to adaptation can be compared with Threespine stickleback to determine if similar, or unique, mechanisms are used to cope with ion poor environments in the Gasterosteidae. However, it is currently unknown if Fourspine Sticklebacks locally adapted to freshwater after the last glaciation, as has been seen in the Threespine Stickleback, or if they are an ancestrally fully euryhaline species (meaning that marine populations, and the common ancestor of extant freshwater populations, are also capable of residing full time in freshwater).

The Fourspine Stickleback has a native range throughout eastern North America (Blouw and Hagen, 1981; Nelson, 1968). While they are primarily cited as a marine and

brackish species (Blouw and Hagen, 1981; Rowland, 1974), freshwater populations have been documented throughout their native range (Baker et al., 2010; Blouw and Hagen, 1981; Blouw and Hagen, 1984; Nelson, 1968). Fourspine Sticklebacks participate in a yearly spring migration moving from the ocean to inland breeding grounds in estuaries or tidal rivers (in May; Audet et al., 1985; Worgan and Fitzgerald, 1981). The peak of breeding is later in the summer for Fourspine Stickleback than other stickleback species, occurring up until the end of July (Worgan and Fitzgerald, 1981). Post-breeding Fourspine Stickleback also tend to remain longer in estuarine waters, until freeze up occurs (in November), whereas other sticklebacks leave after fulfilling reproductive roles (Worgan and Fitzgerald, 1981). Thus, they subject themselves to fluctuating salinities (3 ppt to 20 ppt) for longer periods of time than other stickleback species (Worgan and Fitzgerald, 1981).

In North America, Ninespine, Threespine, Fourspine, and Brook Sticklebacks may live amongst each other in the same estuaries and streams, but the different stickleback species inhabit different salinity niches within these areas (Le Bris and Wroblewski, 2018; McCleave et al., 2018; Worgan and Fitzgerald, 1981). Therefore, it has been suggested that salinity choice may correspond with divisions of suitable habitat among the different species and limit competition and aggression (Audet et al., 1985; Rowland 1983; Worgan and Fitzgerald, 1981). In nature, Fourspine Sticklebacks tend to avoid naturally occurring extreme salinity challenges, such as high salinity tidal pools (Rowland, 1974; Worgan and Fitzgerald, 1981) and strictly freshwater niches (Worgan and Fitzgerald, 1981). Audet et al. (1985 and 1986) showed a significant relationship between experimental photoperiod (to represent seasons) and Fourspine Stickleback salinity preference. Over the summer month photoperiod, 14 hours of light and 10 dark,

salinity preference is between fresh (Audet et al., 1986) and 7ppt water (Audet et al., 1985). A higher salinity preference (21ppt) is seen in Fourspine Stickleback with a decreased light photoperiod (9 hours light and 15 hours dark), resembling autumn conditions (Audet et al., 1986). Salinity preference also seems to be affected by temperature, where increased temperature leads to a decrease in salinity tolerance for Fourspines with a higher salinity tolerance (above 100 ppt) associated with lower temperatures (Nelson, 1968).

These studies show the complimentary nature of experimental cues (temperature and seasonal light exposure) to seasonal migration patterns of marine Fourspine Stickleback (Audet et al., 1985, 1986; Nelson, 1968). However, they do not speak to the overall freshwater tolerance of the species. Audet et al. (1985 and 1986) set up horizontal salinity gradients and preference was assigned based on what salinity in the tank the fish most often occupied. Fish were introduced to the acclimation salinity region (21 ppt) and able to move freely between salinities contained in the tank (0, 7, 14, 21ppt; Audet et al., 1985). Additionally, Audet et al. (1985, 1986) studied individual fish under small time frames (observation based over four hours). While we have some knowledge of preference, we do not know how Fourspines physiologically cope with prolonged confinement to freshwater.

1.4 Current Study Objectives

The present study will: 1) measure freshwater tolerance of Fourspine sticklebacks in general and 2) test for differences in salinity tolerance between a wild-caught marine Fourspine Stickleback population thought to resemble the ancestor of current freshwater populations, and a wild-caught population of Fourspine Stickleback inhabiting a

freshwater lake for at least 35 years (first discovered by Blouw and Hagen, 1984, with at least 1 generation per year, and possibly living in freshwater for up to 10-12,000 years). If I find that fish from a lake population are better able to cope with an acute freshwater transfer than fish from a marine population, then adaptation to freshwater or developmental acclimation may have occurred (Belanger et al., 1986). This would guide further tests for local adaptation (e.g. lab-bred crosses in controlled environments). If I find no differences in freshwater tolerance among lake and marine populations, this would indicate that Fourspine Sticklebacks are generally tolerant to both freshwater and full strength seawater (Nelson, 1968), and potentially no local adaptation was required for successful freshwater invasion.

2. METHODS

2.1 Fish Collection and Lab Acclimation

During the summer and fall of 2019, adult Fourspine Stickleback (*Apeltes quadracus)*

from one freshwater lake (Blair Lake, Nova Scotia) and one brackish-water marine site

(Rainbow Haven Estuary, Nova Scotia) were collected. The collection process included a

combination of dip netting and minnow traps and was conducted under DFO Gulf (SG-

RHQ-19-008) and Atlantic region (#343930) scientific fish collection permits, following

protocols approved by the Saint Mary's University Animal Care committee. Blair

Lake (45.7984° N, 64.2097° W; Fig. 2.1 A), is a eutrophic lake near Amherst, Nova

Scotia. At the time of sampling (August – October 2019), salinity in Blair Lake ranged

from 0.4 – 0.8 ppt (parts per thousand). Rainbow Haven Provincial Park in Cole Harbour,

Nova Scotia (44.6473° N, 63.4224° W; Fig 2.1 B), had salinity at the time of collection of

32 ppt (at high tide, in October 2019) and is known to range from 20 – 32 ppt (A. Dalziel,

personal communication).

The collected fish were brought back to the Saint Mary's University Aquatic

Facility and held in tanks equipped with waterfall filters and maintained at 10 ± 2 ppt

(measured with a Hanna probe; Hanna Instruments, Woonsocket, RI), and ambient room

temperature (16.3-25.6 °C). The two populations were split into separate tanks and left to

acclimate to lab conditions for at least one week prior to the start of the experiment under

a fall photoperiod (10L:14D). Fish were fed to satiation twice daily; the morning feeding

consisted of *Artemia* nauplii and the afternoon feeding of *Mysis* shrimp and bloodworms

(prepared from frozen). Water quality analysis was conducted at least once per week to

test pH, ammonia, nitrite (API, Mars Fishcare Inc., Chalfont, PA), and nitrate levels

(Fluval, Rolf C. Hagen Inc., Montreal, QC), with water changes being performed as necessary. Throughout the experimental period, fish were monitored at least twice daily, during feedings and again when conducting water changes.
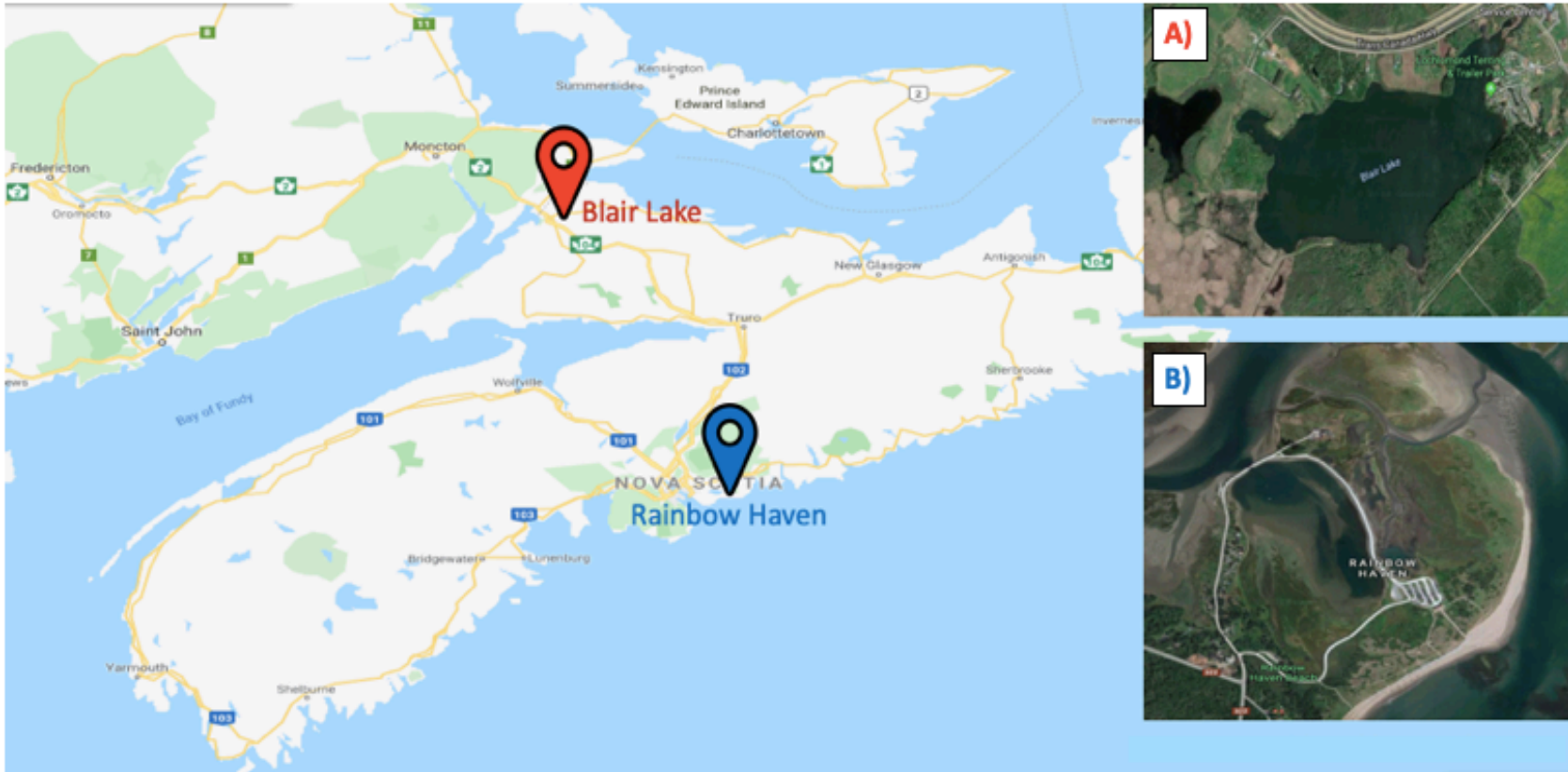
Figure 2.1. Map indicating the location of sampling sites in Nova Scotia. Integrated is an aerial satellite image of the location (both screen shots taken from Google Maps ©). Satellite image A shows Blair Lake. Satellite image B shows Rainbow Haven Beach Provincial park.
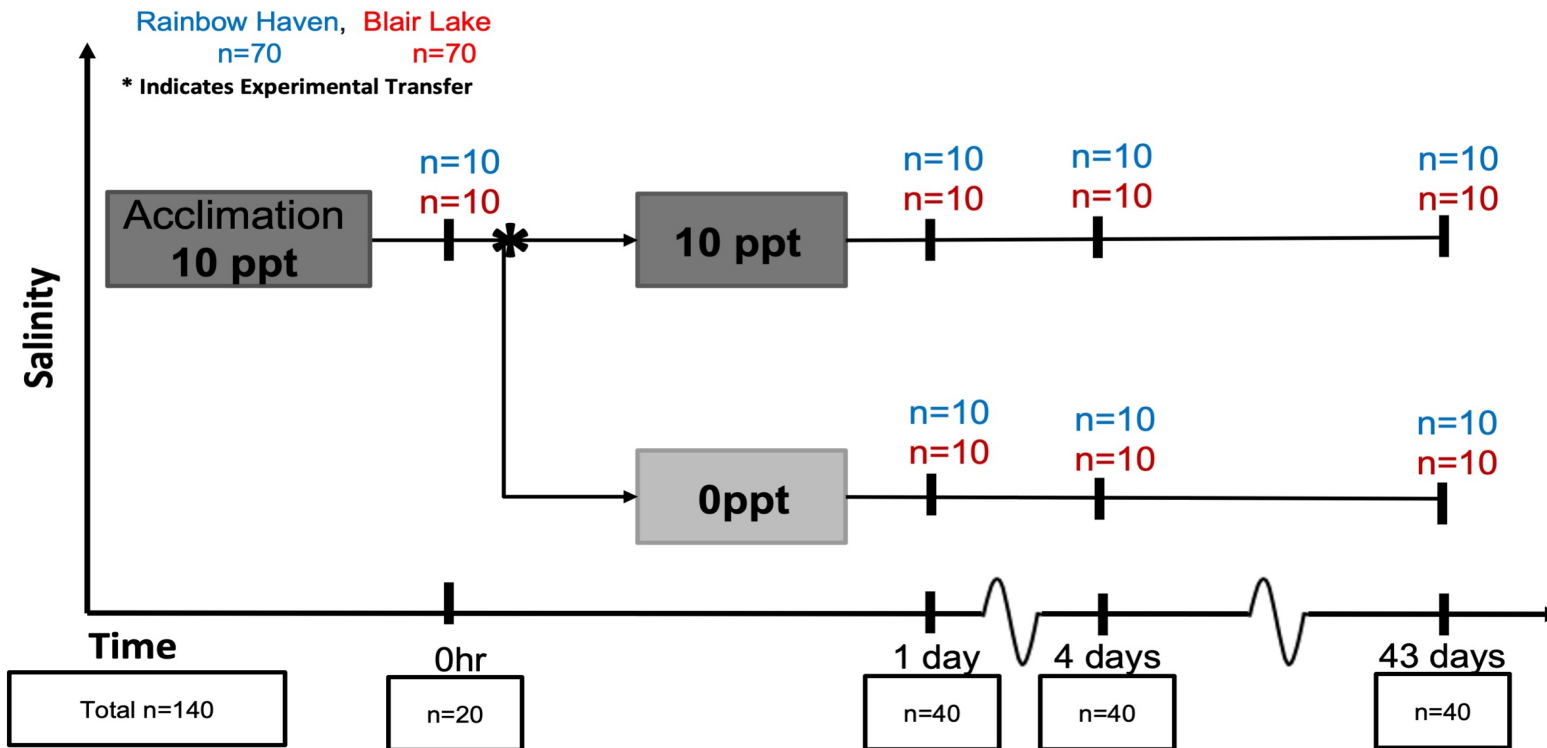
2.2 Freshwater Tolerance Experiment

The freshwater transfer was designed to measure how the two populations of Fourspine Stickleback coped with an acute freshwater challenge (0 ppt) and also if they were able to acclimate to freshwater over time. Prior to the freshwater transfer, six new tanks were prepared for the fish. To account for tank effects, we housed fish from Blair Lake and Rainbow Haven in the same tank, but on different sides of a mesh barrier so that water could easy flow across. I used marine-grade silicone to attach the mesh, dividing the tank in half. For the freshwater challenge, three tanks were maintained at freshwater (0 ppt + 0.6 ppt; freshwater challenge) and three at the control salinity (10 ppt ± 2 ppt; handling control). Freshwater was prepared by reconstituting 1.28 grams of Red Sea salt (Red Sea U.S.A., Houston, TX) with 4.0 grams of alkaline buffer (Seachem, Seachem Laboratories Inc., Madison, GA), and 50.0 mL of stress coat (API, Mars Fishcare Inc., Chalfont, PA) in approximately 55 gallons of RODI (reverse osmosis deionized) water. This mixture resulted in concentrations of 13.4 mg/L Na^+, 9.3 mg/L Cl^-, 0.7 mg/L Ca^{2+}, <0.5mg/L Mg^{2+}, <0.5 mg/L K^+. In comparison, Blair lake water was slightly saltier [69.8 mg/L Na^+, 120 mg/L Cl^-, 21.1 mg/L Ca^{2+}, 5.3 mg/L Mg^{2+}, 3.2 mg/L K^+]. The 10 ppt water for control tanks was prepared using the same Red Sea Salt (Red Sea U.S.A., Houston, TX), by measuring approximately nine cups mixed with 55 gallons of RODI water, and then checking the salinity with a handheld salinity probe (Hanna Instruments HI 98192, Woonsocket, RI). Each of the six experimental tanks were filled with prepared water and equipped with waterfall filters (as explained in section 2.1). The salinity of stock water and tanks was monitored using a Hanna Probe (Hanna Instruments, Woonsocket, RI).

We conducted the transfer by first taking Blair Lake fish from their 10 ppt acclimation tanks and placing them into a well-aerated bucket filled with 10 ppt water. Next, 16 Blair lake fish were placed into the left-hand side of each of the six experimental tanks (three tanks at 0 ppt and three tanks at 10 ppt). This same procedure was then replicated for the Rainbow Haven fish, changing the destination of the fish to the right-hand side of the experimental tank. Over the course of the experiment, tanks were kept at ambient room temperature (18.1 – 20.9 °C), with a winter photoperiod (10L:14D).

2.3 Sampling Procedure and Calculation of Body Water Percentage

Lethal sampling was conducted at 0 hours, 24 hours, 96 hours and 43 days after the freshwater transfer (Figure 2.2). All fish were fasted for 24 hours prior to sampling time points. A total of 140 fish were sampled using this procedure: 30 from each population at the two treatment salinities, plus 10 fish per population to measure pre-transfer parameters (0 hr). For each sampling period, fish were euthanized in a bubbled Tricaine Methanesulfonate (MS222) solution (0.5 g MS222 buffered with 1.0 g of Sodium bicarbonate in 1L of tank water). When signs of life ceased (no opercular beats or response to caudal pinch), fish were removed from the MS222 solution and blotted dry with paper towel. Quickly, using a razor blade the caudal region was severed, and blood was collected from the caudal artery by using hematocrit tubes (sealed and stored on ice after blood collection). After the collection of blood from 8-12 fish, a hematocrit centrifuge (Sorvall Legend Micro 17, ThermoFisher Scientific, Waltham, MA) was used to separate blood plasma from red blood cells by spinning the samples at 10,000 rpm for six minutes. After the separation, the plasma was stored at -20°C for analysis of plasma

ion content in future experiments (data not included in this thesis). Fish standard length and weight were then measured using a ruler (measuring from snout to distal end of the caudal peduncle) and an analytical balance (whole-fish wet mass; Sartorius analytic, model A 120 S). Gill arches were dissected, and flash frozen in liquid nitrogen and later stored at -80 °C for future studies. During dissection, I first removed and discarded the pelvic girdle (and spines) followed by the viscera of the fish. The sex of the Fourspines were visually assigned by assessing colouration of pelvic spines (white pelvic spines indicate a female while red pelvic spines are present on males; Rowland, 1974) and presence of testes or eggs during dissections. The dissected fish carcasses [fish head, flanks and tail region (without caudal fin)] were placed in a 1.5mL centrifuge tube which was pre-weighed using an analytical balance for the weigh by difference method; this value represented the carcass wet weight. The fish carcasses from each sampling day were left to dry in microfuge tubes with caps open at room temperature in a fume hood. Following the methods of Al-Jandal and Wilson (2011), the samples were weighed periodically until the mass was consistent and the lowest obtained value represented the dry mass of the carcass. Water-body content was determined as percent body water content by calculating the original carcass wet mass minus carcass dry mass divided by carcass wet mass and multiplied by 100. The carcass contained in the tube was saved for the later determination of tissue-ion content in future studies.

Figure 2.2. Experimental set-up for freshwater tolerance test. 10 fish from each population (Rainbow Haven marine fish and Blair Lake freshwater fish) were sampled at 0 hours, before placement in experimental tanks (indicated in diagram by *), as a pre-transfer control. There were three replicate tanks maintained at each salinity (0 and 10ppt) and n=3-4 fish were sampled from each replicate tank at each post-transfer sampling point. Samples collected at 10 ppt represent time-matched handling controls. Black vertical lines indicate sampling points, with corresponding times and sample sizes
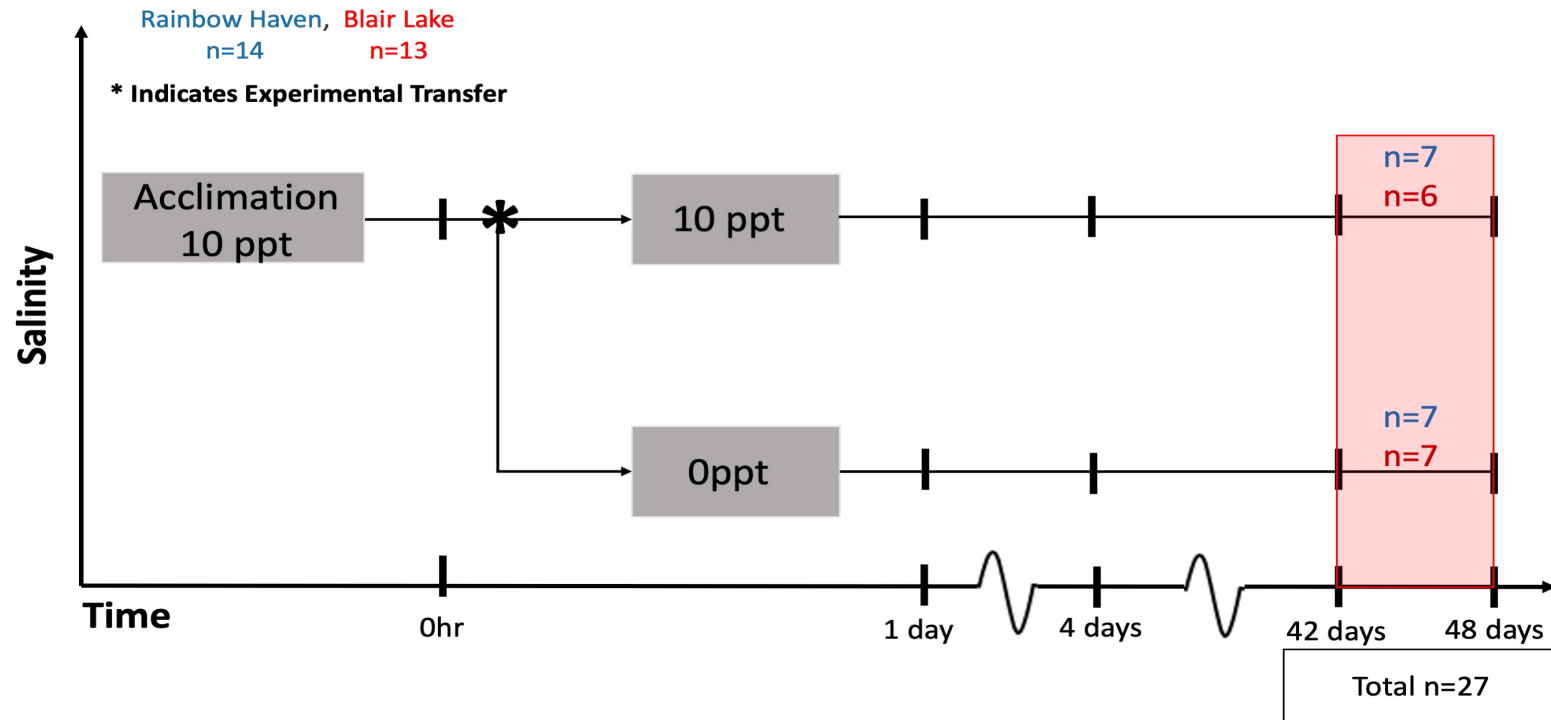
2.4 Standard Metabolic Rate

To address the amount of energy needed to maintain homeostasis in the face of the freshwater challenge, we measured fish standard metabolic rate between days 42 and 48 after freshwater transfer (Figure 2.3). For each salinity, the oxygen consumption of Fourspine Stickleback was measured using a medium glass respirometry chamber set up (45mm inner diameter, volume 250mL, 11mm outer diameter fittings; Loligo® Systems, Tjele, Denmark). When I initially placed Fourspine Sticklebacks in the chamber the flow of the water in the chamber was fast enough to induce swimming activity in the fish over the measurement phase. This was problematic as SMR measurements are dependent on fish being at rest (reviewed by Chabot et al., 2016). To slow the velocity of water moving within the chamber, I outfitted each chamber with glass marbles and sponges and constricted water supply tubing using tube clamps. Four respirometry chambers were utilized in this experiment; two respirometers were set up in freshwater and two in 10 ppt 10 gallon fish tanks. Both tanks were continuously aerated using air stones to ensure oxygenation of water outside of the chambers.

A Witrox 4-channel oxygen measurement system with fiber optic oxygen probes and oxygen flow through cells (Loligo® Systems, Tjele, Denmark) was used to measure oxygen consumption. Oxygen probes were calibrated prior to running respirometry with fish. A low value calibration was conducted using 500 mL of a 0.32 M sodium sulfite solution (purging oxygen from solution) by submerging the oxygen probe and flow through cells into the solution. The high oxygen saturation value calibration was performed using a beaker filled with 500 mL of water, aerated with an air stone for at least 15 minutes to ensure oxygenation (Rosewarne et al., 2016). Calibration was set when phase value readings levelled out across all four sensors (the phase value is the raw

sensor signal from the fibreoptic cable to measure the phase shift in the blue light caused by fluorescence). With this system, four fish were measured simultaneously. A fish from each population (Blair or Rainbow Haven) was assigned to each salinity (0 or 10 ppt).

Fish were fasted for 24 h before introduction into the respirometer between 1700H and 1800H. Prior to introduction to the chamber, we measured fish mass for determination of mass-specific metabolic rate (Chabot et al., 2016; Rosewarne et al., 2016). SMR data was collected overnight for approximately 12-14 h using intermittent flow respirometry with repeated periods of 45 min of measurements in the closed chamber, 12 min of flushing with oxygenated water, and a 2 min wait, resulting in at least 12 measurement periods per individual. Brennan et al. (2016) reported that 14-hours of overnight measures are sufficient to capture accurate SMR measurements. During metabolic rate measurements, the tops and sides of the holding tanks were surrounded with sheets of insulation to ensure darkness. In addition, each chamber was wrapped in black plastic to promote comfort for the fish (Brennan et al., 2016; Chabot, 2016). After the conclusion of the overnight measurement, fish were returned to their original tanks placed in floating mesh-bottomed tupperware containers to ensure sufficient waterflow and separation from unmeasured fish. Next, measurements were collected in absence of the fish from the chambers, to account for bacterial background respiration. After the completion of overnight SMR measurements, I planned to hand select the lowest oxygen consumption measurements to calculate SMR for each fish in AutoResp (2.3.0). These measurements were corrected for body mass and background respiration.

Figure 2.3. Experimental set-up for respirometry to measure standard metabolic rate in Rainbow Haven marine fish and Blair Lake freshwater fish. Seven nights of standard metabolic rate measurements were completed between 42 and 48 days (red shaded time period) post freshwater transfer (indicated in diagram by *). Samples collected at 10 ppt represent time-matched handling controls. Black vertical lines indicate sampling points (see Figure 2.2).

2.5 Statistical Analysis

A three-way Analysis of Variance (ANOVA) was used to test the effect of ecotype (Blair, Rainbow Haven), salinity (0 or 10 ppt) and time (0, 24, 96 or 43 days) on carcass water content. A two-way ANOVA will be conducted to test the effect of ecotype and salinity on standard metabolic rate, after the university COVID-19 closure is complete and data can be accessed. All statistical analysis was conducted with R (R version 3.5.1; packages ggplot2 and devtools).

3. RESULTS

3.1 Fourspine Stickleback Freshwater Survivorship

Figure 3.1 shows a survivorship curve for the marine and lake populations of Fourspine Stickleback, in the two treatment salinities (control of 10 ppt and freshwater). The marine fish fared well in both 0 and 10ppt treatments. In freshwater (0 ppt), survival was higher in the marine fish than the lake fish. In the lake population, five deaths occurred in the first three days of the freshwater challenge, but no fish died after day three (survival rates remained constant apart from one addition death for a control lake fish 48 days post transfer during respirometry trials).
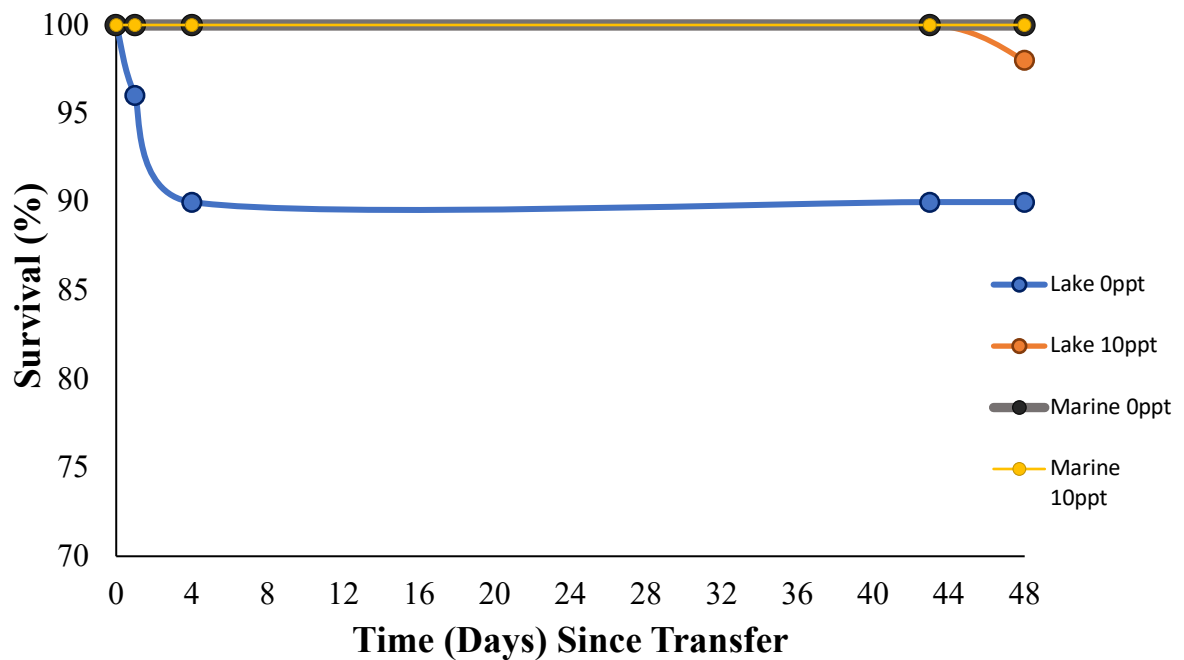
Figure 3.1. Effects of freshwater transfer on survival of lake (Blair) and marine (Rainbow Haven) Fourspine Stickleback populations. All fish were acclimated to 10 ppt (near-isosmotic salinity) for at least one week prior to transfer to freshwater (0 ppt) or back to 10 ppt as a handling control. After transfer fish were observed at least two times daily and deaths were recorded as observed. Yellow and orange lines represent survival in the control salinity (10 ppt) for marine and lake populations, respectively. The grey line represents survival of marine fish at 0 ppt, in the plot this is enlarged behind the survival line of the marine fish at the control salinity (yellow line) with 100% survival for each group. The survival of lake fish at 0 ppt is marked with a blue line (90% survival).

3.1 Body Water Content Maintenance After Freshwater Transfer

Differences in the percent body water content between lake and marine fish over the course of the experiment are shown in Figure 3.2. If a fish is unable to maintain homeostasis during freshwater transfer, body water content should increase due to the influx of water down its concentration gradient. A three-way Analysis of Variance (ANOVA) was run with percent body water as the response variable (as shown in Table 3.1) and population, time and salinity as manipulated variables. There was significant effect of population on body water content (F=17.286, p = 0.001), with lake fish having a significantly higher body water content in than marine fish. There was also a slightly significant effect of time on body water percentage (F=2.679, p = 0.0498). There was no significant interactions among any of the three manipulated variables (salinity x population: F=0.319, P=0.5735; salinity x time: F=1.552, p=0.2157; population x time: F=1.019, p=0.3868 and salinity x population x time: F=1.028, p=0.3606).
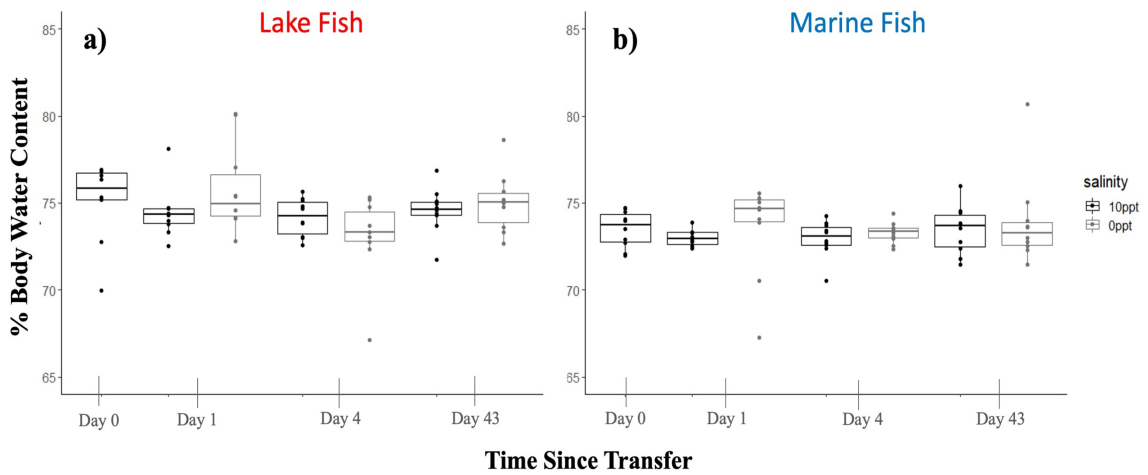
Figure 3.2. Effects of freshwater transfer (0 ppt) or transfer to a control salinity (10 ppt) on percent body water content in Fourspine Sticklebacks from (a) a lake (Blair Lake) and (b) a marine (Rainbow Haven) population. Boxes in black represents data for fish at the control salinity (10 ppt), while light grey represents body water content for fish in freshwater (0 ppt). There was significant effect of population on body water content (F=17.286, p = 0.001and a significant, but small, effect of time on body water percentage (F=2.679, p = 0.0498). There was no significant interactions among any of the three manipulated variables (salinity x population: F=0.319, P=0.5735; salinity x time: F=1.552, p=0.2157; population x time: F=1.019, p=0.3868 and salinity x population x time: F=1.028, p=0.3606).

4. DISCUSSION

We tested freshwater tolerance of Fourspine Stickleback (*Apeltes quadracus*) by studying survival and osmoregulatory performance (muscle water content) in fish from a wild caught derived (freshwater) and ancestral-like population (marine population). Both populations were subjected to either an acute freshwater transfer (0 ppt) or control transfer (10 ppt) after acclimation at a near iso-osmotic salinity of 10 ppt and observed for a period of 48 days. We found limited intraspecific variation in terms of survival and water body content of experimental fish after freshwater transfer. These results suggest Fourspine Stickleback are fully euryhaline. It is therefore likely that ancestral marine Fourspines who became isolated in freshwater faced minimal iono- and osmo-regulatory challenges when populating this novel environment.

4.1 Measuring Osmoregulatory Performance: Survival and Body Water Content

We know that marine Threespine Stickleback normally struggle in freshwater tolerance experiments. Previously, Divino et al. (2016) reported that juvenile freshwater Threespine Stickleback (a newly introduced population with only two generations in freshwater, native to Alaska) exhibited a 67% higher survival rate in freshwater than an anadromous marine population. As well Ishikawa et al. (2016) found that two sympatric marine ecotypes of Threespine sticklebacks (Pacific Ocean and Japan Sea) in Japan also display differential freshwater tolerance. Ishikawa et al. (2016) took migrating adult fish and subjected them to a freshwater transfer. The Japan Sea ecotype has no derived freshwater populations, unlike the Pacific Ocean ecotype (Ishikawa et al, 2016). Ishikawa et al. (2016) found that the Japan sea lineage had higher death rate in freshwater than Pacific Ocean fish (7 of 13 Japan Sea fish compared to 1 of 15 Pacific Ocean fish with no deaths

in either control group). While Divino et al. (2016) reported no difference in body water content between the freshwater and marine population at 0 ppt, both studies reported that the main contributor to death was likely an inability to tightly regulate internal ion content based on carcass ion content (Divino et al., 2016; Ishikawa et al., 2016).

Based on previous research with Threespine Stickleback, we predicted that the transfer to freshwater would be difficult for marine Fourspine Stickleback and measured survival and body water content as indicators of their ability to maintain homeostasis (Divino et al., 2016; Ishikawa et al., 2016). We expected different survival rates of fish between the populations, when exposed to freshwater we believed there would be higher survival in the freshwater population compared to the marine population (Divino et al., 2016; Ishikawa et al., 2016), and a lower ability to maintain body water homeostasis in the marine fish, which would exhibit higher levels of tissue water when acclimating to freshwater. Over the course of the experiment the marine fourspine population had 100% survival (in both 0 and 10 ppt) and showed no significant increase in body water. This was unexpected, as our marine population have never had previous exposure to freshwater and this species was thought to be primarily marine. The only natural deaths we observed in the study were among freshwater fish; these were mainly at 0 ppt (90% survival), with most occurring 3 days post-transfer, as seen in similar studies on Threespine Stickleback (Divino et al., 2016). Again, the freshwater population showed no significant variance in body water content. The lower survival was unexpected as these lake fish were bred and raised in freshwater and percent body water data indicates the freshwater group had no difficulties osmo-regulating at 0 ppt. One explanation for this result is that one of our acclimation tanks of Blair Lake fish experienced some deaths prior to the experimental transfer (predicted to be due to numerous causes such as disease

and senescence), although water quality was good. It is possible that the deaths occurring in our experiment were of fish that were moved into the experiment from this acclimation tank, but as fish were not marked, we do not know. In addition, the ionic components of the water were different between Blair lake and our experimental freshwater: Blair lake water was slightly more ion rich [69.8 mg/L Na^+, 120 mg/L Cl^-, 21.1 mg/L Ca^{2+}, 5.3 mg/L Mg^{2+}, 3.2 mg/L K^+] and our reconstituted freshwater: [13.4 mg/L Na^+, 9.3 mg/L Cl^-, 0.7 mg/L Ca^{2+}, <0.5mg/L Mg^{2+}, <0.5 mg/L K^+]. The more limited ion environment in the experimental treatment may have been stressful for these fish and induced higher death tolls. Apart from this, it appears both populations were able to tolerate the freshwater transfer.

We expected intra-specific variation in freshwater tolerance in this species, which would indicate potential freshwater adaptation among fish of the studied freshwater population. One explanation why marine fish may have been equally good at tolerating freshwater could be the contribution of potential 'freshwater' alleles entering the gene pool via interbreeding of freshwater and marine populations of Fourspine Stickleback. However, our populations are geographically isolated from each other. We do know that in regions such as tidal rivers where fresh and marine Threespine Stickleback populations share occupancy during breeding there is gene flow which is thought to contribute to increased freshwater tolerance of some marine populations (see Jones et al., 2006).

We know that different stickleback species use regions of estuaries with different salinities during the breeding season (Le Bris and Wroblewski, 2018; McCleave et al., 2018; Worgan and Fitzgerald, 1981). Migratory marine populations of Threespine Stickleback and Fourspine Stickleback do migrate to estuaries but avoid freshwater over their breeding season (migratory marine Fourspines in Newfoundland have been found at

salinities as low as 1.8ppt; Le Bris et al., 2018). Therefore, early life stages of the fish also occur at fluctuating salinities (Defaveri and Merilä, 2014; Worgan and Fitzgerald, 1981), which may explain the success of some marine Threespine Stickleback populations reared at lower salinities (Belanger et al., 1987; Defaveri and Merilä, 2014). While migratory marine Fourspine Stickleback have not been observed to breed in the wild at 0 ppt (Worgan and Fitzgerald, 1981), Audet et al. (1986) found a preference for 0 ppt and (Audet et al., 1985) a significant preference for 7 ppt for anadromous marine Fourspine Stickleback acclimated to a summer photoperiod; this supports our finding that Fourspine Stickleback are freshwater tolerant. As 7 ppt is close to a near isosmotic salinity (around 10ppt; Schultz and McCormick, 2013), this may limit the energetic costs osmoregulation. Audet et al. (1985) suggested that the lower salinity preference of Fourspines in their study could be due to sampling from Fourspine Stickleback populations that were not exclusively marine (having been collected from the mouth of the tidal river, facing salinities of 3-20 ppt). For our study, we sampled marine native populations of Fourspine Stickleback at a fully marine collection site, Rainbow Heaven Beach Provincial Park. I suggest that my results show that even fully marine Fourspine face no difficultly acclimating to ion-limiting freshwater (0 ppt).

It is important to note that the life stage of the experimental fish can also modify the effects that salinity can have. In our study we utilized post breeding adult Fourspines, but other life stages (such as embryos or juveniles) have not been studied. It is possible that these groups of Fourspines may show variance in tolerance. In Threespine Stickleback there have been instances where different salinity tolerances can be seen across life stages. Defaveri and Merilä (2014) also observed the highest survival of Threespine Stickleback juveniles, regardless of the populations source salinity (fresh or

saltwater) in low salinity environments. Belanger et al. (1987), reported that marine

Threespine Stickleback eggs reared in a freshwater treatment (0 ppt), leads to higher

survival and growth rates than those maintained in saltwater (28 ppt). This is different

than what studies studying adult Threespine Stickleback tend to see in terms of fish

survival (Ishikawa et al., 2009) and growth rates (Gibbons et al., 2016) when marine

populations are exposed to freshwater. Thus, there is a possibility that embryonic or

juvenile tolerance to freshwater in Fourspine Stickleback may vary among populations,

even though adult tolerance does not.

4.2 Experimental Limitations

4.2.1 Carcass Ion Content

As our marine Fourspine Stickle population experienced no deaths, and no difference in

body water content, an analysis of internal ion content would highlight differences in

iono-regulation of marine and lake Fourspine Stickleback. This could show an increased

iono-regulatory capability or better strategy used by marine Fourspines to handle

hypoosmotic challenges. We were unable to complete this analysis due to Covid-19

shutdowns.

4.2.2 Standard Metabolic Rate

Another goal of the present study was to gage the difficulty the two populations faced in

respect to energetic demands when exposed to freshwater. To do this standard metabolic

rate (SMR) measurements were obtained using Loligo® Systems technology. SMR is a

common proxy for energetic demands in resting fish (Chabot et al., 2016; Ern et al.,

2014). Mass specific metabolic rates calculated for the two populations of Fourspine

Stickleback compared to the 10 ppt control can be used as a measure of how much energy was being expended to iono- and osmo-regulate in freshwater compared to near iso-osmotic salinities. We know that the largest energy cost would be expected to be immediately following direct transfer and can occur up to four days of exposure (Ern et al., 2014). Measuring after this time frame (in our protocol between 42 and 48 days post transfer), will allow us to address the long-term costs of freshwater osmoregulation in marine and freshwater Fourspine Stickleback. It should be noted that this was later than initially designed (two to three weeks post transfer), due to issues with set up of this new equipment.

Due to lack of time, the analysis of this data was not completed. We know from a previous study in Threespine Stickleback there was no difference in SMR measurements of freshwater and marine Norwegian Threespine Stickleback at salinities of 0, 15, and 30ppt (Grøtan et al., 2012). Freshwater, brackish and marine native fish had no detectable metabolic issues acclimating to different environments (fresh and saltwater; Grøtan et al., 2012). Lack of detectable differences in the study by Grøtan et al. (2016) indicate population specific effects among the global Threespine Stickleback ecotypes, as Canadian Pacific Threespine Sticklebacks do show large differences in growth rates that are predicted to be the result of a higher metabolic rate in marine populations (e.g. Gibbons et al., 2016). As previously noted, most cellular changes required in iono- and osmo-regulatory tissues of euryhaline fish in response to salinity challenges, including changes in the types of ionocytes and ion-transporters present within these cells, occur within four days post transfer (Ern et al., 2014). Therefore, in the future it would be interesting to test the difference at earlier experimental stages (i.e., days 0 -4), to gage how energetically challenging it is for different populations to acclimate to freshwater.

Although data analysis was only in the preliminary stage, we noted high background

respiration rates despite only taking seven consecutive nights of respirometry. The major

contributor to this was likely the additional surface area of sponges within our custom

fitted chambers. We added sponges for two reasons, to shrink the size for our fish, and to

minimize the water velocity initially observed within the chamber (which caused the fish

to swim and therefore threatened the quality of our SMR data). In the future, our

chambers should be disinfected frequently to avoid this (at least once a week). We also

did not monitor the activity of the fish overnight (as suggested by Chabot et al., 2016; Ern

et al., 2014). We personally observed minimal to no sudden activity (i.e. swimming) of

Fourspines within respirometry chambers, so do not think this needs to be recorded in

future studies. More realistic winter temperatures may have altered the capability of our

marine populations to iono- and osmo-regulate, possibly indicating local adaptation

among freshwater populations as indicated to play a factor for Threespine Stickleback by

Gibbons et al. (2016). Future studies can test if temperature influences freshwater

tolerance in marine and lake Fourspine Sticklebacks.

4.3 Future Directions

We chose a winter photoperiod for this experiment, which we predicted would increase

the freshwater challenge on the marine population as under this photoperiod the fish

would naturally participate in a migration from their estuarial breeding site (Rainbow

Haven) to deeper sea water. As the freshwater population is isolated and non-migratory,

we originally predicted they would better cope with a freshwater challenge than the

marine fish, who would traditionally be migrating into more saline waters during the

winter (Audet et al., 1986; Worgan and Fitzgerald, 1981). We did not match seasonal

water temperatures to this winter photoperiod in the present experiment. However, the

combination of low temperatures and a winter photoperiod may have led to a difference

in freshwater tolerance of the two populations, as was previously found in Threespine

Stickleback (Gibbons et al., 2016, 2017). Gibbons et al. (2016), indicated that growth rate

was lower for marine Threespine Stickleback facing freshwater exposure at winter

temperature than that of freshwater populations (Gibbons et al. 2016). Nelson (1968) also

found that increased water temperature (16 °C) leads to a decrease in upper salinity

tolerance for Fourspines with a higher salinity tolerance (above 100 ppt) associated with

lower temperatures (8 °C) (Nelson, 1968). We do know from the field observations of

Worgan and Fitzgerald (1981), that post-breeding Fourspine Stickleback tend to remain

longer in estuarine waters (at salinities between 3-20ppt) than other marine Stickleback

species (such as Threespine Sticklebacks). In this regard, marine populations subject

themselves to decreasing water temperatures along the shoreline that accompany the fall

and winter months alongside changes in salinity with tidal flow. This could indicate that

the species may be better equipped to handle colder water temperatures at salinities

greater than 20 ppt (Audet et al., 1986; Nelson, 1968).

 While our marine population was tolerant to freshwater transfer, Fourspines have

been shown to avoid pure freshwater (0 ppt) during the breeding season (Audet et al.,

1986; Worgan and Fitzgerald, 1981). These past findings may indicate that Fourspines

have a hard time breeding in freshwater. I suggest that future studies compare freshwater

tolerance across life-history stages in freshwater and migratory marine populations to

further test for local adaptation. This would also allow us to test if marine Fourspine

Sticklebacks eggs are vulnerable in freshwater in terms of survival and development (see

Belanger et al., 1987; Kassen et al., 1995) as an explanation for why marine Fourspines have not been observed in freshwater regions of estuaries.

Another possible future direction is to study the potential tradeoffs associated increased freshwater tolerance among derived populations of Fourspine Stickleback. In the Threespine Stickleback, some studies show decreased upper salinity tolerance (diminishing ion secretion) in derived freshwater populations for freshwater iono-regulation (McCairns and Bernatchez 2010; Defaveri and Merilä, 2014). However, Divino et al. (2016) reported that juvenile lake Threespine Stickleback showed no significant difference in performance in hypersaline conditions within two generations of residency, as fish of both populations were able to recover control osmolality within 10 days of freshwater exposure.

It is important to note that osmo-regulatory function is ultimately determined at the molecular level, by ion transporters and cell junction proteins. Future studies may use RNA-sequencing to look at the mechanisms leading to freshwater acclimation in the Fourspine Stickleback to test if they acclimate in the same manner as Threespine Sticklebacks (Gibbons et al. 2017). Shimada et al. (2011), found divergence in a complex of physiological relevant genes (for osmoregulation, temperature range, and growth) during freshwater adaptation in European freshwater populations of Threespine Stickleback. This finding has been mirrored in global populations of Threespine Stickleback indicating parallel evolution among global populations of freshwater Threespine Stickleback (Defaveri et al., 2011). However, it is not yet known if other stickleback species use similar mechanisms to adapt to freshwater.

4.4 Conclusions

Species of fish that include fresh and saltwater populations can be used to address questions of salinity acclimation and adaptation by taking a comparative approach (Kültz, 2015; Lee and Bell, 1999). Euryhaline fish have become models to study how freshwater acclimation occurs and can also increase our understanding of freshwater evolution by comparing ancestral and derived forms (Divino et al., 2016; Gibbons et al., 2016; 2017; Kültz, 2015; McCormick et al., 2013; Velotta et al., 2017). In this study I measured salinity tolerance in marine and freshwater Fourspine Stickleback to test overall tolerance and determine if local adaptation to freshwater might have occurred. My results currently indicate no difference (in terms of survival and water retention level) in freshwater tolerance among freshwater and marine populations of wild-caught Fourspine Stickleback. This indicates that all Fourspine Sticklebacks may have the ability to survive in freshwater concurrently with full strength seawater, and local adaptation of iono- and osmo-regulatory mechanisms is not required for successful freshwater colonization. Thus, intra-specific comparisons of lake and marine Fourspine Stickleback cannot be used to study the evolution of freshwater tolerance. However, quantifying freshwater tolerance in this species allows us to further map when freshwater tolerance evolved in the Gasterosteidae phylogeny, and indicates that freshwater tolerance evolved earlier in the phylogeny than previously believed.

5. REFERENCES

Al-Jandal, N. J. and Wilson, R. W. (2011). A comparison of osmoregulatory responses in plasma and tissue of rainbow trout (*Oncorhynchus mykiss*) following acute salinity challenges. *Comparative Biochemistry and Physiology, Part A* **159**, 175-181.

Audet, C., Fitzgerald, G. J., and Guderley, H. (1985). Salinity preferences of four sympatric species of sticklebacks (Pisces: *Gasterosteidae*) during their reproductive Season. *Copeia* **1**, 209-213.

Audet, C., Fitzgerald, G.J. and Guderley, H. (1986). Environmental control of salinity preferences in four sympatric species of sticklebacks: *Gasterosteus aculeatus, Gasterosteus wheatlandi, Pungitius pungitius* and *Apeltes quadracus. Journal of Fish Biology* **28**, 725-739.

Baker, J. A., Dewey, J. E. and Foster, S. A. (2010). Variation in reproductive traits of female fourspine Stickleback (*Apeltes quadracus*) in Nova Scotia, Canada. *Copeia.* **3**, 437–442.

Belanger, G., Guderley, H. and Fitzgerald, G. J. (1987). Salinity during embryonic development influences the response to salinity of *Gasterosteus aculeatus* L. (*trachurus*). *Canadian Journal of Zoology* **65**, 451-454.

Blouw, D. M. and Hagen, D. W. (1981). Ecology of the fourspine stickleback, *Apeltes quadracus,* with respect to a polymorphism for dorsal spine number. *Canadian Journal of Zoology* **59**, 1677-1692.

Blouw, D. M. and Hagen, D. W. (1984). The adaptive significance of dorsal spine variation in the fourspine stickleback, *Apeltes quadracus.* I. Geographic variation in spine number. *Canadian Journal of Zoology* **62(7)**, 1329-1339.

Brennan, R. S., Hwang, R., Tse, M., Fangue, N. A. and Whitehead A. (2016). Local adaptation to osmotic environment in killifish, *Fundulus heteroclitus*, is supported by divergence in swimming performance but not by differences in excess post-exercise oxygen consumption or aerobic scope. *Comparative Biochemistry and Physiology, Part A* **196**, 11–19.

Chabot, D., Steffensen, J. F. and Farrell, A. P. (2016). The determination of standard metabolic rate in fishes. *Journal of Fish Biology* **88**, 81-121.

DeFaveri, J., Shikano, T., Shimada, Y., Goto, A., and Merilä, J. (2011). Global analysis of genes involved in freshwater adaptation in threespine sticklebacks (*Gasterosteus aculeatus*). *Evolution* **65(6)**, 1800- 1807.

DeFaveri, J. and Merilä, J. (2014). Local adaptation to salinity in the three-spined stickleback? *Journal of Evolutionary Biology,* **27(2)**, 290-302.

Divino J. N., Monette M. Y., McCormick S. D., Yancey, P. H., Flannery, K. G., Bell, M. A., Roll, J. L., von Hippel, F. A. and Schultz, E. T. (2016). Osmoregulatory physiology and rapid evolution of salinity tolerance in threespine stickleback recently introduced to freshwater. *Evolutionary Ecology Research* **17**, 179–201.

Dymowska, A. K., Hwang, P. P. and Goss, G.G. (2012). Structure and function of ionocytes in the freshwater fish gill. *Respiratory Physiology & Neurobiology* **184 (3),** 282-292

Edwards, S. L., and Marshall, W. S. (2013). *Principles and Patterns of Osmoregulation and Euryhalinity in Fishes*. In *Fish Physiology* (eds S.D. McCormick, A.P. Farrell and C.J. Brauner), pp. 2–32. New York: Academic Press.

Ern, R., Huong, D. T., Cong, N. V., Bayley, M. and Wang, T. (2014). Effect of salinity on oxygen consumption in fishes: a review. *Journal of Fish Biology* **84**, 1210-1220.

Gibbons, T. C., Rudman, S. M., Schulte, P. M. (2016). Responses to simulated winter conditions differ between threespine stickleback ecotypes. *Molecular Ecology* **25(3)**, 764-775.

Gibbons, T. C., Metzger, D. C., Healy, T. M. and Schulte, P. M. (2017). Gene expression plasticity in response to salinity acclimation in threespine stickleback ecotypes from different salinity habitats. *Molecular Ecology* **26(10),** 2711-2725.

Gilhen, J. and Claridge, E. (1985). Additional records of the brook stickleback, *Culaea inconstans*, from Nova Scotia. *The Canadian Field Naturalist* **99**, 107-109.

Grøtan, K., Østbye, K., Taugbøl, A. and Vøllestad, L. (2012). No short-term effect of salinity on oxygen consumption in threespine stickleback (*Gasterosteus aculeatus)* from fresh, brackish, and salt water. *Canadian Journal of Zoology* **90***, 1386-1393.

Hiroi, J. and McCormick, S. D. (2012). New insights into gill ionocyte and ion transporter function in euryhaline and diadromous fish. *Respiratory Physiology & Neurobiology* **184**, 257-268.

Hwang P. P., Lee T. H. and Lin, L. Y. (2011). Ion regulation in fish gills: recent progress in the cellular and molecular mechanisms. *American Journal of Physiology Regulatory, Integrative and Comparative Physiology* **301**, 28-47.

Ishikawa, A., Jusakabe, M., Kume, M. and Kitano, J. (2016). Comparison of freshwater tolerance during spawning migration between two sympatric Japanese

marine threespine stickleback species. *Evolutionary Ecology Research* **17**, 525-534.

Jones, F., Brown, C., Pemberton, J. and Braithwaite, V. (2006). Reproductive isonaltion in a threespine stickleback hybrid zone. *Journal of Evolutionary Biology* **19**, 1531-1544.

Kassen, R., Schluter, D. and McPhail J. D. (1995). Evolutionary history of threespine sticklebacks (*Gasterosteus spp.*) in British Columbia: insights from a physiological clock. *Canadian Journal of Zoology* **73**, 2154-2158.

Kawahara, R., Miya, M., Mabuchi, K., Near, T. J. and Nishida, M. (2009). Stickleback phylogenies resolved: Evidence from mitochondrial genomes and 11 nuclear genes. *Molecular Phylogenetics and Evolution* **50**, 401-404.

Kültz, D. (2015). Physiological mechanisms used by fish to cope with salinity stress. *Journal of Experimental Biology* **218(12)**, 1907-1914.

Le Bris, A. and Wroblewski J. S. (2018). Species composition and habitat preferences of the nearshore fish fauna of Bonne Bay, Newfoundland. *Marine Biodiversity Records* **11(12).**

Lee, C. E. and Bell, M. A. (1999). Causes and consequences of recent freshwater invasions by saltwater animals. *Trends in Ecology & Evolution* **14(7),** 284-288.

Losos, J. B. (2011). Convergence, adaptation, and constraint. *Evolution* **65(7)**, *1827-1840.*

Marshall, W. S. (2013). *Osmoregulation in Estuarine and Intertidal Fishes*. In *Fish Physiology* (eds S.D. McCormick, A.P. Farrell and C.J. Brauner), pp. 396–423. Elsevier Inc.

McCairns, R. J. S. and Bernatchez, L. (2010). Adaptive divergence between freshwater

    and marine sticklebacks: Insights into the role of phenotypic plasticity from an

    integrated analysis of candidate gene expression. *Evolution* **64(4)**, 1029-1047.

McCleave, J. D., Zydlewski, G. B. and Vieser, J. D. (2018). Biology of four sympatric

    sticklebacks in Cobscook Bay, Maine, a boreal, macrotidal bay. *Environmental*

    Biology of Fishes **101**, 1443–1465.

McCormick, S. D., Farrell, A. P. and Brauner, C. J. (Eds.). (2013). *Euryhaline Fishes*,

    pp xi-xii. New York: Academic Press.

Moyes, C. D. and Schulte, P. M. (2016). *Ion and water balance.* In *Principles of*

    Physiology, pp. 549-559. Toronto: Pearson Canada, Inc.

Nelson, J.S. (1968). Salinity tolerance of brook sticklebacks*, Culaea inconstans*,

    freshwater ninespine sticklebacks, *Pungitius pungitius,* and freshwater fourspine

    sticklebacks, *Apeltes quadracus*. *Canadian Journal of Zoology* **46**, 663-667.

Piersma, T. and Drent, J. (2003). Phenotypic flexibility and the evolution of organismal

    design. *Trends in Ecology & Evolution* **18(5)**, 228-233.

Rosenblum, E. B., Parent, C. E. and Brandt, E. E. (2014). The molecular basis of

    phenotypic convergence. *Annual Review of Ecology, Evolution, and Systematics*

    45, 203-226

Rosewarne, P. J., Wilson, J. M. and Svendsen, J. C. (2016). Measuring maximum and

    standard metabolic rates using intermittent-flow respirometry: a student laboratory

    investigation of metabolic scope and environmental hypoxia in aquatic breathers.

    Journal of Fish Biology **88(1)**, 265–283.

Rowland, W.T. (1974). Reproductive behaviour of the fourspine stickleback, *Apeltes*

    quadracus. *Copeia* **1**, 183-194.

Rowland, W. T. (1983). Interspecific aggression in sticklebacks: *Gasterosteus aculeatus* displaces *Apeltes quadracus*. *Copeia* **1983(2)**, 541-544.

Schultz, E. T. and McCormick, S. D. (2013). Euryhalinity in an evolutionary context. In *Euryhaline Fishes* (eds S.D. McCormick, A.P. Farrell and C.J. Brauner), pp. 477–533. New York: Academic Press.

Scott, G. R., Rogers, J. T., Richards, J. G., Wood, C. M. and Schulte, P. M. (2004). Intraspecific divergence of ionoregulatory physiology in the euryhaline teleost *Fundulus heteroclitus*: possible mechanisms of freshwater adaptation. *Journal of Experimental Biology* **207**, 3399–3410.

Shimada, Y., Shikano, T. and Merilä, J. (2011). A High Incidence of Selection on Physiologically Important Genes in the Three-Spined Stickleback, *Gasterosteus aculeatus*. *Molecular Biology and Evolution* **28(1)**,181–193.

Somero, G. N., Lockwood, B. L. and Tomanek, L. (2017). A Conceptual Foundation for Understanding Biochemical Adaptation. In *Biochemical Adaptation Response to Environmental Challenges from Life's Origins to the Anthropocene,* pp 1-27. Sunderland MA: Sinauer Associates.

Tseng, Y. C. and Hwang, P. P. (2008). Some insights into energy metabolism for osmoregulation in fish. *Comparative Biochemistry and Physiology Part C* **148**, 419-429.

van Vliet, W. H. (1970). Shore and freshwater fish collections from Newfoundland. Natl. Mus. Nat. Sci. (Ottawa) *Publications in Zoology* **3**, l-30.

Velotta, J. P., Wegrzyn, J. L., Ginzburg, S., Kang, L., Czesny, S., O'Neill, R. J., McCormick, S. D., Michalak, P. and Schultz, E. T. (2017). Transcriptomic

imprints of adaptation to fresh water: parallel evolution of osmoregulatory gene expression in the Alewife. *Molecular Ecology* **26(3)**, 831–848.

Whitehead, A. (2010). The evolutionary radiation of diverse osmotolerant physiologies in Killifish *(Fundulus* sp.). *Evolution* **64(7)**, 2070-2085.

Whitehead, A., Roach, J. L., Zhang, S., and Galvez, F. (2011). Genomic mechanisms of evolved physiological plasticity in Killifish distributed along an environmental salinity gradient. *Proceedings of the National Academy of Sciences* **108(15)**, 6193– 6198.

Worgan, J. P. and FitzGerald, G. J. (1981). Habitat segregation in a salt marsh among adult sticklebacks. *Environmental Biology of Fishes* **6(1)**, 105-109.

Woods, A. H. and Harrison, J. F. (2002) Interpreting rejections of the beneficial acclimation hypothesis: when is physiological plasticity adaptive? *Evolution* **56**, 1863–1866.