PHENOTYPIC PLASTICITY AND DIVERGENCE IN PHYSIOLOGICAL TRAITS DURING FRESHWATER COLONIZATION IN THREESPINE STICKLEBACK (*GASTEROSTEUS ACULEATUS*)

by

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Abstract

Colonization of new environments exposes organisms to novel combinations of abiotic factors that have the potential to negatively affect fitness. Organisms may be able to cope with these changes in abiotic factors using existing phenotypic plasticity, or the novel environment may drive adaptive divergence, but the role of phenotypic plasticity in assisting or hindering the process of local adaptation remains unclear. This dissertation contributes to addressing this topic by examining the interactive effects of multiple abiotic factors on phenotypic plasticity and the evolution of physiological traits, which is an area that has received relatively little study. Specifically, I explored the roles of salinity and temperature in driving divergence during freshwater colonization using marine, anadromous, and derived freshwater populations of the threespine stickleback, *Gasterosteus aculeatus*. In north-temperate freshwater habitats, stickleback experience a combination of low salinity and low winter temperatures that is not experienced by the ancestral marine and anadromous forms which overwinter at sea. Overall, the results of this work are consistent with adaptive evolution in response to the interactive effects of low salinity and low temperature during freshwater colonization. My results showed that both salinity and temperature, and the interaction between them, had stronger negative effects on the growth of marine and anadromous populations compared to the freshwater population. Using a whole-transcriptome approach, I also detected differentiation in gene expression patterns between populations, particularly in processes important for changes in gill structure and permeability. Based on these data I hypothesize that freshwater stickleback have less permeable gills in fresh water, which may result in less energy use for osmoregulation, providing a physiological mode by which freshwater stickleback

save energy, resulting in superior growth in cold fresh water. Both marine and freshwater stickleback showed interactive effects of low temperature and salinity on gill morphology, and marine stickleback exhibited substantial increases in the expression of Na^+,K^+-ATPase in cold fresh water, whereas more modest responses were observed in the freshwater ecotype, which may indicate increased energetic costs of osmoregulation in the marine population and potentially contribute to the growth deficits exhibited by these fish in cold fresh water.

Preface

Chapter 2

The experiments performed in Chapter 2 of the thesis were published as the article titled "*Responses to simulated winter conditions differ between threespine stickleback ecotypes*" in the journal *Molecular Ecology*. The design of the experiments for this publication and the interpretation of the data were performed equally by Patricia M. Schulte (my supervisor and co-author) and I. I performed all the experiments for this study, and Seth M. Rudman (co-author) assisted with the data analysis and interpretation. All of the co-authors contributed equally to drafting the manuscript for this publication.

Chapter 3

The experiments performed in Chapter 3 of the thesis have been accepted for publication as the article titled "*Gene expression plasticity in response to salinity acclimation in threespine stickleback ecotypes from different salinity habitats*" in the journal *Molecular Ecology*. Patricia M. Schulte (my supervisor and co-author) and I designed the experiment, with input from David C. H. Metzger (co-author) and Timothy M. Healy (co-author). I performed all the experiments for this study. David C. H. Metzger and Timothy M. Healy analyzed the RNA-seq data, and I analyzed the qRT-PCR data. All authors contributed equally to the data interpretation. Patricia M. Schulte and I wrote the manuscript, with input from David C. H. Metzger and Timothy M. Healy.

Chapter 4

The experiments performed in Chapter 4 of the thesis are in preparation for submission for publication. Patricia M. Schulte (my supervisor and co-author) and I designed the experiment. I performed all the experiments for this study. Tara L. McBryan (co-author) performed the gill microscopy and analyzed the gill morphology data, and I analyzed the qRT-PCR data. Patricia M. Schulte and I interpreted the data, with input from Tara L. McBryan. Patricia M. Schulte and I wrote the manuscript.

Chapter 5

The experiments performed in Chapter 5 of the thesis are in preparation for submission for publication. The design of the experiments and the interpretation of the data were performed equally by Patricia M. Schulte (my supervisor and co-author), Seth M. Rudman (co-author), and I. Seth M. Rudman and I performed all the experiments for this study, and Seth M. Rudman performed the data analysis. All of the co-authors contributed equally to drafting the manuscript for this publication.

All fish husbandry and experimentation for Chapters 2-5 were conducted under approved animal care and breeding protocols (A10-0285 and A11-0372) in compliance with the regulations of the Canadian Council of Animal Care.

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List of Abbreviations

cDNA – complementary DNA

$[Cl^-]$ – chloride concentration

CTmin – critical thermal minimum

DD – degree-days

DNA – deoxyribonucleic acid

ILCM – interlamellar cell mass

LC – Little Campbell River

OL – Oyster Lagoon

ppt – parts per thousand

qRT-PCR – quantitative real-time polymerase chain reaction

RNA – ribonucleic acid

RNA-seq – RNA sequencing

SEM – standard error of the mean

SGR – specific growth rate

TL – Trout Lake

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It was definitely a journey to make it to this point, and I have so many people to thank for helping me along the way. I am extremely lucky to have been afforded the opportunity of being a part of the Department of Zoology and the Comparative Physiology group at the University of British Columbia. The collaborative and supportive environment fostered here is truly one-of-a-kind, and I am so glad I was able to call this place home for my degree.

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Dedication

For Nana, Grandpa, & Grandpop

Chapter 1: Introduction

Plasticity and the colonization of new environments

It is relatively straightforward to survey populations or species that reside in distinct (and often adjacent) habitats, and identify the differentiated traits that might confer adaptive value in their local environment. Determining how the colonization of a new habitat initially occurred and led to this putatively adaptive divergence, however, can be challenging. Clearly, the initial colonists had to be able to tolerate the changes imposed by the foreign environment, either through tolerance due to standing genetic variation or by way of adaptive plasticity.

Although a considerable amount of research has concentrated on the genetic differences associated with divergent natural selection and local ecological adaptation to new habitats, phenotypic plasticity may have also played an important role in colonization and subsequent adaptation to novel habitats. However, the role of plasticity in this adaptation remains unclear (Mery & Kawecki 2004; Paenke *et al*. 2007; Ghalambor *et al*. 2007, 2015). Adaptive plasticity may confer survival in a new environment, facilitating natural selection and adaptive evolution (Hinton & Nowlan 1987; Fontanari & Meir 1990; Ghalambor *et al*. 2007). However, other work suggests that adaptive plasticity actually slows adaptive evolution by weakening the strength of directional selection (Wright 1931; Anderson 1995; Ancel 2000). Regardless, it is likely that phenotypic plasticity plays a role in the colonization of new environments, as well as influencing how evolution proceeds after the initial colonization (Pfennig *et al*. 2010; Ghalambor *et al*. 2015; Schneider & Meyer 2017). Furthermore, phenotypic plasticity

itself can be subject to evolutionary processes after colonization, resulting in a variety of processes and patterns (Pigliucci *et al*. 2006; Crispo 2007).

Colonization of freshwater habitats

Differences in abiotic factors can pose barriers to the colonization of new habitats (Dunson & Travis 1991; Jackson *et al*. 2001; Holway *et al*. 2002; Sexton *et al*. 2009), as many organisms are unable to cope with the changes in single or multiple abiotic factors associated with transitioning from one habitat to another. One such major transition is the move from the ocean to fresh water. The interface between these two habitats presents a major barrier to movement, but many organisms have nonetheless been able to make this transition and have successfully colonized freshwater habitats from the marine environment (Lee & Bell 1999). By investigating these colonization events, we are able to gain insight into the evolutionary processes that have occurred since the initial invasion of these habitats, providing us with clues as to how species adapted to and continue to function in freshwater habitats.

Salinity forms a barrier to colonization by influencing osmoregulation

Seawater and fresh water differ greatly in their concentrations of major ions. Seawater has an osmotic pressure of approximately 1,000 mOsm, while the osmotic pressure of fresh water is much lower, roughly 0.5-15 mOsm (Hill *et al*. 2008). These osmotic and ionic differences pose major challenges for organisms. For example, osmoconforming organisms, such as many aquatic invertebrates, allow the osmolarity of their internal body fluids to track that of the environment. Thus, moving from seawater to

fresh water will cause a large change in the osmotic gradient between the body fluids and intracellular spaces (Hill *et al*. 2008; Bradley 2009), and thus there are no euryhaline osmoconformers. Osmoregulating organisms such as teleost fishes, on the other hand, maintain a relatively stable body fluid osmolarity regardless of external osmolarity, and thus face a large change in osmotic gradient between the external environment and the blood (Hill *et al*. 2008; Bradley 2009). For example, freshwater teleosts are hyperosmotic to the surrounding freshwater of their environment, and maintain an internal osmolality of approximately 250-350 mOsm (Hill *et al*. 2008). Because the osmotic pressure of their blood plasma is higher than that of the surrounding medium, these freshwater fishes face two major problems: 1) gain of water by osmosis, and 2) loss of ions by diffusion (Evans *et al*. 2005; Hill *et al*. 2008; Hwang *et al*. 2011). In contrast, teleosts living in seawater are hyposmotic to the surrounding water of their environment, and maintain an internal osmolality of approximately 300-500 mOsm (Hill *et al*. 2008). Since the osmotic pressure of their blood plasma is sufficiently lower than that of the surrounding seawater, seawater fishes are confronted with the opposite problems of freshwater fishes: 1) loss of water by osmosis, and 2) gain of ions by diffusion (Evans *et al*. 2005; Hill *et al*. 2008; Evans 2011a,b). In teleosts, the main site of water gain and ion loss in fresh water, and water loss and ion gain in seawater, is the gill (Evans *et al*. 2005; Evans & Claiborne 2006). Specifically, the gill epithelium presents a large surface area that renders fish vulnerable to water gain and loss by osmosis and gain and loss of ions by diffusion (Evans & Claiborne 2006).

Since teleosts are osmoregulators, they have the capacity to offset these problems, and are able to maintain a constant osmotic pressure of their blood plasma independent of

the external environment (Hill *et al*. 2008). In fresh water, fish drink very little water (less than in seawater) and produce dilute urine in order to offset diffusive water gain (Hill *et al*. 2008; Hwang 2011). To combat the problem of diffusive ion loss, ions must be actively transported back into the blood plasma against the steep ionic concentration gradient with the fresh water (Hwang *et al*. 2011; Dymowska *et al*. 2012). Therefore, regulation of the composition of the blood in the face of water gain and ion loss is an active process that requires energy (Evans & Claiborne 2006; Hill *et al*. 2008). This ion transport occurs at cells called ionocytes or mitochondrion-rich cells, which are located on the epithelium of the gill filaments (Evans & Claiborne 2006; Hwang 2011; Hwang *et al*. 2011; Dymowska *et al*. 2012). In particular, these cells require energy to actively pump Na^+, Cl^-, and Ca^{2+} from the external freshwater environment across the gill epithelium and back into the blood, while also pumping H^+ and HCO_3^- from the blood and into the external environment in order to maintain internal pH balance and to serve as counter ions for ion regulation (Evans & Claiborne 2006; Hwang 2011; Hwang *et al*. 2011; Dymowska *et al*. 2012). In order to accomplish this ion regulation, ionocytes possess ion transporters that allow transepithelial movement of these ions (Hwang 2011; Hwang *et al*. 2011; Dymowska *et al*. 2012). These ion transporters are located on the apical and basolateral membranes of the ionocytes, enabling movement of ions between the external environment, cytoplasm of the ionocyte, and the blood plasma (Hwang 2011; Hwang *et al*. 2011; Dymowska *et al*. 2012). Extensive research has identified multiple freshwater ionocyte subtypes that vary in the specific composition and location of ion transporters depending on the fish species (Hwang *et al*. 2011; Dymowska *et al*. 2012). While there are many differences between these ionocyte subtypes, the ion transporters

themselves can be separated into three basic categories depending on which ions they move. Ion transporters associated with the regulation of Na^+ uptake and acid secretion include the Na^+/H^+ exchanger (NHE), Na^+/Cl^- co-transporter (NCC), Na^+,K^+-ATPase (NKA), Na^+/HCO_3^- co-transporter (NBC), H^+-ATPase (HA), and the epithelial Na^+ channel (ENaC) (Hwang *et al*. 2011; Dymowska *et al*. 2012). However, there is debate about the precise pathways for sodium uptake in fresh water, which may vary among species and habitats, as the set of transporters required to drive Na^+ uptake may be different at very low concentrations of external ions to maintain thermodynamically favorable sodium uptake (Parks *et al*. 2008). Transporters known or hypothesized to be involved in Cl^- uptake and base secretion include the Na^+/Cl^- co-transporter (NCC), an anion exchanger (AE), and SLC26a anion exchanger family members (Hwang *et al*. 2011; Dymowska *et al*. 2012). Lastly, transporters known or hypothesized to be involved in Ca^{2+} uptake include the epithelial Ca^{2+} channel (ECaC), plasma membrane Ca^{2+}-ATPase (PMCA), and Na^+/Ca^{2+} exchanger (NCX) (Hwang *et al*. 2011; Dymowska *et al*. 2012).

In seawater, fish drink seawater to regain water lost by osmosis, and have low rates of urine production to prevent further water loss by this means (Evans *et al*. 2005; Evans 2011a). By drinking seawater, though, these fish gain additional ions, adding to the ions gained from seawater by diffusion (Evans *et al*. 2005; Evans 2011a,b). As marine teleosts are unable to produce urine that is hyperosmotic to blood plasma, energy is used to actively secrete ions back into the hyperosmotic seawater environment (Evans *et al*. 2005; Hill *et al*. 2008; Evans 2011a,b). Specifically, Cl^- is actively secreted from the blood into the external seawater environment by ionocytes, while Na^+ secretion at this

location occurs via a paracellular pathway with passive diffusion down the electrical and concentration gradients established by the gill epithelium (Evans & Claiborne 2006). In comparison to the multiple freshwater ionocyte subtypes and differences in ion transporter composition and location between species, seawater ionocyte composition and function is not as variable among species. Specifically, seawater ionocytes express these major ion transporters: cystic fibrosis transmembrane conductance regulator (CFTR) on the apical membrane, and Na^+,K^+-ATPase (NKA), Na^+-K^+-$2Cl^-$ co transporter (NKCC), and a K^+ channel that are all located on the basolateral membrane (Evans 2011b).

In addition to adjusting the expression of ion transporters to maintain homeostasis in the face of diffusive ion movement, fish are also able to modulate the permeability or "leakiness" of their gills, regulating ion movement into or out of their blood between ionocytes. For example, increasing the expression of cell tight junction proteins such as claudins and occludins in fresh water decreases paracellular ion permeability, leading to a reduction in diffusive ion loss to this dilute environment (Furuse *et al*. 1993; Van Itallie & Anderson 2006; Brennan *et al*. 2015). Similarly, fish are also able to remodel the morphology of their gills. Morphology of the gill is critically important to managing energy budgets in response to environmental changes. Recent work has shown how some fish modify the size of the gill interlamellar cell mass (ILCM) in response to stressors such as salinity, temperature, oxygen levels, and exercise (Sollid *et al*. 2003, 2005; Mitrovic & Perry 2009; LeBlanc *et al*. 2010; Brauner *et al*. 2011; Fu *et al*. 2011; Nilsson *et al*. 2012; Perry *et al*. 2012; Barnes *et al*. 2014). This ability to adjust gill morphology by increasing or decreasing the size of the ILCM has the potential to save energy required

for processes such as osmoregulation by reducing ion flux due to increases in the diffusion distance across the gill.

Other factors that differ between freshwater and marine environments

Coupled with the salinity differences between the ocean and fresh water, there are also major differences in nutrient availability and biotic interactions between these environments. Specifically, aquatic productivity levels at temperate latitudes are higher in the ocean, while at tropical latitudes they are higher in fresh water (Gross *et al*. 1988). So, even if potential freshwater colonists can withstand the drastic salinity changes experienced when invading a new habitat, coping with subsequent differences in food availability could also exert strong effects on these fish. Furthermore, upon invasion of freshwater habitats, fish may be faced with novel interactions such as competition with new species and differences in predation pressure (Schultz & McCormick 2013).

In addition to salinity, there is another major abiotic factor that differs between the ocean and fresh water at temperate latitudes, and creates a distinct physiological challenge to colonizing fresh water. Specifically, the temperature of freshwater habitats in temperate regions is more variable than the temperature of salt water (Lee & Bell 1999). Temperature is a critical factor for fish, because as poikilothermic ectotherms most fishes do not regulate their internal body temperature, which is instead determined by the temperature of their environment (Hochachka & Somero 2002; Schulte 2011). Therefore, water temperature presents physiological challenges for fish because changes in water temperature in turn directly influence many physiological functions such as rates of chemical reactions and membrane fluidity, as well as the rates of critical functions

such as feeding, growth, respiration, and locomotion (Hochachka & Somero 2002; Moyes & Ballantyne 2011). In the context of freshwater colonization, this connection between salinity and temperature differences in the temperate zone presents a distinct physiological challenge for freshwater colonists in this region. In order to make a successful colonization of freshwater habitats, fish would be required to tolerate the novel, interactive effects of low salinity (influencing ionoregulation) and variable temperatures (influencing rate processes) that were not experienced in their more thermally stable, hyperosmotic ancestral marine environment.

Anadromy and the evolution of freshwater residency

While these differences in salinity, temperature, and nutrient availability between salt and fresh water present major physiological challenges to fish, there are many diadromous fish species that have evolved the ability to tolerate these differences and move between the ocean and fresh water (Gross *et al*. 1988; Schultz & McCormick 2013). Diverse research on north temperate fishes such as brown trout (*Salmo trutta*), Arctic char (*Salvelinus alpinus*), and rainbow trout (*Oncorhynchus mykiss*) supports the hypothesis that differences in salinity, temperature and nutrient availability, and interactions between them, influence migration patterns between these two environments (Gross *et al*. 1988; Rikardsen *et al*. 2000, 2006, McDowall 2001, 2008; Svenning & Gullestad 2002; Thomsen *et al*. 2007; Jensen & Rikardsen 2008; Finstad & Hein 2012; McMillan *et al*. 2012). The higher levels of aquatic productivity in the ocean at temperate latitudes correlate with the general distribution of anadromous fishes: there are more anadromous species at temperate latitudes (Gross *et al*. 1988). Accordingly, research

provides support to the hypothesis that the low productivity of fresh water is a barrier to year-round survival, necessitating migrations to the ocean where food is more abundant (Gross *et al*. 1988; Rikardsen *et al*. 2006; Jensen & Rikardsen 2008), particularly in winter. There are exceptions, however, as populations of anadromous Arctic char migrate to the ocean in the summer to feed, yet overwinter in cold, nutrient-poor freshwater lakes (Rikardsen *et al*. 2000; Svenning & Gullestad 2002).

However, the more common pattern at high latitudes is that anadromous fishes tend to migrate to the ocean before the cold, low-productivity, winter months (McDowall 2008). In the temperate zone, freshwater habitats become colder than the ocean in the winter (Lee & Bell 1999; Willmer *et al*. 2005; Barrett *et al*. 2011). Thus, marine habitats are not only more productive than freshwater habitats, in the north-temperate zone they also do not present the combined challenge of low salinity and low temperature that would occur during the winter months in fresh water (McDowall 2001, 2008).

In the context of permanent colonization of freshwater habitats from the ocean, fish will not only face the novel ionoregulatory challenge that comes with crossing the salinity interface between these habitats, but in order to remain in fresh water year-round they must also perform critical physiological functions that will be directly affected by freshwater temperatures that are colder than any they previously faced in the ocean. It has been shown that the combination of low salinity and low temperature poses a challenge to ionoregulation (Buhariwalla *et al*. 2012) and survival (Schaarschmidt *et al*. 1999), so it is likely that this combination may be a major physiological barrier to the colonization of freshwater habitats in the temperate zone. Furthermore, traits such as growth, cold tolerance, and osmoregulatory gene expression are all affected by changes in

temperature, as they each exhibit plasticity in response to temperature change (Rombough 1997; Beitinger *et al*. 2000; Buhariwalla *et al*. 2012; Handeland *et al*. 2014). In general for fish, high juvenile growth rate is beneficial because it results in higher overwintering survival, higher reproductive potential, and a decreased risk of predation (Arendt 1997; Marchinko & Schluter 2007). Therefore, the effect of decreased growth rates due to low temperature would also be likely to inhibit the ability to colonize fresh water from the ocean.

A model for studying freshwater colonization: the threespine stickleback

The threespine stickleback, *Gasterosteus aculeatus*, is a species with numerous characteristics that allow questions related to freshwater colonization to be addressed. Following the melting and recession of Pleistocene glaciers 10,000-20,000 years ago, a marine or anadromous ancestor invaded and adapted to freshwater habitats (Haglund *et al*. 1992; Bell & Foster 1994; McPhail 1994; Orti *et al*. 1994; Taylor & McPhail 1999, 2000; McKinnon *et al*. 2004; Colosimo *et al*. 2005; Boughman 2007; Jones *et al*. 2012a,b; Kitano *et al*. 2012). More specifically, there was parallel evolution in morphology, physiology, and behavioral traits throughout its distribution during the colonization of fresh water, which has resulted in this species being a model for the study of adaptive divergence (Baker 1994; McPhail 1994; McKinnon & Rundle 2002; McKinnon *et al*. 2004; Colosimo *et al*. 2005; Schluter 2009; Jones *et al*. 2012a,b). Additionally, the ancestral marine and anadromous stickleback are still present today, which makes it relatively easy to study ancestral and derived forms side by side in an experimental setting.

Of the many changes that occurred between marine and freshwater ecotypes, several have major implications when thinking about the selective pressures that may have been present during the colonization of fresh water in this species. For example, repeated morphological changes, such as reduction in armor plating, occurred following freshwater colonization and one of the major factors that may have driven these changes is differences in predators between environments (Colosimo *et al*. 2005; Marchinko 2009). In addition, previous work has also shown that growth has diverged between marine and freshwater stickleback ecotypes (Marchinko & Schluter 2007; Barrett *et al*. 2008, 2009). Growth is a very important trait in stickleback, as body length is positively correlated with reproductive output in this species (Wootton 1984; Schluter 1995). In fresh water, low plated freshwater stickleback have higher juvenile growth rates than completely plated marine stickleback (Marchinko & Schluter 2007). Further work has shown that selection on growth in stickleback is likely to have been present and played a role in the evolution of many of the differences between marine and freshwater ecotypes (Marchinko & Schluter 2007; Barrett *et al*. 2008, 2009). In coastal British Columbia, where marine, anadromous, and freshwater stickleback are all present, freshwater lakes become colder than the ocean in the winter (Barrett *et al*. 2011). This is of note because studies have shown that low temperature and short day-length reduce the capacity for growth in stickleback (Allen & Wootton 1982), which can decrease fecundity (Wootton 1984; Schluter 1995). Perhaps not surprisingly, cold tolerance has diverged between ecotypes, with freshwater stickleback exhibiting superior cold tolerance than marine stickleback (Barrett *et al*. 2011).

In addition to these studies on growth and cold tolerance, there has also been parallel divergence in the sequence and expression of many osmoregulatory genes critical for coping with salinity changes (McCairns & Bernatchez 2010; Shimada *et al*. 2011; Jones *et al*. 2012a,b, DeFaveri *et al*. 2013a,b). As noted earlier, the combination of low salinity and low temperature presented by fresh water in the winter is a novel stressor that may have been a barrier to the colonization of fresh water. Accordingly, work investigating the effects of low salinity and low temperature on ionoregulation in stickleback observed high mortality of brackish water stickleback (Schaarschmidt *et al*. 1999). This has direct implications to the freshwater habitats that were colonized in northern temperate zone areas like those in British Columbia because here the ancestral anadromous stickleback leave fresh water to return to the ocean in the early fall, prior to the onset of cold winter water temperatures (Hagen 1967). Therefore, these differences between ecotypes in osmoregulatory abilities, cold tolerance, and growth provide support for the idea that the combination of low salinity and low temperature may have posed a challenge to the colonization of fresh water for the threespine stickleback. The ancestral colonizers would have had to tolerate fresh water, grow, and overwinter through novel cold temperatures in order to survive and reproduce in the spring.

Colonization of fresh water by stickleback: questions addressed in this dissertation

Although some work on stickleback has investigated differences in physiological traits between ecotypes (Schaarschmidt *et al*. 1999; Barrett *et al*. 2008, 2009, 2011; McCairns & Bernatchez 2010; Jones *et al*. 2012b; Spence *et al*. 2012), much more has focused on divergence in morphological traits, such as lateral plate morphology (see

Colosimo *et al*. (2005) and Marchinko (2009)). Although biotic factors such as predation influence these differences in morphology between ecotypes (Marchinko 2009), the focus of my work centers on the evolution of physiological traits and their impact on freshwater colonization in stickleback. Due to the lack of work investigating the evolution of physiological traits in stickleback and how it relates to freshwater colonization in this species, the most logical place to start is by investigating two environmental factors that differ between the ocean and fresh water and that have major influences on physiology: salinity and temperature. The experiments carried out in this thesis focus on answering questions centered on how salinity (which directly affects ionoregulation), temperature (which directly affects rate processes), and their interaction influence the evolution of physiological traits in stickleback and how these impacts relate to the colonization of fresh water in this species.

Question #1: Do stickleback exhibit plasticity in cold tolerance, growth, and osmoregulatory gene expression in response to cold temperature in fresh water, and are there differences in plasticity between marine and freshwater ecotypes?

Growth, cold tolerance, and osmoregulatory gene expression exhibit plasticity in response to low temperature in many fish species (Rombough 1997; Beitinger *et al*. 2000; Handeland *et al*. 2014), and plasticity in these traits in response to temperature in stickleback could have a great impact on survival in fresh water and colonization of freshwater habitats. Although separate studies have shown how stickleback ecotypes differ in cold tolerance (Barrett *et al*. 2011), growth with respect to salinity (Marchinko &

Schluter 2007), and how low temperature and short day-length decrease growth in general in stickleback (Allen & Wootton 1982), there is much less work aimed at investigating phenotypic plasticity in stickleback and determining its possible role in the adaptation to fresh water.

In addition to the lack of studies on phenotypic plasticity in stickleback, there has been very little work investigating the basic physiology of ionoregulation in stickleback (Schaarschmidt *et al*. 1999; McCairns & Bernatchez 2010), especially when looking at the combined effects of low salinity and low temperature on ionoregulation (Schaarschmidt *et al*. 1999). However, we do know that the combination of low salinity and low temperature results in high mortality in brackish water stickleback (Schaarschmidt *et al*. 1999), which may relate to differences in osmoregulatory mechanisms and/or osmoregulatory plasticity in response to low salinity and low temperature between ecotypes.

The work listed above documents divergence in traits (such as cold tolerance and growth) in stickleback, yet without investigating the effects of acclimation to cold winter conditions in fresh water, we cannot truly delineate whether these phenotypic differences are actually evolved differences or simply plastic changes. Therefore, in this chapter of my thesis (Chapter 2) I determined whether stickleback ecotypes exhibit evolved differences and/or differential plasticity in cold tolerance, growth, and osmoregulatory gene expression. This information allowed the determination of whether cold winter conditions in fresh water presented a physiological challenge to the colonization of freshwater habitats. In addition to filling this knowledge gap, these results provided new insight into whether plasticity facilitates or constrains local adaptation to new

environments (Wright 1931; Hinton & Nowlan 1987; Fontanari & Meir 1990; Anderson 1995; Ancel 2000; Mery & Kawecki 2004; Pigliucci *et al*. 2006; Crispo 2007; Paenke *et al*. 2007; Ghalambor *et al*. 2007, 2015; Pfennig *et al*. 2010), which is necessary information for determining how stickleback have colonized and adapted to freshwater habitats.

Question #2: Do changes in salinity influence patterns of gene expression plasticity in the gill across the entire transcriptome?

Salinity is the clearest and most obvious abiotic difference between saltwater and freshwater habitats, and thus this chapter of my thesis (Chapter 3) focuses on the responses of threespine stickleback to salinity change, independent of other factors. Threespine stickleback are euryhaline fish that have the capacity to transition between salt water and fresh water, and in this chapter I examined variation between marine and freshwater ecotypes in the physiological response to transfer between these two conditions.

Chapter 2 of my thesis enabled the determination of how a limited number of osmoregulatory genes varied in expression between ancestral and derived stickleback ecotypes in response to winter conditions in fresh water. Before my work there was no information on how overall gene expression differs between ecotypes of this euryhaline species in salt water versus fresh water. Therefore, in Chapter 3 of my thesis I assessed phenotypic plasticity's role in the adaptation of stickleback to fresh water by examining variation in gene expression in the gill of marine and freshwater stickleback in salt water

versus fresh water using RNA-seq. This facilitated the first examination of broad patterns of gene expression plasticity across the entire transcriptome in marine and freshwater ecotypes in response to salinity change.

Following the discovery of differential expression of several gill ionocyte ion transporters between salinities, I examined the expression of these specific genes using quantitative real-time PCR (qRT-PCR). This allowed me to better describe the shape of the reaction norm of gene expression for these candidate ion transporters at five different salinities, identifying fine-scale patterns of exactly where along the salinity spectrum gene expression changed. This combination of techniques not only allowed thorough comparison of gene expression plasticity between marine and freshwater ecotypes in the context of freshwater colonization, but also provided evidence as to whether evolutionary processes have shaped patterns of gene expression plasticity in the gill after colonization (Pigliucci *et al*. 2006; Crispo 2007).

RNA-seq provides an effective and efficient method to obtain an unbiased survey of changes in mRNA levels in response to salinity change, and is an excellent tool for developing hypotheses regarding the physiological mechanisms involved in coping with salinity change, as mRNA levels often correlate with protein levels (Koussounadis *et al*. 2015). However, it is important to keep in mind that mRNA levels, protein amounts, and protein activities are not always correlated (Chen *et al*. 2002; Greenbaum *et al*. 2003; Pascal *et al*. 2008; de Sousa Abreu *et al*. 2009; Vogel & Marcotte 2012; Koussounadis *et al*. 2015). Therefore, RNA expression patterns provide hypotheses that can subsequently be tested by measuring the protein levels and activities of the protein products of these genes.

Question #3: Did the interactive effects of low salinity and low temperature present a physiological challenge to freshwater colonization by stickleback?

In British Columbia, anadromous stickleback leave fresh water to migrate to the ocean in the early fall prior to the onset of cold winter temperatures in fresh water (Hagen 1967), avoiding the combination of low salinity and low temperature that is present in freshwater habitats in winter. Although work on other fish species has shown the challenge that the combination of low salinity and low temperature poses for ionoregulation (Buhariwalla *et al*. 2012), only one study has investigated the effects of these factors on stickleback (Schaarschmidt *et al*. 1999). This work showed that the combination of these two factors resulted in high mortality of brackish water stickleback, providing preliminary evidence that the combined effects of low salinity and low temperature may have posed a barrier to the colonization of freshwater habitats from the ocean.

While differential mortality was observed in this previous work (Schaarschmidt *et al*. 1999), in this chapter of my thesis (Chapter 4) I examined the individual and combined effects of low salinity and low temperature on gene expression of critical gill ion transporters in marine, anadromous, and freshwater stickleback. My work provides crucial evidence to better answer the question of whether the combined effects of these factors actually presented a physiological challenge to colonizing fresh water. In addition to this molecular perspective of the gill, I also investigated the effects that low salinity and low temperature have on gill morphology in stickleback. By examining gill gene

expression and morphology in response to the interactive effects of salinity and temperature, I was able to isolate the individual and combined effects of these abiotic factors, differentiating between temperature-induced versus salinity-induced differences between ecotypes. This enabled me to discern whether the combination of low salinity and low temperature posed a physiological challenge to freshwater colonization and if it may have driven adaptive evolution in these important functional gill traits.

Question #4: Do natural changes in photoperiod, salinity, and temperature elicit differences in growth and survival between marine, anadromous, and freshwater stickleback?

The results of Chapters 2-4 of my thesis provided new information about how marine and freshwater stickleback ecotypes exhibit differential plasticity and/or evolved differences in physiological traits critical for freshwater colonization. However, each of these experiments utilized lab conditions that did not incorporate the differences in photoperiod that occur in nature during the transition from spring to winter. Changes in photoperiod are a crucial preparatory signal for initiating modifications in physiology and energy metabolism that facilitate survival through the cold winter months and reproduction in the spring (Beamish 1964; Evans 1984; Bradshaw & Holzapfel 2007; Shuter *et al*. 2012).

Accordingly, the goal of this chapter of my thesis (Chapter 5) was to determine whether the impacts of cold winter conditions on growth (Chapter 2) are also observed when stickleback are exposed to natural photoperiods. Growth rate and body size are

critical traits for fish, as a faster juvenile growth rate results in higher overwintering survival, greater reproductive potential, and decreased risk of predation (Arendt 1997; Marchinko & Schluter 2007). Therefore, in this experiment of my thesis, growth of marine, anadromous, and freshwater stickleback was monitored from hatching in both salt water and fresh water in simulated seasonal conditions that mimicked the temperature and photoperiod that stickleback experience from summer through winter in nature. Although previous work has investigated the effects of salinity (Marchinko & Schluter 2007), and the combination of low temperature and short-day length at low salinity (Allen & Wootton 1982), on stickleback growth, no single experiment has investigated how interactions between salinity, temperature, and photoperiod impact stickleback growth. Consequently, this experiment allowed the determination of differences in growth rate and mortality between ecotypes through seasonal conditions, providing insight into survival and fitness implications, and whether adaptive evolution in growth occurred in response to these factors during freshwater colonization.

Chapter 2: Responses to simulated winter conditions differ between threespine stickleback ecotypes

Introduction

When an organism moves into a new environment it may be faced with a novel combination of abiotic environmental factors that can act as a barrier to colonization (Dunson & Travis 1991; Jackson *et al*. 2001; Holway *et al*. 2002; Sexton *et al*. 2009) and exert divergent natural selection leading to local ecological adaptation (Kawecki & Ebert 2004; Schluter 2009; Keller & Seehausen 2012). Existing phenotypic plasticity has the potential to facilitate the colonization of new environments, but has also been suggested to constrain ecological adaptation; thus, the role of phenotypic plasticity in local adaptation remains unclear (Wright 1931; Hinton & Nowlan 1987; Fontanari & Meir 1990; Anderson 1995; Ancel 2000; Mery & Kawecki 2004; Pigliucci *et al*. 2006; Crispo 2007; Paenke *et al*. 2007; Ghalambor *et al*. 2007, 2015; Pfennig *et al*. 2010).

Here, we use threespine stickleback (*Gasterosteus aculeatus*) to explore the role of plasticity and adaptive divergence during the colonization of novel environments. This species provides one of the best-known examples of local ecological adaptation in vertebrates (Jones *et al*. 2012a,b). Following the last ice age and the retreat of Pleistocene glaciers 10,000 to 20,000 years ago, marine sticklebacks invaded freshwater streams and lakes, adopting a freshwater-resident lifestyle (Bell & Foster 1994; McPhail 1994; Colosimo *et al*. 2005; Boughman 2007; Jones *et al*. 2012a,b). With the colonization of these newly formed freshwater habitats, there has been widespread parallel evolution of morphological, physiological, and behavioral traits in multiple populations of freshwater stickleback which provides strong evidence that these traits are adaptations to freshwater

habitats (Baker 1994; McPhail 1994; McKinnon & Rundle 2002; McKinnon *et al*. 2004; Colosimo *et al*. 2005; Schluter 2009; Jones *et al*. 2012a,b). One of the key advantages of stickleback as a model system is that the putative ancestral forms are still present, making it possible to assess the relationship between plasticity and adaptation using both the ancestral and derived forms (McCairns & Bernatchez 2010; Morris *et al*. 2014).

In stickleback, colonization of fresh water likely occurred via an anadromous ancestor that was capable of moving between salt water and fresh water (Haglund *et al*. 1992; Orti *et al*. 1994; Taylor & McPhail 1999, 2000; McKinnon *et al*. 2004; Colosimo *et al*. 2005; Kitano *et al*. 2012). In British Columbia, and across much of the species range, these anadromous stickleback return to freshwater streams to breed in the spring and juveniles typically migrate to the marine environment in the early fall. As a result, anadromous stickleback only experience the freshwater environment during the summer when water temperatures are warm (Hagen 1967). Ionoregulation in fresh water is particularly challenging in the cold, as some anadromous and euryhaline species exhibit decreased ability to maintain plasma ion levels in cold fresh water (Stanley & Colby 1971; McCormick *et al*. 1997; Buhariwalla *et al*. 2012). Furthermore, freshwater habitats tend to become colder than marine habitats in the winter in the temperate zone (Lee & Bell 1999; Willmer *et al*. 2005). Thus, adaptation to fresh water in this part of the species range may be tied to the ability to osmoregulate in cold fresh water. Surprisingly, there have been relatively few studies on the physiology of ion regulation in stickleback (Schaarschmidt *et al*. 1999; McCairns & Bernatchez 2010) particularly with respect to freshwater ionoregulation in the cold (Schaarschmidt *et al*. 1999).

Here, we assess plasticity and divergence in two whole-organism phenotypes – growth and acute cold tolerance – that have previously been shown to have diverged among stickleback ecotypes (Marchinko & Schluter 2007; Barrett *et al*. 2008, 2009, 2011), and that are known to exhibit substantial plasticity in response to low temperature in a variety of fish species (Rombough 1997; Beitinger *et al*. 2000). For example, low temperatures and short day-length reduce the capacity for growth in fish, including stickleback (Allen & Wootton 1982), which could have important negative consequences because size at first reproduction is strongly associated with stickleback fecundity (Wootton 1984; Schluter 1995).

In addition, we examined plasma [Cl^-] as an indicator of osmoregulatory ability, and the expression of a variety of key osmoregulatory genes in the gill because there is evidence of parallel divergence in the sequence and/or expression of these genes in stickleback (McCairns & Bernatchez 2010; Jones *et al*. 2012a,b; DeFaveri *et al*. 2013a,b). Osmoregulatory genes also demonstrate substantial plasticity in response to thermal acclimation in fish (Buhariwalla *et al*. 2012; Handeland *et al*. 2014), suggesting that plasticity could play a role in facilitating or constraining local adaptation for these genes in stickleback. Previous work on stickleback from the Baltic Sea (Schaarschmidt *et al*. 1999) has shown that brackish water stickleback have substantially higher mortality than do freshwater stickleback when exposed to the combination of cold and fresh water, which could relate to differences between ecotypes in osmoregulatory mechanisms or osmoregulatory plasticity in response to temperature change.

These experiments allow us to address the following key questions: 1) Would the combination of cold and fresh water have presented a barrier to colonization of

freshwater habitats by marine stickleback? and 2) Is there evidence in this system that plasticity facilitated or constrained local adaptation to freshwater habitats?

Materials and methods

Stickleback populations & acclimation conditions

Adult stickleback were collected from two sites in British Columbia in May and June of 2010: a marine population from Oyster Lagoon (49°36'43.53"N, 124°01'52.12"W), and a freshwater population from Paxton Lake (49°42'22.37"N, 124°31'24.76"W) on Texada Island (benthic individuals only). These populations were chosen to represent a typical marine phenotype and a highly derived freshwater phenotype, with divergence in fresh water occurring approximately 12,000 years ago (Schluter & McPhail 1992; McPhail 1993; Kassen *et al*. 1995; Taylor & McPhail 2000). Fish were transported to the University of British Columbia and housed in 100 L glass aquaria. All fish were acclimated to a salinity of 2 ± 0.5 ppt (with Instant Ocean® sea salt), a water temperature of 17°C, at a photoperiod of 12L:12D, and were fed daily with a mixture of bloodworms and Mysis shrimp. These fish were used as parents to generate progeny using artificial fertilization as outlined in Marchinko & Schluter (2007).

We made genetic crosses in July-September of 2010 to yield the following families: five marine families (Oyster Lagoon x Oyster Lagoon), five freshwater families (Paxton Lake benthic x Paxton Lake benthic), and four hybrid families (two each of the reciprocal hybrid crosses) as in Barrett *et al*. (2011). Briefly, eggs were fertilized in a petri dish and then transferred to a 500 ml cup with a mesh bottom. Cups were suspended in 100 L glass aquaria. At hatch, the larvae pass through the mesh into the tank. At this

point, the cup and any unhatched eggs were removed. All fish were raised at a salinity of 2 ± 0.5 ppt, a water temperature of 17°C, at a photoperiod of 12L:12D, at a density of approximately 60 fish per tank. Larval fish were fed twice daily with live brine shrimp until they reached a size of approximately 3 cm, at which point they were transitioned to a diet of bloodworms. Fish were fed bloodworms once a day, to satiation. Fish were held under these conditions until March 28, 2011. At this point, approximately 20 individuals from each marine and freshwater family and 40 individuals from each hybrid family were randomly selected from the rearing aquaria and split into two groups (simulated winter and simulated spring treatments). In each treatment, fish were held at equal densities of 20-25 fish per 100 L aquarium at a salinity of 2 ± 0.5 ppt. Each marine and freshwater family was split across two aquaria in each environment with approximately 10-12 freshwater and 10-12 marine individuals housed together in each aquarium. Because hybrid fish are very difficult to reliably distinguish from the other two genotypes by visual inspection, each hybrid family was housed in a single aquarium at a density of 20 fish per 100 L. The environments differed in both temperature and photoperiod, with one simulating spring conditions (14°C; 12L:12D) and one simulating winter conditions (4°C; 9L:15D). To achieve simulated spring and winter conditions, temperature was gradually lowered from 17°C to 14°C or 4°C by 1°C per day and photoperiod was changed in equal increments from 12L:12D to 9L:15D during the same 13-day period. Shortly after transfer to the experimental aquaria, high mortality occurred in one aquarium containing one marine and one freshwater family in the simulated spring condition. Therefore, these two families were removed from the experiment in both the simulated spring and simulated winter conditions. As a result, the total number of

families in the experiment was four marine, four freshwater, and four hybrid families. All fish were exposed to the simulated spring or winter conditions for at least 8 months prior to testing. Mortality was low throughout the experiment and there was no significant difference in mortality between ecotypes or acclimation conditions (Appendix A, Table A1). Fish husbandry and experimentation were conducted under approved animal care and breeding protocols (A10-0285 and A11-0372) according to the regulations of the Canadian Council on Animal Care.

Critical thermal minimum (CTmin) testing

After acclimation to the "spring" and "winter" conditions, a subset of the fish (three fish per family/acclimation condition; four marine and freshwater families, and two families of each reciprocal hybrid cross; 72 fish total) were randomly selected and used to determine acute thermal tolerance. Tolerance was assessed as critical thermal minimum (CTmin), which is defined as the temperature at which a fish can no longer perform coordinated movements (Beitinger *et al.* 2000). Here, we determined CTmin using loss of equilibrium (LOE) as the endpoint, as this metric has been shown to be appropriate for stickleback (Barrett *et al*. 2011).

The CTmin testing apparatus consisted of twelve 1 L beakers suspended in a rectangular plastic water bath. Fish were introduced into the beakers at their acclimation temperature (14°C or 4°C) and acclimation salinity (2 ppt), and after a 15 minute acclimation period to the apparatus, the temperature was lowered at a constant rate of -0.3°C/min. The temperature in the water bath was lowered by adding dry ice to a header tank filled with ethylene glycol, and controlling the flow of this liquid into the water bath.

Each beaker was equipped with a digital thermometer to monitor temperature and aerated with an airstone to prevent thermal stratification. The airstone was removed when the water in the beaker reached 1°C so that the fish could be easily observed to determine the temperature at which they lost equilibrium. Two individuals failed to lose equilibrium above the freezing point of the water. We assigned these individuals a CTmin of -3.0°C because in this apparatus the water typically remained liquid down to -3.0°C as a result of supercooling. Once a fish reached CTmin it was removed from the apparatus and allowed to recover in an aquarium at its original acclimation conditions for at least two weeks. After recovery, fish were weighed and euthanized with an anesthetic overdose (using MS-222 at a concentration of 0.5g/L, buffered to a pH of 7.0-7.5 with sodium bicarbonate (0.5-1g/L)). For all fish, the right gill basket was excised and snap-frozen in liquid N_2 and stored at -80°C for later analysis of gene expression.

Plasma chloride concentration

Fish that had not been tested for CTmin were used for determination of plasma $[Cl^-]$ (Spring conditions: marine: four families, 23 total fish; freshwater: four families, 17 total fish; hybrid: four families, 50 total fish; Winter conditions: marine: three families, 15 total fish; freshwater: four families, 22 total fish; hybrid: four families, 66 total fish). Note that one marine family held under winter conditions experienced high mortality late in the experimental period and was not available for plasma $[Cl^-]$ determination. Fish were euthanized as described above and the caudal peduncle was immediately severed, and blood was collected in heparinized capillary tubes. Plasma was obtained by centrifugation for three minutes in a BD/Clay Adams Autocrit Ultra 3 Microhematocrit

Centrifuge (Becton, Dickinson and Company, Mississauga, ON, Canada) and stored at -80°C. [Cl^-] was measured using the colorimetric mercuric thiocyanate method (Zall *et al*. 1956; De Boeck *et al*. 2013).

Total RNA extraction, reverse transcription, and gene expression

Frozen gill tissue was homogenized using a Next Advance Bullet Blender 24 with ten 1.0-mm-diameter Ceria Stabilized Zirconium Oxide beads at an instrument speed of nine for three minutes (Next Advance Inc., Averill Park, NY, USA). Total RNA was extracted using TRIzol® Reagent (Life Technologies Inc., Burlington, ON, Canada) according to the manufacturer's protocol. Eight fish per ecotype/acclimation condition were used for RNA isolation (two fish per family/acclimation condition; four families of each ecotype; three ecotypes; 48 fish total). RNA concentration was measured spectrophotometrically on a SpectraMax 190 Microplate Reader (Molecular Devices, LLC., Sunnyvale, CA, USA) and RNA quality was assessed using agarose gel electrophoresis. All RNA samples were then stored at -80°C. RNA (2 μg) was then reverse-transcribed using a High Capacity cDNA Reverse Transcription Kit (Life Technologies Inc., Burlington, ON, Canada) and gene expression was assessed by quantitative real-time PCR (qRT-PCR) using a Bio-Rad CFX96 (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada) using SYBR® Green PCR Master Mix (Life Technologies Inc., Burlington, ON, Canada) according to the manufacturer's protocol. Forward and reverse primers were at a concentration of 10 μM (Integrated DNA Technologies, Toronto, ON, Canada). Real-Time PCR cycling conditions were as follows: one cycle of 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds followed by

55°C for one minute, 95°C for 10 seconds, followed by ramping from 65°C to 95°C by 0.5°C increments for five seconds each.

Gene-specific primers (Table 2.1) were designed for the Na^+,K^+-ATPase α-subunit (NKA atp1a1a), epithelial Ca^{2+} channel (ECaC), H^+-ATPase (two isoforms: V Type ATPase 1 and V Type ATPase 2), the electroneutral Na^+/H^+ exchanger (NHE; two isoforms: NHE2 and NHE3) and beta-actin as a control gene using Primer Express (Life Technologies Inc., Burlington, ON, Canada). Primer sequences for two additional control genes (eukaryotic elongation factor 1 alpha (EEF1A), and L13A ribosomal binding protein (RPL13A)) were obtained from Hibbeler *et al*. (2008). Note that the stickleback genome contains duplicate copies of NKA atp1a1a that have been annotated as atp1a1a 203 and atp1a1a 204. Preliminary experiments indicated that isoform 204 was not expressed in gills at detectable levels, and thus only isoform 203 was assessed. Expression of the ion transporters was normalized to the geometric mean of the expression of the three control genes (beta-actin, EEF1A, and RPL13A) (Vandesompele *et al*. 2002). A standard curve was generated for each gene assayed using an equal mixture of cDNA (2μL) from all samples to create the following dilution series: undiluted, 1:5, 1:25, 1:125, 1:625, which was included on all plates. Thus gene expression is reported as the expression of a sample relative to the average expression of that gene across all samples, normalized to the expression level of the control genes. Non-reverse transcribed controls were used to assess levels of genomic DNA contamination in RNA samples, which were less than 2% on average.

Statistical analysis

To test for the effects of stickleback ecotype, acclimation condition, and the interaction between ecotype and acclimation condition, we used a linear mixed models approach implemented in the 'lme4' package in R (Bates *et al*. 2015). Ecotype and acclimation condition were treated as fixed effects and stickleback family was treated as a random effect. We examined each response variable for normality; if the data did not appear normal we used a Shapiro test to confirm deviation from normality (Shapiro & Wilk 1965), and log-transformed if necessary before carrying out statistical analyses. Separate linear mixed models were generated for each stickleback trait (CTmin, mass, plasma [Cl^-]) and gene expression data set (NHE2, NHE3, ECaC, V Type 1, V Type 2, NKA 203). Model fits for both fixed effects and the interaction were visualized using the 'visreg' package in R (Breheny & Burchett 2013). Statistical significance of fixed effects and interactions was determined using two alternative approaches: 1) ANOVA and a Wald Chi-Square test implemented using the 'car' package in R (Fox & Weisberg 2011), and 2) likelihood ratio tests to compare models and generate a p-value for each fixed effect and the interaction between them. These two approaches yielded similar results; so we present only values from the Wald Chi-Square test here. Post-hoc multiple comparison tests (Tukey's HSD) were used to detect significant differences among groups. All data are presented as mean ± SEM and alpha was set at 0.05.

Results

Critical thermal minimum (CTmin)

CTmin was significantly affected by acclimation conditions ($p<0.00001$) and ecotype ($p=0.002$), with no significant interaction ($p=0.48$). Acclimation to winter conditions improved cold tolerance in both ecotypes and their hybrids. When acclimated to spring conditions, freshwater stickleback had superior cold tolerance compared to marine stickleback, and hybrid stickleback had intermediate cold tolerance. Similar patterns were observed under winter conditions, although differences between ecotypes could not be detected with post-hoc tests (Fig. 2.1A).

We calculated the slope of the CTmin reaction norm for each family as the change in CTmin per 1°C change in acclimation temperature. This slope did not differ significantly among ecotypes (Mean ± SEM; 0.25 ± 0.04 for marine, 0.26 ± 0.02 for hybrid, and 0.19 ± 0.04 for freshwater stickleback; n= 4 families per ecotype; One-way ANOVA; $p = 0.3947$).

Effects of acclimation conditions on mass

Final mass was significantly affected by acclimation conditions ($p<0.00001$) and ecotype ($p<0.00001$), and there was a significant interaction between the two factors ($p=0.002$). Both ecotypes and their hybrids had lower final mass when exposed to winter conditions than when exposed to spring conditions (Fig. 2.1B). Comparing between ecotypes, freshwater stickleback had the highest mass and marine stickleback had the lowest, with hybrid stickleback being intermediate under both spring and winter conditions (Fig. 2.1B). Acclimation to winter conditions had a more negative effect on

final mass in marine stickleback than in freshwater stickleback, with hybrid fish showing intermediate impacts. As a percent of mass under spring conditions, mass under winter conditions was 28 ± 2% for marine, 43 ± 3% for freshwater, and 36 ± 1% for hybrid stickleback families (n=4 families per ecotype).

Plasma chloride concentration

There was a significant effect of acclimation conditions (p=0.00002) and ecotype (p=0.0004) on plasma [Cl^-], with no significant interaction (p=0.22). At 14°C, the freshwater ecotype maintained higher plasma [Cl^-] than the other two ecotypes, but at 4°C only the freshwater ecotype and the hybrid fish differed (Fig. 2.2). Cold acclimated fish generally had higher plasma [Cl^-] than did warm acclimated fish.

Gene expression of ion transporters in gill tissue

There was a significant effect of acclimation conditions (p=0.00004) on gene expression for NKA atp1a1a 203, with no significant effect of ecotype (p=0.83) or interaction (p=0.8). In general, winter acclimated fish had higher expression than did spring acclimated fish (Fig. 2.3A).

For ECaC, there was a significant effect of ecotype on gene expression (p=0.002), with no significant effect of acclimation conditions (p=0.5) and no significant interaction (p=0.58). Freshwater and hybrid stickleback had higher expression than marine stickleback across both acclimation conditions, and these differences were detected as significant in *post hoc* tests (Fig. 2.3B).

For NHE2 expression there was a significant effect of acclimation conditions ($p=0.022$), but no effect of ecotype ($p=0.42$) or interaction ($p=0.45$) (Fig. 2.3C). In general, winter-acclimated fish had slightly higher NHE2 expression compared to spring-acclimated fish.

For NHE3 expression there was a significant effect of acclimation conditions ($p=0.00007$) but no significant effect of ecotype ($p=0.67$) and a significant interaction ($p=0.007$). In general, winter acclimated fish had higher NHE3 expression compared to spring acclimated fish. This difference was detected as significant in *post hoc* tests in the marine ecotype (Fig. 2.3D).

For V Type ATPase 1 there was no significant effect of acclimation conditions ($p=0.39$), but a significant effect of ecotype ($p=0.012$) and a significant interaction ($p=0.013$) (Fig. 2.3E). Under winter conditions, freshwater stickleback had higher expression than did marine and hybrid stickleback, whereas under spring conditions hybrid stickleback had greater expression than did the other two ecotypes.

For V Type ATPase 2 there was a significant effect of acclimation conditions ($p=0.039$), but no significant effects of ecotype ($p=0.19$), or interaction ($p=0.421$) (Fig. 2.3F). Expression under winter conditions was generally higher than under spring conditions.

Discussion

The first key question we addressed in this study was whether the combination of cold temperature and low salinity might have presented a barrier to the colonization of freshwater habitats by marine stickleback. Following acclimation to winter conditions,

marine stickleback acquire the ability to tolerate acute exposure to temperatures at or below the freezing point of fresh water. These data suggest that acute cold tolerance is unlikely to have presented a barrier to freshwater colonization. However, winter conditions reduced the growth of marine stickleback to a greater degree compared to freshwater stickleback. Because size at first reproduction is strongly associated with fecundity in stickleback (Wootton 1984; Schluter 1995), these data suggest that winter conditions during the first year of life could represent a challenge to colonization of freshwater habitats by marine stickleback in the north-temperate zone and may have acted as a factor driving ecological adaptation.

The second key question we addressed was whether there was evidence that ancestral plasticity could have facilitated or impeded local adaptation during freshwater colonization in stickleback. There was no compelling support for any particular relationship between plasticity and local adaptation in the traits we examined. Three traits (CTmin, growth, and plasma [Cl^-]) displayed both ancestral plasticity and divergence between ecotypes; four traits (the expression of the NKA 203, NHE2, NHE3, and V Type ATPase 2) displayed ancestral plasticity but no divergence between ecotypes; one trait (the expression of the ECaC) demonstrated divergence but no plasticity. Previous work on gene expression plasticity in response to temperature in threespine stickleback (Morris *et al*. 2014) observed a larger number of genes exhibiting plasticity in the freshwater ecotype than in the marine ecotype in muscle tissue. Taken together, these data provide little support for the importance of ancestral plasticity in facilitating colonization of freshwater in stickleback. In fact, the most striking difference between ecotypes we observed was the higher expression of the epithelial calcium channel (ECaC) in the gills

of the freshwater ecotype under both spring and winter conditions (Fig. 2.3B). These data suggest that the freshwater ecotype may have improved ability to take up calcium at low salinity, which could play a role in the superior growth performance of this ecotype under both spring and winter conditions at low salinity.

Stickleback cold tolerance

Consistent with previous observations (Barrett *et al*. 2011), we found that freshwater stickleback had better tolerance of acute cold temperatures than did marine stickleback, when acclimated to spring conditions. Note, however, that our estimates of CTmin are substantially lower than those measured by Barrett *et al*. (2011). These differences are likely due to the fact that the fish tested in previous work were acclimated to 17°C, whereas the fish tested here were acclimated to 14°C, although differences due to apparatus design cannot be ruled out. Acclimation temperature influences temperature tolerance in many fish species (Beitinger *et al*. 2000), and we observed clear improvement in cold tolerance with acclimation to winter conditions in stickleback. However, the slopes of the CTmin reaction norms for stickleback are at the lower end of the range for fishes (Beitinger *et al*. 2000), indicating that stickleback demonstrate relatively modest plasticity in acute cold tolerance. For example, the slope of the reaction norm for freshwater stickleback (0.19 per 1°C change in acclimation temperature) is similar to that of the Amargosa pupfish (*Cyprinodon nevadensis*), which (at 0.17) is the lowest known for fish (Beitinger *et al*. 2000). The ancestral plasticity in CTmin in marine stickleback is sufficient to allow them to withstand acute exposures to temperatures below the freezing point of pure fresh water when they are acclimated to winter

conditions; thus, acute cold tolerance may not have posed a severe barrier to colonization of freshwater habitats. However, previous work using experimental evolution in semi-natural ponds (Barrett *et al*. 2011) indicates that cold tolerance undergoes rapid adaptive evolution following freshwater colonization, which suggests that this phenotype could be highly correlated with other phenotypes under strong selection. Alternatively, the results of Barrett *et al*. (2011) could potentially be due to epigenetic effects of prior cold exposure on cold tolerance, rather than rapid adaptive evolution.

Growth differences between stickleback ecotypes

Because freshwater populations of stickleback are thought to have derived from an anadromous ancestor, and anadromous fish do not normally overwinter in freshwater habitats in British Columbia (Hagen 1967; Haglund *et al*. 1992; Orti *et al*. 1994; Taylor & McPhail 1999, 2000), adaptation to freshwater habitats may have involved selection on overwinter survival and growth. In stickleback, several studies have provided strong evidence showing that selection on growth probably played a role in the evolution of the prominent differences between marine and freshwater stickleback ecotypes (Marchinko & Schluter 2007; Barrett *et al*. 2008, 2009). In addition, differences in growth rate and growth rate plasticity exist between marine and freshwater stickleback raised in fresh water versus salt water (Robinson 2013). However, no previous studies have explicitly assessed how conditions during winter in low salinity impact growth in stickleback ecotypes.

The data presented here strongly suggest that either marine stickleback have lower metabolic capacity than do freshwater stickleback, or that marine stickleback are

unable to allocate as much energy toward growth in low salinity during winter as can freshwater-resident stickleback because of increased maintenance costs. One possible mechanism that could account for increased maintenance costs in marine stickleback is the challenge of ionoregulation in cold temperature and low salinity.

Ionoregulation and gene expression

Environmental calcium levels have been suggested to be an important selective factor during the colonization of fresh water by stickleback (Spence *et al*. 2012). Ancestral marine and anadromous fish have extensive bony lateral plates, whereas many of the derived freshwater populations lack these plates, suggesting the possibility of differences in calcium requirements between the morphs (Giles 1983). At low calcium concentrations the growth of completely plated fish is inhibited to a greater degree than the growth of low plated fish (Spence *et al*. 2012). Consistent with this observation, stickleback morph distribution coincides with levels of dissolved calcium across a natural environmental calcium gradient (Spence *et al*. 2013).

Calcium uptake at the gill involves an epithelial calcium channel (ECaC), which is located in the apical membrane of gill mitochondrion-rich cells (Hwang *et al*. 2011). Our data suggest that there is limited ability to adjust the expression of this transporter in the cold in either the marine or freshwater stickleback ecotype, but that the freshwater ecotype maintains high levels of this transporter under all conditions (Fig. 2.3B). Thus, the novel acquisition of high expression levels of this transporter may have been a key step in the evolution of effective freshwater ionoregulation in stickleback, and could be associated with the growth differences between freshwater and marine stickleback under

both spring and winter conditions. However, these differences in ECaC expression between ecotypes cannot account for the larger negative effects of winter conditions on the mass of marine stickleback.

Similarly, the patterns we observed in plasma [Cl^-] do not provide clear support for our hypothesis of reduced ionoregulatory ability in the marine ecotype under winter conditions. Under spring conditions, the freshwater ecotype had a significantly higher plasma [Cl^-] compared to the marine ecotype (Fig. 2.2) suggesting that the freshwater ecotype has superior ionoregulatory ability under these conditions. Acclimation to winter conditions resulted in an increase in plasma [Cl^-] in the marine ecotype, such that there were no differences in plasma [Cl^-] between the marine and freshwater ecotypes under winter conditions (Fig. 2.2). Increases in plasma [Cl^-] at low temperatures are consistent with data from other temperate teleosts (DeVries 1971, Davenport 1992), but the causes and consequences of this pattern are not well understood. However, increases in plasma [Cl^-] in the cold are speculated to have beneficial consequences such as lowering of blood freezing point (DeVries 1971, Davenport 1992).

Isoforms of the electroneutral Na^+/H^+ exchanger (NHE) are important for osmoregulation in dilute fresh water, as the NHE excretes H^+ into the external environment while taking up Na^+ (Hwang *et al*. 2011). This transporter has been implicated in local adaptation along a natural salinity gradient in stickleback (DeFaveri *et al*. 2013a; DeFaveri & Merilä 2014), emphasizing its likely importance. We observed cold-induced plasticity in the expression of NHE3 in marine stickleback and no equivalent plasticity in freshwater stickleback, which maintained intermediate expression levels under both spring and winter conditions (Fig. 2.3D). This pattern is consistent with

evolution via a process such as genetic assimilation, in which a phenotype that is originally plastic becomes fixed (or canalized) in a derived population (Pigliucci *et al*. 2006). Genetic assimilation has been suggested to play an important role in adaptation to novel environments (Pigliucci *et al*. 2006; Ghalambor *et al*. 2007). However, the expression of the NHE3 was the only trait for which a clear pattern consistent with genetic assimilation was detected.

The NKA is an energy-dependent pump that maintains low intracellular Na^+ and a highly negative charge within the ion pumping cells of the gill. These gradients are then used to facilitate the transport of various other ions to maintain internal homeostasis in the face of diffusive ion gain or loss (Hwang 2011; Hwang *et al*. 2011; Dymowska *et al*. 2012), and this protein is thus a key player in ionoregulation in both fresh and salt water. There is strong evidence of parallel evolution of the genomic region encoding this gene in multiple freshwater populations of stickleback (Jones *et al*. 2012a,b). But despite the probable importance of this locus, there is remarkably little data on NKA expression or activity in stickleback gills, and the few studies that are available do not show a consistent pattern of differences between freshwater and marine ecotypes (Schaarschmidt *et al*. 1999; McCairns & Bernatchez 2010; Judd 2012). We did not detect any differences between ecotypes in NKA expression, and expression increased with acclimation to winter conditions in all groups (Fig. 2.3A), consistent with patterns in a variety of fish species (Zaugg *et al*. 1972; McCarty & Houston 1977; Stuenkel & Hillyard 1980; Paxton & Umminger 1983; Staurnes *et al*. 1994). The NKA is a major consumer of energy in the gill, which may contribute to increased costs of ionoregulation in the cold (Buhariwalla *et*

al. 2012), suggesting that the combination of cold temperature and low salinity is challenging for both marine and freshwater stickleback ecotypes.

Conclusions

Body size at first reproduction is a critical trait for stickleback, as body size is positively correlated with reproductive output in this species (Wootton 1984; Schluter 1995). Accordingly, a faster juvenile growth rate during the cold winter months would benefit stickleback in the spring because they would be larger and have a greater reproductive output at the time of reproduction (Marchinko & Schluter 2007). The growth differences that we observed are thus consistent with the hypothesis that the combination of winter conditions and low salinity imposed a challenge to freshwater residency resulting in divergence of ecologically relevant traits. Although we only investigated a single marine and freshwater population, the results of this study show that gill ion transporter gene expression patterns have the potential to differ between stickleback ecotypes. Overall, these contrasting gene expression patterns between ecotypes, particularly in the epithelial calcium channel (ECaC), are indicative of alternative ionoregulatory strategies that may reflect differences in physiological costs associated with growth at low salinity. We observed a variety of patterns in plasticity and divergence across the traits measured, which illustrates the complexity of the roles that plasticity may play in the colonization of novel environments.

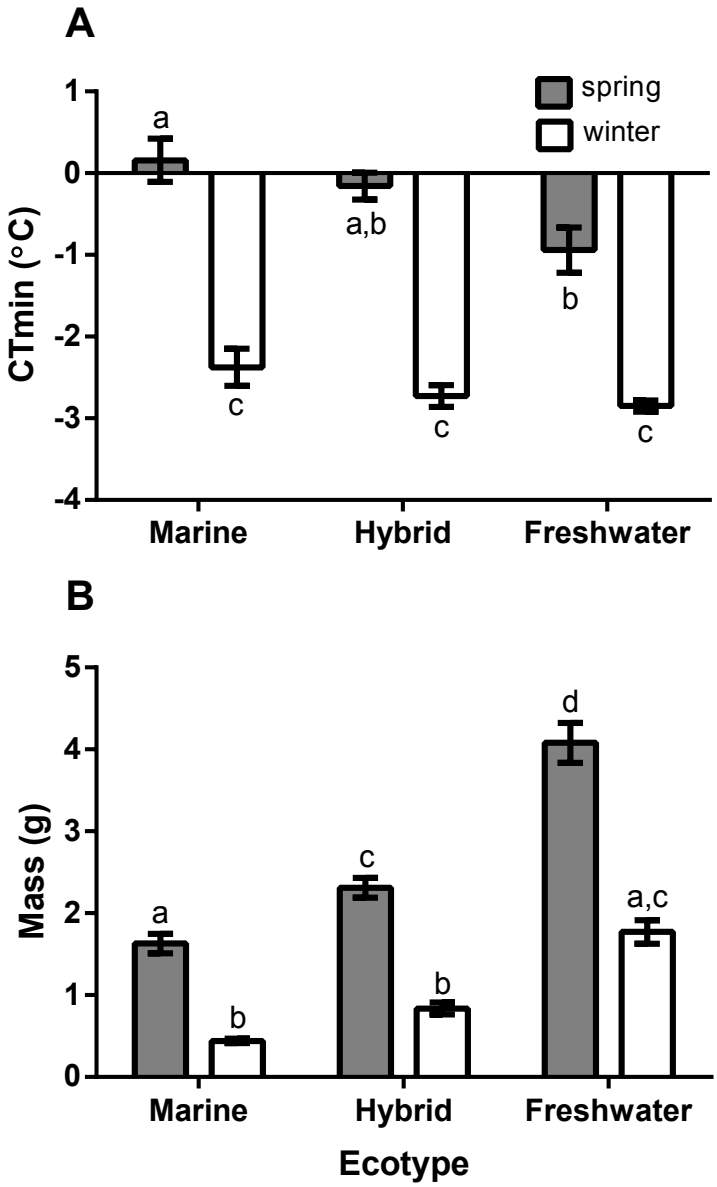

Figure 2.1. (A) Acute thermal tolerance (CTmin) of marine, hybrid, and freshwater stickleback acclimated to spring (dark grey bars; 12L:12D at 14°C) and winter conditions (white bars; 9L:15D photoperiod at 4°C). Values are means ± SEM; (*n*=12 individuals per ecotype/acclimation condition). (B) Final mass (in g) of marine, hybrid, and freshwater stickleback acclimated to spring (dark grey bars; 12L:12D at 14°C) and winter conditions (white bars; 9L:15D photoperiod at 4°C). Values are means ± SEM; (*n*=12 individuals per ecotype/acclimation condition). Groups sharing the same letter do not differ significantly ($p>0.05$). All fish were raised at a salinity of 2 ± 0.5 ppt.

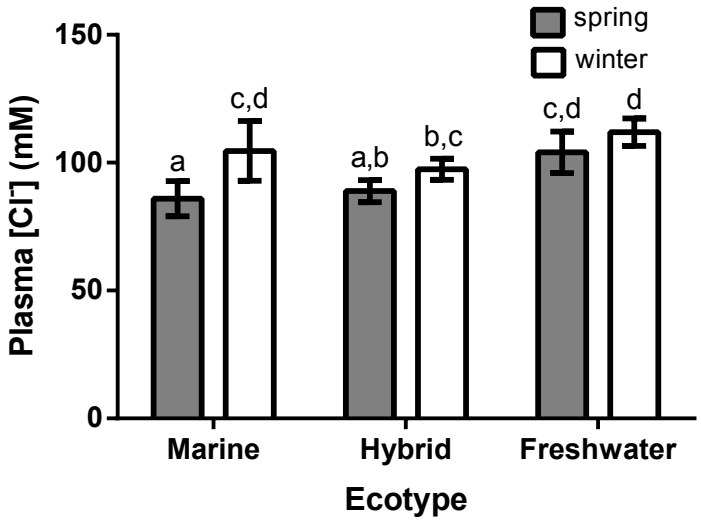

Figure 2.2. Plasma [Cl^-] (mM) of marine, freshwater, and hybrid stickleback acclimated to spring (dark grey bars; 12L:12D at 14°C) and winter conditions (white bars; 9L:15D photoperiod at 4°C). Values are means ± SEM. Spring conditions: marine: *n*=26 individuals; freshwater: *n*=18; hybrid: *n*=50. Winter conditions: marine: *n*=19; freshwater: *n*=32; hybrid: *n*=66. Groups sharing the same letter do not differ significantly ($p>0.05$). All fish were raised at a salinity of 2 ± 0.5 ppt.

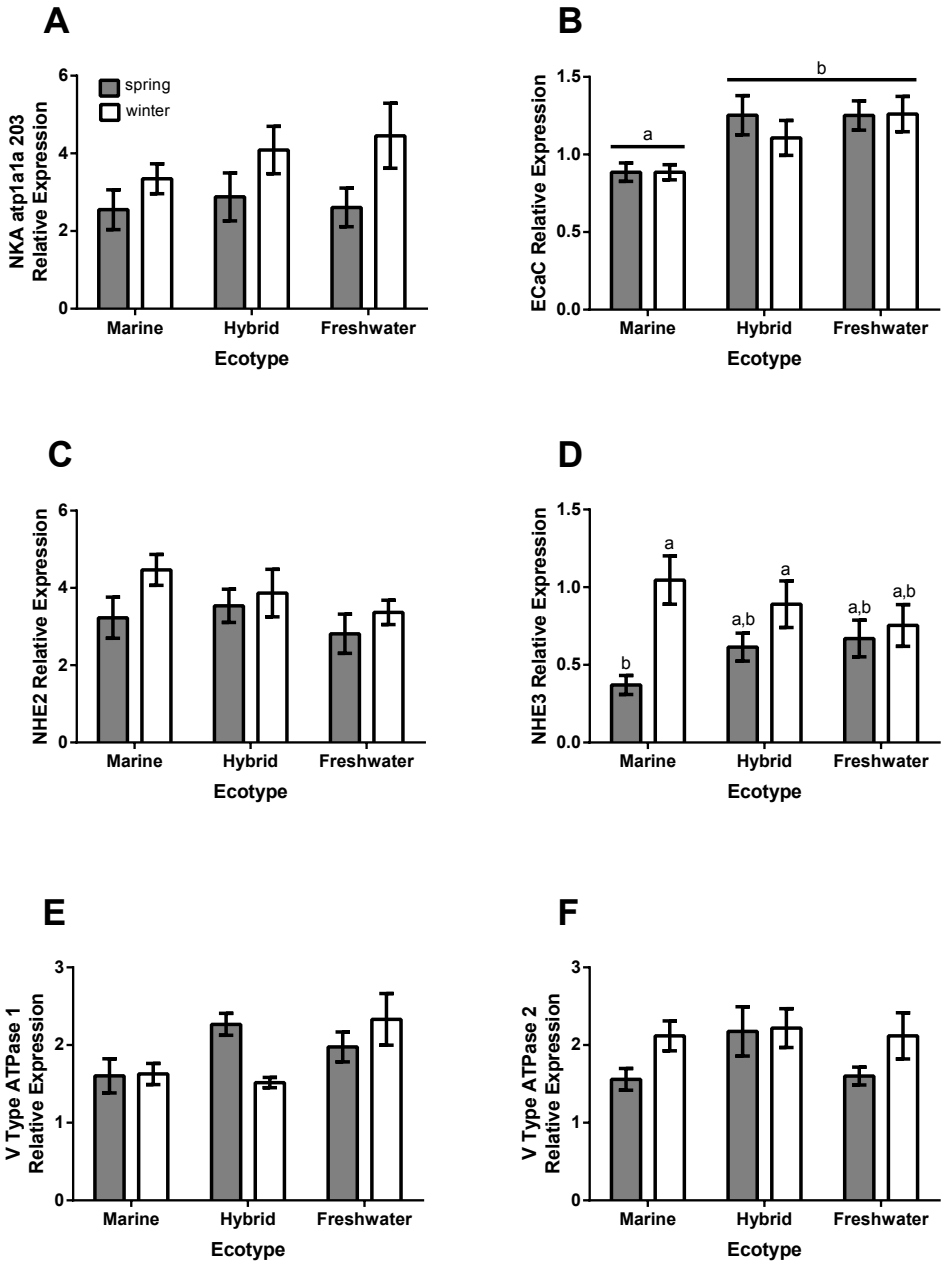

Figure 2.3. mRNA levels in gill tissue of marine, freshwater, and hybrid stickleback acclimated to spring (dark grey bars; 12L:12D at 14°C) and winter conditions (white bars; 9L:15D photoperiod at 4°C). (A) Na^+,K^+-ATPase α-subunit isoform 203 (NKA atp1a1a 203), (B) epithelial calcium channel (ECaC), (C) Na^+/H^+ exchanger isoform 2 (NHE2), (D) Na^+/H^+ exchanger isoform 3 (NHE3), (E) V Type H^+-ATPase isoform 1 (V Type ATPase 1), and (F) V Type H^+-ATPase isoform 2 (V Type ATPase 2). Expression is relative to the geometric mean of the expression of three control genes: Beta-actin, eukaryotic elongation factor 1 alpha (EEF1A), and L13A ribosomal binding protein (RPL13A). Values are means ± SEM (*n*=8 individuals per ecotype/acclimation condition). Groups sharing the same letter do not differ significantly ($p>0.05$). All fish were raised at a salinity of 2 ± 0.5 ppt.

Table 2.1. Primer sets used for qRT-PCR

Gene	Sequence (5'– 3')	Ensembl Transcript ID or source
Beta-actin	F: TCAAGATCATTGCCCCACCA R: ATCCACATCTGCTGGAAGGT	ENSGACG00000007836
ECaC	F: TGCAGGGTGGCAGGTGAT R: TCGAGCGGCTGCATCTC	ENSGACT00000013861
EEF1A	F: CCACCGTTGCCTTTGTCC R: TGGGACTGTTCCAATACCTCC	from Hibbeler *et al*. 2008
NHE2	F: TGCGCTGCCCAACGA R: TGGCGTGGCGTCTTATCAA	ENSGACT00000003889
NHE3	F: TCCTACCTGACCGCTGAGATG R: CGCCACAGAAGGTGATCGA	ENSGACT00000003204
NKA atp1a1a 203	F: ACCTGGACGATCACAAGTTAACC R: TGGAAAGACCCCTGGCTAGA	ENSGACT00000018954
RPL13A	F: CACCTTGGTCAACTTGAACAGTG R: TCCCTCCGCCCTACGAC	from Hibbeler *et al*. 2008
V Type ATPase 1	F: AAATCCAATGAGACGAGCCTATTT R: GAAAACCGCAAAGCCTTCAC	ENSGACT00000004257
V Type ATPase 2	F: CAACATAGTGCGGATTGAATGG R: GGACGTAAACAACAAGGAATAAAAGG	ENSGACT00000027401

Chapter 3: Gene expression plasticity in response to salinity acclimation in threespine stickleback ecotypes from different salinity habitats

Introduction

Phenotypic plasticity is thought to play an important role in facilitating colonization of new habitats and in shaping patterns of evolution following colonization (Pfennig *et al*. 2010; Schneider & Meyer 2017), and patterns of plasticity can themselves be subject to selection and may evolve during colonization of novel environments (Crispo 2007). A variety of changes in plasticity have been hypothesized to occur following colonizing. For example, the Baldwin effect occurs when an initially plastic trait confers increased survival allowing the organism to persist in the novel habitat long enough that selection can act upon this trait (Crispo 2007; Hendry 2016). This results in either an increase in plasticity (increased slope of the reaction norm) or a shift in trait value in the same direction as the plasticity (change in the intercept of the reaction norm) (Crispo 2007; Hendry 2016), resulting in a phenomenon known as cogradient variation, which accentuates the phenotypic differences between populations when compared in their native environments (Conover & Schultz 1995; Conover *et al*. 2009). Genetic assimilation occurs when a plastic ancestral trait becomes canalized in the derived population, resulting in a loss of plasticity and a decrease in the slope of the reaction norm (Pigliucci *et al*. 2006; Crispo 2007; Pfennig *et al*. 2010; Schneider & Meyer 2017). Alternatively, decreases in reaction norm slope can also occur in the case where ancestral plasticity is maladaptive (i.e. causes the phenotype to move away from the phenotypic optimum in the new environment), resulting in strong selection for a decrease in this

maladaptive plasticity (Crispo 2007; Ghalambor *et al*. 2015; Hendry 2016). Similarly, maladaptive plasticity may result in selection for a shift in the intercept of the reaction norm in the opposite direction of the ancestral plasticity, causing a pattern termed countergradient variation, which minimizes the phenotypic differences between populations when compared in their native environments (Conover & Schultz 1995; Conover *et al*. 2009). Although each of these phenomena has been hypothesized to be important following colonization of novel environments, their relative prevalence remains unclear (e.g. Lande 2015).

One colonization event that requires a major evolutionary transition, and in which plasticity may play an important role, is the invasion of freshwater habitats from marine environments (Lee & Bell 1999). In fresh water, osmoregulators such as teleost fishes face the challenge of diffusive ion loss and water gain (Evans *et al*. 2005; Hwang *et al*. 2011; Dymowska *et al*. 2012), whereas in seawater, fish are confronted with the opposite challenge – gain of ions and loss of water (Evans *et al*. 2005; Evans 2011a,b; Hwang *et al*. 2011). Thus, the ability to make the physiological changes needed to transition between seawater and fresh water requires extensive plasticity in key osmoregulatory tissues such as the gill epithelium, as they must be completely restructured to cope with changes in the osmotic environment between these habitats (Evans *et al.* 2005; Hwang *et al.* 2011). The ability to make this physiological transition is rare among teleost fishes, as most fishes are stenohaline and confined to either fresh water or salt water, and truly euryhaline taxa are relatively rare (Schultz & McCormick 2013). However, these relatively rare euryhaline taxa are thought to be important sources

of evolutionary diversity, as landlocking and associated radiations into fresh water are common in ancestrally euryhaline taxa (Schultz & McCormick 2013).

Recent studies have provided evidence for salinity-induced plasticity in gills of euryhaline fish (Evans & Somero 2008; McCairns & Bernatchez 2010; Whitehead *et al*. 2012; Lam *et al*. 2014; Norman *et al*. 2014; Taugbøl *et al*. 2014; Velotta *et al*. 2014, 2015; Kavembe *et al*. 2015; Zhang *et al*. 2015). However, there are relatively few studies comparing the extent of gene expression plasticity between related marine and freshwater forms (although see McCairns & Bernatchez (2010), Whitehead *et al*. (2011), Velotta *et al*. (2014, 2015), and Kozak *et al*. (2014)).

The threespine stickleback (*Gasterosteus aculeatus*) provides an excellent system in which to address the evolution of phenotypic plasticity following the colonization of novel environments because this species has undergone repeated colonization of freshwater habitats from the marine environment. After the retreat of Pleistocene glaciers 10,000-20,000 years ago, ancestral marine stickleback colonized newly available freshwater habitats around the Northern hemisphere – with subsequent adaptation and parallel evolution of morphological, physiological, and behavioral traits (Baker 1994; Bell & Foster 1994; McPhail 1994; McKinnon & Rundle 2002; McKinnon *et al*. 2004; Colosimo *et al*. 2005; Boughman 2007; Schluter 2009; Jones *et al*. 2012a,b). Indeed, there is strong evidence that freshwater stickleback have a reduced ability to survive in seawater, and this trait is associated with a significant quantitative trait locus located on chromosome 16 and with changes in the expression of several candidate genes in response to seawater challenge (Kusakabe *et al*. 2017). The extant marine forms are thought to be representative of the original ancestor of the derived freshwater ecotype,

and thus this system provides an opportunity to compare plasticity between the ancestral and derived forms following colonization of a new environment (McCairns & Bernatchez 2010; Morris *et al*. 2014).

In this study, we used a combination of RNA-seq and quantitative real-time polymerase chain reaction (qRT-PCR) assays to examine levels of plasticity in gene expression in the gill of freshwater and marine stickleback ecotypes in response to changes in salinity. Whole-transcriptome studies of gene expression are potentially a fruitful avenue to explore the evolution of phenotypic plasticity during colonization of novel environments (e.g. Morris *et al*. 2014; Dayan *et al*. 2015), and evidence is accruing for a variety of patterns including the Baldwin effect (Morris *et al*. 2014) and selection against maladaptive plasticity (Ghalambor *et al*. 2015).

Because adult marine and freshwater stickleback ecotypes are euryhaline fish that can tolerate both fresh and salt water, we predicted that both stickleback ecotypes would exhibit salinity-induced plasticity in the expression of many genes. However, we also predicted that the colonization of fresh water would be associated with both divergence in gene expression between ecotypes independent of salinity acclimation, and changes in the shapes of the reaction norms for gill gene expression between the ecotypes either via the loss of pre-existing plasticity or the evolution of novel phenotypic plasticity in the derived freshwater form.

Materials and methods

Stickleback populations & acclimation conditions

Adult threespine stickleback (*Gasterosteus aculeatus*) from a marine and a freshwater population were collected in British Columbia, Canada in June of 2013 (marine population: Oyster Lagoon (49°36'43.53"N, 124°01'52.12"W); freshwater population: Trout Lake (49°30'29"N, 123°52'29"W)). Fish husbandry and experimentation were conducted under an approved animal care protocol (A10-0285) according to the regulations of the Canadian Council on Animal Care. All fish were transported to the University of British Columbia and acclimated in 100 L glass aquaria under conditions similar to those of the collection sites: temperature: 14°C; photoperiod: 12L:12D; salinities: 20 ± 0.5 ppt for Oyster Lagoon fish and 2 ± 0.2 ppt for Trout Lake fish. Water salinities were achieved by mixing Instant Ocean® sea salt with dechlorinated Vancouver, BC municipal tap water (0.0 ppt; 0.06 mmol l^{-1} Na^{+}). All fish were kept at densities of 11-12 fish per tank and fed bloodworms once daily to satiation. All fish were held in the laboratory under these conditions for at least four weeks after collection.

At the start of the experimental period salinity was changed gradually (at a rate never exceeding 0.5 ppt per day) to the following experimental salinities: 0.0 ppt, 0.3 ppt, 2 ppt, 11 ppt, and 30 ppt, with two replicate tanks for each ecotype at each salinity. These salinities were chosen because at 30 ppt euryhaline fish maintain a specific "seawater" gill morphology and physiology. Eleven ppt is isosmotic to the body fluids of stickleback, which should represent the energetically least challenging environment for a euryhaline fish (Schaarschmidt *et al*. 1999; Bœuf & Payan 2001), whereas 2 ppt is well below the isosmotic point, and thus requires a transition of the gill to ion uptake. 0.3 ppt

represents a physiological barrier at which the suite of freshwater ion transporters must change to maintain thermodynamically favorable ion uptake, particularly of Na^+ and Cl^- (Boisen *et al*. 2003; Parks *et al*. 2008; Brix & Grosell 2012). All fish were allowed to acclimate at these treatment salinities for at least three months prior to sampling. At this point, fish were euthanized with an anesthetic overdose (with MS-222 at a concentration of 0.5g/L, buffered to a pH of 7.0-7.5 with sodium bicarbonate (0.5-1g/L) and the right gill basket was excised, snap-frozen in liquid N_2, and stored at -80°C for subsequent RNA extraction.

Total RNA extraction

Eight fish per ecotype/salinity treatment were randomly selected from each group for RNA isolation, except for the marine ecotype at 0 ppt where seven fish were used because of higher mortality in this treatment group (see Appendix B, Table B1). Total RNA was isolated by homogenizing the gill basket from each individual fish using a Bullet Blender 24 with ten 1.0 mm diameter Ceria Stabilized Zirconium Oxide beads per sample at an instrument speed setting of nine for three minutes (Next Advance Inc., Averill Park, NY, USA) in TRIzol® Reagent (Life Technologies Inc., Burlington, ON, Canada) according to the manufacturer's protocol.

Sample and library preparation for RNA-seq

We performed RNA-seq using fish acclimated to 0 ppt and 30 ppt. Five of the RNA samples isolated above were randomly selected from each ecotype and salinity combination. Prior to RNA-seq library preparation, RNA was treated to remove genomic

DNA contamination using an RNeasy Mini Kit and RNase-Free DNase Set (Qiagen Sciences Inc., Germantown, MD). Total RNA concentration was determined with a Molecular Probes™ Qubit® RNA BR Assay Kit and an Invitrogen™ Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA). RNA integrity was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA), according to the manufacturers' instructions. cDNA library synthesis was performed by the Nucleic Acid Protein Service (NAPS) Unit at the University of British Columbia. RNA pools were enriched for mRNA by pull down with NEXTflex™ Poly(A) Beads (BIO-O Scientific, Austin, TX), and the resulting enriched samples were quantified using a Quant-iT™ RiboGreen® RNA Assay Kit (Thermo Fisher Scientific, Waltham, MA). cDNA libraries were synthesized using NEXTflex™ Rapid RNA Sequencing Kits (BIO-O Scientific, Austin, TX) and quantified using a Quant-iT™ PicoGreen® dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, MA).

Libraries were paired-end sequenced using an Illumina HiSeq2000 (Illumina, Inc., San Diego, CA) at the University of British Columbia Biodiversity Research Centre NextGen Sequencing Facility. Samples were multiplexed in two groups of ten and sequenced on two flow cells. Samples were evenly distributed across the two flow cells with five individuals from each ecotype on each flow cell and either two or three of each ecotype/salinity combination on each flow cell.

Analysis of RNA-seq read counts

Illumina reads were mapped to the *Gasterosteus aculeatus* genome (BROAD S1 Ensembl release 83) using CLC genomics Workbench v8.5 (CLC bio Qiagen®, Aarhus,

Denmark), and total exon counts were exported for further analysis. Table B2 in Appendix B provides summary coverage and mapping statistics for all libraries.

Analysis of total read counts was performed in R v3.2.2 with edgeR v3.12.0 (Robinson *et al.* 2010), following the approach suggested by Lin *et al.* (2016). The data set was filtered to remove genes with no mapped reads, relative log expression (RLE) normalized (Anders & Huber 2010), and then filtered to remove genes with low expression (< 1 count per million, which is equivalent to ten reads in the sample with the smallest library). Robust tagwise dispersions (Zhou *et al.* 2014) were then estimated for each gene that remained in the data set (14,829 genes). Expression differences were assessed by principal component analysis (PCA) using the prcomp() function from the base package in R on $\log_2$ counts per million expression values, and the significance of differences between the groups for each of the major principal components was analyzed using t-tests. Differential gene expression was analyzed using negative binomial generalized linear models that tested the effects of ecotype, acclimation salinity, and interactions between these factors for each gene.

Functional enrichment of gene ontology (GO) pathway annotations of the differentially expressed genes was conducted using the goseq (v1.22.0) R package as previously described (Metzger & Schulte 2016).

For all analyses, alpha was set at 0.05 and p-values were corrected for the consequences of multiple testing using the Benjamini-Hochberg method (Benjamini & Hochberg 1995). Unless stated otherwise, the p-values reported below reflect these corrections.

Quantitative real-time PCR

All samples from both ecotypes and all acclimation salinities (including those used for RNA-seq) were used to examine the shape of the reaction norm for changes in gene expression across a range of salinities using quantitative real-time PCR (qRT-PCR). Expression of the following genes was monitored: epithelial Ca^{2+} channel (ECaC, *trpv6*), Na^+,K^+-ATPase α-subunit (NKA, *atp1a1a.5*), and electroneutral Na^+/H^+ exchanger (NHE3, *slc9a3.2*). Primer sequences were as in Gibbons *et al*. (2016) and are reported here in Appendix B, Table B3. qRT-PCR was performed essentially as in Gibbons *et al*. (2016). Briefly, 2 μg of the total RNA isolated above was reverse-transcribed using a High Capacity cDNA Reverse Transcription Kit (Life Technologies Inc., Burlington, ON, Canada) and qRT-PCR was performed using a Bio-Rad CFX96 (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada) with SYBR® Green PCR Master Mix (Life Technologies Inc., Burlington, ON, Canada) according to the manufacturer's protocol. Forward and reverse primers were at a concentration of 10 μM (Integrated DNA Technologies, Toronto, ON, Canada). Real-time PCR cycling conditions were: one cycle of 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds followed by 55°C for one minute, 95°C for 10 seconds, followed by ramping from 65°C to 95°C by 0.5°C increments for five seconds each. Gene expression was determined relative to a standard curve generated from a serial dilution of an equal mixture of all cDNA samples that was included on all plates, and then normalized to the expression of the control gene ribosomal protein L13a (*rpl13a*). Levels of genomic DNA contamination were assessed using non-reverse transcribed RNA samples, and were less than 3% of the signal from reverse transcribed cDNA in all samples (mean = 0.48 ± 0.59%). qRT-PCR data were

analyzed by two-way ANOVA using ecotype and acclimation salinity as fixed factors, using GraphPad Prism 6.0 with alpha set at 0.05. All data met the assumptions of normality and homogeneity of variance.

Results

Principal component (PC) analysis of all expressed genes separated the samples into groups based on ecotype and salinity acclimation conditions (Fig. 3.1A). PC1 explained 21.6% of the variation in the data and significantly separated the two salinity acclimation treatments ($p = 0.002$). PC2 explained 15.1% of the variation in the data and significantly separated the marine and freshwater ecotypes ($p = 3.4 \times 10^{-5}$). These results suggest that ecotype and salinity were the major factors explaining variation in gene expression in our study.

Likelihood ratio tests identified 2,684 genes that were differentially expressed in response to salinity acclimation, 2,515 genes that were differentially expressed between the marine and freshwater stickleback ecotypes, and 87 genes that exhibited a significant interaction between ecotype and salinity acclimation (Fig. 3.1B).

Genes differentially expressed in response to salinity

2,684 genes were differentially expressed in response to salinity acclimation. Of these genes, approximately equal numbers were up-regulated and down-regulated in fresh water (51.1 and 48.9%, respectively; Appendix B, Fig. B1). GO-enrichment analysis revealed enrichment for processes such as "positive regulation of epithelial cell migration" and "transmembrane transport" (Table 3.1), consistent with the known re-

structuring of fish gills during salinity transfer (Hwang *et al*. 2011). Among the genes that were up-regulated with acclimation to fresh water in the transmembrane transport term were many that are known to be critical for ion regulation in fresh water, such as isoforms of the Na^+/H^+ exchanger (*slc9a3.2, slc9a5, slc9a6a, slc9a8*), the Na^+,K^+-ATPase α-subunit (*atp1a1*), and the epithelial calcium channel (*trpv6*). Within this GO-term we also detected upregulation of several genes involved in bicarbonate transport (such as the chloride-bicarbonate exchangers, *slc4a1a* and *slc4a1b*, and the sodium-bicarbonate exchanger, *slc4a4a*), that are known to participate in acid-base regulation in freshwater fish (Hwang *et al.* 2011). In contrast, freshwater acclimation was associated with the down-regulation of key "salt water" ion transporters such as the $Na^+,K^+,2Cl^-$ cotransporter (*slc12a2*) (Hwang *et al*. 2011). Consistent with observations in many fish species (Brennan *et al*. 2015), freshwater acclimation was also associated with increases in the expression of multiple isoforms of cell tight junction proteins, such as claudins and occludins. These proteins reduce paracellular ion permeability, decreasing the efflux of critical ions in fresh water (Furuse *et al.* 1993; Van Itallie & Anderson 2006).

To refine our description of the shape of the reaction norm for gene expression across salinities and to detect potential threshold effects, we used quantitative real-time PCR (qRT-PCR). Here, we examined the expression of three candidate ion transporters that were differentially expressed between salinities in the RNA-seq experiment: the epithelial Ca^{2+} channel (ECaC, *trpv6*), the electroneutral Na^+/H^+ exchanger (NHE3, *slc9a3.2*), and the Na^+,K^+-ATPase α-subunit (NKA, *atp1a1a.5*).

There were significant effects of salinity on the expression of ECaC, NKA and NHE3 ($p < 0.0001$ for all genes). A significant effect of ecotype was detected for ECaC

and NKA expression ($p = 0.0007$ and 0.0057 respectively) but not NHE3 ($p = 0.9595$). A significant interaction between salinity and ecotype was detected for ECaC ($p = 0.0001$) but not for NKA or NHE ($p = 0.9393$ and 0. 9466 respectively) (Fig. 3.2).

ECaC expression increased at salinities of 0.3 ppt and below in the freshwater ecotype, whereas in the marine ecotype there was a modest increase in expression from 30 ppt to 0.3 ppt but not at the lowest salinity (Fig. 3.2A). Both ecotypes increased NKA gene expression at salinities of 0.3 ppt and below, and the freshwater ecotype maintained slightly higher expression than the marine ecotype across salinities (Fig. 3.2B). Changes in the expression levels of NHE3 with salinity were similar to the patterns observed for NKA with increased expression at salinities of 0.3 ppt and below (Fig. 3.2C).

Genes differentially expressed between ecotypes

Of the 2,515 genes that differed in expression, approximately equal numbers had higher expression in each ecotype (49.9% upregulated in the freshwater ecotype and 50.1% upregulated in the marine ecotype; Appendix B, Fig. B2). The majority of the genes that differed between ecotypes did not overlap with those that were differentially expressed in response to salinity (Fig. 3.1B). GO-enrichment analysis of the genes that were differentially expressed between the marine and freshwater ecotypes demonstrated enrichment of multiple terms associated with the extracellular matrix and cell adhesion, suggesting the possibility of differences in gill structure between the ecotypes (see Table 3.2 for the top ten biological process GO-terms and Table B4 in Appendix B for a complete list). In addition, the GO-terms that were enriched among the genes that

differed in expression between ecotypes were not the same as those that were enriched among the genes that responded to salinity acclimation (Table 3.1).

Evolution of plastic gene expression

The evolution of phenotypically plastic traits can involve changes in the slope or intercept of a reaction norm (Crispo 2007). We detected 500 genes with significant effects of both acclimation salinity and ecotype and no significant interaction between these factors (Fig. 3.1B), which are indicative of changes in the intercept of the salinity reaction norm between ecotypes without significant changes in reaction norm slope. We also detected 87 genes with significant interactions between salinity and ecotype (Fig. 3.1B), suggesting a change in the slope of the salinity reaction norm between ecotypes.

Within the 500 genes with significant effects of both acclimation salinity and ecotype and no significant interaction between these factors, four general categories of expression pattern are possible: higher expression in the marine vs. freshwater ecotype or vice versa at both salinities, and higher expression in fresh water vs. salt water or vice versa in both ecotypes. As can be seen from the heat map of the expression of these genes (Fig. 3.3), no single pattern of expression was dominant, and all four possible combinations were approximately equally common. GO-enrichment analysis on this subset of genes did not reveal significant enrichment of any specific biological process within this gene set; however, "transmembrane transport" was one of the most frequently occurring biological process terms annotated within this gene set, with 25 genes annotated within this term (see Table B5 in Appendix B for a summary of all biological process GO-terms and their frequencies in this set of genes).

Eighty-seven genes demonstrated a significant interaction between salinity and ecotype (Fig. 3.4). As was the case for the genes with significant effects of acclimation salinity and ecotype, but no interaction, no single pattern of plasticity in gene expression dominated within the genes exhibiting significant interactions between acclimation salinity and ecotype, and there was no significant enrichment of GO biological process terms within this gene set. However, similar to the genes with effects of both salinity and ecotype with no interactions, the most commonly represented GO-terms included "transmembrane transport" and "ion transmembrane transport" (eight and seven genes, respectively; see Appendix B, Table B6).

Discussion

Our data demonstrate that salinity acclimation changes the expression of a substantial number of genes in the gill transcriptome of both marine and freshwater ecotypes of threespine stickleback, highlighting a large and highly conserved "core" response to environmental salinity. We also detected divergence in the expression of many genes between ecotypes. However, in contrast to our prediction, only a relatively small number of genes that exhibited plasticity in expression showed evidence of the evolution of either divergence between ecotypes in expression level or in the extent of phenotypic plasticity, and no particular pattern of change in reaction norm slope or intercept dominated among this group of genes. Thus, our data do not support a role for a single or primary mode of evolution of patterns of phenotypic plasticity in gene expression in stickleback gills. This stands somewhat in contrast to the conclusions of previous candidate-gene studies that have suggested an important role for genetic

assimilation in the evolution of the gill transcriptome in stickleback (McCairns & Bernatchez 2010) and highlights the complexity of patterns of phenotypic plasticity following the colonization of novel environments.

Mechanisms of salinity acclimation

Salinity acclimation in euryhaline fishes involves a substantial transformation of gill structure and function, as this tissue must transition from performing ion secretion in salt water to ion uptake in fresh water (Evans *et al*. 2005; Evans 2011a,b; Hwang *et al*. 2011). Consistent with previous studies of the response of the gill transcriptome to freshwater acclimation across a variety of fish species (Whitehead *et al*. 2011, 2012; Lam *et al*. 2014; Brennan *et al*. 2015; Nguyen *et al*. 2016) we observed changes in the expression of genes involved in key processes important for maintaining physiological homeostasis in fresh water. The common core salinity response is enriched for processes such as transmembrane transport and regulation of epithelial cell migration, suggesting that it is possible that both ecotypes have substantial capacity to remodel their gills and alter ion transport physiology following salinity transfer. Most stickleback ecotypes are euryhaline as adults, and can be held in either fresh water or salt water in the laboratory (Marchinko & Schluter 2007). The ability to remodel the gills and alter transmembrane transport of ions may play a key role in establishing these euryhaline capabilities.

Changes in the nature and direction of ion transport are key components of the response to changes in environmental salinity in fish (Hwang *et al*. 2011) and, not surprisingly, transmembrane transport was a significantly enriched biological process GO-term in response to salinity acclimation. Freshwater acclimation resulted in the up-

regulation of multiple transporters that are known to be critical for ion or acid-base regulation in fresh water and the down-regulation of key "salt water" ion transporters. Taken together, these data suggest that the changes in ion transporter gene expression in stickleback in response to salinity are typical of those observed in many species of fish (Hwang *et al.* 2011). Similarly, maintaining cell volume is a critical component of the ability to transition between saltwater and freshwater habitats (Whitehead *et al*. 2012), which involves both altering membrane permeability and inducing mechanisms to restore cell volume. We observed increases in the expression of multiple isoforms of cell tight junction proteins (such as claudins and occludins) in response to freshwater acclimation. Increases in tight junction proteins would be expected to reduce paracellular ion permeability, decreasing the efflux of critical ions in fresh water (Furuse *et al.* 1993; Van Itallie & Anderson 2006). We also observed up-regulation of multiple members of the aquaporin (AQP) gene family in fresh water. Aquaporins are membrane proteins that function as water channels (Cerda & Finn 2010). Although the precise role of AQP in freshwater acclimation remains unknown, it is thought to be an important component of either cell volume regulation or sensing, as AQP has been observed to be up-regulated in fresh water in studies in other species (Cutler & Cramb 2002; Lignot *et al.* 2002; An *et al.* 2008; Whitehead *et al.* 2012). Similarly, genes involved in osmolyte synthesis increased in expression in freshwater acclimated fish, which may contribute to cell volume regulation (Whitehead *et al.* 2012).

To further assess the shape of the reaction norms of gene expression in response to salinity acclimation, we used quantitative real-time PCR (qRT-PCR) to examine the expression of three candidate ion transporters that were significantly up-regulated with

freshwater acclimation in the RNA-seq experiments: ECaC, NKA, and NHE3. ECaC encodes a calcium channel that is crucial for uptake of calcium from dilute freshwater environments (Hwang *et al*. 2011). Calcium levels are likely to have been a selective factor during the colonization of fresh water by stickleback (Spence *et al*. 2012, 2013), as low levels of environmental calcium have been shown to inhibit the growth of completely plated stickleback more than the growth of low plated stickleback (Spence *et al*. 2012). The NKA plays an important role in generating the electrochemical gradients that are necessary for uptake of ions in fresh water (Hwang *et al*. 2011), and has undergone parallel evolution in multiple populations of freshwater stickleback (Jones *et al*. 2012a,b; DeFaveri *et al*. 2013b). Similarly, NHE3 has been identified as being under directional selection in response to salinity in stickleback (Shimada *et al*. 2011; DeFaveri *et al*. 2013a).

For all three genes, the qRT-PCR results supported the results of the RNA-seq analysis, as all three genes increased in expression at lower salinities. In addition, the qRT-PCR data demonstrate that increases in gene expression in response to salinity acclimation occur when salinity drops below 2 ppt. Our data suggest that stickleback may maintain a "seawater-type" gill morphology and physiology down to salinities as low as 2 ppt. Similar patterns have been observed in a distantly-related euryhaline teleost, the Atlantic killifish (Whitehead *et al*. 2012), which undergo a significant change in gill gene expression with transition to salinities below ~0.4 ppt, and highlight the non-linear shape of the reaction norms for changes in gill gene expression with acclimation to salinity change. Interestingly, the increase in NKA expression that we observed in response to low salinity acclimation stands in contrast to observations of changes in NKA protein

activity following freshwater transfer (Divino *et al*. 2016). Although this experiment examined acute effects of transfer over seven days compared to the ~6 month acclimation period utilized here, these data highlight the possibility that mRNA and protein expression can be decoupled (Chen *et al*. 2002; Greenbaum *et al*. 2003; Pascal *et al*. 2008; de Sousa Abreu *et al*. 2009; Vogel & Marcotte 2012; Koussounadis *et al*. 2015). Alternatively, it is possible that the short-term response of the gill to high salinity transfer requires increased activity of NKA that is not required in the longer term following the morphological adjustment of the gill during acclimation.

In general, expression patterns detected by RNA-seq were similar to those detected by qRT-PCR at 0 ppt and 30 ppt (Appendix B, Fig. B3). However, with the larger sample sizes and increased number of acclimation salinities possible with qRT-PCR, we were able to detect some subtle effects that were not evident in the RNA-seq data. For example, for ECaC we detected a significant effect of ecotype and an interaction between ecotype and salinity that were not statistically significant in the RNA-seq data. Although both marine and freshwater ecotypes showed increased ECaC expression at low salinities, this increase was greater in the freshwater ecotype in the qRT-PCR data. This pattern is consistent with our previous study that showed that the freshwater ecotype maintains higher ECaC expression at low salinity than the marine ecotype (Gibbons *et al*. 2016), and suggests the evolution of enhanced plasticity in the freshwater ecotype, potentially resulting in improved uptake of this ion and in a pattern of cogradient variation in the expression of this gene. Similarly for NKA, qRT-PCR detected a significant difference in gill gene expression between the two ecotypes, which was not detected in the RNA-seq data, with the freshwater ecotype having slightly higher

expression at each acclimation salinity. This pattern is consistent with the Baldwin effect, and suggests the possibility of selection acting on the expression of this gene in the gill following the colonization of fresh water. This pattern is in contrast to a previous study in a different set of stickleback populations (McCairns & Bernatchez 2010), which detected a slight decrease in the extent of plasticity in NKA expression in the freshwater ecotype, consistent with the process of genetic assimilation. Taken together, these data suggest that despite the evidence for repeated parallel evolution at this locus (DeFaveri *et al*. 2011, 2013b, Jones *et al*. 2012a,b), changes in the plasticity of NKA expression may not be consistent among populations following freshwater colonization in stickleback.

Divergence between ecotypes

More than 2,500 genes differed in expression between ecotypes, but the majority of these (79.8%) did not overlap with those that were differentially expressed in response to salinity (Fig. 3.1B). In addition, the GO-terms that were enriched among the genes that differed in expression between ecotypes were not the same as those that were enriched among the genes that responded to salinity acclimation (compare Table 3.2 to Table 3.1). Examination of the enriched GO-terms for genes that diverged in expression between the ecotypes demonstrates that there has been expression divergence in genes associated with cell adhesion and the extracellular matrix. Interestingly, within these GO-terms a much larger number of genes were upregulated in the freshwater ecotype compared to the marine ecotype. For example, of the 266 genes in the enriched "extracellular matrix organization" term (GO:0030198) 82 had higher expression in the freshwater ecotype, while only nine had higher expression in the marine ecotype (see Table B4 in Appendix

B for a complete list of the enriched GO-terms). In contrast, across all genes, a roughly equal number had higher expression in each ecotype (1,254 genes with higher expression in the freshwater ecotype and 1,261 genes with higher expression in the marine ecotype). These data suggest that there has been a general up-regulation of genes involved in the extracellular matrix and cell adhesion in the freshwater ecotype, which could indicate potential decreases in gill permeability associated with colonization of freshwater habitats.

Evolution of phenotypically plastic gene expression

We detected gene expression divergence between ecotypes involving changes in both the intercept and slope of the salinity acclimation reaction norm. The largest group of these (500 genes) had a significant main effect of both salinity and ecotype, which suggests a change in the intercept, rather than the slope, of the reaction norm. Thus, we observed divergence between ecotypes in the level of expression of approximately 20% of the genes involved in the response to salinity acclimation, without a significant change in the extent of plasticity in either ecotype. Patterns in which the plasticity and the relationship between expression in the derived and ancestral forms are in the same direction (e.g. higher expression in fresh water conditions and higher expression in the freshwater ecotype) are potentially consistent with the patterns indicative of the Baldwin effect (Crispo 2007) and cogradient variation (Conover & Schultz 1995; Conover *et al*. 2009; Hendry 2016). Among these 500 genes, approximately half had expression patterns consistent with Baldwin effects, suggesting that this class of effect does occur in our data set. In addition, a previous study demonstrated that Baldwin effects were observed for

genes that respond to low temperature acclimation in stickleback white muscle and suggested these effects play an important role in evolutionary change following freshwater colonization (Morris *et al*. 2014). However, all four of the potential relationships in this group of 500 genes (higher in fresh water or higher in salt water, and higher expression in the freshwater or marine ecotype) are detected in similar numbers (Fig. 3.3). Consequently, our data suggest that expression patterns consistent with Baldwin effects are not more common than other patterns of expression plasticity in response to salinity in stickleback. Furthermore, different isoforms of genes with known functions in fish gills demonstrated opposite patterns of expression. For example, three of the claudin isoforms that increase in expression in fresh water also have significant differences in expression between the ecotypes. Two of these (*cldnh* and *cldni*) have higher expression in the freshwater ecotype, which is potentially consistent with the Baldwin effect, while the other (*cldnf*) has higher expression in the marine ecotype. Similarly, the two aquaporins that increase expression with freshwater acclimation differ between ecotypes, but in opposite directions.

Although only 500 genes had significant main effects of both ecotype and salinity, this analysis may underestimate the functional overlap between plasticity and divergence, because many genes are members of large families and it is possible that divergence and plasticity could occur in different isoforms within the same family that may play similar biochemical roles. For example, the growth hormone (GH)/insulin-like growth factor I axis is known to be involved in salinity acclimation in euryhaline fish (McCormick 2001; Sakamoto & McCormick 2006). One isoform of the insulin-like growth factor binding protein (*igfbp6b*) had significant effects of both ecotype and

acclimation salinity, with expression being higher in the freshwater ecotype than in the marine ecotype at both salinities, but with expression being down-regulated by freshwater acclimation in both ecotypes. Several other isoforms in this gene family had higher expression in the freshwater ecotype than in the marine ecotype (*igfbp1a, igfbp4, igfbp5, igfbp6b*), but were unaffected by salinity acclimation, and another isoform (*igfbp6a*) was down-regulated by freshwater acclimation in both ecotypes (for a complete list of differentially expressed genes, see Table S4 in Gibbons *et al*. (2017)). Interestingly, *igfbp5* has been previously identified as having undergone parallel expression divergence across multiple fresh and saltwater stickleback populations (Kusakabe *et al*. 2017). The varying patterns across multiple genes within this family highlight the possibility for overlap between gene expression plasticity and gene expression divergence at the level of the gene family. However, we did not detect any overlap in enriched biological process GO-terms between the genes that were significantly affected by ecotype and the genes that were significantly affected by acclimation salinity (compare Table 3.2 to Table 3.1), suggesting that this is not a major pattern within our data set.

Only a limited number of genes (87) had a significant interaction between the main effects of acclimation salinity and ecotype (Fig. 3.1B), consistent with limited divergence in the extent of plasticity in the gill transcriptome between these ecotypes. Several studies have suggested that evolution via genetic assimilation could play an important role in the evolution of freshwater colonization in stickleback (hatching success (Marchinko & Schluter 2007); gene expression plasticity in response to salinity (McCairns & Bernatchez 2010)), but no single expression pattern dominated among the genes with a significant interaction between acclimation salinity and ecotype (Fig. 3.4).

Thus, our data suggest that neither the evolution of novel phenotypic plasticity nor the loss of phenotypic plasticity in the gill transcriptome has been a major component of the evolution of freshwater residency in stickleback. These data stand in contrast to patterns in other species, where loss of phenotypic plasticity in gene expression has been detected in freshwater resident forms (Whitehead *et al*. 2011; Velotta *et al*. 2014; Kozak *et al*. 2014). In general, evolution of freshwater residency in fish has been associated with loss of tolerance of high salinity habitats (Whitehead 2010), whereas we do not detect any clear signature of this effect in our RNA-seq data set. In contrast, our observations in adult stickleback suggest that, at least at this life stage, the freshwater ecotype retains the ability to osmoregulate in salt water. However, this may not be the case at all life stages, as some authors have reported low hatching success and survivorship of freshwater stickleback ecotypes reared in salt water (Marchinko & Schluter 2007).

Although our overall patterns contrast with those observed in previous studies, we did detect patterns consistent with a loss of plasticity in the freshwater form in a few genes that may play important roles in osmoregulation. For example, the transmembrane transporter *trpm6* (ENSGACG00000011569; a transient receptor potential cation channel that is responsible for magnesium transport) increases in expression in response to freshwater acclimation in the marine ecotype, but has high expression under all conditions in the freshwater ecotype. Magnesium is essential for the growth of bone and bony structures, and magnesium levels are low in freshwater habitats, suggesting the possibility that increases in the expression of this transporter could have functional importance. For example, reductions in the extent of bony lateral plates in freshwater

stickleback are correlated with (among other factors) water ion levels (Bourgeois *et al*. 1994).

Conclusions

The results of this study highlight that the response of the gill transcriptome to environmental salinity is largely shared among stickleback ecotypes, and that it is induced at salinities below 2 ppt. In contrast, differentiation in gill gene expression between the ecotypes has occurred in a different set of processes that could be implicated in changes in the gill structure and decreases in gill permeability with freshwater colonization. However, there was a subset of genes that exhibited both plasticity in response to salinity acclimation and differentiation between the ecotypes, although no specific processes were enriched among this gene set. No single pattern of relative expression dominated among these genes, suggesting that gill gene expression is not evolving in a way consistent with a single process such as the Baldwin effect. A smaller set of genes showed evidence of changes in the extent of plasticity, either due to the evolution of novel plasticity or the loss of plasticity, but neither gains nor losses of plasticity dominated the changes in expression patterns among different genes. Although this study examined only a single marine and freshwater population, these data demonstrate the complexity of the evolution of gene expression patterns in gill during colonization of freshwater habitats.

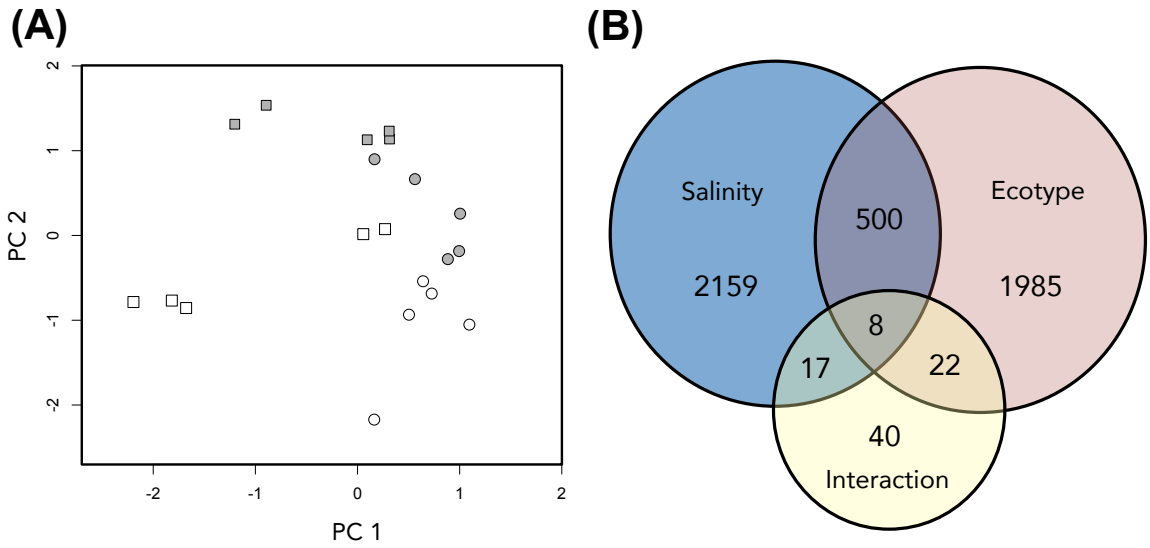

Figure 3.1. Differential gene expression in the gills of threespine stickleback ecotypes acclimated to fresh water and salt water. A. Principle components 1 and 2 of expressed genes in gill tissue of freshwater (open symbols) and marine (filled symbols) stickleback ecotypes acclimated to 0 ppt (circles) or 30 ppt (squares) water salinity. B. Venn diagram illustrating the total number of differentially expressed genes for which a main effect of ecotype, acclimation salinity or an interaction was detected.

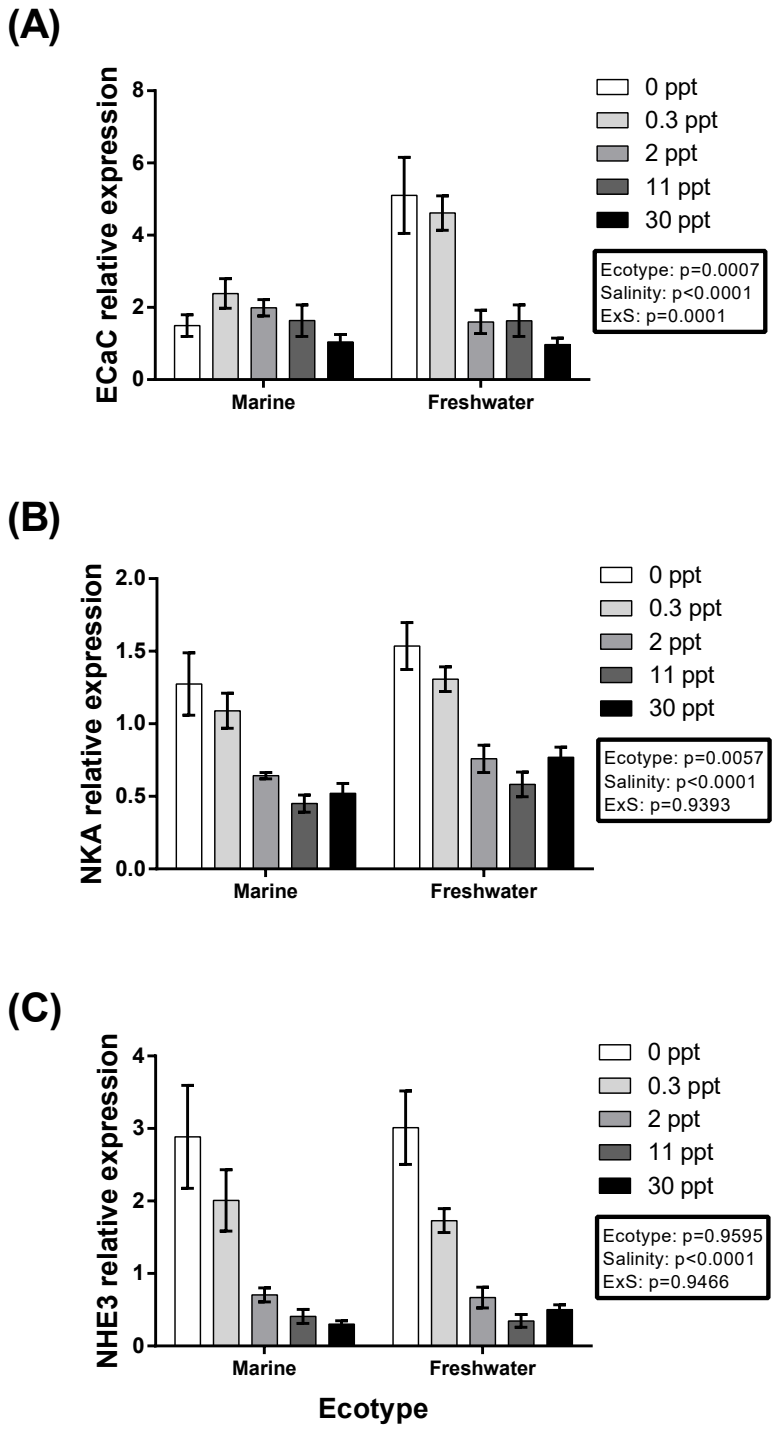

Figure 3.2. mRNA levels of candidate genes in gill tissue of freshwater and marine stickleback ecotypes acclimated to a range of salinities. A. ECaC (*trpv6*), the epithelial calcium channel. B. NKA (*atp1a1a.5*), the Na^+,K^+-ATPase α-subunit. C. NHE3 (*slc9a3.2*), the electroneutral Na^+/H^+ exchanger. Expression is relative to a standard curve of a pool of all samples, normalized to the expression of the control gene ribosomal protein L13a (*rpl13a*). Data are expressed as mean ± SEM (n = 7-8).

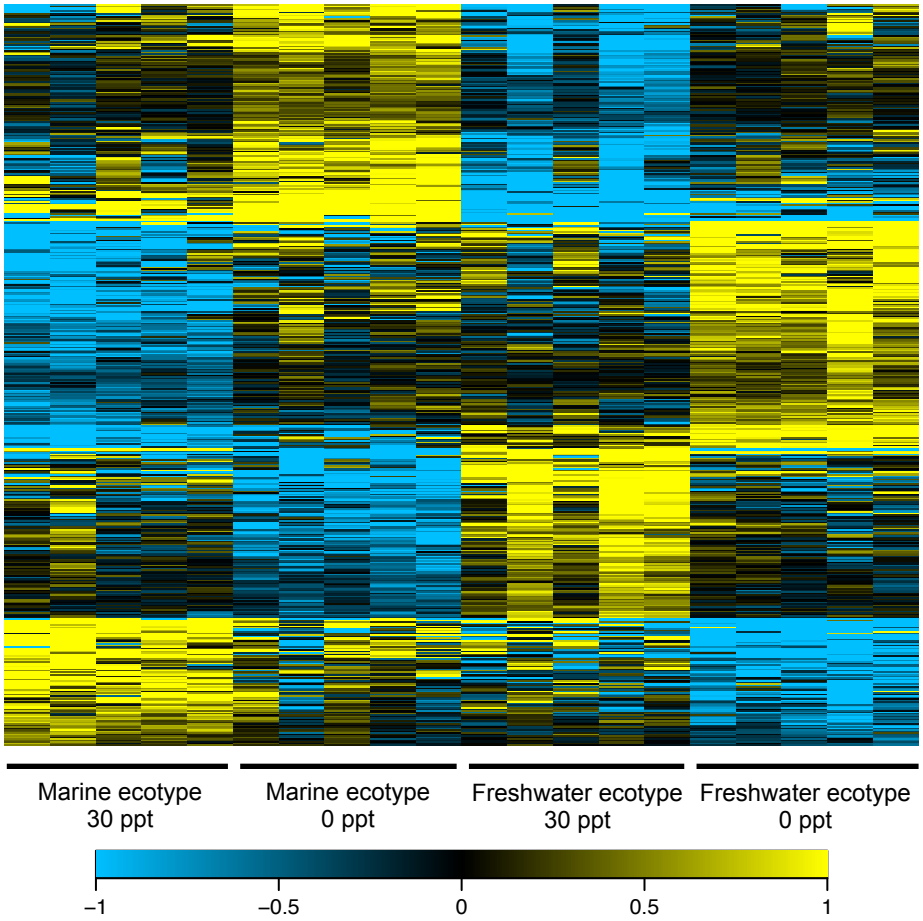

Figure 3.3. Heat map displaying expression patterns of genes with significant effects of both acclimation salinity (0 ppt and 30 ppt) and ecotype (freshwater and marine). Blue indicates genes with expression levels lower than the mean of all samples. Yellow indicates genes with expression levels higher than the mean of all samples. Each column indicates one individual (n = 5 per group).

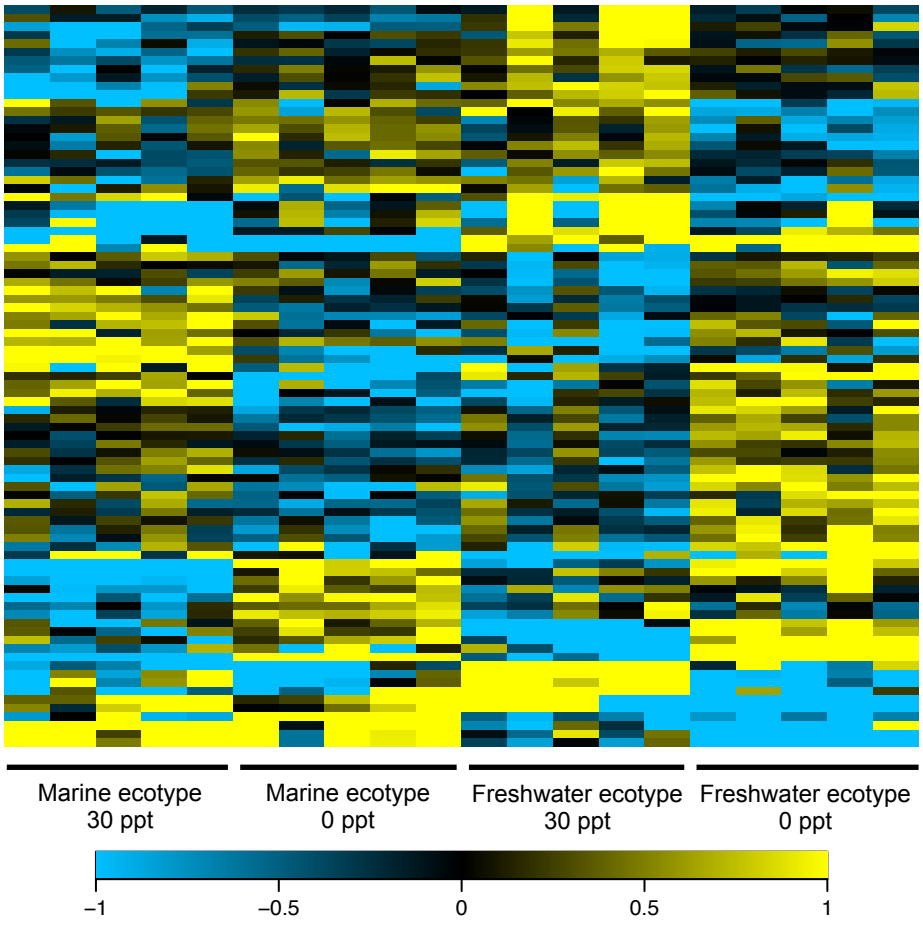

Figure 3.4. Heat map displaying expression patterns of genes with a significant interaction between acclimation salinity (0 ppt and 30 ppt) and ecotype (freshwater and marine). Blue indicates genes with expression levels lower than the mean of all samples. Yellow indicates genes with expression levels higher than the mean of all samples. Each column indicates one individual (n = 5 per group).

Table 3.1. Biological Process GO-terms enriched among genes that were differentially expressed in response to salinity acclimation in stickleback

GO-ID	Term	Number of genes up-regulated at 0 ppt	Number of genes down-regulated at 0 ppt	p-value (over-representation)
GO:0010634	positive regulation of epithelial cell migration	8	9	1.78×10^{-5}
GO:0055085	transmembrane transport	63	57	4.92×10^{-5}
GO:0008645	hexose transport	0	18	6.08×10^{-5}
GO:0005975	carbohydrate metabolic process	27	53	6.27×10^{-5}
GO:0016042	lipid catabolic process	14	8	6.30×10^{-5}
GO:0006364	rRNA processing	2	30	8.81×10^{-6}

(All terms significantly enriched with FDR <0.1 shown)

Table 3.2. Top ten Biological Process GO-terms enriched among genes that were differentially expressed between stickleback ecotypes.

GO-ID	Term	Number of genes up-regulated in the freshwater ecotype	Number of genes down-regulated in the freshwater ecotype	p-value (over-representation)
GO:0030198	extracellular matrix organization	82	9	2.93×10^{-16}
GO:0032897	negative regulation of viral transcription	2	38	6.79×10^{-15}
GO:0051092	positive regulation of NF-kappaB transcription factor activity	9	45	1.98×10^{-8}
GO:0007155	cell adhesion	68	14	2.79×10^{-8}
GO:0022617	extracellular matrix disassembly	35	4	4.57×10^{-8}
GO:0030574	collagen catabolic process	26	1	4.80×10^{-8}
GO:0007186	G-protein coupled receptor signaling pathway	45	17	1.14×10^{-7}
GO:0051091	positive regulation of sequence-specific DNA binding transcription factor activity	3	43	1.76×10^{-7}
GO:0045087	innate immune response	55	112	1.91×10^{-7}
GO:0045766	positive regulation of angiogenesis	19	13	5.16×10^{-6}

(All terms significantly enriched with FDR <0.05; see Table B4 in Appendix B for complete list of all significantly enriched terms)

Chapter 4: Interactive effects of salinity and temperature acclimation on gill morphology and gene expression in threespine stickleback

Introduction

Saltwater and freshwater habitats present opposing challenges to organisms, and the interface between these habitats creates a barrier to movement between them (Lee & Bell 1999). In salt water, aquatic organisms are hyposmotic to the surrounding water, resulting in a gain of ions by diffusion from the environment (Evans *et al*. 2005; Hill *et al*. 2008; Evans 2011a,b). In fresh water, however, organisms are hyperosmotic to the dilute environment and face the opposite problem – loss of ions by diffusion into their environment (Evans *et al*. 2005; Hwang *et al*. 2011). In addition to different salinity conditions, freshwater habitats may also have different temperature regimes compared to adjacent marine habitats. For example, in temperate regions, the temperature of fresh water is more variable than that of seawater (Lee & Bell 1999), and in the north-temperate zone, freshwater lakes become colder than the ocean in the winter (Barrett *et al*. 2011). Because most fish are poikilothermic ectotherms, changes in environmental temperature affect fish by altering biochemical and physiological processes including the fluidity of biological membranes, and the rates of enzymatic reactions, respiration, feeding, growth, and locomotion, which can strongly affect fitness (Hochachka & Somero 2002; Moyes & Ballantyne 2011).

The threespine stickleback (*Gasterosteus aculeatus*) is one of many organisms that have been able to overcome the barriers presented by the transition between freshwater and saltwater habitats (Lee & Bell 1999), successfully colonizing numerous

freshwater habitats in the Northern Hemisphere. In stickleback, this colonization has been associated with repeated adaptation and parallel evolution of a variety of traits since marine stickleback colonized freshwater habitats 10,000-20,000 years ago following the recession of Pleistocene glaciers (Baker 1994; Bell & Foster 1994; McPhail 1994; McKinnon & Rundle 2002; McKinnon *et al*. 2004; Colosimo *et al*. 2005; Boughman 2007; Schluter 2009; Jones *et al*. 2012a,b). The ancestral stickleback that colonized fresh water were likely anadromous (Haglund *et al*. 1992; Orti *et al*. 1994; Taylor & McPhail 1999, 2000; McKinnon *et al*. 2004; Colosimo *et al*. 2005; Kitano *et al*. 2012), and so would have been able to tolerate changes in salinity. However, in British Columbia, anadromous stickleback return to the ocean before the winter, and therefore do not experience the cold winter temperatures that occur in fresh water (Hagen 1967). Therefore, in order to colonize fresh water, anadromous stickleback had to overcome not only the change in salinity of moving from salt water to fresh water, but also had to survive and overwinter in the novel combination of low salinity and low temperature. Recent work on the euryhaline Atlantic killifish (*Fundulus heteroclitus*) (Buhariwalla *et al*. 2012) and other anadromous species (Stanley & Colby 1971; McCormick *et al*. 1997) has shown that the combination of low salinity and low temperature may be particularly challenging for fish ionoregulation by causing a decreased capacity to maintain plasma ion levels. This suggests that the combination of these two abiotic factors may have posed a significant challenge to colonization of freshwater habitats in the north-temperate zone.

Only a single study has addressed the combined effects of both low salinity and low temperature on ionoregulation in stickleback (Schaarschmidt *et al*. 1999). These

authors examined brackish water and freshwater populations of stickleback acclimated to fresh water and brackish water under warm and cold conditions. There were only minor differences in the activity of a few genes involved in ionoregulation between stickleback native to either brackish water or freshwater habitats, yet the combination of low salinity and low temperature resulted in high mortality in stickleback from brackish water habitats (Schaarschmidt *et al*. 1999). This differential mortality provides support for the idea that the combination of low salinity and low temperature may have posed a challenge to colonization of fresh water by stickleback from the marine environment.

In addition to the osmoregulatory functions carried out by specific ion transporters located in fish gills (Evans *et al*. 2005; Hwang *et al*. 2011), the morphology of the gill itself can have major physiological impacts for fish. The gill lamellae are the major structural sites of ion flux and oxygen uptake, and alterations in lamellar surface area directly impact these processes (Nilsson *et al*. 2012). In addition to modifying lamellar surface area by alterations in blood perfusion (Nilsson *et al*. 2012), it has been shown that changes in the size of an interlamellar cell mass (ILCM) directly modify lamellar surface area (Sollid *et al*. 2003). In response to hypoxia (Sollid *et al*. 2003), warm water (Sollid *et al*. 2005; Mitrovic & Perry 2009; Barnes *et al*. 2014), and exercise (Brauner *et al*. 2011; Fu *et al*. 2011; Perry *et al*. 2012), some fish species are able to remodel their gills by reducing the size of the ILCM. This decrease in ILCM increases lamellar surface area, and is likely beneficial for the increased oxygen uptake required in response to these factors (Nilsson *et al*. 2012). Additionally, the opposite may be true – when oxygen demand is low, increasing the size of the ILCM may limit energy expended on osmoregulation (the "osmorespiratory compromise"; but see caveats in Nilsson *et al*.

(2012)), and one study in the mangrove killifish *Kryptolebias marmoratus* has shown that salinity changes also elicit changes in ILCM size (LeBlanc *et al*. 2010).

Here we examined the effects of the combination of low salinity and low temperature on gill ion transporter gene expression and the morphology of the ILCM in marine, anadromous, and freshwater populations of stickleback. The combined effects of low salinity and low temperature are likely to have posed a challenge to colonization of freshwater habitats from the ocean following glacial retreat, and thus may have driven adaptive evolution in gill functional traits. Consequently, we predicted that freshwater-resident stickleback would demonstrate novel plasticity in gene expression and gill morphology in response to changes in both salinity and temperature, whereas anadromous stickleback, which do not experience fresh water in the winter, would lack the temperature-induced component of this response, and that marine stickleback would show the smallest response to both of these factors.

Materials and methods

Stickleback populations, acclimation conditions, & time course

Adult stickleback were collected from three populations in British Columbia, Canada in June and July of 2013. Marine (Oyster Lagoon (49°36'43.53"N, 124°01'52.12"W)), anadromous (from the mouth of the Little Campbell River (49°00'52"N, 122°45'33"W)), and freshwater (Trout Lake (49°30'29"N, 123°52'29"W)) stickleback were transported to the laboratory at the University of British Columbia and housed in 100 L glass aquaria with recirculating filtered water. All experimentation and fish husbandry were performed in compliance with the Canadian Council of Animal

Care, with an approved animal care protocol (A10-0285). Oyster Lagoon (marine) and Little Campbell River (anadromous) stickleback were initially acclimated to a salinity of 20 ± 0.5 ppt (with Instant Ocean® sea salt), while Trout Lake (freshwater) stickleback were acclimated to 2 ± 0.2 ppt, which are similar to the salinities at their collection locations. All fish were acclimated to a water temperature of 14°C at a photoperiod of 12L:12D. Fish were held at a density of 25 fish per aquarium, and were fed bloodworms once daily. We collected 200 marine stickleback and 200 anadromous stickleback (eight aquaria each), but we were only able to collect 100 freshwater stickleback (four aquaria). All fish were acclimated to these conditions for at least 27 days; at which point the acclimation salinities of all fish were changed to 11 ppt (temperature and photoperiod were not altered). Salinity changes were performed gradually (over two to three days), and all fish acclimated in these conditions (14°C and 11 ppt) for at least 32 days prior to experimental acclimations.

After acclimating at 14°C and 11 ppt for ≥32 days, temperature and/or salinity were gradually changed over the course of one day to reach the following experimental conditions: 1) no change in temperature or salinity (14°C and 11 ppt); 2) salinity change only (14°C and 0.3 ppt); 3) temperature change only (4°C and 11 ppt); and 4) salinity and temperature change (4°C and 0.3 ppt) (Fig. 4.1). Fish were then acclimated to these conditions for 31 days.

At 31 days after the salinity/temperature changes, fish from all four groups were euthanized with an anesthetic overdose (with MS-222 at a concentration of 0.5g/L, buffered to a pH of 7.0-7.5 with sodium bicarbonate (0.5-1g/L)). Immediately after euthanasia, fish were weighed, standard length was measured, and the right and left gill

baskets were excised. The right gill basket was snap-frozen in liquid N_2 and stored at -80°C (for RNA extraction), while the left gill basket was preserved for microscopy. For microscopy, left gill baskets were immediately placed in Karnovsky's Fixative following excision and stored at 4°C. After 24 hours in Karnovsky's Fixative, these gills were transferred to Sodium cacodylate and stored at 4°C.

Total RNA extraction, reverse transcription, and gene expression

Total RNA was isolated by homogenizing the frozen right gill basket (with a Next Advance Bullet Blender 24 with ten 1.0 mm diameter Ceria Stabilized Zirconium Oxide beads per sample at an instrument speed of nine for three minutes (Next Advance Inc., Averill Park, NY, USA)) in TRIzol® Reagent (Life Technologies Inc., Burlington, ON, Canada) according to the specifications of the manufacturer. Eight fish per ecotype per salinity/temperature group were used for RNA isolation for the marine ecotype. Higher mortality occurred for the anadromous and freshwater ecotypes, so three to eight (anadromous) and four to eight (freshwater) fish per salinity/temperature group were used for these ecotypes (see Table C1 in Appendix C for details of sample sizes). High mortality occurred for the anadromous ecotype in the cold freshwater group (4°C and 0.3 ppt), with no fish remaining at the end of the experiment (Appendix C, Table C1). RNA concentration, assessment of RNA quality, reverse-transcription, and measurement of gene expression by quantitative real-time PCR (qRT-PCR) were performed as in Gibbons *et al*. (2016).

Primer Express (Life Technologies Inc., Burlington, ON, Canada) was used to design gene-specific primers for the following genes: the epithelial Ca^{2+} channel (ECaC,

trpv6), electroneutral Na^+/H^+ exchanger (NHE3, *slc9a3.2*), and Na^+,K^+-ATPase α-subunit (NKA, *atp1a1a.5*), and the control gene eukaryotic translation elongation factor 1 alpha 1b (*eef1a1b*) (see Table C2 in Appendix C for primer sequences). As in Gibbons *et al.* (2016), a High Capacity cDNA Reverse Transcription Kit (Life Technologies Inc., Burlington, ON, Canada) was used to reverse-transcribe 2 μg of RNA per sample. qRT-PCR was then was carried out with a Bio-Rad CFX96 system (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada), using SYBR® Green PCR Master Mix (Life Technologies Inc., Burlington, ON, Canada), and the forward and reverse qRT-PCR primers synthesized above at a concentration of 10 μM each (Integrated DNA Technologies, Toronto, ON, Canada). For qRT-PCR, the following cycling conditions were used: one cycle of 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds followed by 55°C for one minute, 95°C for 10 seconds, followed by ramping from 65°C to 95°C by 0.5°C increments for five seconds each. To assay gene expression, a standard curve was created by mixing an equal amount of cDNA from each sample (2μL), and this mixture was serially diluted to make the following standard curve: undiluted, 1:5, 1:25, 1:125, 1:625. All qRT-PCR plates, for all genes assayed, included this standard curve, and expression of each gene is reported relative to this standard curve and normalized to the expression of the control gene, *eef1a1b*. Individual cDNA samples were diluted 1:20 prior to assaying for gene expression, and genomic DNA contamination was generally undetectable and always less than 0.1%, assessed by carrying out qRT-PCR on non-reverse-transcribed RNA samples. qRT-PCR data were analyzed by three-way ANOVA, with ecotype, temperature, and salinity as fixed factors. Because some data failed to meet the assumption of homogeneity of variance, all data were log-transformed before carrying

out the three-way ANOVA, which resulted in all assumptions of ANOVA being met. Because no anadromous fish survived until the end of the experiment in the combined cold and freshwater treatment group (4°C and 0.3 ppt), only the data for the marine and freshwater ecotypes were included. Sample sizes were unequal, so Type III sums of squares were used for ANOVA, implemented in the 'car' package in R. A full factorial model was run including all interactions followed by model reduction, hierarchically dropping non-significant interactions and alpha was set at 0.05. To assess the effects of salinity and/or temperature treatment, we next analyzed gene expression within each ecotype separately using one-way ANOVA with Type III sums of squares. Because some data failed to meet the assumption of homogeneity of variance, all data were log-transformed before carrying out one-way ANOVA. When significant effects were detected, Dunnett's multiple comparison tests were performed to detect significant changes in expression relative to the 14°C and 11 ppt treatment (control condition).

Gill microscopy

In preparation for microscopy, gills were removed from Sodium cacodylate buffer (pH= 7.5) and the second gill arch was detached from the rest of the gill basket for analysis. Due to the small size of the gills, the entire second gill arch was embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin by Wax-it Histology Services Inc. (Vancouver, BC, Canada). Each sample was sectioned three times and the most intact sample was selected for imaging, using a Motic AE31 microscope at 400X magnification with Motic Images Plus 2.0 software (Motic, Richmond, BC, Canada). Image analysis was performed in ImageJ (version 1.47) using a micrometer to set the

scale. For each sample, interlamellar cell mass (ILCM) area was measured at five lamellae situated in equally spaced sections along the gill arch, at the base, middle, and tip of each filament, and care was taken to ensure that the lamellae selected for analysis were in the same plane of sectioning. For each fish, these fifteen measurements were then averaged, and gill samples from the marine and freshwater ecotypes were compared. Evaporation of Sodium cacodylate during refrigeration at 4°C occurred for some gill samples from the freshwater ecotype at 14°C and 11 ppt and 14°C and 0.3 ppt, therefore sample sizes for these treatments were low (see Table C3 in Appendix C for gill sample sizes in all treatments). All data analyses were performed using R (version 3.3.1). Data were analyzed via three-way ANOVA with ecotype, temperature, and salinity as fixed factors (as above), and data met the assumptions of normality and homogeneity of variance as assessed using the Shapiro-Wilk test of normality and Levene's test for homogeneity of variance. We used planned comparisons to make biologically meaningful comparisons between pairs of samples using t-tests and adjusted the results for multiple comparisons using the Benjamini-Hochberg method (Thissen *et al*. 2002).

Results

Quantitative real-time PCR

ECaC expression demonstrated a significant effect of ecotype ($p = 0.0008$), but no significant effect of salinity ($p = 0.4181$) or temperature ($p = 0.9233$) (Fig. 4.2A), and there were no significant interactions in the full factorial model so these terms were dropped as described in the Materials & Methods section. In general, the freshwater ecotype had higher ECaC expression levels than the marine ecotype across treatment

groups, and although we did not directly test the difference between expression in the anadromous ecotype relative to either the marine or freshwater ecotypes, expression in the anadromous ecotype was generally similar to the levels of expression in the marine fish.

NHE3 expression demonstrated a significant effect of salinity ($p = 0.0003$), but no significant effect of temperature ($p = 0.0543$) or ecotype ($p = 0.2252$) (Fig. 4.2B), and there were no significant interactions in the full factorial model. A Dunnett's multiple comparison test detected a significant increase in expression in the warm freshwater treatment group (14°C and 0.3 ppt) and the combined cold and freshwater treatment group (4°C and 0.3 ppt) compared to the control group (14°C and 11 ppt) in the marine ecotype. A similar trend, although statistically non-significant, was apparent in the freshwater ecotype. In the anadromous ecotype, NHE3 expression was elevated in the warm freshwater treatment group (14°C and 0.3 ppt) compared to the control group (14°C and 11 ppt).

NKA expression demonstrated a significant effect of temperature ($p = 6.24 \times 10^{-5}$) and salinity ($p = 0.0219$), but no significant effect of ecotype ($p = 0.3625$) (Fig. 4.2C), and there were no significant interactions in the full factorial model. Marine stickleback had higher NKA expression levels in the cold freshwater treatment group (4°C and 0.3 ppt) than in the control group (14°C and 11 ppt), and a similar, although not statistically significant, pattern was evident in the freshwater ecotype. In the anadromous ecotype, NKA expression was higher in the cold isosmotic treatment group (4°C and 11 ppt) compared to the control group (14°C and 11 ppt).

Gill interlamellar cell mass (ILCM)

For ILCM area, there was no significant three-way interaction in the full factorial model so this term was dropped. ILCM area demonstrated a significant effect of temperature ($p = 3.36 \times 10^{-9}$) and salinity ($p = 5.29 \times 10^{-6}$), but no significant effect of ecotype ($p = 0.3019$). In addition, there was a significant interaction between the effects of temperature and salinity ($p = 4.27 \times 10^{-7}$) (Fig. 4.3). ILCM area was lowest in fish from the control group (14°C and 11 ppt; Fig. 4.3). Samples sizes were too low to make planned comparisons at 14°C and 11 ppt, and 14°C and 0.3 ppt for the freshwater ecotype (see Table C3 in Appendix C for gill sample sizes in all treatments), but planned comparisons within the marine ecotype revealed that relative to the control conditions (14°C and 11 ppt), ILCM area was larger in all other treatment groups. ILCM area increased the most in response to the cold isosmotic treatment group (4°C and 11 ppt), while ILCM area increased to a lesser and equivalent extent in both the warm freshwater treatment group (14°C and 0.3 ppt) and the cold freshwater treatment group (4°C and 0.3 ppt) (Fig. 4.3). Representative gill images for the marine ecotype in each of the four treatment groups are presented in Figure 4.4.

Discussion

The individual effects of salinity and temperature have well documented effects on ionoregulation and gill morphology in fish, yet little work has focused on how the interaction of these two abiotic factors impact these basic processes. In this study, for the first time in a single experiment, we illustrated how the combination of low salinity and

low temperature elicited non-additive responses in gene expression of NKA in the gill and changes in gill morphology, suggesting that this combination of environmental factors is likely a physiological challenge for marine and anadromous stickleback.

Salinity and temperature influence gill ion transporter gene expression

Environmental salinity has broad impacts on plasticity of gene expression in fish gills, influencing processes such as ion transport (Evans & Somero 2008; McCairns & Bernatchez 2010; Whitehead *et al*. 2012; Lam *et al*. 2014; Norman *et al*. 2014; Taugbøl *et al*. 2014; Velotta *et al*. 2014, 2015; Kozak *et al*. 2014; Kavembe *et al*. 2015; Zhang *et al*. 2015), cell junctions (Whitehead *et al*. 2011, 2012; Lam *et al*. 2014; Norman *et al*. 2014; Kozak *et al*. 2014; Kavembe *et al*. 2015), and signaling pathways (Evans & Somero 2008; Whitehead *et al*. 2011, 2012; Lam *et al*. 2014; Kozak *et al*. 2014; Kavembe *et al*. 2015), which are all critical physiological components of surviving changes in salinity. As ectotherms, the water temperature of a fish's habitat directly influences body temperature (Hochachka & Somero 2002; Schulte 2011), and work has also begun to explore temperature's effect on gene expression in the gill (Chou *et al*. 2008; Mladineo & Block 2009; Logan & Somero 2010; Rebl *et al*. 2013; Narum & Campbell 2015). Although there are relatively few studies of the impacts of the combination of these two stressors on gill gene expression or morphology, it is clear that the combination of low salinity and low temperature presents a challenge to ionoregulation (Stanley & Colby 1971; McCormick *et al*. 1997; Buhariwalla *et al*. 2012) and survival (Schaarschmidt *et al*. 1999). While no previous studies have examined the interactive effects of salinity and temperature on stickleback ionoregulation, the isolated

effects of salinity exert directional selection and result in divergence in multiple genes involved in osmoregulation in stickleback (Shimada *et al*. 2011; DeFaveri *et al*. 2011, 2013a,b; Jones *et al*. 2012a). Furthermore, changes in salinity result in differences in gene expression in the stickleback gill (McCairns & Bernatchez 2010; Jones *et al*. 2012b; Taugbøl *et al*. 2014; Kusakabe *et al*. 2017). Accordingly, we identified three candidate ion transporters (all located in gill mitochondrion-rich cells, or ionocytes) that are physiologically essential for ionoregulation and movement between the ocean and fresh water, and assessed the effects of low salinity and low temperature on their gene expression: the Na^+,K^+-ATPase α-subunit (NKA), epithelial Ca^{2+} channel (ECaC), and the electroneutral Na^+/H^+ exchanger (NHE3) (Hwang *et al*. 2011). The NKA is critical for generating electrochemical gradients essential for transporting ions between the environment and the blood plasma, and the ECaC and NHE3 are necessary for taking up calcium and sodium, respectively, from dilute fresh water (Hwang *et al*. 2011). Additionally, it has been shown that there has been selection and parallel evolution of NKA in freshwater stickleback populations (Shimada *et al*. 2011; DeFaveri *et al*. 2011; Jones *et al*. 2012a,b; DeFaveri *et al*. 2013b). Further, dissolved calcium levels have differential effects on growth of stickleback ecotypes (Spence *et al*. 2012), and there has been directional selection on NHE3 in response to salinity in stickleback (Shimada *et al*. 2011; DeFaveri *et al*. 2013a).

In agreement with previous work that showed ECaC expression in stickleback does not vary in response to cold winter conditions in fresh water (Gibbons *et al*. 2016 (Chapter 2)), here we saw divergent patterns in ECaC expression between ecotypes, with higher expression levels across all treatment conditions in the freshwater ecotype (Fig.

4.2A). These data strongly suggest that high ECaC expression is a fixed trait in freshwater populations of stickleback that differentiates them from marine stickleback. If high ECaC expression is associated with high expression of the associated ion channel, this could indicate that freshwater populations of stickleback are better able to take up calcium in freshwater habitats. The hypothesis that low water calcium may be a key abiotic factor driving evolution in stickleback following freshwater colonization is supported by the observation of repeated parallel reductions in bony structures such as lateral plates, spines and the pelvic girdle across multiple populations of stickleback (Giles 1983; Bell *et al*. 1993; Spence *et al*. 2012, 2013). In addition, experimental work has demonstrated that low water calcium concentration inhibits the growth of completely plated stickleback to a greater degree than that of low plated stickleback, which suggests that low calcium concentration may have been an important agent driving divergence between marine and freshwater ecotypes of stickleback (Spence *et al*. 2012).

We have previously shown that, in response to the effects of cold winter conditions in fresh water (Gibbons *et al*. 2016) and low salinity (Gibbons *et al*. 2017), NKA and NHE3 exhibit increased expression levels, which may be beneficial for ion uptake in fresh water. However, the interactive effects of salinity and temperature on ion transporter gene expression have not previously been examined, which is critical for understanding how freshwater colonization occurred in this species. In this study, the combination of low temperature and low salinity (4°C and 0.3 ppt) had non-additive effects on the expression of NHE3 (Fig. 4.2B), such that fish exposed to both low temperature and low salinity had expression levels similar to those of fish exposed to either factor in isolation. In contrast, NKA expression in fish exposed to both low

temperature and low salinity was greater than expression levels in fish exposed to either factor in isolation in the marine ecotype (Fig. 4.2C), with similar, but less extreme, changes occurring in the freshwater ecotype. If this increase in gene expression is associated with increases in the amount and activities of the proteins, this may help marine stickleback to maintain ion homeostasis in cold fresh water. However, such a response is likely to result in higher physiological costs for marine stickleback compared to freshwater stickleback, providing a physiological explanation for the reduced growth they exhibit in cold fresh water compared to freshwater stickleback (Gibbons *et al*. 2016; Chapter 5). Freshwater stickleback did not exhibit statistically significant increases in NKA expression in cold freshwater conditions (relative to control conditions). We hypothesize that this difference in gene expression pattern may be due to differences in gill permeability between ecotypes. We have previously shown that freshwater stickleback up-regulate more genes involved in cell adhesion and the extracellular matrix in response to low salinity (e.g. claudins; Gibbons *et al*. 2017). This divergence in expression in the freshwater ecotype could result in lower gill permeability in the freshwater ecotype than in the marine ecotype (Gibbons *et al*. 2017), resulting in less diffusive ion loss in cold fresh water, making an increase in NKA expression less critical for the freshwater ecotype when exposed to the combination of cold and fresh water.

In addition, the anadromous ecotype, which avoids facing the combined effects of low salinity and low temperature by migrating back to the ocean prior to winter (Hagen 1967), was severely affected by this combination of environmental factors and was unable to survive the duration of the experiment when exposed to cold fresh water. Similar patterns of poor survival in cold fresh water in brackish water populations of

stickleback have been observed in European populations (Schaarschmidt et al. 1999), suggesting that this may be a common characteristic of anadromous stickleback populations.

The large increase in the expression level of NKA in the marine ecotype in cold fresh water, and the high mortality of the anadromous ecotype in response to these conditions suggest that the combination of low salinity and low temperature could be a challenge for ionoregulation and survival in the marine and anadromous fish. Thus, these factors may have posed a physiological challenge for ancestral marine or anadromous stickleback when colonizing freshwater habitats.

Interactive effects of low temperature and salinity on gill morphology

By modifying the size of the interlamellar cell mass (ILCM), some fish are able to change gill lamellar surface area, which alters ion flux and oxygen uptake and directly affects energy expenditures (Sollid *et al*. 2003; Nilsson *et al*. 2012). Consistent with previous work on modifications of the ILCM in response to exposure to cold temperatures (Sollid *et al*. 2005; Mitrovic & Perry 2009; Barnes *et al*. 2014), we observed increases in ILCM area in both marine and freshwater ecotypes in response to the cold when fish were held at 11 ppt (Fig. 4.3, 4.4). This suggests that these fish are able to reduce gill lamellar surface area as oxygen demand decreases in response to temperature reduction, likely preventing unnecessary energy from being used for osmoregulation by limiting lamellar surface area in contact with the surrounding water. Likewise, consistent with previous work on the effects of salinity on the ILCM in mangrove killifish (LeBlanc *et al*. 2010), we observed an increase in ILCM area in

response to a decrease in salinity when fish were held at warm temperature (Fig. 4.3, 4.4), which is likely beneficial because it limits ion loss to dilute fresh water, hence preventing energy expenditure for ion uptake.

Here for the first time, we were able to detect interactive effects of these two factors on gill morphology. When fish were exposed to the combination of low salinity and low temperature, ILCM area increased to the level observed when fish were acclimated to low salinity alone, but the increase was not as large as that observed when fish were acclimated to low temperature alone (Fig. 4.3, 4.4). This suggests that the combined effects of low salinity and low temperature prevent both marine and freshwater stickleback from increasing ILCM area to the extent expected for low temperature exposure. Although the physiological causes and consequences of this effect are unknown, this observation suggests that interactions between low salinity and low temperature may play critical roles in the response of the gill.

We observed no differences between the ecotypes in the response of the ILCM to cold freshwater conditions, but it is possible that the ecotypes could differ in the number and location of ionocytes in response to changing temperature or salinity. In goldfish acclimated to warm fresh water, transfer to cold temperature resulted in an increase in the number and size of ionocytes (Mitrovic & Perry 2009). If the same were true in marine stickleback, this could potentially result in higher overall levels of the NKA protein in the gill of the marine ecotype in cold fresh water, which could produce the higher expression levels of NKA mRNA that we saw in our study. Further work investigating changes in ionocyte size, number, and location in relation to the ILCM (Mitrovic & Perry 2009; Barnes *et al*. 2014) could uncover potential differences between ecotypes that might

provide a mechanistic explanation for the functional differences that are present between stickleback ecotypes at low temperature (for example, growth differences in cold winter conditions in fresh water (Gibbons *et al*. 2016) and in the combination of low salinity and low temperature (Chapter 5)). Differences between ecotypes in any or all of these features would help provide more mechanistic physiological evidence for how the divergence and widespread adaptation to freshwater habitats occurred in stickleback.

Conclusions

Although multiple studies have shown that there has been repeated parallel evolution of stickleback in freshwater habitats, until recently very little work has investigated whether differences in abiotic factors between the ocean and fresh water may have been selective factors driving local adaption of physiological traits to freshwater habitats. Cold winter conditions (Gibbons *et al*. 2016) and low salinity (Marchinko & Schluter 2007; Chapter 5) have differential effects on the growth of stickleback ecotypes, highlighting the physiological challenges associated with colonizing fresh water. However, this is the first study to specifically examine how the interactive effects of salinity and temperature influence gene expression of ion transporters in gill ionocytes and gill morphology in stickleback (but see Schaarschmidt *et al*. (1999)). Connecting these new data with previous studies investigating growth and gill transcriptome differences between ecotypes (Gibbons *et al*. 2016; Gibbons *et al*. 2017), it is apparent that overwintering in cold freshwater habitats was likely a major physiological challenge for ancestral stickleback during this widespread post-glacial colonization.

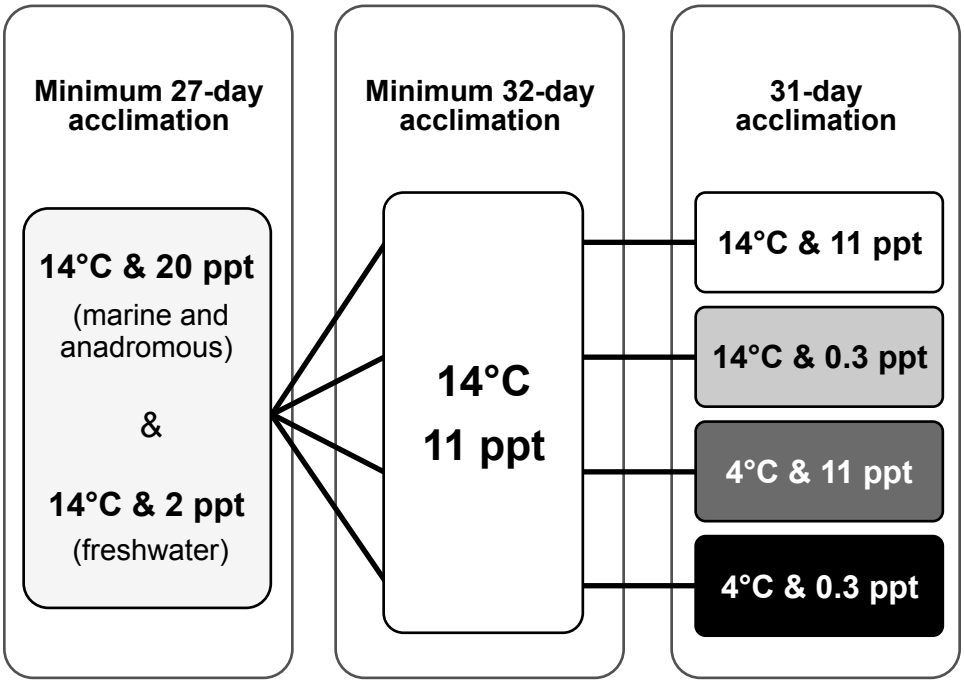

Figure 4.1. Experimental Design. Marine, anadromous, and freshwater stickleback were caught from the wild: marine and anadromous stickleback were initially acclimated to 14°C and a salinity of 20 ± 0.5 ppt; freshwater stickleback were initially acclimated to 14°C and a salinity of 2 ± 0.2 ppt. All fish acclimated in these conditions for at least 27 days. Acclimation salinities of all fish were then gradually changed (over 2-3 days) to 11 ppt; temperature remained at 14°C. All fish acclimated in these conditions for at least 32 days. Temperature and/or salinity were then gradually changed (over 1 day), resulting in the following conditions: 1) 14°C and 11 ppt (control conditions); 2) 14°C and 0.3 ppt; 3) 4°C and 11 ppt; 4) 4°C and 0.3 ppt.

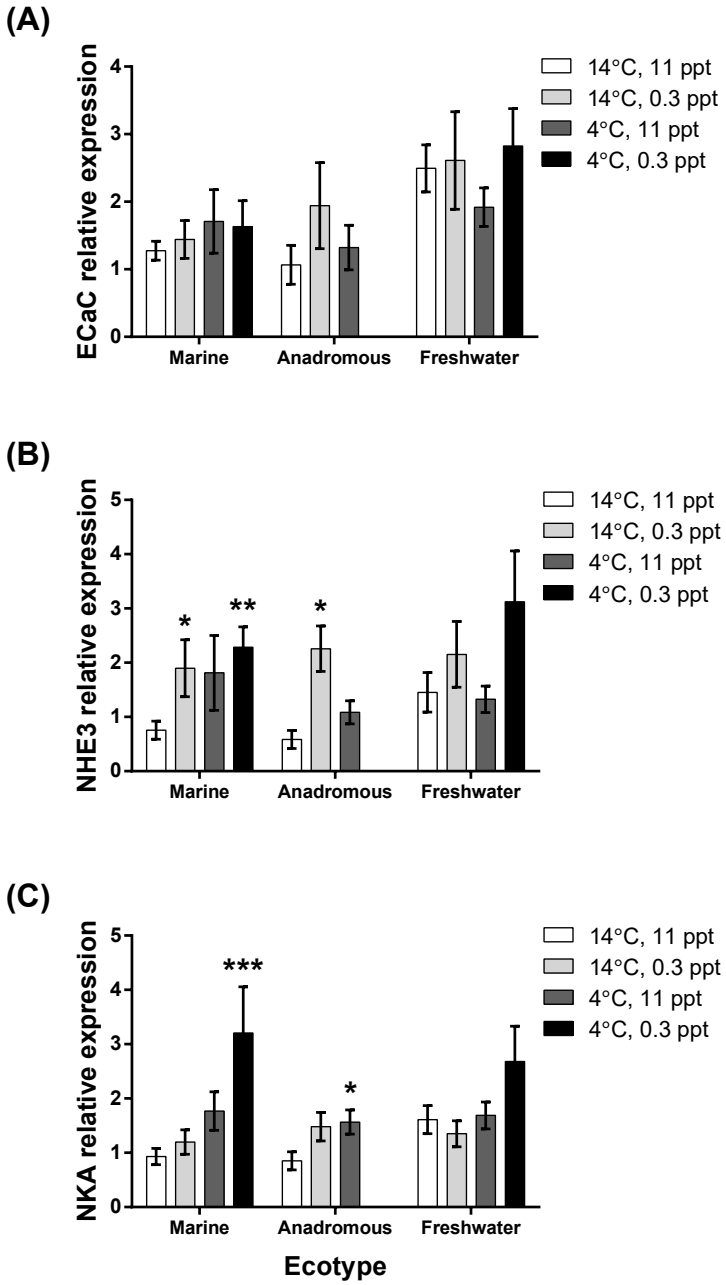

Figure 4.2. mRNA levels of ion transporters in gill tissue of marine, anadromous, and freshwater stickleback ecotypes acclimated to four different conditions. A. ECaC (*trpv6*), the epithelial calcium channel. B. NHE3 (*slc9a3.2*), the electroneutral Na^+/H^+ exchanger. C. NKA (*atp1a1a.5*), the Na^+,K^+-ATPase α-subunit. Expression is relative to a standard curve of a pool of all samples normalized to the expression of the control gene eukaryotic translation elongation factor 1 alpha 1b (*eef1a1b*). Values are presented as mean ± SEM. (*) denotes a significant difference in expression relative to the 14°C and 11 ppt treatment (control condition) within an ecotype. (*), (**), and (***) denote a significant difference in expression relative to the 14°C and 11 ppt treatment (control condition) within an ecotype. (*): $p \leq 0.05$; (**): $p \leq 0.01$; (***): $p \leq 0.001$.

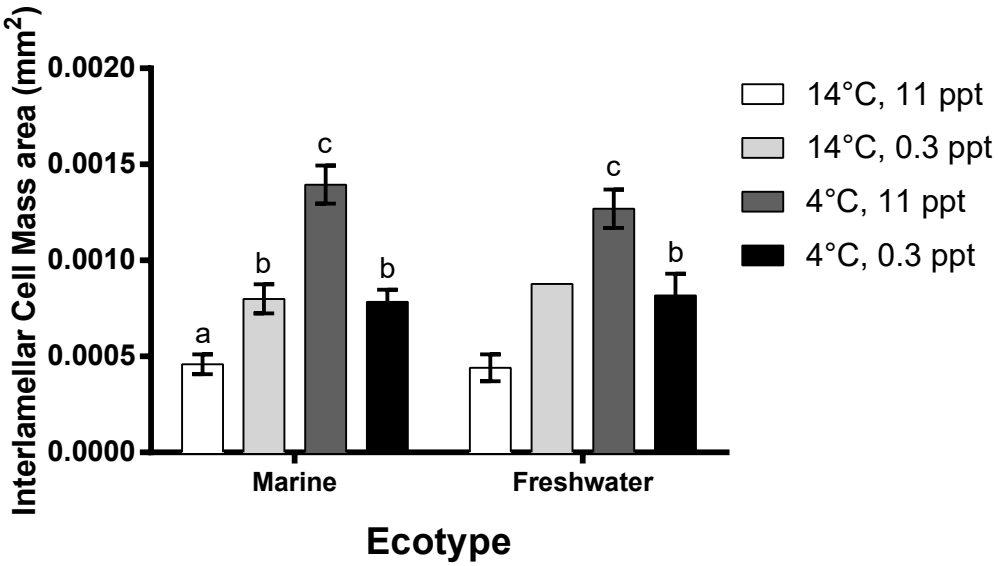

Figure 4.3. Interlamellar cell mass (ILCM) area of marine and freshwater stickleback ecotypes acclimated to four different conditions. Values are presented as mean ± SEM. Groups sharing the same letter do not differ significantly. Sample sizes were too low to make planned comparisons for the freshwater ecotype at (14°C, 11 ppt) and (14°C, 0.3 ppt) (Table C3, Appendix C).

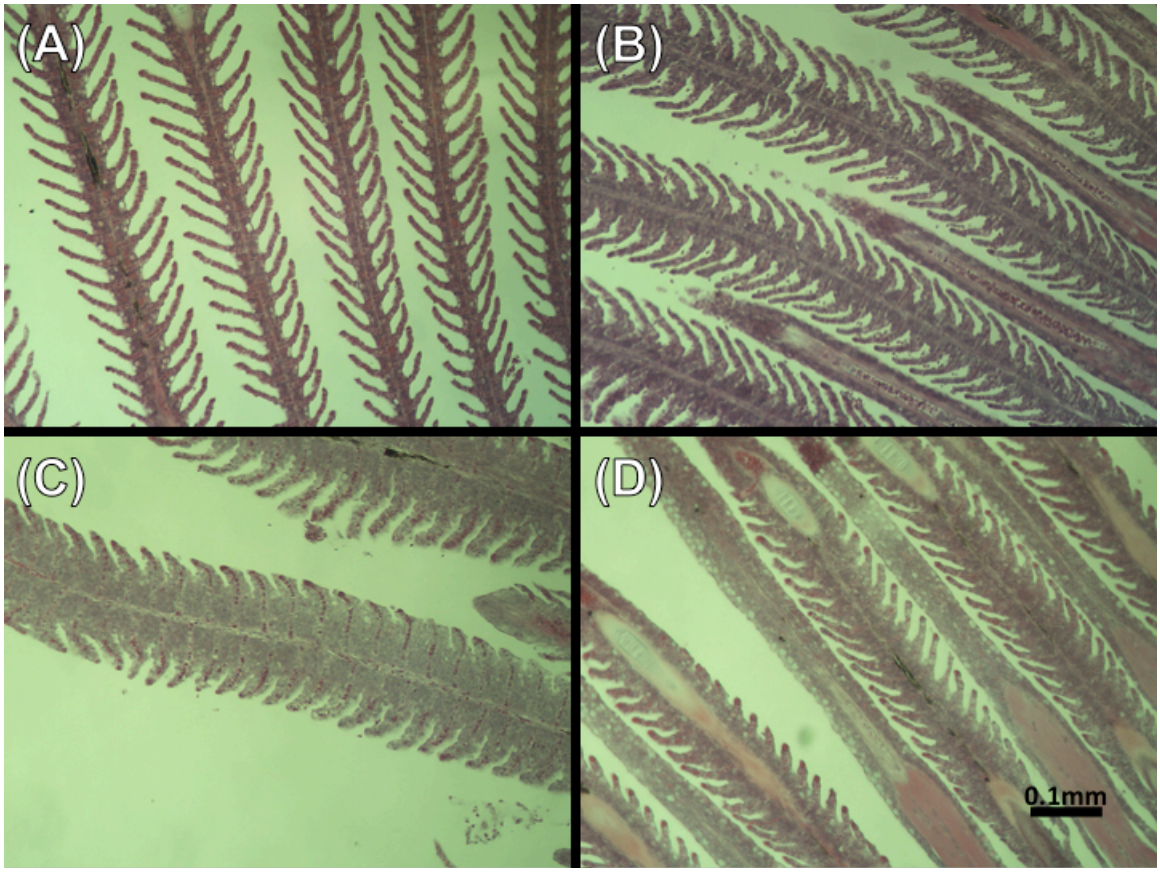

Figure 4.4. Representative images of gill filaments, and their associated lamellae, on the second gill arch of marine stickleback from all four treatment groups: A) 14°C and 11 ppt; B) 14°C and 0.3 ppt; C) 4°C and 11 ppt; D) 4°C and 0.3 ppt. Magnification is at 400X and the scale bar is 0.1 mm.

Chapter 5: Cold and diluted: Evidence for evolution in response to the interactive effects of temperature and salinity in threespine stickleback

Introduction

A growing number of studies have demonstrated that changes in abiotic factors can lead to local adaptation in physiological traits (Kitano *et al*. 2010; Fraser *et al*. 2011; Sanford & Kelly 2011; Cheviron & Brumfield 2012; Culumber *et al*. 2012; Savolainen *et al*. 2013). Yet most of these studies either do not address the specific abiotic factors driving divergence (i.e. they look for adaptation between environments that differ in many factors) or consider only a single abiotic factor in isolation. In natural environments multiple abiotic factors can co-vary between localities, and divergent natural selection is likely the result of responses to differences in multiple factors between environments (Macel *et al*. 2007). Thus, understanding local adaptation requires investigating both how individual factors can act as agents of selection and how interactive effects between factors drive divergence (Kawecki & Ebert 2004).

North-temperate zone fish species present an ideal test case for the investigation of the multifaceted nature of local adaptation to new environments. As Pleistocene glaciers receded, marine and anadromous fishes in the north-temperate zone would have been able to move into newly created freshwater habitats that provided "…diverse niche opportunities, including habitat diversity, habitat complexity, food-chain structure and other variables" (McDowall 2008), and adopt a freshwater-resident life history. Although freshwater habitats presented these benefits to the new colonists, they also presented a suite of biotic and abiotic challenges such as differences in salinity, ion availability,

nutrients, and temperature, which are known to influence migration patterns between the ocean and fresh water and the evolution of freshwater residency (Gross *et al*. 1988; Rikardsen *et al*. 2000, 2006; McDowall 2001, 2008; Svenning & Gullestad 2002; Thomsen *et al*. 2007; Jensen & Rikardsen 2008; Finstad & Hein 2012; McMillan *et al*. 2012). An interaction of particular importance for the adoption of year-round freshwater residency in the north-temperate zone is the combination of low salinity and cold winter temperatures in fresh water, which may present difficulties for fish growth and survival (Schaarschmidt *et al*. 1999). For anadromous fish, migration back to the sea during the winter allows them to avoid these harsh winter conditions (McDowall 2001, 2008). As such, evolution in response to cold winter conditions and low salinity might be critical for the long-term persistence of temperate freshwater fish populations.

The threespine stickleback (*Gasterosteus aculeatus*) is a species that can give us insight into the evolution of freshwater residency. After the recession of Pleistocene glaciers in the Northern hemisphere 10,000-20,000 years ago, ancestral marine or anadromous stickleback colonized fresh water from the ocean (Bell & Foster 1994; McPhail 1994; Colosimo *et al*. 2005; Boughman 2007; Jones *et al*. 2012a,b). This was followed by adaptation to freshwater habitats, demonstrated by the parallel evolution of morphological, physiological, and behavioral traits in freshwater populations (Baker 1994; McPhail 1994; McKinnon & Rundle 2002; McKinnon *et al*. 2004; Colosimo *et al*. 2005; Schluter 2009; Jones *et al*. 2012a,b). Additional work has provided strong evidence that selection on growth is likely to have been present and may have played a role in the evolution of the prominent differences between marine and freshwater stickleback ecotypes (Marchinko & Schluter 2007; Barrett *et al*. 2008, 2009).

During their colonization of freshwater habitats from the ocean, it is likely that stickleback faced not only the challenge of a change in salinity, but also a change in temperature. In the north-temperate zone, the temperature of freshwater habitats is more variable than the temperature of the ocean (Lee & Bell 1999), and, in particular, lakes in British Columbia become colder than the ocean in the winter (Barrett *et al*. 2011). Previous studies have suggested that marine stickleback are at a growth disadvantage compared to freshwater stickleback in fresh water at an intermediate temperature (Marchinko & Schluter 2007), and that cold winter conditions reduce the growth of marine stickleback more than freshwater stickleback (Gibbons *et al*. 2016). These two studies separately investigated different abiotic factors that may act as agents of divergent natural selection in stickleback when they evolve freshwater residency, but did not assess the interactive effects of salinity and temperature or follow the effects of these environmental differences across a winter season. In the present study, we monitored the growth and survival of marine, anadromous, and freshwater stickleback from hatching to an age of nine months in a factorial experiment that manipulated both salinity and winter temperature. The factorial manipulation confers three crucial benefits over previous work: 1) the design mimics ecologically realistic conditions (e.g. cold fresh water and warm salt water); 2) the manipulation of two factors allows for a direct comparison of their relative effects; 3) manipulating two factors allows for a test of their interactive effects. We predicted that freshwater stickleback would show local adaptation to both low salinity and cold winter temperatures when compared to the marine and anadromous ecotypes.

Materials and methods

Stickleback populations, acclimation conditions, & time course

Adult stickleback were collected from three populations in May and June of 2014. All collection sites were in British Columbia, and included one marine population from Oyster Lagoon (49°36'43.53"N, 124°01'52.12"W), one anadromous population from the mouth of the Little Campbell River (49°00'52"N, 122°45'33"W), and one freshwater population from Trout Lake (49°30'29"N, 123°52'29"W). Only male and female stickleback that were in breeding condition were collected, and they were used to generate progeny using the artificial fertilization techniques outlined in Marchinko & Schluter (2007). All experiments were conducted under University of British Columbia approved animal care protocols (#A10-0285; A11-0372).

Genetic crosses from wild-caught adults were performed at two different salinities immediately after collecting the fish: 0 ppt (dechlorinated Vancouver tap water) and 20 ppt (made using Instant Ocean ® sea salt), yielding the following numbers of families: 0 ppt: five families Oyster Lagoon x Oyster Lagoon (OL x OL), four families Trout Lake x Trout Lake (TL x TL), four families Little Campbell River x Little Campbell River (LC x LC); 20 ppt: six families OL x OL, four families TL x TL, six families LC x LC. Individual families were raised in 100 L glass aquaria at the University of British Columbia at the same salinity at which the genetic crosses were performed, at a water temperature of 17°C and a photoperiod of 12L:12D. Larval stickleback were fed live brine shrimp twice daily for the first four months, a mixture of brine shrimp and finely chopped bloodworms (Chironomid larvae) to satiation for the next three months, and roughly chopped bloodworms to satiation for the remaining two months of the

experiment. Approximately one month after hatching, fish families (one family in each aquarium) were split and evenly redistributed amongst aquaria so each new aquarium contained an even mixture of fish from each individual family (from the same ecotype and salinity). From this point forward, individual families were not tracked, and thus family is not included as a unit of replication in subsequent analyses. Each aquarium had a starting fish density of 27-28 fish, with the following total numbers of aquaria: 0 ppt: eight OL x OL, four TL x TL, eight LC x LC; 20 ppt: eight OL x OL, four TL x TL, eight LC x LC. Half of the aquaria from each ecotype/salinity combination were housed in one of two environmental chambers; both chambers had an initial temperature of 17°C and photoperiod of 12L:12D. Five months after hatching, 10 fish from each aquarium were individually marked so that growth, in both length and mass, of individual fish could be tracked over the course of the experiment. Body size is positively correlated with reproductive output in stickleback (Wootton 1984; Schluter 1995) and is therefore a proxy for fitness in each environment.

Five months after hatching, the photoperiod in both chambers was switched to mimic that of the natural environment (photoperiod was gradually transitioned from 12L:12D to 8L:16D by reducing the amount of light by 20 minutes per week). Photoperiod was altered to simulate seasonal conditions during fall and winter, as changes in photoperiod that precede winter are a critical cue for changes in physiology and energy metabolism that are important for winter survival and preparation for reproduction (Beamish 1964; Evans 1984; Bradshaw & Holzapfel 2007; Shuter *et al*. 2012). Temperature was maintained at 17°C for the entirety of the experiment in the 'control' environmental chamber, but five months after hatching, temperature was

gradually decreased by 0.13°C per day to reach a minimum of 4°C in the 'experimental' environmental chamber (Fig. 5.1). Note that because there was only one environmental chamber designated as the control environment and one environmental chamber designated as the treatment environment, temperature and chamber effects are confounded.

Fish length and mass were measured on all fish in each aquarium at four points in both environments (Fig. 5.1): 1) Five months (151 days) post-hatching, immediately before temperature reduction; 2) 57 days after the start of temperature reduction at which point the temperature in the experimental condition had reached 9.5°C; 3) at the end of the temperature reduction phase (44 days after the second sampling); 4) 28 days after reaching the minimum temperature of 4°C.

Analysis of growth data and growth in relation to degree-days

To investigate the effects of salinity, temperature, and stickleback ecotype on mass and length, we carried out linear mixed effects models (LME) (Pinheiro & Bates 2000) at the time of first sampling and the final sampling, implemented in R. LME models for mass and length at first sampling included salinity and stickleback ecotype as fixed effects and rearing tank as a random effect to explicitly investigate the influence of salinity on early growth of each ecotype. To assess the overall effects of salinity, temperature, and ecotype we included all three variables as fixed effects in LME models with final mass and length data, and we also included rearing tank as a random effect. These LME models were used to assess stickleback size both at the early and late growth stages of the study.

To test explicitly for the effects of experimental treatments on growth rate, we calculated specific growth rate (SGR; for both mass and length) as the difference in log-transformed mass and length between sample periods, divided by the time of growth (in days). We used LME models with salinity, temperature, stickleback ecotype, and sample period as fixed effects and individual and rearing tank as random effects, to determine the effects of experimental treatments over time on SGR. We examined each response variable for normality (Shapiro & Wilk 1965) and transformed the data if necessary before carrying out statistical tests. Individually marked fish within each tank were the unit of replication for mass and length data.

To assess stickleback growth relative to temperature conditions, stickleback growth was standardized to degree-days (DD). Degree-days are a measure of the amount of ambient thermal energy that an ectotherm experiences (Chezik *et al*. 2014), and thus expressing growth relative to degree-days allows the explicit investigation of growth independent of ambient thermal energy. We calculated the number of degree-days experienced by the control and experimental treatments between the last two sampling periods when the temperatures were the most divergent. Degree-days were calculated using the following formula:

$$\mathrm{DD} = \sum_{d=0}^{n} T_d - T_0$$

Where DD = cumulative degree-days, T_d is the temperature on a given day, and T_o is the base temperature, which is the temperature at which growth no longer occurs (Chezik *et al*. 2014). Previous studies estimated values of T_o in stickleback as 3°C (Allen & Wootton 1982) and 3.5°C (Lefébure *et al*. 2011). As such, and based on the results

observed in the current study, we used a value of 3°C for T_o. We employed an LME model with salinity, temperature, and stickleback ecotype as fixed effects and used rearing tank as a random effect.

Mortality

We also tested for the effects of experimental treatments on mortality. We used a generalized linear mixed model approach implemented in the 'lme4' package in R (Bates *et al*. 2015) with salinity, temperature, and stickleback ecotype as fixed effects to understand how these treatments, and the interactions between them, altered total mortality. Mortality data were counts and showed a pattern of overdispersion, so we specified a quasi-Poisson distribution, which is a term that fits overdispersion, for the distribution in the model (Venables & Ripley 2002).

All LME models in the study were analyzed using a Wald chi-square test to calculate chi-square and p-values for each LME (Fox 2008) ('Anova' command, 'car' package in R (Fox & Weisberg 2011)).

Results

Mass at the first and last sampling

Mass across time during the experiment is presented in Fig. D1 in Appendix D. At the initial sampling point we observed a direct effect of salinity (df=1,28, Chisq=12.92, p=0.0003) on mass (Fig. 5.2A). At the final sampling point we observed significant effects of ecotype (df=2,22, Chisq=7.95, p=0.019), temperature (df=1,22, Chisq=4.24,

p=0.040), and the interaction between ecotype and salinity (df=2,22, Chisq=6.54, p=0.038) on final mass (Fig. 5.2B).

Length at the first and last sampling

Fish length across time during the experiment is presented in Fig. D1 in Appendix D. For initial length, we observed a significant effect of salinity (df=1,34, Chisq=11.40, p=0.0007) and ecotype (df=1,34, Chisq=3.25, p=0.039) with no significant interaction (Fig. 5.2C). In contrast, for final length, we uncovered both significant effects of ecotype (df=2,22, Chisq=8.70, p=0.0123) and an interaction between salinity and ecotype (df=2,22, Chisq=7.18, p=0.028) (Fig. 5.2D).

Specific growth rate

Fig. 5.3 presents the effects of temperature, salinity, and ecotype on specific growth rate (SGR) for mass. SGR for mass was significantly affected by ecotype (df=2,22, Chisq=12.80, p=0.0017) and temperature (df=1,22, Chisq=7.39, p=0.006). In addition, there was an ecotype by time interaction (df=4,424, Chisq=10.02, p=0.04), with marine and anadromous fish showing a steeper decline in SGR over the course of the study. Furthermore, there was a temperature by time interaction (df=2,424, Chisq= 60.30, p<0.0001), with SGR decreasing more slowly in the 17°C control conditions than in the experimental conditions. There was also a salinity by time interaction (df=2,424, Chisq=7.23, p=0.027), with fish showing a higher SGR in 20 ppt early in the first two growth periods. We also observed a salinity by temperature interaction (df=1,22, Chisq=3.97, p=0.047), with fish kept at 17°C having a higher SGR when exposed to 0 ppt

in contrast to fish kept at 4°C experimental conditions having a higher SGR when kept at 20 ppt. Furthermore, there was a temperature by ecotype by time interaction (df=4,424, Chisq=22.66, p=0.0001), driven by rapidly declining SGR of both marine and anadromous fish when they were kept in cold experimental winter conditions. We also observed a temperature by salinity by time interaction (df=2,424, Chisq=15.18, p=0.0005) that was driven by rapidly declining SGR when fish experienced 4°C experimental winter conditions and 0 ppt. This was in contrast to 17°C control conditions, where fish kept at 0 ppt maintained higher SGRs than those kept at 20 ppt throughout the study. Finally, we found an ecotype by salinity by temperature by time interaction (df=2,424, Chisq=22.40, p=0.022) that stemmed from differences in SGR between marine and freshwater fish increasing over time when held in the 4°C and 0 ppt experimental winter conditions.

Growth in relation to degree-days

The number of degree-days (DD) differed substantially between temperature treatments during the last growth period between sample points 3 and 4 (experimental conditions = 29 DD; control conditions = 406 DD) (Fig. 5.4). There was an effect of ecotype (df=2,22, Chisq=19.73, p<0.0001) and winter temperature (df=1,22 Chisq=26.15, p<0.0001) on growth expressed relative to degree-days. In addition, there was a significant interaction between ecotype and winter temperature (df=2,22, F=18.46, p<0.0001), with the freshwater ecotype showing a much greater ability to grow in cold conditions than the anadromous or marine ecotypes.

Mortality

There were 2,782 total fish in the experiment and 232 total mortalities during the study. Salinity had an effect on mortality (df=1, Chisq=40.50, p<0.0001), as tanks with a salinity of 0 ppt had an average of 3.20 mortalities compared to 0.50 mortalities for tanks with a salinity of 20 ppt (Fig. 5.5). In addition, there was a non-significant trend toward an interactive effect of salinity and ecotype on mortality (df=2, Chisqi=5.15, p=0.064), with tanks of marine fish kept at 0 ppt having an average of 3.67 mortalities compared to 0.21 mortalities on average for anadromous fish kept at 20 ppt.

Discussion

When colonizing novel environments, populations may immediately face changes in a number of abiotic conditions, each of which may impose selection. In addition, combinations of novel abiotic factors may pose particularly strong barriers to residence in novel environments. Disentangling the effects of each abiotic difference and their interactions is a crucial step in identifying the abiotic challenges associated with the colonization of novel environments. Ancestral anadromous stickleback leave fresh water to migrate to the ocean prior to the onset of cold winter temperatures (Hagen 1967), and thus do not naturally experience the combination of low temperature and low salinity that are characteristic of freshwater habitats in the north-temperate zone. Here we show that both salinity and temperature, and the interaction between these abiotic factors, differentially alter the growth and size of stickleback ecotypes with different evolutionary histories. Our data suggest that growing in low salinity conditions is a challenge for marine and anadromous fish. Temperature also caused profound differences in growth

between ecotypes, as marine fish showed a substantial reduction in growth at low temperatures, while freshwater stickleback were able to maintain relatively high growth rates at the coldest winter temperatures. The striking ability of freshwater stickleback to maintain growth rates in cold fresh water, in concert with previous work on differences in thermal tolerance among the ecotypes (Barrett *et al*. 2011; Gibbons *et al*. 2016), suggests that cold overwintering temperatures may be a key agent of natural selection for marine fish colonizing temperate freshwater environments.

Growth in fresh water

We observed an interaction between salinity and ecotype on both mass and length in stickleback at later life stages. This interactive effect likely stemmed from faster growth over time by freshwater stickleback in 0 ppt conditions. For example, at the time of first sampling, freshwater stickleback were 20% heavier than marine stickleback in fresh water (Fig. 5.2A), but by the end of the study this difference had increased to 33% (Fig. 5.2B). Low salinity also had a negative effect on survival, with both the marine and anadromous ecotypes having much higher mortality at low salinity than at high salinity (Fig. 5.5). The superior growth of the freshwater ecotype relative to the marine ecotype prior to temperature reduction (Fig. 5.2C) may be beneficial by allowing higher initial levels of pre-winter energy storage (Shuter *et al*. 2012). This is critical because smaller fish have higher mass-specific metabolic rates and lower lipid storage capacity, contributing to a high risk of winter mortality (Ultsch 1989; Shuter & Post 1990; Shuter *et al*. 2012). Therefore, freshwater stickleback may have an advantage during overwintering in fresh water due to a superior ability to store energy prior to winter and

deplete these energy stores more slowly than marine and anadromous ecotypes (Shuter *et al*. 2012).

In addition, a key component of the negative effects of low salinity on the growth of the fully plated marine ecotype may be the low concentrations of calcium associated with fresh water, as freshwater colonization is associated with reductions in bony structures such as lateral plates, spines, and the pelvic girdle in stickleback (Giles 1983; Bell *et al*. 1993; Spence *et al*. 2012, 2013). For example, the growth of completely plated stickleback is inhibited to a greater degree than the growth of low plated stickleback at low calcium concentrations, providing evidence that low calcium concentration may have been an important selective agent driving major differences between marine and freshwater ecotypes (Spence *et al*. 2012). Furthermore, stickleback ecotypes may differ in their ability to take up calcium from their environment. In dilute fresh water, where stickleback are confronted with the problem of diffusive ion loss, the epithelial calcium channel (ECaC) is an ion transporter located in the fish gill that is utilized to actively pump calcium from dilute fresh water back into the blood plasma (Evans *et al*. 2005; Hwang *et al*. 2011). Freshwater stickleback exhibit higher gene expression of ECaC than marine stickleback in fresh water, independent of decreases in temperature and photoperiod associated with winter conditions (Gibbons *et al*. 2016). Increased ECaC expression in the freshwater ecotype may play a beneficial role in calcium uptake and the improved growth of freshwater stickleback in fresh water.

Interactions between low temperature and low salinity

During the last sampling period in our experiment, when temperatures were the most divergent between the two treatment groups (Fig. 5.1), we observed striking differences in growth between ecotypes. Specific growth rate (SGR) (Fig. 5.3) showed an ecotype, by temperature, by salinity, by time interaction, suggesting that the interaction between ecotype, winter temperature, and salinity plays a major role in dictating daily growth of stickleback. Because of the reduced thermal energy in the environment at low temperatures, the growth rate and metabolic rate of ectotherms is expected to decline in the cold, and this effect is evident when examining SGR for both mass and length during the final sampling period (Fig. 5.3 and Fig. D2 in Appendix D, respectively). However, these effects of low temperature differed among ecotypes. Freshwater stickleback showed only a small (~20%) reduction in SGR for mass when experiencing 4°C winter conditions (i.e. between the third and final sampling period), while marine and anadromous stickleback showed sharp declines (82% and 74% respectively) under these same 4°C winter conditions (Fig. 5.3B). This difference cannot be explained by inherent differences in growth rate among the ecotypes, as freshwater, marine, and anadromous stickleback showed similar small declines in growth rate during this same life stage when kept at 17°C (8%, 2%, and 5% respectively) (Fig. 5.3A). These population level differences in cold conditions were magnified in fresh water, where anadromous populations showed extreme reductions in growth (84%) and marine fish showed a negative growth rate (-36%). The relatively small reduction in SGR in the freshwater ecotype in the cold is unexpected, because in the absence of physiological compensation, rate processes such as growth would be expected to decline by ~2-3 fold for each 10°C decrease in temperature.

Our data strongly suggest that freshwater stickleback are able to induce physiological compensatory mechanisms to reduce or prevent the expected thermodynamically-driven reduction in growth, and that these mechanisms are not available, or not as effective, in marine or anadromous stickleback. Previous work has suggested differential gene expression plasticity in response to temperature among stickleback ecotypes (Morris *et al*. 2014), and these or other differences in gene expression plasticity have the potential to play a role in shaping the growth differences we observe here. Furthermore, freshwater stickleback may have less permeable gills than marine stickleback in fresh water (Gibbons *et al*. 2017), potentially resulting in higher energetic costs associated with ion uptake for marine stickleback in fresh water. These higher costs could result in lower growth rates for marine stickleback in fresh water.

The differences in growth between ecotypes at low temperature and low salinity are most evident when growth is examined in terms of "degree-days" (Fig. 5.4). Degree-days represent the cumulative thermal energy available for growth (Chezik *et al*. 2014). During the last sampling period growth per degree-day was very similar among the ecotypes and was not affected by salinity when fish were held at 17°C (Fig. 5.4). In contrast, when fish were exposed to low temperature, the freshwater ecotype had much higher growth per degree-day than did the other two ecotypes, clearly demonstrating that this ecotype has superior ability to grow in the cold. In addition, the growth of the marine ecotype was severely affected by the combination of cold temperature and low salinity, whereas the growth of the other two ecotypes was not negatively affected by low salinity at either temperature (Fig. 5.4). These divergent patterns among the ecotypes strongly

suggest that overwintering in fresh water may have presented a challenge to freshwater colonization for stickleback in the north-temperate zone.

Conclusions

Our results are consistent with previous work that has suggested that the combination of cold and fresh water may have presented a challenge to freshwater colonization in stickleback. For example, freshwater stickleback have evolved superior acute cold tolerance relative to that of the ancestral marine form (Barrett *et al*. 2011; Gibbons *et al*. 2016), suggesting that low temperatures act as a selective force driving divergence in stickleback. However, differences in cold tolerance between ecotypes are abolished after acclimation to winter conditions (Gibbons *et al*. 2016), indicating that poor cold-tolerance per se cannot be a major barrier to freshwater colonization. The data presented here suggest that cold temperatures drive differences in growth rate between marine and freshwater ecotypes during the winter in fresh water, which represents a potential fitness cost during freshwater colonization. Although we have examined only single marine, anadromous, and freshwater populations in this study, our results suggest that these freshwater fish may have adapted to growing at cold winter temperatures likely only experienced in fresh water, which could represent a case of fairly rapid adaptation in a key physiological process in response to a challenging thermal regime.

As a whole, our study demonstrates that these two abiotic factors, and the interaction between them, can shape the performance of closely related populations. In addition to temperature and salinity, previous work has identified nutrient availability (El-Sabaawi *et al*. 2016) as a potential axis of variation, and many more factors could

also be important. As such, investigations into what drives physiological adaptation should most often include a manipulation of multiple factors; simply because environments often differ along a number of axes. This view may seem daunting, as experiments quickly become less tractable as more treatments are added, but in many cases it adds crucial realism to the investigation of physiological adaptation.

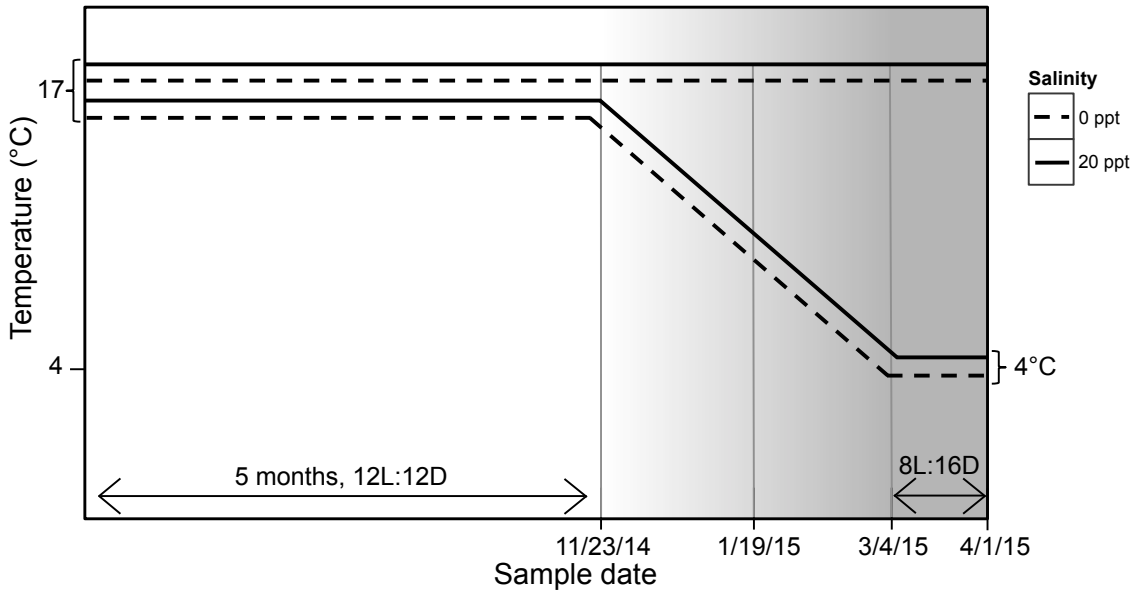

Figure 5.1. Experimental Design. All ecotypes (anadromous (Little Campbell River), marine (Oyster Lagoon), freshwater (Trout Lake)) were hatched and raised in salinities of 0 ppt and 20 ppt for 5 months at 12L:12D photoperiod. After 5 months, starting on 11/23/14, the light period was decreased at a rate of 20 minutes per week and reached a minimum of 8L:16D (solid grey shading) at 2/24/15 for all groups. Over the same period, the temperature in the experimental tanks was decreased by 0.13°C per day, reaching a minimum of 4°C on 3/4/15, while the control tanks were maintained at 17°C.

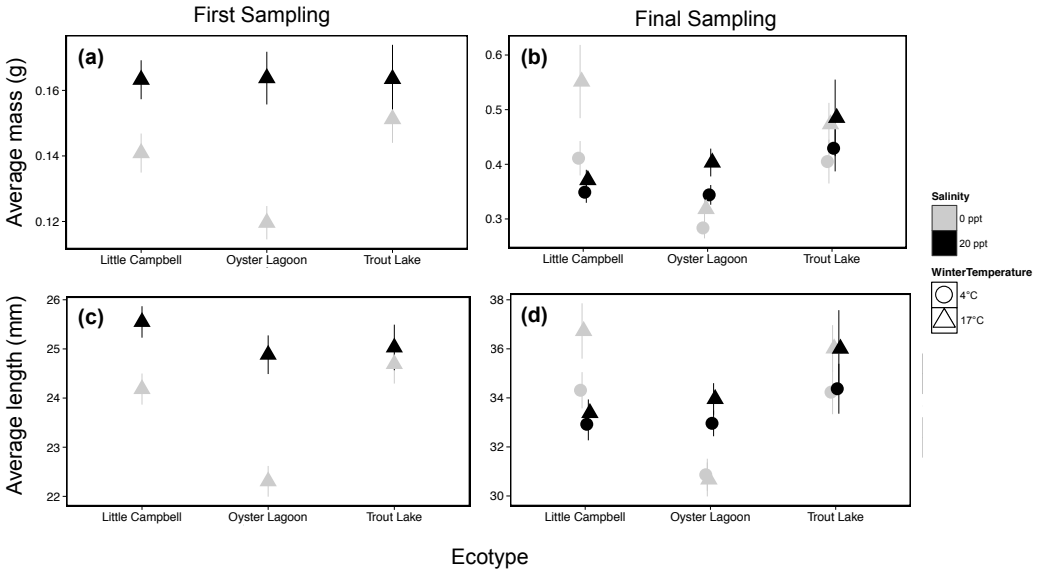

Figure 5.2. Mean mass and length of stickleback at the first and final sampling periods. Panel A, C: mass and length, respectively, of stickleback at the first sampling period. Panel B, D: mass and length, respectively, of stickleback at the final sampling period. Little Campbell River = anadromous ecotype; Oyster Lagoon = marine ecotype; Trout Lake = freshwater ecotype.

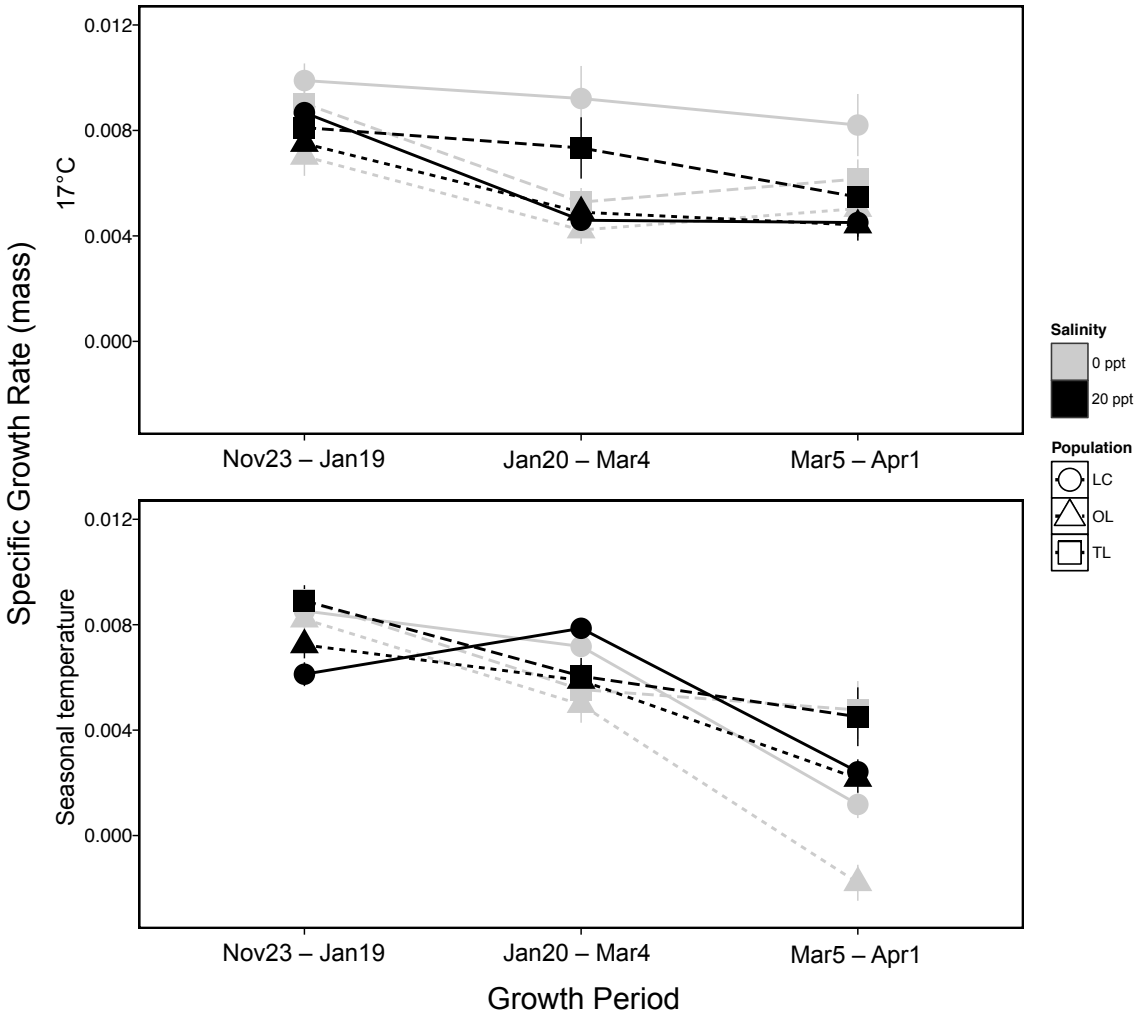

Figure 5.3. Specific growth rates (SGR) for mass between sampling points. Panel A: stickleback held at 17°C for the duration of the study. Panel B: stickleback that experienced declining temperatures. All data are expressed as mean ± SEM. (See Fig. D2 in Appendix D for SGR for length). LC = Little Campbell River (anadromous ecotype); OL = Oyster Lagoon (marine ecotype); TL = Trout Lake (freshwater ecotype).

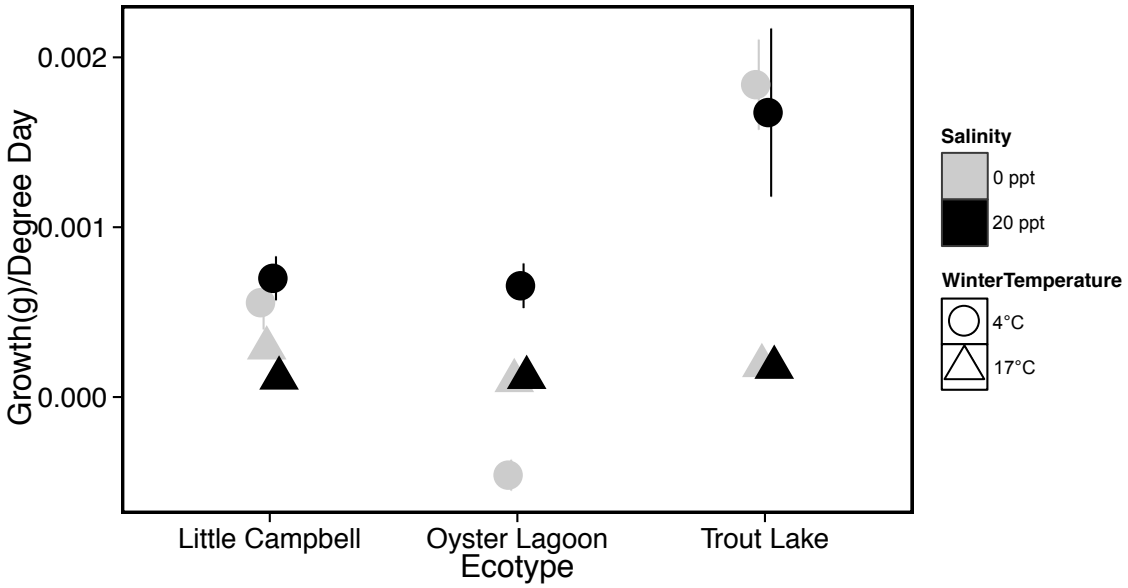

Figure 5.4. Growth per degree-day during the final sampling period (3/5/15-4/1/15). All data are expressed as mean ± SEM. Little Campbell River = anadromous ecotype; Oyster Lagoon = marine ecotype; Trout Lake = freshwater ecotype.

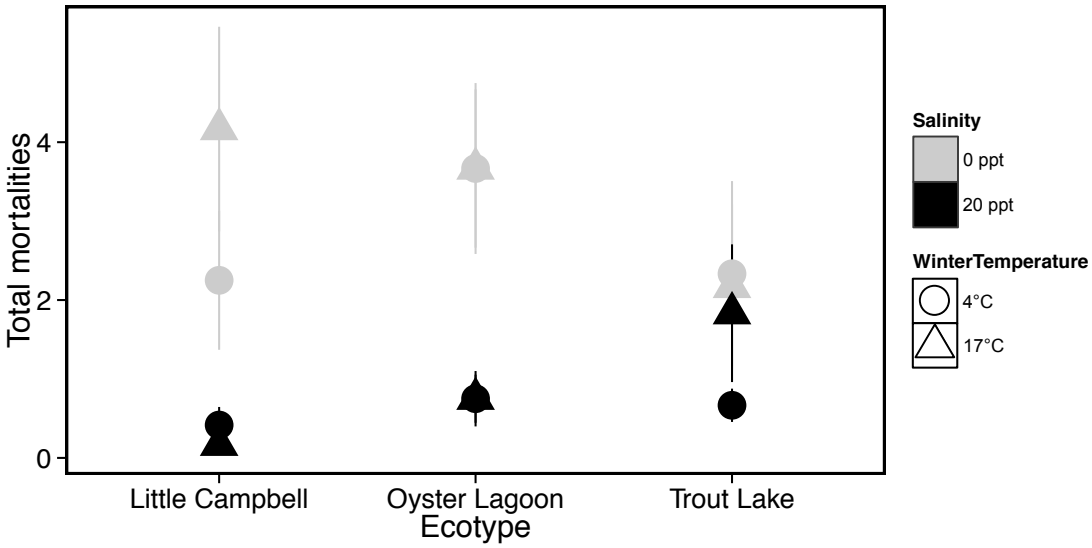

Figure 5.5. Mortalities over the course of the experiment. Data are presented as mean number of mortalities per tank ± SEM. Little Campbell River = anadromous ecotype; Oyster Lagoon = marine ecotype; Trout Lake = freshwater ecotype.

Chapter 6: Conclusion

Major findings & implications

Decades of research on stickleback has provided important insights into the evolutionary processes involved in the colonization of new habitats. By taking advantage of one key attribute of this system, that current marine populations are likely very similar to the putative ancestor, researchers have examined variation between ancestral marine and derived freshwater stickleback populations in genetic, physiological, morphological, and behavioral traits, highlighting numerous traits that exhibit parallel divergence and adaptation to freshwater habitats. However, prior to the work presented in this thesis very little research had looked specifically for potential physiological differences in ionoregulation between stickleback ecotypes that could provide an adaptive benefit to freshwater stickleback in these recently colonized habitats. Although there are many biotic and abiotic differences between the ocean and fresh water, I focused my dissertation research on the two most obvious, simple abiotic differences between these habitats that might pose challenges to colonization of freshwater habitats: salinity and temperature.

I focused on stickleback populations in the north-temperate zone, where the substantial differences in salinity and temperature between the ocean and lakes allowed me to develop the following hypothesis: the combination of low salinity and low temperature in freshwater lakes, which was not experienced by ancestral marine or anadromous stickleback, likely posed a physiological challenge to the colonization of freshwater habitats. The overall goal of my thesis was to test this hypothesis by examining which physiological traits or mechanisms are differentiated between

stickleback ecotypes. In this way, I aimed to provide physiological insight into the adaptive evolution of stickleback in fresh water.

One of the first traits that I investigated was acute cold tolerance, or critical thermal minimum (CTmin). Previous work had shown that freshwater stickleback in British Columbia have superior cold tolerance to marine stickleback, and in experiments done in artificial ponds, this cold tolerance evolved in the marine ancestor over the course of just a few generations (Barrett *et al*. 2011). Therefore, this was consistent with rapid adaptive evolution of acute cold tolerance, and suggested that the poor acute cold tolerance of marine stickleback could have posed a challenge to colonization of freshwater habitats in the northern parts of the species range. However, cold tolerance in fish is known to exhibit plasticity in response to low temperature in many fish species (Beitinger *et al*. 2000). So, it was also possible that CTmin in stickleback may be a plastic trait that could improve through acclimation within a single generation.

In this first experiment I found that stickleback exhibited phenotypic plasticity in cold tolerance, and that after acclimation to moderately low temperatures, the acute cold tolerance (CTmin) of marine and freshwater stickleback did not differ, and was lower than they likely experience in nature. Therefore, I concluded that cold tolerance alone was unlikely to present a challenge to colonization of fresh water in British Columbia (Chapter 2). However, in this experiment I also monitored fish size as an index of growth and found that cold winter conditions in fresh water reduced the growth of marine stickleback more than freshwater stickleback. Growth (which has important fitness implications in stickleback) in cold winter conditions in fresh water habitats was therefore highlighted as a likely physiological challenge to the colonization of freshwater

habitats. Additionally, expression of the gill ionocyte ion transporter ECaC showed clear divergence between stickleback ecotypes, with the freshwater ecotype exhibiting higher expression levels regardless of temperature. This result suggests that the freshwater ecotype might have a superior ability to take ions up from their dilute freshwater environment, and given that calcium is critical for the growth of bony skeletal elements this could account for the observed differences in growth.

The results of this experiment established the major areas upon which I focused for the remainder of my work: growth and gene expression in the gill in response to cold freshwater conditions. The goal was to answer the question of whether the combined effects of low salinity and low temperature presented a physiological challenge to the colonization of freshwater habitats for ancestral marine or anadromous stickleback. After performing three additional, inter-related experiments, the results showed that the combined effects of low salinity and low temperature likely did present a physiological challenge to the colonization of freshwater habitats.

Possibly the most compelling data to illustrate this is the growth differences I found between ecotypes during the cold winter conditions that were simulated in Chapter 5. Marine and anadromous stickleback exhibited large decreases in growth (82% and 74% reductions, respectively) in cold fresh water during simulated winter conditions, while freshwater stickleback did not (exhibiting only a ~20% reduction in growth). This inability to compensate physiologically by the marine and anadromous ecotypes shows that the combination of low salinity and low temperature was likely a physiological challenge for overwintering and survival in fresh water, which would have been a mandatory first step for a successful freshwater colonization.

In Chapter 3, I investigated gene expression patterns in the gill across the whole transcriptome in marine and freshwater ecotypes. These data demonstrated distinct differences in gene expression between ecotypes that could play a role in the whole organism growth differences identified in Chapter 2 and 5. Specifically, in response to fresh water, both ecotypes remodeled their gills by increasing the expression of genes associated with transmembrane transport and epithelial cell migration. In addition to increasing the expression of genes that maintain cell volume and membrane permeability (aquaporin, claudins, occludin), qRT-PCR work enabled the discovery that both ecotypes only increase the expression of critical ion transporters once salinity becomes very low (below 2 ppt), something not previously known in stickleback. However, ecotypes did exhibit major divergence in the expression of genes involved in cell adhesion and the extracellular matrix of the gill, with the freshwater ecotype up-regulating many more of these genes in response to fresh water. Consequently, it is probable that the freshwater ecotype has "tighter" gills, with lower gill permeability than the marine ecotype, particularly when exposed to fresh water. If these mRNA expression patterns are consistent with patterns at the level of protein amounts and activities, this could result in higher energetic costs for the marine ecotype in fresh water, providing a physiological mechanism for why the marine ecotype has a lower growth rate in cold fresh water.

While this gene expression in the gill provided a great deal of new data about the challenge posed by the combined effects of low salinity and low temperature, there is also the possibility that morphological changes in the gill could play a role in the response to the combination of low temperature and freshwater exposure. By investigating changes in the gill ILCM and ion transporter gene expression (Chapter 4), I

showed that the combination of low salinity and low temperature likely prevented both marine and freshwater stickleback from increasing ILCM area, as increases in ILCM area were not as large as in response to low temperature alone. However, only the marine ecotype exhibited significant increases in NKA expression in response to the combined effects of low salinity and low temperature. Combined with the finding that freshwater stickleback likely have lower gill permeability than marine stickleback in fresh water (Chapter 3), the increased NKA expression in marine stickleback may be in compensation for increased ion loss. This could lead to higher energetic costs, providing a physiological explanation for the reduced growth of marine stickleback in cold freshwater conditions (Chapter 2 and 5).

Strengths & limitations

One of the major strengths of this work is the questions it is able to answer, filling a major gap in the literature for this species and providing a physiological perspective for this already well-described system. Specifically, we were able to design a series of experiments that built sequentially upon one another to isolate how individual and combined abiotic factors were likely to have presented challenges to the colonization of fresh water by stickleback. Beyond the specifics of the experiments and their findings, my work shows how basic principles of comparative physiology can be positioned within an evolutionary context to provide new insight in an already well-studied model organism. While much research focuses on either mechanistic physiology or molecular genetics, I believe that the research provided here unites these two often differentiated

fields of study to illuminate the physiological mechanisms that may have played a part in this repeated parallel divergence that molecular genetics has already described so well.

Although this evolutionary physiological approach provides powerful information and addresses the questions posed here, that does not discount the fact that there are limitations in this work. The stickleback system is an excellent model for studying adaption because of the widespread, repeated parallel evolution of numerous features in many freshwater populations throughout the globe. Although the experiments here were conducted to sequentially isolate the effects of two abiotic variables and the challenges they posed for freshwater colonization by ancestral stickleback, we only focused on two freshwater, one anadromous, and one marine stickleback population. In order to be sure that the findings here apply generally to stickleback populations in the north-temperate zone, rather than simply to the populations we studied, replicating these experiments on several populations of marine, anadromous, and freshwater stickleback is necessary. Unfortunately, the time required to attain sufficient sample sizes, space to house these fish in one facility, and lab work required to perform these additional studies would far exceed the scope of one PhD. However, it is my hope that the conclusions of this work and the line of questioning it uses inspire a similar style of research in this framework of evolutionary physiology to follow up on the results found here, and answer new questions in this way in stickleback and other species.

An additional limitation of this work is that although we determined mRNA expression levels using qRT-PCR and RNA-seq, we did not determine protein levels of any of the genes we assayed or discussed. Although the levels of expression of differentially expressed mRNAs can correlate with their protein levels (Koussounadis *et*

al. 2015), much research has shown that there is not always a correlation between mRNA expression levels and protein levels (Chen *et al*. 2002; Greenbaum *et al*. 2003; Pascal *et al*. 2008; de Sousa Abreu *et al*. 2009; Vogel & Marcotte 2012; Koussounadis *et al*. 2015). Events such as degradation of mRNA or post-translational modifications may account for a lack of correlation between mRNA expression and protein levels. Since the action of the protein directly determines the phenotype of interest, it is necessary to measure protein levels in order to verify the relationship observed at the mRNA expression level.

Future research directions

In addition to replicating this work in additional stickleback populations, there are a number of other areas that I think are worthy of follow-up studies. First, while the divergence we found in ECaC expression between marine and freshwater ecotypes is consistent with differences in ionoregulatory mechanisms between ecotypes, no one has yet measured calcium uptake kinetics in stickleback. Superior ability to take up calcium ions from dilute fresh water in the freshwater ecotype would corroborate the gene expression differences we found here and provide a more comprehensive analysis of the physiological differences between ecotypes. In addition, although the RNA-seq data from Chapter 3 is consistent with the freshwater ecotype having "tighter", less permeable gills in fresh water, gill permeability has not been measured in stickleback. Measuring gill permeability to determine whether freshwater stickleback have lower gill permeability than marine stickleback in fresh water (and cold fresh water) would provide additional physiological evidence to complement the gene expression, gill morphology, and growth data presented here.

An additional area to focus on would be the morphology of the stickleback gill. My work provides the first look at plasticity in the size of the gill ILCM and related changes in the expression of ion transporters in stickleback gills in response to low salinity and low temperature. No research, however, has been performed to investigate whether there are also changes in ionocyte number, size, and/or location in relation to the ILCM in response to low salinity and low temperature in stickleback. These characteristics, and potential differences in them between ecotypes, would provide a more comprehensive physiological description of how stickleback ecotypes respond to changes in the environment, enabling a more nuanced explanation of the challenges that freshwater colonization exerts on stickleback.

On a broader, more conceptual scale, it would be interesting to see how the physiological challenges associated with freshwater colonization differ in other parts of the stickleback species range. From Alaska to southern California, for example, there are likely major differences in how marine and freshwater stickleback habitats vary in comparison to those in British Columbia. While the work presented here shows that the combination of low salinity and low temperature likely posed a challenge to freshwater colonization, these major temperature differences between the ocean and fresh water during the winter are not present in southern California. Therefore, it remains to be seen if freshwater colonization in these more southern latitudes posed different or additional challenges to those seen in the north-temperate zone.

Conclusion

In summary, these data provide solid evidence that the combination of low salinity and low temperature likely presented a physiological challenge to the colonization of freshwater habitats by ancestral marine or anadromous stickleback in British Columbia. Freshwater stickleback exhibited gill transcriptome divergence that likely decreases gill permeability in fresh water, as well as improved growth and divergence in ion transporter gene expression (especially in ECaC) in cold fresh water. Furthermore, this work was the first to thoroughly investigate phenotypic plasticity in these traits in stickleback in response to cold freshwater conditions. Consequently, my work adds to the growing body of work investigating whether adaptive plasticity facilitates or constrains adaptation to new environments (Wright 1931; Hinton & Nowlan 1987; Fontanari & Meir 1990; Anderson 1995; Ancel 2000; Mery & Kawecki 2004; Paenke *et al*. 2007; Ghalambor *et al*. 2007, 2015). I found plasticity in cold tolerance and the ability to remodel the gill in both marine and freshwater stickleback. Although plasticity itself has the potential to evolve following colonization (Crispo 2007), I did not find evidence to support this for stickleback, as there was a lack of divergence between ecotypes in plasticity of the gill transcriptome in response to salinity change. Therefore, evolution of plasticity in the gill transcriptome was likely not an integral part of the evolution of freshwater residency in this species. However, the results presented here are consistent with adaptive divergence in ion transporter gene expression, gill permeability, and growth in response to the interactive effects of low salinity and low temperature, illustrating some of the likely adaptive changes that occurred in freshwater stickleback following their colonization of freshwater habitats.

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Appendices

Appendix A: supplementary information for chapter 2

Table A1. Mortality during acclimation.

Ecotype	Winter conditions	Spring conditions
Freshwater ecotype	12% (5/41)	15% (5/34)
Marine ecotype	8% (7/88)	8% (6/72)
Hybrid crosses	4% (2/49)*	3% (1/39)

* In the marine ecotype under winter conditions, 10 additional fish from one family in one tank died late in the experimental period.
Statistical analysis: ecotype p=0.7; acclimation conditions p=0.65; interaction p=0.22

Appendix B: supplementary information for chapter 3

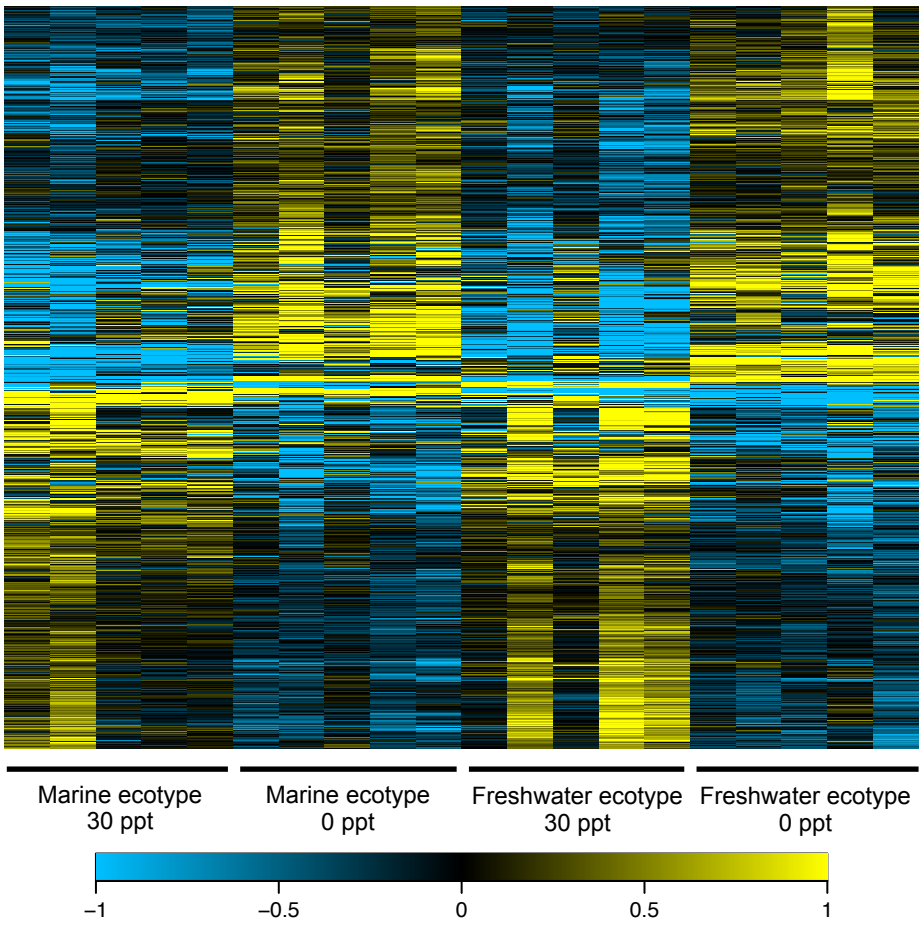

Figure B1. Heat map displaying expression patterns of genes with a significant effect of acclimation salinity. Blue indicates genes with expression levels lower than the mean of all samples. Yellow indicates genes with expression levels higher than the mean of all samples. Each column indicates one individual (n = 5 per group).

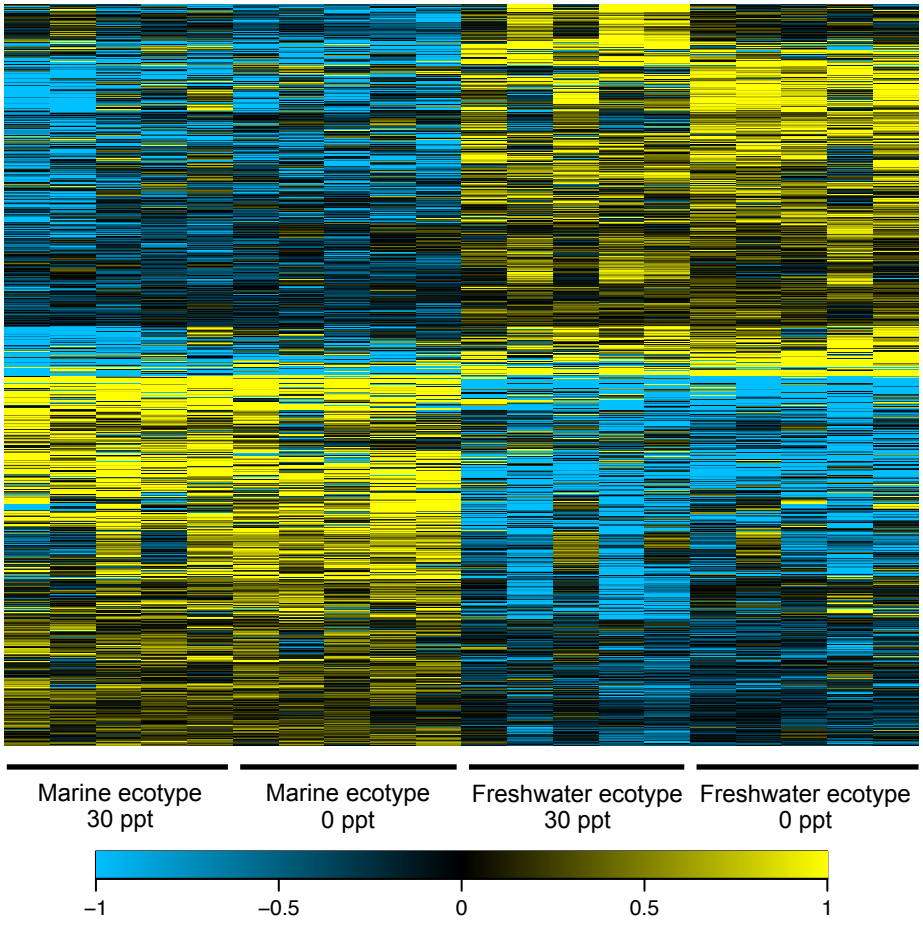

Figure B2. Heat map displaying expression patterns of genes with a significant effect of ecotype. Blue indicates genes with expression levels lower than the mean of all samples. Yellow indicates genes with expression levels higher than the mean of all samples. Each column indicates one individual (n = 5 per group).

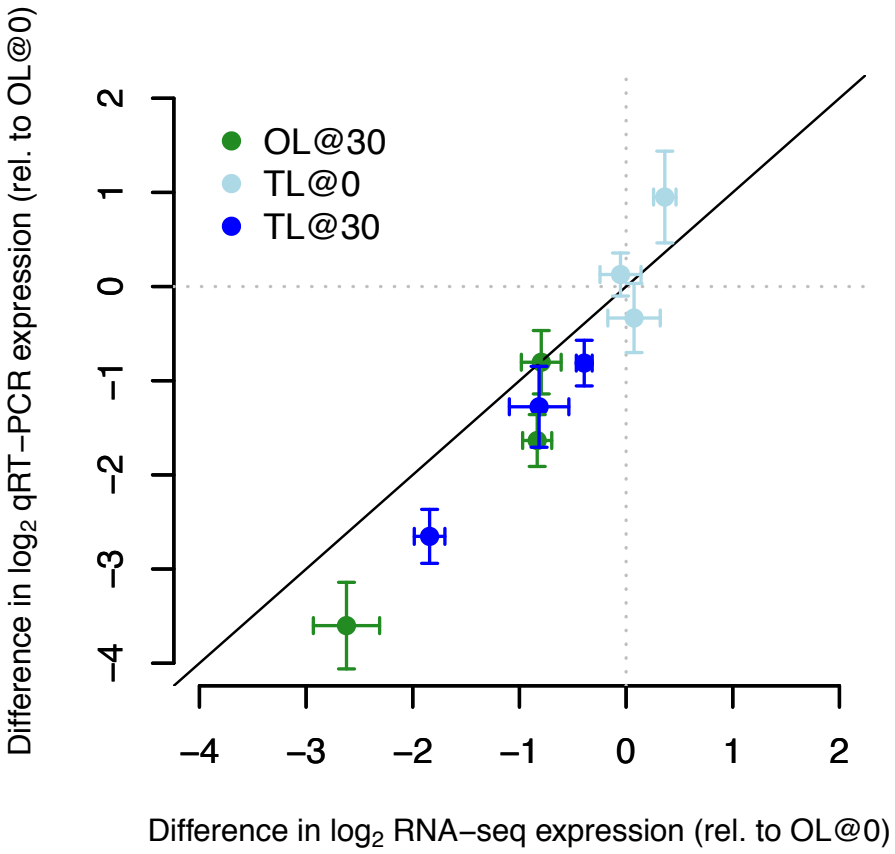

Figure B3. Correlation between the results of RNA-seq and qRT-PCR analysis.

Table B1. Percent mortality over the 6 months of the experiment.

	Acclimation salinity				
	0 ppt	0.3 ppt	2 ppt	11 ppt	30 ppt
Oyster Lagoon (Marine Ecotype)	70.8	50.0	37.5	41.7	54.2
Trout Lake (Freshwater Ecotype)	54.6	27.3	63.6	31.8	54.6

Table B2. Coverage and mapping statistics for RNA-seq libraries

Ecotype	Salinity	Sample ID	# of Reads	Reads mapped as pairs	Reads mapped as broken reads	Total # of reads mapped	% Reads mapped
Oyster lagoon	0 ppt	OL0.100	33853714	21641846	7058370	28700216	84.8
Oyster lagoon	0 ppt	OL0.97	34478242	22055084	7318740	29373824	85.2
Oyster lagoon	0 ppt	OL0.91	33411702	21156400	6895637	28052037	84.0
Oyster lagoon	0 ppt	OL0.80	28617278	18015570	6134425	24149995	84.4
Oyster lagoon	0 ppt	OL0.5	34233716	21556086	7520797	29076883	84.9
Oyster lagoon	30 ppt	OL30.30	32891108	19673704	7506007	27179711	82.6
Oyster lagoon	30 ppt	OL30.96	36757846	23968726	6620026	30588752	83.2
Oyster lagoon	30 ppt	OL30.67	44964134	29691020	8688244	38379264	85.4
Oyster lagoon	30 ppt	OL30.31	41367076	26296284	8035027	34331311	83.0
Oyster lagoon	30 ppt	OL30.20	35520050	22924446	6903322	29827768	84.0
Trout Lake	0 ppt	TL0.59	29932430	19212694	5858203	25070897	83.8
Trout Lake	0 ppt	TL0.58	30984474	20836114	6055451	26891565	86.8
Trout Lake	0 ppt	TL0.51	45951172	30588572	8024036	38612608	84.0
Trout Lake	0 ppt	TL0.9	36243720	23872596	6721044	30593640	84.4
Trout Lake	0 ppt	TL0.6	38060678	24919596	7734285	32653881	85.8
Trout Lake	30 ppt	TL30.23	42739352	28555550	7493732	36049282	84.3
Trout Lake	30 ppt	TL30.71	38011980	24620392	7500317	32120709	84.5
Trout Lake	30 ppt	TL30.64	37487530	24079044	7644909	31723953	84.6
Trout Lake	30 ppt	TL30.16	33592196	21529288	7209125	28738413	85.6
Trout Lake	30 ppt	TL30.15	31822124	20971654	6379503	27351157	86.0
Average:			36046026	23308233	7165060	30473293	84.6

Table B3. Primer sets used for qRT-PCR

Gene	Sequence (5'– 3')	Ensembl Transcript ID or source
ECaC, ***trpv6***	F: TGCAGGGTGGCAGGTGAT R: TCGAGCGGCTGCATCTC	ENSGACT00000013861
NHE3, ***slc9a3.2***	F: TCCTACCTGACCGCTGAGATG R: CGCCACAGAAGGTGATCGA	ENSGACT00000003204
NKA, ***atp1a1a.5***	F: ACCTGGACGATCACAAGTTAACC R: TGGAAAGACCCCTGGCTAGA	ENSGACT00000018954
rpl13a	F: CACCTTGGTCAACTTGAACAGTG R: TCCCTCCGCCCTACGAC	from Hibbeler *et al*. 2008

Table B4. Enriched GO-terms for genes differentially expressed between ecotypes

Category	Term	Ontology	P value	Number of genes with higher expression in the Freshwater ecotype	Number of genes with lower expression in the Freshwater ecotype	Total number of genes in GO category	FDR cutoff
GO:0030198	extracellular matrix organization	BP	2.93E-16	82	9	266	0.05
GO:0032897	negative regulation of viral transcription	BP	6.79E-15	2	38	68	0.05
GO:0051092	positive regulation of NF-kappaB transcription factor activity	BP	1.98E-08	9	45	162	0.05
GO:0007155	cell adhesion	BP	2.79E-08	68	14	312	0.05
GO:0022617	extracellular matrix disassembly	BP	4.57E-08	35	4	111	0.05
GO:0030574	collagen catabolic process	BP	4.80E-08	26	1	64	0.05
GO:0007186	G-protein coupled receptor signaling pathway	BP	1.14E-07	45	17	216	0.05
GO:0051091	positive regulation of sequence-specific DNA binding transcription factor activity	BP	1.76E-07	3	43	135	0.05
GO:0045087	innate immune response	BP	1.91E-07	55	112	777	0.05
GO:0045766	positive regulation of angiogenesis	BP	5.16E-06	19	13	95	0.05
GO:0060346	bone trabecula formation	BP	1.05E-05	6	0	6	0.05
GO:0010811	positive regulation of cell-substrate adhesion	BP	1.33E-05	16	1	39	0.05
GO:0008217	regulation of blood pressure	BP	1.90E-05	12	6	43	0.05
GO:0006954	inflammatory response	BP	2.16E-05	30	35	265	0.05
GO:0030199	collagen fibril organization	BP	2.30E-05	17	0	41	0.05
GO:0001501	skeletal system development	BP	2.79E-05	26	5	100	0.05
GO:0032967	positive regulation of collagen biosynthetic process	BP	7.54E-05	6	2	12	0.05
GO:0006508	proteolysis	BP	7.86E-05	39	23	261	0.05
GO:0006805	xenobiotic metabolic process	BP	8.45E-05	13	18	104	0.05
GO:0007165	signal transduction	BP	9.56E-05	84	59	716	0.05
GO:0015793	glycerol transport	BP	0.000103	2	3	5	0.05
GO:0051607	defense response to virus	BP	0.000117	4	26	101	0.05
GO:0019835	cytolysis	BP	0.000118	10	5	35	0.05
GO:0006968	cellular defense response	BP	0.000155	9	6	37	0.05
GO:0007044	cell-substrate junction assembly	BP	0.00022	7	0	11	0.1
GO:0006518	peptide metabolic process	BP	0.000253	6	2	14	0.1
GO:0010812	negative regulation of cell-substrate adhesion	BP	0.00029	6	2	14	0.1
GO:0001523	retinoid metabolic process	BP	0.000308	10	3	32	0.1
GO:0010575	positive regulation of vascular endothelial growth factor production	BP	0.000316	9	3	28	0.1
GO:0007601	visual perception	BP	0.00034	23	4	94	0.1

Category	Term	Ontology	P value	Number of genes with higher expression in the Freshwater ecotype	Number of genes with lower expression in the Freshwater ecotype	Total number of genes in GO category	FDR cutoff
GO:0005576	extracellular region	CC	7.64E-25	141	44	593	0.05
GO:0005615	extracellular space	CC	3.37E-17	133	48	669	0.05
GO:0005578	proteinaceous extracellular matrix	CC	1.33E-12	51	7	154	0.05
GO:0031012	extracellular matrix	CC	2.53E-12	54	3	153	0.05
GO:0005886	plasma membrane	CC	2.07E-10	271	165	2289	0.05
GO:0070062	extracellular exosome	CC	1.56E-09	249	175	2213	0.05
GO:0005622	intracellular	CC	3.03E-08	39	62	394	0.05
GO:0016021	integral component of membrane	CC	7.78E-08	216	158	1982	0.05
GO:0009986	cell surface	CC	4.40E-07	69	25	390	0.05
GO:0005887	integral component of plasma membrane	CC	6.69E-07	93	53	682	0.05
GO:0005788	endoplasmic reticulum lumen	CC	2.97E-06	40	5	155	0.05
GO:0005581	collagen trimer	CC	5.64E-06	13	1	27	0.05
GO:0005604	basement membrane	CC	2.40E-05	24	1	74	0.05
GO:0001726	ruffle	CC	6.15E-05	23	19	157	0.05
GO:0005614	interstitial matrix	CC	0.000126	8	0	13	0.05
GO:0008270	zinc ion binding	MF	3.18E-07	79	130	1030	0.05
GO:0004930	G-protein coupled receptor activity	MF	4.88E-06	24	10	104	0.05
GO:0004181	metallocarboxypeptidase activity	MF	5.48E-06	8	2	15	0.05
GO:0005509	calcium ion binding	MF	1.09E-05	71	21	410	0.05
GO:0005518	collagen binding	MF	2.04E-05	17	4	56	0.05
GO:0048407	platelet-derived growth factor binding	MF	2.27E-05	8	0	11	0.05
GO:0008201	heparin binding	MF	2.31E-05	29	3	102	0.05
GO:0005201	extracellular matrix structural constituent	MF	2.80E-05	16	0	38	0.05
GO:0004185	serine-type carboxypeptidase activity	MF	5.73E-05	7	2	15	0.05
GO:0003674	molecular_function	MF	9.55E-05	34	75	505	0.05
GO:0004601	peroxidase activity	MF	0.000182	6	4	19	0.1
GO:0004252	serine-type endopeptidase activity	MF	0.000332	14	9	74	0.1

Table B5. GO-terms associated with genes significantly differentially expressed with both salinity acclimation and between ecotypes (only terms containing 10 or more genes shown)

GO-ID	Term	Number of differentially expressed genes with this GO-ID
GO:0044281	small molecule metabolic process	61
GO:0007165	signal transduction	25
GO:0055085	transmembrane transport	25
GO:0006351	transcription, DNA-templated	21
GO:0043547	positive regulation of GTPase activity	21
GO:0045087	innate immune response	19
GO:0007411	axon guidance	18
GO:0045944	positive regulation of transcription from RNA polymerase II promoter	18
GO:0007166	cell surface receptor signaling pathway	17
GO:0042493	response to drug	16
GO:0005975	carbohydrate metabolic process	15
GO:0006355	regulation of transcription, DNA-templated	15
GO:0007186	G-protein coupled receptor signaling pathway	15
GO:0008152	metabolic process	15
GO:0055114	oxidation-reduction process	14
GO:0000122	negative regulation of transcription from RNA polymerase II promoter	13
GO:0006468	protein phosphorylation	13
GO:0006915	apoptotic process	13
GO:0007264	small GTPase mediated signal transduction	13
GO:0008150	biological_process	13
GO:0016567	protein ubiquitination	13
GO:0030198	extracellular matrix organization	13
GO:0043066	negative regulation of apoptotic process	13
GO:0006508	proteolysis	12
GO:0007155	cell adhesion	12
GO:0007596	blood coagulation	12
GO:0044267	cellular protein metabolic process	12
GO:0045893	positive regulation of transcription, DNA-templated	12
GO:0006954	inflammatory response	11
GO:0007283	spermatogenesis	11
GO:0008283	cell proliferation	10
GO:0008285	negative regulation of cell proliferation	10
GO:0015031	protein transport	10
GO:0030154	cell differentiation	10
GO:0043065	positive regulation of apoptotic process	10
GO:0045892	negative regulation of transcription, DNA-templated	10

Table B6. GO-terms associated with genes with significant interactions between the effects of salinity acclimation and ecotype (only terms containing 3 or more genes shown)

GO-ID	Term	Number of differentially expressed genes with this GO-ID
GO:0044281	small molecule metabolic process	9
GO:0055085	transmembrane transport	8
GO:0007165	signal transduction	7
GO:0034220	ion transmembrane transport	7
GO:0001525	angiogenesis	5
GO:0045087	innate immune response	5
GO:0055114	oxidation-reduction process	5
GO:0006897	endocytosis	4
GO:0007411	axon guidance	4
GO:0008284	positive regulation of cell proliferation	4
GO:0030198	extracellular matrix organization	4
GO:0030574	collagen catabolic process	4
GO:0043066	negative regulation of apoptotic process	4
GO:0044267	cellular protein metabolic process	4
GO:1902476	chloride transmembrane transport	4
GO:0006810	transport	3
GO:0006811	ion transport	3
GO:0006954	inflammatory response	3
GO:0016567	protein ubiquitination	3
GO:0022617	extracellular matrix disassembly	3
GO:0032355	response to estradiol	3
GO:0042060	wound healing	3
GO:0042493	response to drug	3
GO:0043065	positive regulation of apoptotic process	3
GO:0045766	positive regulation of angiogenesis	3
GO:0045944	positive regulation of transcription from RNA polymerase II promoter	3
GO:0048661	positive regulation of smooth muscle cell proliferation	3
GO:0071277	cellular response to calcium ion	3

Appendix C: supplementary information for chapter 4

Table C1. Number of fish sampled per ecotype for each salinity/temperature group

	Treatment group			
	14°C & 11 ppt	14°C & 0.3 ppt	4°C & 11 ppt	4°C & 0.3 ppt
Oyster Lagoon (marine ecotype)	8	8	8	8
Little Campbell River (anadromous ecotype)	8	3	5	0
Trout Lake (freshwater ecotype)	4	5	8	4

Table C2. Primer sets used for qRT-PCR

Gene	Sequence (5'– 3')	Ensembl Transcript ID
ECaC, *trpv6*	F: TGCAGGGTGGCAGGTGAT R: TCGAGCGGCTGCATCTC	ENSGACT00000013861
eef1a1b	F: CAGAGATGGGAAAGGGTTCCT R: CGCTCTGCCTTCAGCTTGTC	ENSGACT00000023950
NHE3, *slc9a3.2*	F: TCCTACCTGACCGCTGAGATG R: CGCCACAGAAGGTGATCGA	ENSGACT00000003204
NKA, *atp1a1a.5*	F: ACCTGGACGATCACAAGTTAACC R: TGGAAAGACCCCTGGCTAGA	ENSGACT00000018954

Table C3. Gill sample sizes for measurement of ILCM area (marine and freshwater ecotypes)

	Treatment group			
	14°C & 11 ppt	14°C & 0.3 ppt	4°C & 11 ppt	4°C & 0.3 ppt
Oyster Lagoon (marine ecotype)	5	5	6	5
Trout Lake (freshwater ecotype)	2	1	4	4

Appendix D: supplementary information for chapter 5

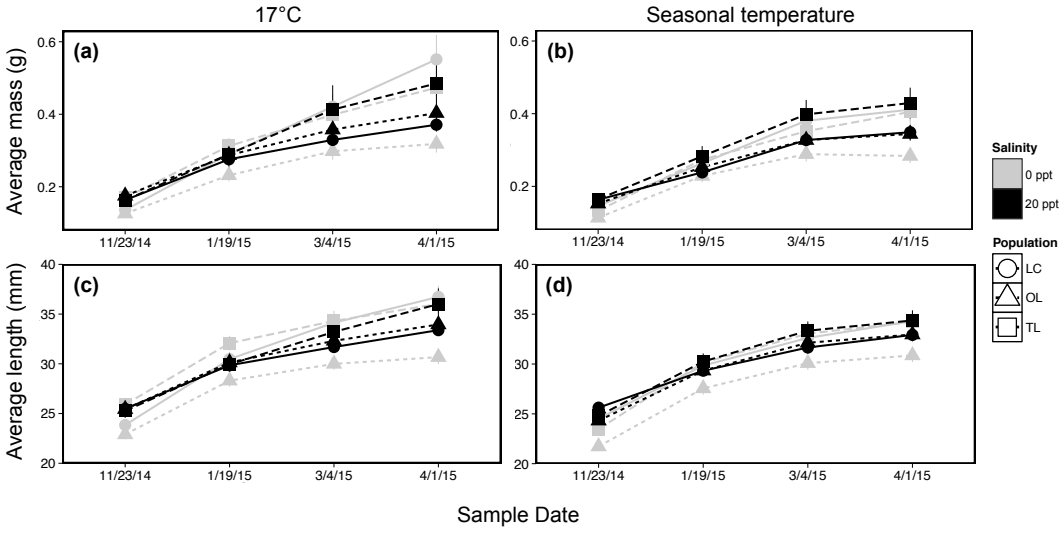

Figure D1. Mean mass and length of stickleback during the course of the study. Panel A, C: mass and length, respectively, of stickleback held at 17°C for the duration of the study. Panel B, D: mass and length, respectively, of stickleback that experienced declining temperatures. Note: the x-axis is not to scale and connecting lines are provided for visual clarity only. LC = Little Campbell River (anadromous ecotype); OL = Oyster Lagoon (marine ecotype); TL = Trout Lake (freshwater ecotype). All data are presented as mean ± SEM.

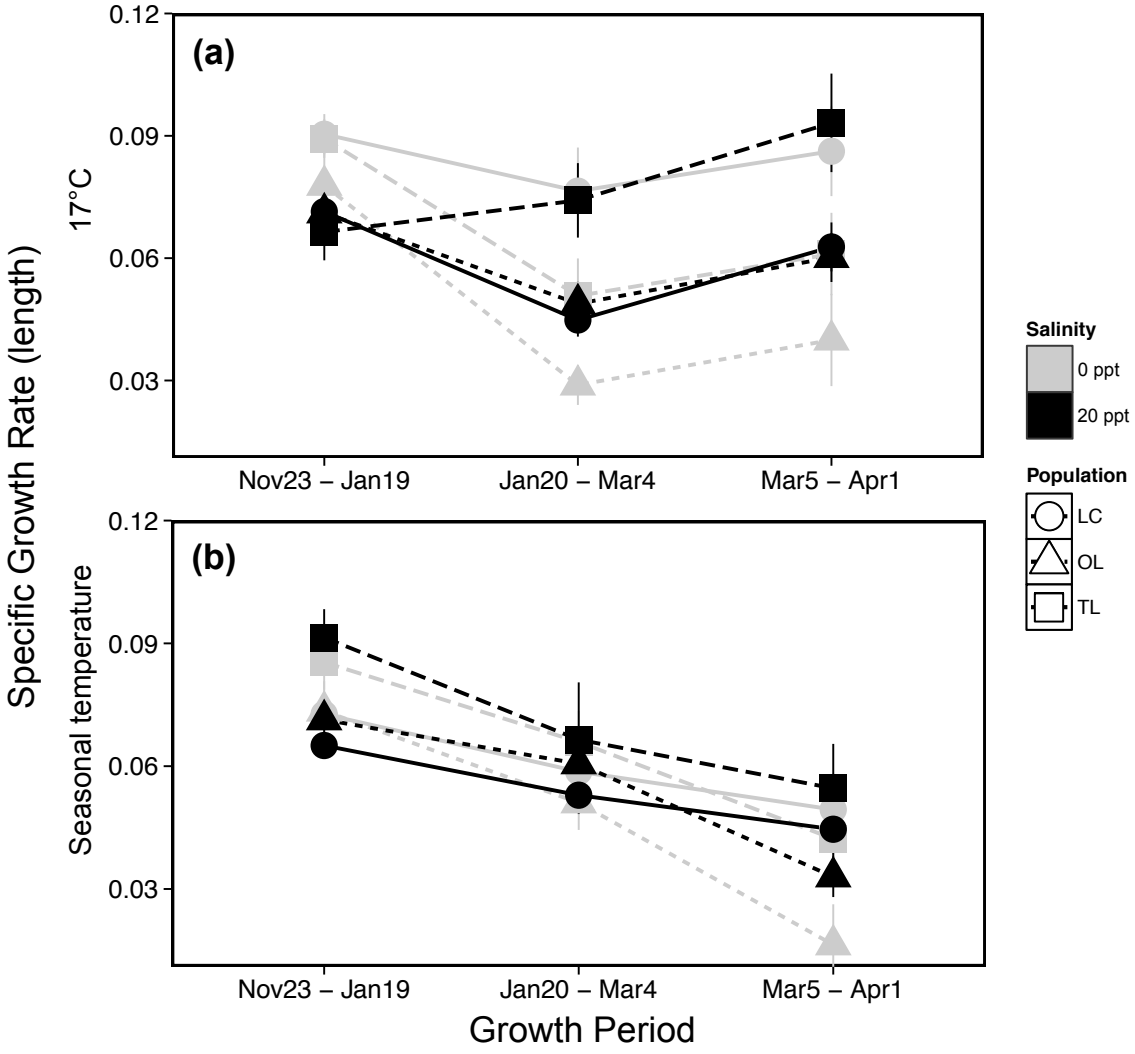

Figure D2. Specific growth rates (SGR) for length between sampling points. Panel A: stickleback held at 17°C for the duration of the study. Panel B: stickleback that experienced declining temperatures. All data are expressed as mean ± SEM. LC = Little Campbell River (anadromous ecotype); OL = Oyster Lagoon (marine ecotype); TL = Trout Lake (freshwater ecotype).