Freshwater tolerance and mechanisms of freshwater acclimation in marine sticklebacks

(*Gasterosteus* spp.)

By
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Abstract

Freshwater tolerance has evolved repeatedly in fishes, including in the *Gasterosteidae* family. However, Blackspotted Stickleback (*Gasterosteus wheatlandi*) and the endemic Nova Scotian "white" Threespine Stickleback (*Gasterosteus aculeatus*) ecotype are largely limited to marine environments. I tested if low freshwater tolerance might limit the distribution of these fishes. Freshwater tolerance was assessed in wild caught fish acclimated to 10 ppt and then transferred to freshwater (0 ppt) or control conditions (10 ppt) for a period of 21 days. There was no effect of salinity on survivorship or tissue water content (a proxy for osmoregulatory homeostasis) in either species. mRNA content of candidate gill ion transporters was measured via qPCR in Blackspotted Stickleback, and I found this marine species can initiate the transcriptional changes associated with successful freshwater acclimation in other sticklebacks. These data suggest that acute freshwater tolerance does not limit initial freshwater colonization in these species.

August 25, 2021

Dedication

This thesis is dedicated to my late mother, Lena Bernhardsson, who not only taught me to stay curious, but she was also very supportive of my education and personal development. She greatly enabled my learning by providing me with educational tools as well as valuable life experiences, experiences that has fueled my interest within the field of biology. It is with great sorrow that I have had to say a distant farewell to her during my studies at Saint Mary's University and the pandemic.

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Table of Contents

List of tables

List of figures

1. Introduction

Organisms face many stressors that they must cope with to maintain physiological homeostasis (*i.e.,* a relatively constant internal environment), and these stressors can be both abiotic and biotic. In coping with stressors, organisms have evolved different physiological mechanisms to maintain homeostasis. Organisms in different environments face different types of stressors, and for fishes, one of the main abiotic factors is the ion content of the water environment in which they live (Evans *et al.*, 2014; McCormick, 2001; Schultz & McCormick, 2012). Environmental salinity plays an important role in influencing the distribution of fishes. Due to the physiological changes that are required to maintain homeostasis during environmental shifts in salinity levels, saltwater species rarely occur in freshwater, or vice versa (Kültz, 2015). While most fish species are restricted to either fresh- or saltwater environments, euryhaline fishes have evolved the ability to cope with the physiological challenges that come with living in both environments (Kültz, 2015).

With increasing climate change due to combined effect from natural climate change and anthropogenic activities, it is expected that the natural environment that fish live in will change, a change that may include changes in water salinity. Adding solutes to saltwater through natural processes such as chemical weathering and erosion, runoff water from rivers and estuaries, and thermal activity on the ocean floor, combined with the increased evaporation of water due to global warming increases the salinity of saltwater environments (Stocker *et al.*, 2013). Global warming may also contribute to decreasing salinity in both fresh- and saltwater areas (Stocker *et al.*, 2013). For example in Arctic waters, the melting of polar ice deposits freshwater into the oceans, leading to a decrease in salinity through dilution (Curry *et al.*, 2003; Stott *et al.*, 2008). As these

processes contribute to a more rapid change in fishes' natural habitat, it is important to better understand the mechanisms involved in acclimation and adaptation to environmental change. With the environment constantly changing, it is interesting to find out how salinity tolerance has evolved in the past, which can then help in understanding how it may evolve in the future as a response to climate change. In addition, understanding how salinity tolerance evolved can help in predicting how fish will adapt to the changing environment.

This study will focus on how fish evolve to cope with exposure to low salinity water by studying the physiological responses initiated to maintain homeostasis during freshwater (0 ppt) acclimation in sticklebacks. The *Gasterosteidae* family (sticklebacks) are widely distributed, with different species distributed around the coast-lines in the Northern Hemisphere (McKinnon & Rundle, 2002; Scott & Scott, 1988). This family includes euryhaline species as well as species and ecotypes restricted to freshwater or saltwater. Therefore, the *Gasterosteidae* family is a suitable model system for studying both the ability to acclimate to saltwater and freshwater in fish as well as the evolution of freshwater tolerance among populations and species (Kawahara *et al.*, 2009; Kültz, 2015; McKinnon & Rundle, 2002).

In particular, this dissertation investigates the freshwater acclimation abilities in two primarily marine and brackish water species, for which there may be no extant freshwater populations: the Blackspotted Stickleback (*Gasterosteus wheatlandi*) (Garside & Kerekes, 1969) and the white Threespine Stickleback, a primarily marine ecotype of the Threespine Stickleback (Blouw & Hagen, 1990; Blouw, 1996). Both species are closely related to the euryhaline Threespine Stickleback (*e.g.,* Baube, 2008; Garside & Kerekes, 1969; Gibbons *et al.*, 2017; Jones *et al.*, 2012; Kawahara *et al.*, 2009) which has

repeatedly colonized hundreds of freshwater lakes, streams and rivers (McKinnon & Rundle, 2002) and evolved to better tolerate low salinities (*e.g.,* Gibbons *et al.*, 2017). The results from the present research could provide more information about the freshwater acclimation abilities of the primarily marine Blackspotted Stickleback and white Threespine Stickleback ecotype. With this information, it could be possible to see if a lower ability to acclimate to freshwater may be associated with potential limitations in their ability to colonize freshwater.

1.1 Why salinity change is stressful for fishes

Seawater contains a high concentration of many ions, with the most abundant being chloride (Cl^-), sodium (Na^+), sulfate (SO_4^{2-}), magnesium (Mg^{2+}), calcium (Ca^{2+}) and potassium (K^+) (Mackenzie, Howard, Alyn, & Duxbury, 2020). Seawater (~30-35 ppt dissolved ions) and freshwater (< 0.5 ppt dissolved ions) have very different ion concentrations and compositions, which leads to differences in the passive transport of water and ions into the surrounding water from the internal environment. Seawater is hypersaline compared to the fish's cells and extracellular fluids (around 10-14 ppt dissolved ions) (Evans, 2011), while the salinity of freshwater is much lower than the internal salinity of fishes at < 0.5 ppt (Evans *et al.*, 2014). To maintain homeostasis during transfer from hypersaline to hyposaline environments, fish must actively change the types of cells as well as ion and water transporters in osmoregulatory tissues (*e.g.,* the gills, intestine and kidneys) as they acclimate to their external environment (Evans *et al.*, 2014; Evans *et al.*, 2005). To survive, bony fishes must maintain internal ion and water content within a narrow range (Evans *et al.*, 2014).

In saltwater, water is passively transported down the osmotic gradient out of the body and ions passively flow into the fish. Therefore, fish must actively drink seawater to gain water and transport excess ions out of the body (Evans *et al.*, 2014). In freshwater, fishes face the reverse issue of passive ion loss and water gain through osmosis (Evans *et al.*, 2005) and actively excrete water through increased urine levels. Freshwater fishes must therefore actively take up ions from the external environment to maintain ion homeostasis (Evans *et al.*, 2014; Evans, 2011, 2008). Failure to maintain homeostasis can have severe consequences for fish. For example, in saltwater shrivelling of the cells due to increased water transport out of the cells down the osmotic gradient would occur without active ionic regulation. In contrast, freshwater fish struggle with the opposite issue of water gain into the cells, due to higher solute concentration in the cells. This leads to expansion of the cells, which could rupture (Evans *et al.*, 2014; Evans, 2011).

Most fishes live in relatively stable salinity throughout their lifetime. However, some species have found a way to cope with the challenges that come with living in both freshwater and saltwater (Schultz & McCormick, 2012). Despite possessing the physiological machinery required to live in these different environments, even euryhaline fish often have some difficulty coping with very low salinity levels in freshwater (Evans *et al.*, 2014; Shrimpton, 2012). This may be because most fish species evolved in a marine environment (Lee & Bell, 1999).

To deal with the challenges that come with living in freshwater, euryhaline fishes have evolved a number of different mechanisms to acclimate to freshwater environments (Kültz, 2015). Recent work has found that the specific mechanisms that fish use to regulate ion levels (*i.e.* ionocyte types, ionocyte combinations and combinations of specific ion transporters in gills) vary among species of fishes (Dymowska *et al.*, 2012;

Hwang *et al.*, 2011), highlighting that there are multiple mechanisms by which distantly related fish species can evolve to tolerate freshwater (Hwang *et al.*, 2011). However, little is known about if, and how, more closely related fish species (such as the different species within the *Gasterosteidae* family; Kawahara et al., 2009) vary in their freshwater acclimation mechanisms. To better understand the evolutionary constraints and facilitations that might influence the evolution of freshwater tolerance, it is important to find out if closely related species use convergently evolved mechanisms to obtain freshwater tolerance (Losos, 2011; Rosenblum *et al.*, 2014). As well, the importance of freshwater acclimation capacity, relative to other biotic and abiotic factors, in limiting colonization of lakes and streams is largely unknown (but see Gibbons *et al.*, 2017; Ishikawa *et al.*, 2019). Thus, comparative studies of freshwater tolerance in clades of fish that vary in their salinity distribution in the wild can help to inform us if it is freshwater tolerance, or other factors, that may limit fish distributions.

1.2 Mechanisms of acclimation to freshwater in fish.

There are four major ionoregulatory tissues that undergo remodelling during freshwater acclimation in adult fishes: the gills, operculum, kidneys and gastrointestinal tract (Dymowska *et al.*, 2012; Kültz, 2015; Schultz & McCormick, 2012). Physiological changes in ionoregulatory tissues in freshwater include the upregulation of ionocytes involved in ion uptake and downregulation of ionocytes involved in ion excretion in saltwater (Al-Jandal & Wilson, 2011; Dymowska *et al.*, 2012; Gibbons *et al.*, 2018; Gibbons *et al.*, 2017). Ionocytes are cells that regulate the intracellular and extracellular ion content and contain specialized ion transporters. Remodelling of the types of

ionocytes as well as the ion transporter content of ionocytes in these ionoregulatory tissues contributes to the acclimation in freshwater fishes (Dymowska *et al.*, 2012).

The fish gill is one of the main ion exchange sites between the internal and external environment and the associated mechanisms of ion exchange and transport are well-studied (*e.g.* Evans *et al.*, 2014; Evans *et al.*, 2005; Hiroi & McCormick, 2012) in some species, such as the Japanese Seabass (Inokuchi *et al.*, 2017) and Medaka (Hsu *et al.*, 2014). Gill ionocytes possess a variety of ion transporters and channels that are involved in transporting key ions, such as Na^+, K^+, Ca^{2+}, and Cl^-, across the epithelial membrane. In addition, claudins (tight junction proteins) control the passive transport of Na^+ out of the body through paracellular pathways (Kolosov *et al.*, 2013). The type of ionocytes, as well as the specific ion transporters or channels in and around each ionocyte vary depending on water salinity (Dymowska *et al.*, 2012; Hiroi & McCormick, 2012). In saltwater, the successful maintenance of homeostasis is dependent upon the excretion of ions by the combined action of these transporters and flow through tight junction proteins including, but not limited to, the transporters displayed in *Figure 1* (Dymowska *et al.*, 2012; Evans *et al.*, 2005). The structure and function of the seawater gill ionocytes is very similar among fish species, as this cell type likely evolved in a common ancestor to all teleost fishes (Shrimpton, 2012).

To successfully acclimate to freshwater, a fish must reduce the number of saltwater specific ionocytes or remodel the function (by changing the types of ion transporters) of the ionocytes to better suit a freshwater environment (*Figure 1a*). Therefore, the cystic fibrosis transmembrane conductance regulator (CFTR) and $Na^+/K^+/2Cl$ cotransporter 1 (NKCC1), two transporters unique to seawater ionocytes, are downregulated (Dymowska *et al.*, 2012; Gibbons *et al.*, 2017; Nakamura *et al.*, 2021). The fish must also increase the

number of freshwater ionocytes, which often contain the Na^+/Cl^- cotransporter 2 (NCC2), or remodel the existing ionocytes to include freshwater specific ion transporters (*Figure 1b*). This should lead to an upregulation of NCC2 to help in the additional uptake of ions that is needed in this environment (Breves *et al.*, 2020; Dymowska *et al.*, 2012). There are also common changes in the mRNA content of transporters found in both fresh and saltwater gill ionocytes as euryhaline fish acclimate to freshwater, including increases in Na^+/H^+ exchanger 3 (NHE3), Na^+,K^+-ATPase (NKA) alpha subunit, and epithelial Ca^{2+} channel (ECaC) in freshwater (Breves *et al.*, 2020; Gibbons *et al.*, 2017; Scott *et al.*, 2004). The downregulation of seawater ionocytes and ion transporters and upregulation of freshwater transporters in the gill is a critical process in acclimation to freshwater (Breves *et al.*, 2020; Dymowska *et al.*, 2012; Gibbons *et al.*, 2017). In addition, changes in the specific claudin isoforms expressed in the gill epithelium also reduce the permeability for Na^+ in freshwater to help maintain ion homeostasis (Kolosov *et al.*, 2013). Note that the model depicted in *Figure 1* does not include all ion transporters and enzymes involve in ion transport and acid-base regulations. Hwang *et al.* (2011) reviewed the recent progress on the molecular mechanisms involved in ionic and acid-base regulation. Also see Perry *et al.* (2003) and Evans *et al.* (2005) for further information on additional gill ion transporters involved in acid-base and ion regulation. The present study will focus on Na^+ and Cl^- ion transporters in freshwater and saltwater ionocytes to provide more information about the role of these proteins in freshwater acclimation and adaptation in Blackspotted Stickleback to determine if this primarily marine species is able to effectively downregulate 'seawater ion transporters' and upregulate 'freshwater ion transporters' during freshwater acclimation.

In freshwater, the types of ionocytes and combination of transporters used by fish to take up ions and successfully osmoregulate varies among species (Dymowska *et al.*, 2012; Hwang *et al.*, 2011). Dymowska *et al.* (2012) reviews the different types of ionocytes and the ion transporter content for each type of ionocyte in the gill epithelium, which often varies between different teleost fish species (Dymowska *et al.*, 2012; Evans *et al.*, 2005; Furukawa *et al.*, 2014; Gibbons *et al.*, 2017; Hwang *et al.*, 2011; Inokuchi *et al.,* 2017). It is important to note that no immunohistological studies of gill ionocytes have been conducted in the *Gasterosteidae*, therefore there is little information on the specific types of ionocytes and exact ion transporter composition in each ionocyte in stickleback beyond measures of mRNA content (*e.g.* Gibbons *et al.*, 2017; Inokuchi *et al.*, 2017). Therefore, *Figure 1* depicts the composition that is predicted for sticklebacks (Infraclass Teleostei, Euteleostei, Neoteleostei, Acananthopterygii, Percomorphaceae, Eupercaria; Near *et al.*, 2012), based on the knowledge of ion transporter composition in two other fish in the Percomorphaceae: the Tilapia (*Oreochromis mossambicus*; Percomorphaceae, Ovalentaria) and Japanese Seabass (*Lateolabrax japonicus*; Percomorphaceae, Eupercaria), for which ionocyte types have been studied via immunohistochemistry (Inokuchi *et al.*, 2017).

In the Japanese Seabass, Inokuchi *et al.* (2017) observed three different types of ionocytes (one seawater type and two freshwater types: NHE3 and NCC2 containing, *Figure 1.*) and found evidence that that some seawater type ionocytes transform into NHE3-containing freshwater type ionocytes post freshwater transfer. Inokuchi *et al.* (2017) also observed that NCC2, which was absent in the gill filament ionocytes of fish exposed to seawater, appeared in freshwater exposed fish, potentially via the differentiation of new cells. They also observed that while NCC2 was more predominant

in freshwater gill filaments, a small number of NCC2 appeared in the lamellae after 10 days of freshwater exposure, suggesting that some of the NCC2 ionocytes migrate to the lamellae (Inokuchi *et al.*, 2017). We predict that sticklebacks also have the two freshwater and single seawater ionocyte types found in the Japanese Seabass and Tilapia and the proposed ionocyte types can be viewed in *Figure 1*.

Key ion transporters that will be measured in the present study include Na^+/H^+ exchanger 3 (NHE3; present in freshwater and seawater ionocytes), Na^+, K^+-ATPase (NKA; present in freshwater and seawater ionocytes), epithelial Ca^{2+} channel (ECaC; present in freshwater and seawater ionocytes), Na^+/K^+2Cl cotransporter 1 (NKCC1;present only in seawater ionocytes), Na^+/Cl^- cotransporter 2 (NCC2; present only in freshwater ionocytes), and the Cl^- channel: cystic fibrosis transmembrane conductance regulator (CFTR; present only in seawater ionocytes). See *Figure 1* for the proposed location of these ion transporters in freshwater and saltwater gill ionocytes respectively. It is worth noting that not all salinity-responsive ion transporters are included in *Figure 1*, such as those involved in ammonia transport, and that the identity of some key transporters remains unknown (Hwang *et al.*, 2011). The proposed location and function of ion transporters in the saltwater gill ionocyte is the same in all fish species studied to date, however, the location and function of ion transporters in the freshwater gill ionocyte varies among the fish species and remains unknown for many species, including Gasterosteiformes (Dymowska *et al.,* 2012; Hwang *et al.,* 2011 Kolosov *et al*., 2013).

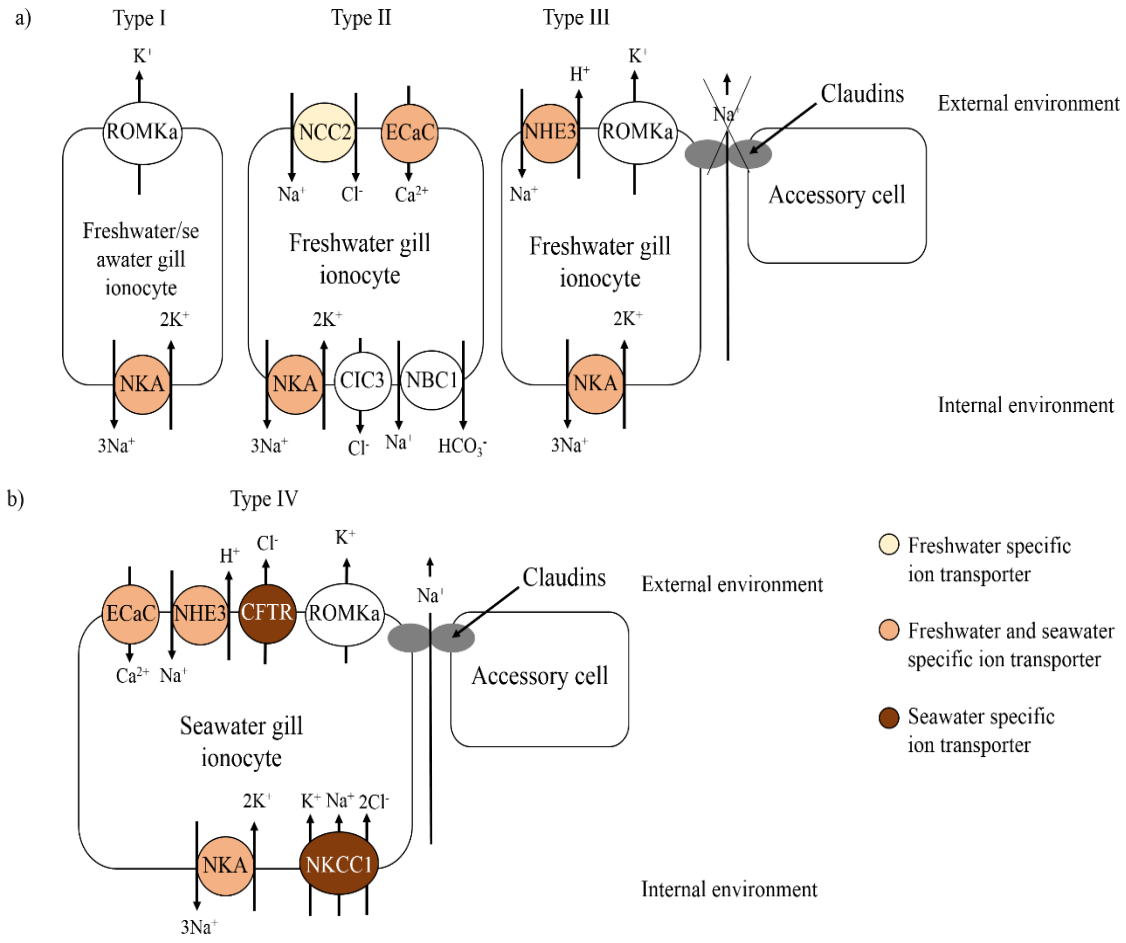

Figure 1. Schematic drawing of the proposed ionocyte and ion transporter composition in percomorph fish gill ionocytes based upon data from the Mozambique Tilapia (*Oreochromis mossambicus*: Furukawa *et al.*, 2014) and Japanese Seabass (Inokuchi *et al.*, 2017) in freshwater (a) and seawater (b), with the addition of predicted ECaC ion transporter locations (Dymowska *et al.,* 2012; Evans *et al.,* 2005). Accessory cells are included to clarify how transcellular transport occurs. Claudins are included to emphasize the passive movement of Na^+ out of fish in seawater, as well as the downregulation of this function in freshwater, likely via the insertion of different claudin isoforms (Kolosov *et al.,* 2013). Arrows indicate the direction of movement of ions across the cell membrane (Dymowska *et al.,* 2012; Kolosov *et al*., 2013). It is worth noting that Type I cells are present in seawater as well as freshwater.

1.3 Evolution of freshwater tolerance in fish

It is commonly believed that fishes evolved in marine environments and have since colonized freshwater multiple times (Dymowska *et al.*, 2012; Evans *et al.*, 2005; Lee & Bell, 1999; McKinnon & Rundle, 2002). Previous research has found that tolerance to freshwater has evolved differently in different fish species (reviewed by Dymowska *et al.*, 2012). The differences in the molecular mechanisms of ion and acid-base regulation covered by Dymowska *et al.* (2012) in Rainbow Trout, Killifish, Tilapia and Zebrafish, highlights that freshwater tolerance has evolved multiple times in different ways in fish species.

Freshwater acclimation in fish is highly dependent on the fish's ability to upregulate ion transporters involved in freshwater acclimation as well as the ability to downregulate ion transporters involved in seawater acclimation (Dymowska *et al.*, 2012). The importance of changes in gene expression in regulating the key transporters involved in freshwater acclimation has been documented by multiple studies and in a wide variety of fish species, including Rainbow Trout, Killifish, Tilapia, Zebrafish, Threespine Stickleback, *Takifugu* and Alewife (DeFaveri *et al.*, 2011; Dymowska *et al.*, 2012; Gibbons *et al.*, 2017; Nakamura *et al.*, 2021; Velotta *et al.*, 2017). Freshwater ionocyte morphology varies between different species (Dymowska *et al.*, 2012), but there is some consistency in the up- and downregulation of candidate ion transporter genes, suggesting that transporters such as the CFTR and NKCC1 should be downregulated in all freshwater fishes, while the NCC2 should be upregulated (DeFaveri *et al.*, 2011; Dymowska *et al.*, 2012; Gibbons *et al.*, 2017; Nakamura *et al.*, 2021; Velotta *et al.*, 2017). The ability to acclimate to freshwater can therefore be estimated through gene expression analysis for the genes involved in freshwater acclimation, as studies have

found that evolving increased freshwater tolerance requires the ability to transcriptionally upregulate genes related to freshwater tolerance and downregulate genes related to seawater tolerance (Gibbons *et al.*, 2018, 2017, 2016; Nakamura *et al.*, 2021; Velotta *et al.*, 2017). By analyzing candidate gene expression, a potential inability to acclimate to freshwater can be estimated to be due to either failure to downregulate 'saltwater genes' (*e.g.* CFTR and NKCC1), or failure to upregulate 'freshwater genes' (*e.g.* NCC2). Therefore, the inability to upregulate genes related to freshwater and downregulate genes related to seawater acclimation may limit freshwater evolutionary potential in fish, but few studies have tested this hypothesis among closely related species and populations (but see DeFaveri *et al.*, 2011; Gibbons *et al.*, 2018, 2017, 2016; Nakamura *et al.*, 2021; Velotta *et al.*, 2017).

1.4 *Gasterosteidae* as a model system to study the evolution of freshwater tolerance

The sticklebacks (*Gasterosteidae*) are a group of small teleost fishes that includes species with a variety of salinity tolerances that are thought to have evolved from a marine ancestor (*Figure 2*; Kawahara *et al.*, 2009). Within the *Gasterosteidae* family, some species mainly live in marine to brackish waters and cannot tolerate acute transfers to freshwater (*e.g.* Fifteenspine Stickleback; Raffy, 1953) while others not only manage the challenges that come with living in saltwater, but also have populations that live in freshwater year-round (*e.g.* Threespine Stickleback, Fourspine Stickleback and Ninespine Stickleback; Arai & Goto, 2005; Blouw & Hagen, 1984; Kawahara *et al.*, 2009; Nelson, 1968). This raises questions such as: Did the common ancestor of all *Gasterosteidae*

possess some freshwater tolerance or did different populations and species evolve freshwater tolerance independently?

Previous studies have looked at the freshwater tolerance in 1- to 5-week-old juvenile Blackspotted Stickleback and Threespine Stickleback (Belanger *et al.*, 1987; Campeau *et al.*, 1984). Campeau *et al.* (1984) found one and five week old Blackspotted Stickleback can survive freshwater, but are more lethargic and have a decreased growth rate in freshwater compared to saltwater (Campeau *et al.*, 1984). As well, adult Blackspotted Stickleback showed a preference for brackish water (7 ppt or 14 ppt), while Threespine Stickleback showed no preference from 0 to 35 ppt (Campeau *et al.*, 1984). At present, we do not know if adult marine and brackish living stickleback species, such as the Blackspotted Stickleback and white ecotype of Threespine Stickleback, are able to acclimate to freshwater or if they are intolerant like the Fifteenspine Stickleback (Belanger *et al.*, 1987; Raffy, 1953). Thus, a further characterization of freshwater tolerance abilities in all stickleback species is needed. There is little evidence in the existing literature regarding the ability of adult Blackspotted Stickleback to colonize freshwater (e.g., Campeau *et al.*, 1984 only find adult Blackspotted fish at higher salinities in Rivière des Vases, Québec), but it should be noted that Blackspotted Stickleback have been collected from at least one estuarine site at 0 ppt (Blouw, 1978; van Vliet, 1970) suggesting that it may be possible for this species to successfully acclimate to freshwater, or adapt to be able to tolerate freshwater. It is therefore of interest to test the freshwater tolerance of the adult Blackspotted Stickleback. Furthermore, by comparing the molecular mechanisms underlying freshwater acclimation

in stickleback species, we can determine if these species use similar molecular mechanisms to acclimate to freshwater.

In addition to the Blackspotted Stickleback, the "white" Threespine Stickleback (*Gasterosteus aculeatus*) has only been observed in saltwater environments along the Nova Scotian coastline (Blouw & Hagen, 1990). This marine ecotype of the Threespine Stickleback species is suggested to have evolved from a "common" Threespine Stickleback ancestor through sexual selection for the white breeding colouration of the white Threespine Stickleback (Blouw & Hagen, 1990; Blouw, 1996). While the common Threespine Stickleback is widely distributed in marine, brackish and freshwater, the white Threespine Stickleback appeared to be restricted to marine environments (Blouw & Hagen, 1990). Therefore, data on the freshwater tolerance in this ecotype is required to better determine the evolution of freshwater tolerance within the Threespine Stickleback model system (McKinnon & Rundle, 2002), as well as the *Gasterosteidae* family.

The mechanisms underlying freshwater acclimation and adaptation has been studied in freshwater, anadromous and marine populations of the euryhaline Threespine Stickleback (DeFaveri *et al.*, 2011; Divino, 2016; Gibbons *et al.*, 2018, 2017; Kusakabe *et al.*, 2017). Threespine Stickleback and Ninespine Stickleback can be found throughout much of the northern hemisphere (Scott & Scott, 1988) and many ecotypes of both species have repeatedly and independently evolved increased freshwater tolerance during post-glacial colonization of lakes and streams (*e.g.* DeFaveri *et al.*, 2011; Divino, 2016; Gibbons *et al.*, 2017; Kusakabe *et al.*, 2017). The evolution of increased freshwater tolerance in Threespine Stickleback populations is thought to have evolved as a result of post-glacial invasion by marine fish after the Pleistocene epoch. As the ice that covered large parts of North America receded after the Pleistocene epoch, sticklebacks were

trapped in lakes that were created from the melting ice water. These trapped marine populations were then forced to acclimate and adapt to the new environment or face extinction (Lee & Bell, 1999). With the new environment having much lower salinity, the sticklebacks faced challenges with water gain and ion loss (section 1.2). To cope with these challenges, species within the *Gasterosteidae* family have undergone parallel divergence in morphology, including the adaptation of physiological mechanisms needed to cope with freshwater (Dymowska *et al.*, 2012; Kültz, 2015). This local adaptation is thought to have occurred in fish that did have some ancestral tolerance to freshwater; the present-day marine ecotype of Threespine Stickleback also shows impressive, but slightly lower, tolerance to freshwater (Gibbons *et al.*, 2017).

The upregulation of ion transporters related to freshwater acclimation (NCC2) in many species (Brennan *et al.*, 2016; Dymowska *et al.*, 2012; Hwang *et al.*, 2011), indicates that this transporter protein would also be upregulated in the freshwater tolerant species within the stickleback family *Gasterosteidae* when exposed to freshwater. In addition, most fish downregulate the seawater-related ion transporters CFTR and NKCC1 in freshwater (e.g., Breves et al. 2020). However, the data for Threespine stickleback is equivocal; Divino et al. (2016) found decreased NKCC1 gill protein content in fresh compared to seawater gill homogenates, but Gibbons et al. (2018) did not detect lower mRNA content in 0 ppt compared to 30 ppt exposed Threespine Stickleback. Thus, we will further explore these transporters in Blackspotted Stickleback to test the generalities of these transcriptional response in the sticklebacks. Gibbons *et al.*, (2017, 2018) also observed increased expression of some transporters present in both freshwater and seawater ionocytes in Threespine Stickleback exposed to freshwater. In particular, NKA and NHE3 were found to have higher expression levels in both marine and freshwater

ecotype exposed to freshwater compared to saltwater exposed fish (Gibbons *et al.*, 2017). In contrast, while ECaC was found to be expressed in both freshwater and saltwater, the expression levels were slightly higher in freshwater exposed fish from a lake population (freshwater adapted fish) compared to a marine population (Gibbons *et al.*, 2017). These results are generally similar to the way other species acclimate to freshwater (Breves *et al*., 2020; Dymowska *et al.*, 2012; Furukawa *et al.*, 2014). However, the transcriptomic changes associated with freshwater tolerance, or lack of tolerance, in other stickleback species (*i.e.* Blackspotted Stickleback) have not yet been measured. Therefore, this thesis investigated the following questions: Can Blackspotted stickleback acclimate to freshwater? If so, do they use similar transcriptional mechanisms as the Threespine Stickleback? If they cannot tolerate freshwater, what transcriptional mechanisms may malfunction to prevent acclimation?

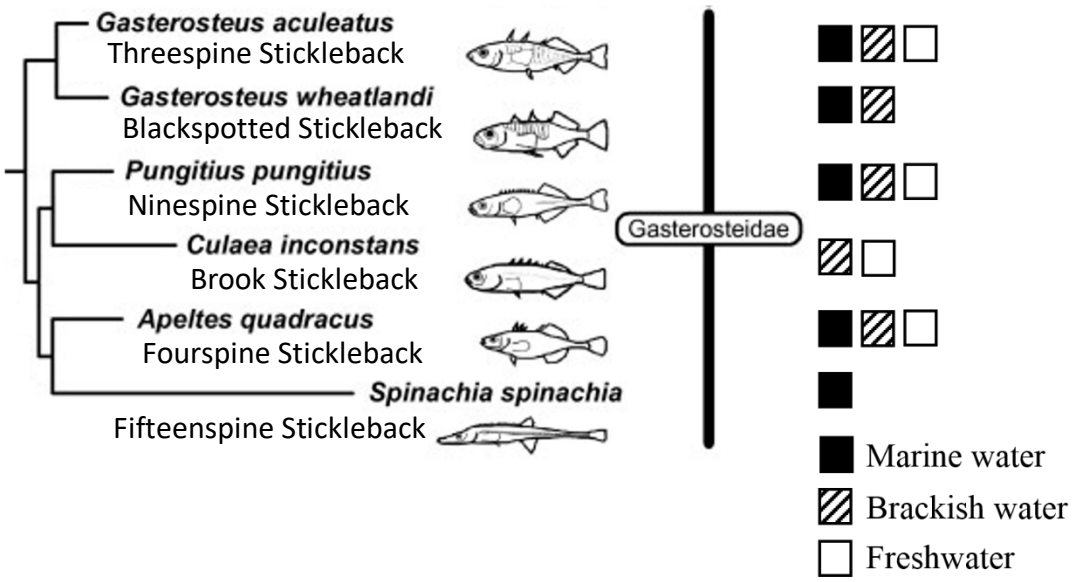

Figure 2. Gasterosteidae phylogeny from Kawahara *et al.* (2009) with corrected habitat distribution information from Blouw and Hagen (1984), Gibbons *et al.* (2016), Shimada *et al.* (2011), Worgan and Fitzgerald (1981) and Dr. A Dalziel (personal communications regarding species distributions in Nova Scotia). The habitat (marine, brackish and freshwater) in which populations of each species are commonly found is indicated on the right.

1.5 Aims and hypothesis

The present research was designed to first test the freshwater acclimation capacity of two almost entirely marine fishes, the Blackspotted Stickleback and "white" Threespine Stickleback, to better understand the evolution of freshwater tolerance in the *Gasterosteidae*. This was assessed by measuring survival rate after freshwater transfer and measuring muscle water content, which is an indicator of the fish's ability to maintain osmoregulatory homeostasis (Evans *et al.*, 2014; Whitehead *et al.*, 2012). The next goal was to measure the expression of ion transporter genes in the gills in Blackspotted Stickleback to test: i) if specific genes or suites of genes are associated with freshwater tolerance or the lack thereof, and ii) if similar ion transporter proteins are up-regulated,

and upregulated to the same extent, in the Blackspotted Stickleback upon freshwater transfer as the freshwater-tolerant Threespine Stickleback (Gibbons *et al.*, 2017), suggesting a similar capacity for, and mechanisms of, freshwater acclimation.

By determining freshwater tolerance levels, and the mechanisms associated with tolerance in each species, we can better understand the evolution freshwater tolerance in the Gasterosteidae (McKinnon & Rundle, 2002; Pfennig *et al.*, 2010; Wolf, 2013). Seeing as Blackspotted Stickleback and white Threespine Stickleback are closely related to the common Threespine Stickleback (Kawahara *et al.*, 2009) and Ninespine, Brook and Fourspine Stickleback also possess freshwater tolerance, I predict that the Blackspotted Stickleback and white Threespine Stickleback would be able to tolerate freshwater. This prediction is based on the assumption that freshwater tolerance evolved early in the evolutionary history of the Gasterosteidae giving all *Gasterosteus* spp., *Pungitius*, *Culaea* and *Apeltes*, the underlying genetics for ion transporters related to freshwater tolerance, as well as the ability to upregulate freshwater related ion transporters (*e.g.* NCC2, ECaC, NKA, and NHE3; Breves *et al.*, 2020; Dymowska *et al.*, 2012; Furukawa *et al.*, 2014; Gibbons *et al.*, 2017) transporters (*e.g.* CFTR and NKCC1: Breves *et al.*, 2020; Li & Kültz, 2020; Taugbøl *et al.*, 2014). Therefore, I predicted survivorship to be equal between 0 ppt and 10 ppt, indicating that the Blackspotted Stickleback and white Threespine Stickleback can live in both fresh- and saltwater. Similarly, the Blackspotted Stickleback as well as the white Threespine Stickleback tissue water content is expected to remain the same in fish exposed to 0 ppt compared to fish exposed 10 ppt.

If the Blackspotted Stickleback are freshwater tolerant, it is predicted that Blackspotted Sticklebacks exposed to 0 ppt water conditions will show an upregulation of NCC2 expression (a transporter aiding in Na^+ and Cl^- uptake) and a slight increase in

NHE3 and NKA alpha subunit expression in freshwater relative to 10 ppt, similarly to the euryhaline Threespine Stickleback tested at a range of salinities, including 0 and 11 ppt (Gibbons *et al.*, 2017). I do not expect ECaC to change in expression levels in fish exposed to the 0 ppt test condition compared to 10 ppt, as Gibbons *et al.* (2017) only found an increase in ECaC gene expression in 0 ppt compared to 11 ppt in a freshwater-adapted population, not in the marine Threespine Stickleback population (Gibbons *et al.*, 2017). In addition, no changes in CFTR and NKCC1 (two saltwater ionocyte ion transporters that help excrete ions) mRNA levels are expected as the Threespine Stickleback does not transcriptionally downregulate these ion transporters to successfully colonize freshwater (Gibbons et al., 2017; Taugbøl *et al.*, 2014), and instead appears to use post-transcriptional mechanisms to reduce NKCC1 protein content (Divino *et al.*, 2016).

2. Methods

2.1 Fish collection

Fish collections were performed in June 2020 at Rainbow Haven Beach Provincial Park, Nova Scotia, Canada (44.654779, -63.421304), under the Department of Fisheries and Oceans Canada Maritime Region fishing licence #343930. Minnow traps were placed in the shallow waters close to shore and left for 3-24 hours. Fishes were collected from their natural habitat with a salinity of 30 ppt and ~18°C.

Fishes were placed in buckets with their native water and a bubbler to aerate the water. After identification of the fish, individuals to be brought back to Saint Mary's University (SMU) were placed in water in buckets with constant aeration using bubblers. The buckets were placed in a cooler with ice packs. Fish were brought back to the SMU

fish facilities and were cared for following an animal care protocol, approved by the SMU Animal Care Committee (AUPF 18-07).

2.2 Freshwater tolerance experiment

2.2.1 Acclimation

Fish were kept at 10 ppt (approximately isosmotic to fish extracellular fluids) at room temperature (17-23°C) and a photoperiod of 16L:8D, as this mimics the natural summer conditions, for an acclimation period of 1-2 weeks prior to start of the experiment. These temperature and light conditions were maintained throughout the experiment. Tanks at 10 ppt and 0 ppt (freshwater) were set up 2 weeks prior to transfer of the fish, allowing for a healthy community of nitrifying bacteria to grow. Three tanks with 0 ppt and three tanks with 10 ppt were used to account for potential tank effects, and fish were kept at a density of 10-12 fish per tank in 3 tanks at 0 ppt and 3 tanks at 10 ppt. Blackspotted Stickleback and white Threespine Sticklebacks were kept in the same tanks, and the two species were separated by a mesh net, *i.e.* 5-6 individuals of each species in each tank.

2.2.2 Freshwater transfer

Fish were transferred from the acclimation tanks into the experimental tanks using nets. For every Blackspotted Stickleback transferred a white Threespine Stickleback was also transferred (randomly), from each of the acclimation tanks to reduce tank effect prior to salinity transfer. The first time point (day 0) was directly before fish were transferred to the test conditions. The other three sampling points were performed at days 1, 4, and 21 post transfer (*Figure 3*). This experimental time frame was chosen to allow for the study

of short-term exposure effects (acute responses; day 1 and 4) as well as prolonged exposure effect (acclimation; day 21).

Survivorship was measured by monitoring the death of individuals from salinity transfer until the end of the experiment at day 21. When a death occurred, the species, tank number, salinity and gender was recorded. The death counts were then used to calculate the percentage of fish that survived the full trial period. The calculations included the assumption that sampled fish would have survived the full trial period. The percent Blackspotted Stickleback and white Threespine Stickleback that survived the duration of the experiment in each salinity (0 and 10 ppt) was calculated by subtracting the fish that were sampled for gene expression and tissue water content studies. The data for survivorship was analyzed in the binomial test to compare two proportions in R (R version 4.0.4, 2021 in RStudio © 2009-2021 version 1.4.1106, “Tiger Daylily” for macOS).

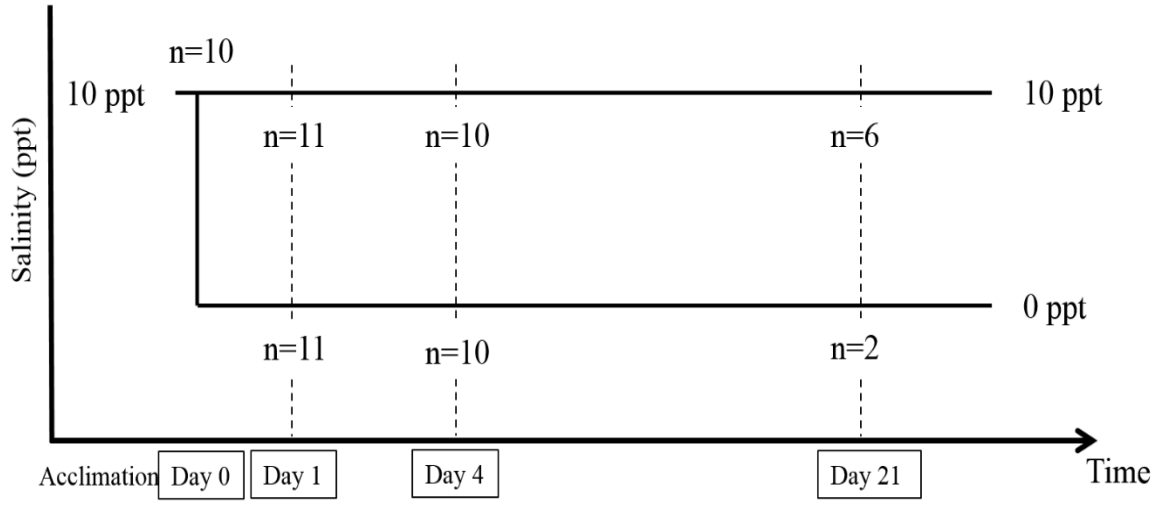

Figure 3. Diagram of the freshwater tolerance experimental sampling time frame. The salinity is presented along the y-axis in ppt and the sampling times (day 0, 1, 4, and 21) post salinity-transfer are presented on the x-axis. At day 0, fish were either transferred to test conditions of 0 ppt or a handling control tank at 10 ppt. 3 tanks at 0 ppt and 3 tanks at 10 ppt were used to account for tank effect. The Blackspotted Stickleback sample sizes are indicated: n = 10 fish were sampled at day 0 for a pre-transfer control. The number of fish (n) sampled at each sampling point is depicted above.

2.2.3 Fish sampling and tissue collection

Fish were euthanized to collect tissue samples from the gills at four different time points. (*Figure 3*: days 0, 1, 4, and 21 post-transfer). For each sampling day, both species of fish (white Threespine Stickleback n = 6 for day 0, n = 6 for day 1 at 0 ppt and 10 ppt respectively, n = 4 for day 4 at 0 ppt, n = 6 for day 4 at 10 ppt, no white Threespine Sticklebacks were sampled on day 21; for Blackspotted Stickleback sample sizes see *Figure 3.*) were randomly selected from each of the three tanks at each salinity to minimize the effects of sampling order, and it was noted which tank each sample came from. Each fish was removed from their tank and immediately put into a lethal dose of anesthetic at a concentration of 3.8 mM (1 g tricaine methanesulfonate (MS222) buffered

with 0.5 g sodium bicarbonate ($NaHCO_3$) in 1 L of water). The MS222 solution was constantly aerated using a bubbler to reduce stress during euthanasia. Once the fish had been euthanized, a scalpel was used to remove the caudal peduncle from the rest of the body. A heparanized microhematocrit capillary tube (Fisherbrand™, Fisher Scientific) was used to collect blood from the body through the caudal artery, but due to the small fish size, not enough blood was collected to measure plasma ion content. The fish was then placed on a ruler along with the caudal peduncle and body length was recorded. The full fish body was then placed on an analytical scale and the wet body weight was recorded. The head was removed using a scalpel and spring scissors were used to remove the full gill basket. Directly after dissection, gills were wrapped in tin foil and flash frozen in liquid nitrogen. In addition, the intestine was dissected, and any remaining organic materials were removed before flash-freezing the intestine samples in liquid nitrogen. All internal organs were removed from the body and the wet weight without internal organs, head and fins was recorded as the wet body mass for later calculation of tissue water content (section 2.4). The carcass was placed in a sample tube and left to air dry in a fume hood. Gill and intestine samples were stored in a -80°C freezer for RNA extractions (section 2.6).

2.3 Tissue water content

After allowing for the fish carcasses to air dry until the weight remained constant (~ 14 days), the dry weight was recorded using an analytical scale. The percent muscle water content was calculated using the following equation:

$$\%\, muscle\ water\ content = \frac{fish\ wet\ weight - fish\ dry\ weight}{fish\ wet\ weight} \times 100$$

2.4 RNA extractions from Blackspotted Stickleback gill samples

RNA was extracted from the whole gills using the GeneJet Purification kit (Thermo Scientific), with some minor modifications. To extract RNA, gills stored at -80°C were thawed and immediately placed in a homogenization solution made by using 300 µL lysis buffer from the Thermo Scientific GeneJet Purification kit and 2M dithriothreitol (Sigma-Aldrich) dissolved in RNase free water (Thermo Scientific) on ice. Gill samples were then homogenized using a roto-stator homogenizer (Variac autotransformer, 7 mm blade, Kinematic Switzerland model GMpH) 3 times in 30 seconds intervals at 60-80 % power, or until homogenized. Homogenized samples were then stored at -80°C.

Following homogenization, RNA purification was executed using 10 µL proteinase K mixed with 590 µL TE buffer as well as wash buffer 1 and 2 from the kit, following the steps in the GeneJet protocol, with the addition of an on-column DNase treatment that was performed using the PureLink™ Invitrogen kit, to remove contaminating DNA. RNA samples were eluted in 100 µL RNase free water and the extracted RNA was divided into three separate 33 µL aliquots: one for quantitation and quality check, one for cDNA synthesis and one extra aliquot.

2.5 RNA quantitation and quality verification

RNA quantity was assessed using a Spectromax M3 spectrophotometer (Molecular Devices) and the Softmax Pro 7 software. 2.5 µL of each sample was loaded in triplicate onto a 24-well MVMP SpectraDrop™ Micro-Volume Microplate (Molecular Devices) with a 0.5 mm cover slip. The nucleic acid concentration was measured at an absorbance

of 260 nm and protein contamination was estimated by absorbance at 280 nm. RNase free water was loaded onto each plate in triplicate as a blank.

The quality of the RNA samples was tested by running out and visualizing samples on an agarose bleach gel (1 % agarose: Tris-acetate EDTA w/v, 1 % bleach v/v, stained with ethidium bromide) following the methodology by Aranda *et al.* (2012). Up to 1 μg of each RNA sample was loaded in each well. Samples were run in a Bio-Rad agarose gel electrophoresis system for 50 minutes. The gel was then removed from the electrophoresis rig and transferred to a Bio Molecular Imager® Gel Doc™ XR+ (Bio-Rad) imaging system. The gel was photographed using the Imager and the Image Lab™ software (Version 6.0, 2017, Bio-Rad). This software was also used to analyze the ratio of 28S rRNA and 18S rRNA band density. The ratio was calculated by assessing the adjusted volume (excluding the background noise) of the 28S and 18S rRNA bands respectively. High quality RNA should have a ratio of 2:1 (Taylor *et al.*, 2010).

2.6 Reverse transcription (RT) and quantitative polymerace chain reaction (qPCR) analysis of genes of interest in Blackspotted Stickleback gill

2.6.1 cDNA synthesis

RNA samples for complementary DNA (cDNA) synthesis were selected by analysing the results from RNA quantification and quality verification assays (section 2.6). Only samples with >16 ng/μL RNA concentration and a 28S:18S ratio of 2:1 were selected for cDNA synthesis.

cDNA synthesis was performed using 6 μL reverse transcription (RT) supermix from the iScript™ cDNA Synthesis kit (Bio-Rad) along with 2 μL no reverse

transcription supermix for the No-RT series, following the protocol provided by manufacturer. A 30 µL cDNA reaction (using 500 ng RNA) was completed for each sample along with a 10 µL non-reverse transcribed (No-RT) reaction. The samples were stored at -80°C for qPCR analyses (see section 2.11).

2.6.2 Selection of genes of interest and qPCR primer design

Genes of interest (GOI) were selected based on findings in previous studies reporting the regulation of ECaC, NHE3 and NKA in response to salinity transfer (Dymowska *et al.*, 2012; Gibbons *et al.*, 2017). Other studies have found that NCC2 is upregulated in teleost fish in freshwater (Breves *et al.*, 2020; Hiroi *et al.*, 2008; Inokuchi *et al.*, 2017; Wang *et al.*, 2009), and the NCC2-1a and NCC2-1b transcripts were therefore selected to test the regulation of ion transporters related to freshwater acclimation. In addition, the NKCC1 and CFTR ion transporters in gills were selected as "seawater ion transporters" for the present study as these ion transporters have been observed to be related to seawater acclimation (Zimmer *et al.*, 2021).

Primers for amplification of genes of interest were designed according to the conditions outlined by Taylor *et al.* (2010), which include, but are not limited to, optimal amplicon length, GC content, and melting temperature. The primers were designed to amplify a 75 – 150 bp long amplicon, have a GC content between 50 – 60 %, melting temperature (T_m) of 55 – 65°C, span an exon-exon boundary, and be in a consensus region of a closely related species within the group of teleost fish. Sequence information for genes of interest, including genes predicted to be upregulated during freshwater acclimation (NHE3, NKA and ECaC), genes involved in seawater acclimation (NKCC1

and CFTR) that are predicted to be downregulated in freshwater, along with the reference genes L13A ribosomal binding protein (RPL13A), ubiquitin (UBA), hypoxanthine phosphoribosyltransferase 1 (HPRT) and eukaryotic elongation factor 1 alpha (EEF1a) (Hibbeler *et al.*, 2008; Wang *et al.*, 2009), were retrieved from Ensembl for the Threespine Stickleback. The Threespine Stickleback genes were used for primer design, since it is a closely related species to the Blackspotted Stickleback and the Blackspotted Stickleback genome is not yet available online. The cDNA sequence was downloaded in FASTA format and aligned with sequences from two other closely related teleost species within the *Actinopterygii* class (wolf-eel, *Anarrhichthus ocellatus* and lump fish, *Cyclopterus lumpus*) using the Multalin online tool (Mitchell, 1993) to find consensus sequences within the gene among the three species.

The NCBI Primer-Blast online tool (Ye *et al.*, 2012) was used to find the primers for each GOI. Among the primer results, the primers that met the conditions outlines above were selected. Where not all conditions could be met, the primers that met the most conditions were selected. Multiple primer sets were designed and used in the primer optimization for determination of the better suited primer sets for the present study (Table 1).

2.6.3 qPCR optimization

Following primer design, primers were ordered from Integrated DNA Technologies Inc. The DNA oligos were order in a lab-ready solution normalized to 100 μM in IDTE buffer at pH 8.0 with standard desalting. For primer optimization, the primers were diluted to 20 μM in sterile nuclease free water (VWR Life Science). The primers were then tested on

cDNA from the meta population, *i.e.,* reverse transcribed pooled RNA from all samples. The reverse transcription from RNA into cDNA was conducted following the procedures described in section 2.7 and then diluted to an eight step 2-fold dilution series starting at a ¼ dilution.

Master mix solution for qPCR was prepared using 5 µL SsoAdvanced™ Unversal SYBR® Green Supermix (Bio-Rad), 0.5 µL forward primer (10 mM) and 0.5 µL reverse primer (10 mM) for each well. Six (6) µL master mix was added to a Multiplate™ 96 well PCR plate (Bio-Rad). Four (4) µL of the serially diluted cDNA was added in triplicate. The plate was then sealed using an adhesive optically clear Microseal 'B' PCR plate sealing film (Bio-Rad). The plate was then centrifuged and placed in a C1000 Touch Bio-Rad Thermal Cycler (Bio-Rad) for a qPCR assay. Standard curves were generated for all primer sets and the best suited primer sets were determined by assessing the acquired qPCR data following the recommendations of Taylor *et al.* (2010). In assessing the qPCR data, the Cq values were used to verify that the different dilutions in the dilution series were separated by 1 cycle. Melt curves were assessed to verify that one single PCR product was being amplified. The melt curve should be uniform and there should be one single melt peak for each primer set (Taylor *et al.*, 2010). Reaction efficiency was estimated and accepted if it fell between 90 – 110 %, in accordance with the MIQE guidelines (Bustin *et al.*, 2009; Taylor *et al.*, 2010). In addition, the R^2 value was verified, and should be >0.98, confirming that the samples conform to the regression line in the standard curve. *Table 1* include the primers that were selected based on the primer optimization results.

Table 1. Primer sequences for genes of interest in qPCR. * indicates reference genes. **indicates potential reference genes that were tested, but not used in the study due to a lack of stability. Primers were designed during this experiment following the procedures from section 2.9. Genes include the Na^+/Cl^- cotransporter 2 (NCC2), Na^+/H^+ exchanger 3 (NHE3), epithelial Ca^{2+} channel (ECaC), Na^+,K^+-ATPase (NKA) alpha subunit, $Na^+/K^+/2Cl$ cotransporter 1 (NKCC1), cystic fibrosis transmembrane conductance regulator (CFTR), ubiquitin (UBA), and L13A ribosomal binding protein (RPL13A). The respective gene transcript, gene ID reference for Ensembl, forward/reverse strands, and primer sequence in the 5' to 3' directions, qPCR product size in base pairs and the melting temperature (T_m) in Celsius are included.

Gene name	**Transcript**	**Gene ID (Ensembl)**	**F/R**	**Primer sequence (5' -> 3')**	**qPCR Product size (bp)**	**T_m (°C)**
slc12α10.1 (NCC2)	NCC2-1a	ENSGACT00000025101.1	F R	GCAACGTCACGGTGATAACG GAAGGGAGCGACACTTTCCA	83	59.91 59.97
slc12α10.1 (NCC2)	NCC2-1b	ENSGACT00000025108.1	F R	CCCATTAGGTTTGGCTGGGT CACGGACATCGCGAGGATAA	141	59.96 59.97
slc9α3.2 (NHE3)	slc9a3.2-201	ENSGACG00000002446.1	F R	CGTTTGTGTCACTGGGAGGA ACACAAGGCCCAAAAGGGAA	105	59.89 60.03
trpv6 (ECaC)	trpv6-201	ENSGACT00000013861.1	F R	TATCCGCGTCCAGAAAACCC ATCTCCAGCAACAGGATGGC	114	60.11 60.11
atp1α1.2 (NKA)	atp1a1-203	ENSGACT00000018954.1	F R	AGCCCAGAAACCCCAAAACA GTCATTGGGGAGGAAACCGT	143	60.03 59.96
slc12α2 (NKCC1)	slc12a2-201	ENSGACT00000024304.1	F R	GGCCTGATGATGTGTGGTCA GGCCTTGGTCTCGTTCTTCA	114	60.04 59.97
cftr (CFTR)	cftr-201	ENSGACT00000011967.1	F R	CCTACGACGAGTTCCGCTAC CCCTCCAGGAGAAGCGTTTT	100	59.97 59.96
*uba52** (UBA)	uba52-201	ENSGACT00000010662.1	F R	ACGGGGAAGACCATCACTCT AGACAGTGTCCGTCCATCCT	147	60.25 60.25
*rpl13α** (RPL)	rpl13a-201	ENSGACT00000012319.1	F R	GGACCGTACCACTTCAGAGC GAGGGATACCGTCGAACACC	124	60.11 59.90
*hprt1*** (HPRT)	hprt1-201	ENSGACT00000024687.1	F R	GTGGACTTCATCCGCCTCAA ACGTCTGCATTGTCTTCCCT	148	60.04 59.31
eef1α ** (EEF1a)	eef1a1l2-201	ENSGACT00000023950.1	F R	CTCAGGCAAATCCACCACCA CTCTGCCTTCAGCTTGTCCA	148	60.25 59.96

2.7 qPCR for Blackspotted Stickleback gill filament tissues

For the qPCR assay for the Blackspotted Stickleback gill cDNA samples, a master mix solution was prepared according to the process described in section 2.9 and 6 µL master mix was added to a 96 well PCR plate. All cDNA and NRT samples were diluted to a 1/8 concentration to prevent inhibition of amplification, as suggested by Bio-Rad (Taylor *et al.*, 2010). Four (4) µL of 1/8 diluted cDNA was added in triplicate for each biological replicate to the designated wells, accompanied by a single 4 µL NRT control. The plate was then sealed using the sealing film and centrifuged before starting the qPCR assay.

Thermal cycles for the qPCR assay were as follows: 50°C for 120 seconds, 95°C for 60 seconds, followed by a repeating step of 95°C for 15 seconds and 60° for 30 seconds. This was repeated 40 times. An additional cycle was added to get the melt curve to assess the purity of the qPCR product: 95°C for 10 seconds, then a repeating step at 60°C with an increase of 0.5°C for each cycle until the temperature reached 95°C. The results from the qPCR assay were analyzed in accordance with the MIQE guidelines (Bustin *et al.*, 2009). Analyzing the qPCR data included checking the melt curve to make sure a single product was being amplified and setting the threshold value for the standard curve on the amplification curve to match the point of exponential amplification. As well, the efficiency and R^2 value was checked and determined to be 90-110 % (efficiency) and > 0.98 respectively.

2.8 Statistical analyses

2.8.1 Reference gene selection

Within the CFX Maestro™ Software (Bio-Rad), the Reference Gene Selection tool was used to determine the stability of the reference genes. The two genes with the highest stability were selected for statistical analyses. Only genes rated as "Acceptable" or higher were selected for further statistical analyses (see Appendix A, *Table A1*). For the present study, RPL and UBA were determined to be acceptable reference genes. In addition, significant effects of time and salinity on gene expression was tested using a two-way ANOVA.

The Gene Study tool in CFX Maestro™ was used to analyse the ΔΔCq (normalized expression) for each gene of interest (GOI). Here, two reference genes (RPL and UBA) from the reference gene stability test were used as reference, and the GOI's (see *Table 1*) were compared to the reference genes.

2.8.2 Statistical analyses: survivorship, tissue water content and gene expression

Chi-square tests were conducted using the software R (R version 4.0.4, 2021 in RStudio © 2009-2021 version 1.4.1106, "Tiger Daylily" for macOS) to test for difference in survivorship between Blackspotted Stickleback and white Threespine Stickleback for each treatment (0 or 10 ppt).

Two-way ANOVAs (analysis of variance) were conducted in R to test for an effect of salinity (0 ppt or 10 ppt) on tissue water content in the two species, Blackspotted Stickleback and white Threespine Stickleback respectively. All assumptions of the ANOVA, including homogeneity of variance and a normal distribution of data, were

tested and met, using a Normal Quantile-Quantile plot, Shapiro-Wilk test and a Residuals vs Fitted plot in R.

Two-way ANOVAs were conducted in R to test for an effect of salinity (0 ppt or 10 ppt) treatment and time (1- and 4-days exposure period) on mRNA content in Blackspotted Stickleback. The data for the GOI's (ECaC, NKA, NHE3, NCC2, CFTR and NKCC1) were transformed to fit the assumptions of a two-way ANOVA using the Tukey's Ladder of Power. The 21 days exposure for Blackspotted Stickleback was excluded due to a low sampling size as a result of deaths during the experiment and the Day 0 control was excluded because reference genes did not show stability at this time point. The *TukeyHSD* package was used for *post-hoc* Tukey multiple comparisons to compare differences between salinity (10 or 0 ppt) and exposure time (1 day and 4 days). The *ggplot2* package was used for data visualization of each GOI.

3. Results

3.1 Survivorship

During the experiment (21 days), 65.7 % of Blackspotted Stickleback survived the 0 ppt treatment (freshwater environment) and 81.8 % survived the 10 ppt treatment (isosmotic environment). The white Threespine Stickleback has a survivorship of 52.6 % in freshwater (0 ppt) and 63.2 % in the control group (10 ppt). These results assume that all fish that were sampled in day 1, 4 and 21 would have survived the 21 days of freshwater exposure. 81 % of the Blackspotted Sticklebacks used in the present study were male fish and 19 % were female fish. Out of the white Threespine Sticklebacks used in the present study, 63 % were male fish and 37 % were females.

While both species had slightly higher survival at 10 ppt, there was no significant difference in survivorship between the freshwater (0 ppt) and the control group (10 ppt) in Blackspotted Stickleback ($p = 0.0662$ $\chi^2 = 2.2631$, $df = 1$) or white Threespine Stickleback ($p = 0.2555$, $\chi^2 = 0.4218$, $df = 1$) (*Figure 4*). During the acclimation period pre salinity transfer and during the experiment (21 days freshwater exposure), a high mortality rate was also observed in the white Threespine Stickleback.

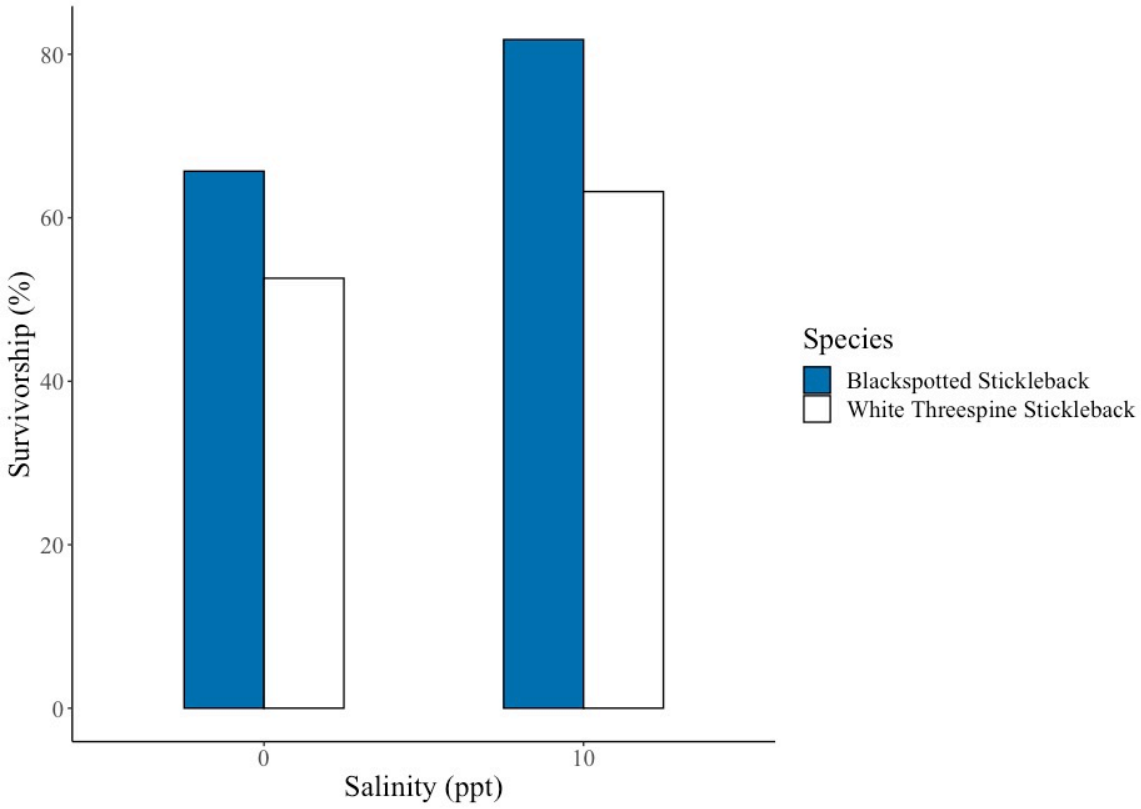

Figure 4. The percent survival of Blackspotted Stickleback and white Threespine Stickleback in freshwater (0 ppt) and the brackish water handling control (10 ppt) after 21 days.

3.2 Tissue water content

There was no effect of salinity (0 ppt or 10 ppt; $F = 1.254$, $p = 0.275$, $df = 1$) or time (day 0, 1, or 4; $F = 2.361$, $p = 0.118$, $df = 2$) on tissue water content in white Threespine

Stickleback (*Figure 5*). There was also no effect of salinity (0 ppt and 10 ppt; F = 0.566, p = 0.455, df = 1) or time (day 0, 1, and 4; F = 1.831, p = 0.171, df = 2) on tissue water content in Blackspotted Stickleback (*Figure 6*).

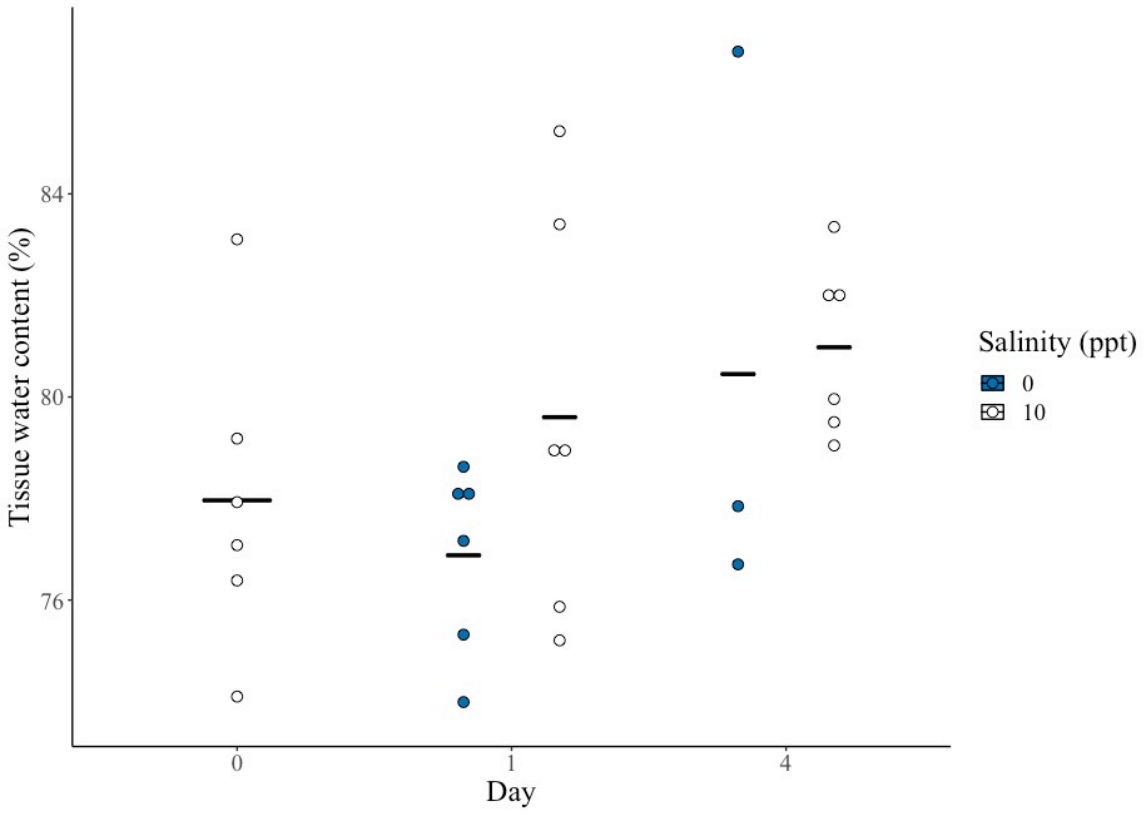

Figure 5. Percent tissue water content in white Threespine Stickleback in freshwater (0 ppt) and isosmotic control conditions (10 ppt) 0, 1 and 4 days after salinity transfer. n = 6 on day 0, n = 6 (0 ppt and 10 ppt) on day 1, n = 4 (0 ppt) and n = 6 (10 ppt) on day 4.

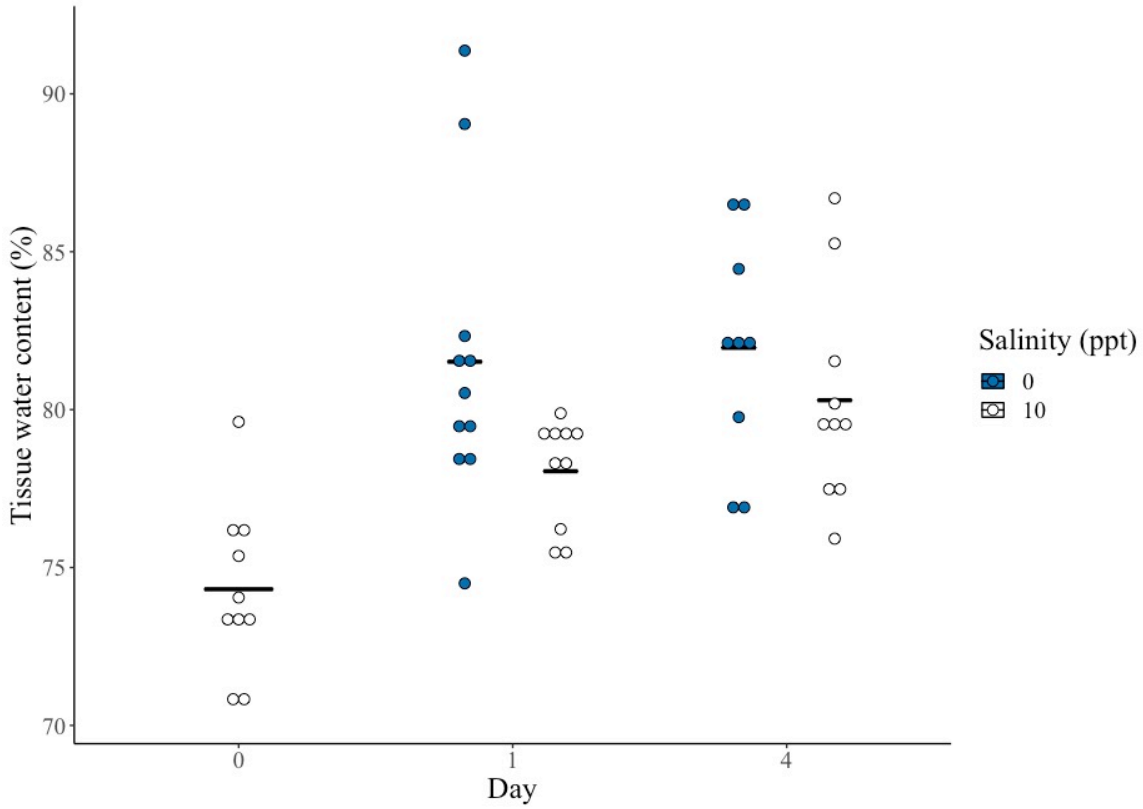

Figure 6. Percent tissue water content in Blackspotted Stickleback in freshwater (0 ppt) and isosmotic control condition (10 ppt) 0, 1 and 4 days after salinity transfer. n = 10 on day 0, n = 11 (0 ppt and 10 ppt) on day 1, n = 10 (0 ppt and 10 ppt) on day 4.

3.3 Gene expression

From the CFX Maestro™ gene stability test (CFX Maestro™ Software Reference Gene Selection tool, Bio-Rad), RPL and UBA were found to be the most stable genes and were therefore used as reference genes (Appendix A). An M value of 0.7 was obtained for both RPL and UBA and they were deemed "acceptable" as reference genes by the software (*Table 1A*). The RPL and UBA Cq values were then used in a two-way ANOVA to test if there was a significant effect of time or salinity on the expression of these genes. A significant effect of day on the average Cq for RPL was detected ($F = 4.484$, $p = 0.017$, $df = 2$), see Appendix A, *Figure A2*. When day 0 was excluded from the statistical analyses there was no significant effect of salinity (0 or 10 ppt) or time (day 1 or 4) on the average

Cq values for the reference genes RPL (effect by salinity: F = 0.184, p = 0.671, df = 1; effect by day: F = 1.769, p = 0.192, df = 1) and UBA (effect by salinity, F = 0.251, p = 0.620, df = 1; effect by day: F = 3.293, p = 0.0784, df = 1), see Appendix A, *Figure A1*. Therefore, the time-matched handling controls were used in our analysis and the pre-transfer gene expression data (reduced stress) was not included due to the large effect of handling on all reference genes. Thus, the analysis focuses on the effects of salinity on mRNA content only.

In addition, hypoxanthine phosphoribosyltransferase 1 (HPRT1) and eukaryotic elongationfactor 1 alpha (EEF1a) were included in the gene stability test. However, EEF1a was deemed "unstable" (*Table 1A*) by the Reference Gene Selection tool (Bio-rad) and was therefore not included as a reference gene in the present study. HPRT1 was deemed "acceptable", but a slightly higher M value was obtained for HPRT1 compared to RPL and UBA (see *Table A1*). As well, a significant effect by day was detected in the ANOVA test statistics (F = 4.003, p = 0.026, df = 2, Tukey *a posteriori*: p = 0.058) and HPRT was therefore not included as a reference gene in the present study.

A difference in relative gill gene expression (relative to the reference genes RPL and UBA) between the two salinity treatments was observed for transcripts encoding transporters present in two fresh- and saltwater ionocytes (NKA and NHE3: *Figure 7*). There was no effect of salinity on ECaC gill mRNA content (F = 3.254, p = 0.080, df = 1). A significant effect of salinity was found for NKA alpha gill mRNA content (F = 7.163, p = 0.012, df = 1, Tukey *a posteriori*: p = 0.011), but no significant difference was detected in the specific interactions of day and salinity (0 ppt compared to 10 ppt on day 1, *etc.*). NHE3 gill expression was also increased at 0 ppt (effect of salinity: F = 11.795, p

= 0.003, df = 1) on day 1 (Tukey *a posteriori*: p = 0.009), but this increase in NHE3 gill mRNA content was no longer significant by day 4.

In addition, a difference in relative gene expression between the two different salinity treatments (10 and 0 ppt) was observed in the transcripts encoding transporters expressed in freshwater gill ionocytes (NCC2-1a: *Figure 8A*). There was an effect of salinity on NCC2-1a gill mRNA content (F = 29.443, p = 1.24e-05, df = 1) with an increase in freshwater on day 1 compared to 10 ppt (p = 0.002) and an increase at day 4 (p = 0.003). A large increase in NCC2-1b gill gene expression was also observed at 0 ppt (*Figure 8B*), with a significant effect of salinity detected (F = 39.344, p = 5.66e-07, df = 1) with a significant difference between 0 and 10 ppt at day 1 (p = 0.001) and at day 4 (p = 0.0003) (*Figure 8B*).

No difference between the two salinity treatments was found in the expression of genes associated with seawater type ionocytes in the gill (CFTR and NKCC1: *Figure 9*). The relative gene expression remained relatively stable for CFTR (F = 0.951, p = 0.336, df = 1) and NKCC1 (F = 0.190, p = 0.666, df = 1) throughout the experiment for both test conditions.

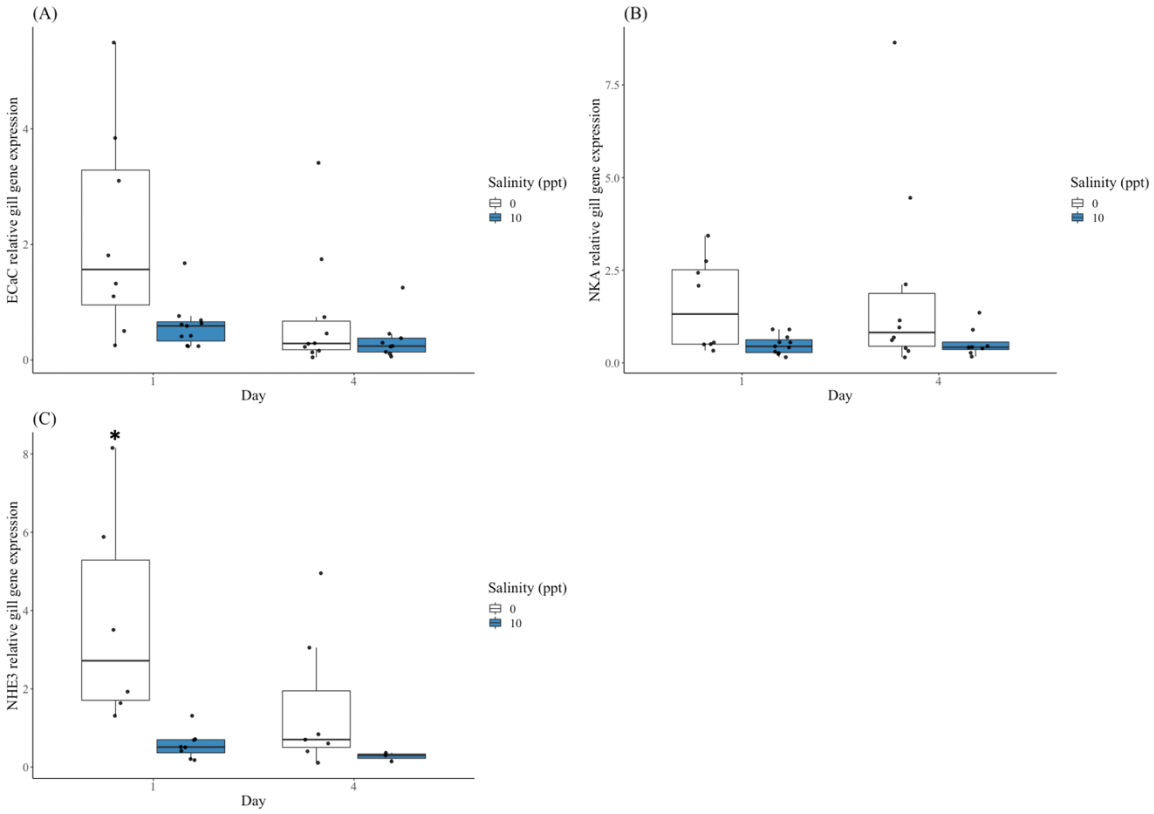

Figure 7. Effect of salinity (10 and 0 ppt) on genes related to freshwater and seawater acclimation in Blackspotted Stickleback gills at days 1 and 4 post salinity transfer. The relative gene expression is presented on the y axis; however, the statistical analyses were performed on transformed data (using the Tukey's Ladder of Power). (A) relative expression of ECaC during the 4-day exposure (n = 11 at 10 ppt on day 1, n = 8 at 0 ppt on day 1, n = 9 at 10 ppt on day 4, n = 10 at 0 ppt on day 4). (B) relative expression of NKA (n = 11 at 10 ppt on day 1, n = 9 at 0 ppt on day 1, n = 9 at 10 ppt on day 4, n = 10 at 0 ppt on day 4). (C) relative expression of NHE3 (n = 8 at 10 ppt on day 1, n = 6 at 0 ppt on day 1, n = 3 at 10 ppt on day 4, n = 7 at 0 ppt on day 4). The "*" indicates significant difference from the 10 ppt treatment on the same day.

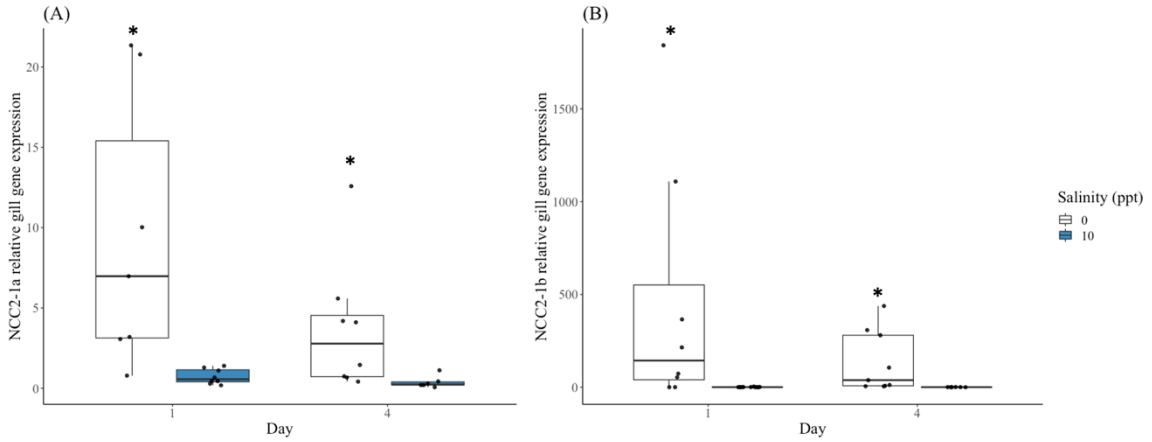

Figure 8. Effect of salinity (10 and 0 ppt) on genes related to freshwater acclimation in Blackspotted Stickleback gills at days 1 and 4 post salinity transfer. The relative gene expression is presented on the y axis; however, the statistical analyses were performed on transformed data (using the Tukey's Ladder of Power). (A) relative expression of NCC2-1a transcript during the 4-day exposure. n = 8 at 10 ppt on day 1, n = 7 at 0 ppt on day 1, n = 6 at 10 ppt on day 4, n = 8 at 0 ppt on day 4. (B) relative expression of NCC2-1b transcript. n = 11 at 10 ppt on day 1, n = 8 at 0 ppt on day 1, n = 7 at 10 ppt on day 4, n = 9 at 0 ppt on day 4. The "*" indicates significant difference from the 10 ppt treatment on the same day.

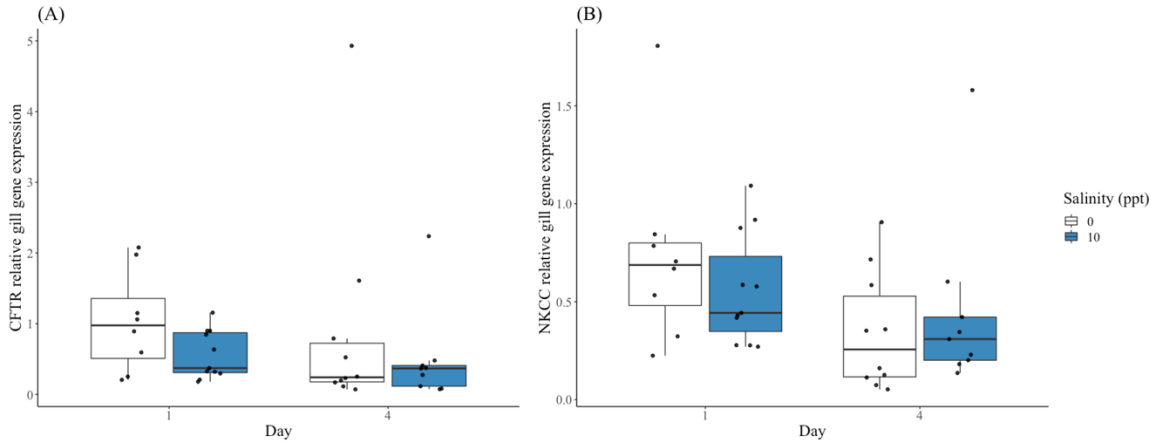

Figure 9. Effect of salinity (10 and 0 ppt) on genes related to seawater acclimation in Blackspotted Stickleback gills at days 1 and 4 post salinity transfer. The relative gene expression is presented on the y axis; however, the statistical analyses were performed on transformed data (using the Tukey's Ladder of Power). (A) relative expression of CFTR during the 4-day exposure. n = 11 at 10 ppt on day 1, n = 8 at 0 ppt on day 1, n = 9 at 10 ppt on day 4, n = 10 at 0 ppt on day 4. (B) relative expression of NKCC1. n = 11 at 10 ppt on day 1, n = 8 at 0 ppt on day 1, n = 9 at 10 ppt on day 4, n = 10 at 0 ppt on day 4.

4. Discussion

The colonization of marine fish in freshwater brings physiological challenges for fish (*e.g.* Pfennig *et al.*, 2010). These freshwater associated physiological challenges include, but are not limited to, warmer temperatures in summer, freezing in winter, lower salinity, as well as differences in nutrients and predators (Pfennig *et al.*, 2010; Stocker *et al.*, 2013). Many studies have assessed the ability to tolerate, and colonize, freshwater in Threespine Stickleback (DeFaveri *et al.*, 2011; Gibbons *et al.*, 2017; Kusakabe *et al.*, 2017; Metzger *et al.*, 2016). However, other species within the stickleback family, such as the Blackspotted Stickleback and the white Threespine Stickleback, are very rarely observed in freshwater (Blouw, 1978; van Vliet, 1970). The paucity of Blackspotted Stickleback and white Threespine Stickleback in freshwater lakes and rivers could be due to low freshwater tolerance as lower freshwater tolerance has been observed in juvenile Blackspotted Stickleback (Campeau *et al.*, 1984). Mechanisms for freshwater acclimation have been studied in Threespine Stickleback (Gibbons *et al.*, 2017), but little is known about the freshwater tolerance and underlying physiological mechanisms potentially allowing freshwater survival in Blackspotted Stickleback (Audet *et al.*, 1986; Campeau *et al.*, 1984) and the white Threespine Stickleback. The present study therefore used a comparative approach to investigate the molecular response to freshwater in the adult Blackspotted Stickleback and white Threespine Stickleback to gain a better understanding of the level of freshwater tolerance in these fish. I found that adult white Threespine Stickleback and Blackspotted Stickleback can tolerate an acute freshwater transfer. However, further studies should be conducted on juvenile and embryos at winter temperatures to further investigate if a lack of freshwater tolerance may have limited the white Threespine Stickleback's ability to colonize post-glacial lakes as the "common"

Threespine Stickleback ecotype has successfully done (Defaveri & Merilä, 2014; Gibbons *et al.*, 2016; Hohenlohe & Magalhaes, 2019). Due to the high mortality rate in the present study, gill gene expression data for the white Threespine Stickleback could not be measured and the mechanisms for freshwater tolerance for these fish could not be determined.

I observed that the Blackspotted Stickleback responds to acute freshwater transfer by upregulation of NCC2 ion transporters that have been found to be related to freshwater acclimation (Breves *et al.*, 2020; Inokuchi *et al.*, 2017). In addition, upregulation of ion transporters related to both freshwater and seawater (ECaC, NKA and NHE3) was observed, however, no downregulation of transporters related to seawater (CFTR and NKCC1) was observed. No significant changes in the expression of CFTR has been observed in Threespine Stickleback after transfer from seawater to freshwater (Taugbøl *et al.*, 2014), nor was the expression of NKCC1 decreased after freshwater transfer from seawater in Threespine Stickleback (Gibbons *et al.*, 2017). Therefore, Blackspotted Stickleback appear to utilize similar mechanisms for freshwater acclimation as other euryhaline sticklebacks. Furthermore, these data suggest that sticklebacks do not transcriptionally downregulate CFTR and NKCC1 in freshwater (Gibbons *et al.*, 2017; Taugbøl *et al.*, 2014; current study) as many other teleost often do (Breves *et al.*, 2020; Hiroi *et al.*, 2008; Scott *et al.*, 2004). Instead, it is possible that post-transcriptional mechanisms lead to a downregulation of "seawater" ionocyte ion transporters (i.e., Divino et al., 2016).

4.1 Freshwater tolerance in two primarily marine sticklebacks: the white Threespine Stickleback and Blackspotted Stickleback

The wide range of salinity tolerances observed within the species of the *Gasterosteidae* family (Gibbons *et al.*, 2016; Kawahara *et al.*, 2009; Nelson, 1968; Raffy, 1953) gives ground to questions regarding the evolution of freshwater tolerance among marine species within the family. The Blackspotted Stickleback is a marine species, with only a few observations in freshwater (Blouw, 1978; van Vliet, 1970), and the present study looked at their capacity for freshwater tolerance in adult Blackspotted fish. Understanding freshwater tolerance, and the mechanisms used to tolerate freshwater, can help us determine if different populations and species of Gasterosteidae evolved freshwater tolerance independently using unique mechanisms, or if common ancestral mechanisms might be commonly redeployed during local adaptation.

I found no effect of salinity on either survivorship (section 3.1) nor tissue water content (section 3.2) in adult white Threespine Stickleback and Blackspotted Stickleback. A high mortality rate was observed in the white Threespine Stickleback during the acclimation period as well as the experimental period, causing a low sampling number for each sampling time (day 1, 4 and 21). The deaths could be due to the fish being collected from the wild and reared in the laboratory during their natural breeding season, causing extra stress for these fish seeing as the breeding state takes a lot of energy (Evans *et al.*, 2014). In addition, the difference in capture site conditions, such as salinity and temperature, could have played a role in the high mortality rate. It is worth noting that similar temperatures were used in the laboratory setting to the natural temperature. As

well, a previous study has observed minimal differences in gene expression of key ion transporters in fish exposed to 30 ppt and 11 ppt (Gibbons *et al.*, 2017). The white Threespine Stickleback were excluded from the qPCR assay due to the low sampling numbers, and no further analyses of the freshwater tolerance in these fish were conducted. The results from the survivorship assay (section 3.1) give no indication of the survivorship being affected by salinity as no significant difference was found in the mortality between the different treatments (10 and 0 ppt). As well, no significant difference was found between the different treatments in the tissue water content assay, indicating that these fish can maintain osmotic homeostasis in both salinities (10 and 0 ppt), as the ability of maintaining water balance within the cells is critical for transitioning between saltwater and freshwater (Whitehead *et al.*, 2012). It is worth noting that previous studies have found higher freshwater growth rates in anadromous Threespine stickleback hatched at 0 ppt compared to 20 ppt (Belanger *et al.*, 1987), while a different study found higher mortality in 20 ppt hatched Threespine Stickleback later exposed to lower (0 ppt) and higher (28 ppt) salinities compared to fish transferred to 7, 14, and 21 ppt (Campeau *et al.*, 1984). Therefore, hatching in a freshwater environment could predispose the development of freshwater tolerance via developmental acclimation (Belanger *et al.*, 1987; Campeau *et al.*, 1984; Defaveri & Merilä, 2014). While I found high survival and tolerance to 0 ppt in our wild caught fish, future studies could breed wild caught fish in common conditions, including freshwater, to test if hatching salinity affects growth and metabolism in marine white Threespine Stickleback and Blackspotted Stickleback and determine if these species differ in tolerance from other sticklebacks that have colonized freshwater.

To address the potential effects of breeding condition on lab survival in white Threespine Stickleback, future studies could look at the freshwater tolerance in healthier, non-breeding, juvenile white Threespine Stickleback before they migrate to the ocean for the winter season. By using juvenile fish, the breeding condition would be eliminated as a factor in the freshwater tolerance experiment. Campeau *et al.* (1984) found that Threespine Stickleback and Blackspotted Stickleback fry (one week old) are less tolerant to freshwater than 5 week old fish of the same species (Campeau *et al.*, 1984). The decreased ability to acclimate to freshwater among fry and juvenile fish could be the reason for the paucity of both Blackspotted Stickleback and white Threespine Stickleback in freshwater lakes as their growth appears to be affected by salinity (Campeau *et al.*, 1984). Seeing as freshwater tolerance seems to be lower in younger fish, another suggestion would be to breed white Threespine Sticklebacks in a laboratory setting and look at the freshwater tolerance in adult fish outside of the breeding season and in less heavily parasitized fish (wild stickleback are consistently parasitized). By looking at laboratory bred adult fish, breeding condition and parasite load can be eliminated as a potential factor leading to mortality.

4.2 Mechanisms of acclimation to freshwater: the effect of freshwater transfer on gene expression in gills

Previous studies have also found that the types of ionocytes used by fish to acclimate to freshwater vary greatly among species, but that certain ion transporters seems to be commonly utilized in acclimation to freshwater, even when their combinations within ionocytes varies (Breves *et al.*, 2020; Dymowska *et al.*, 2012; Hwang *et al.*, 2011;

Inokuchi *et al.*, 2017). Therefore, the present study investigated if the Blackspotted Stickleback use similar mechanisms of freshwater acclimation as the Threespine Stickleback, as well as if the Blackspotted Stickleback acclimate to freshwater as well as the Threespine Sticklebacks do. Studies looking at the gill transcriptional responses in Tilapia, Killifish, Zebrafish and Mummichog were used as reference for their findings on freshwater acclimation in these fishes (Breves *et al.*, 2020; Dymowska *et al.*, 2012; Gibbons *et al.*, 2017).

The primarily marine Blackspotted Stickleback was predicted to possess the ability to acclimate to freshwater, however, Blackspotted Stickleback was also expected to have a weaker freshwater acclimation response than the Threespine Stickleback (Gibbons *et al.*, 2017) that has a relatively high ancestral freshwater acclimation capacity and has also repeatedly colonized freshwater (Bell & Foster, 1994). Thus, it was predicted that Blackspotted Stickleback possess the underlying mechanisms to freshwater tolerance in a similar fashion as the Threespine Stickleback, but that the response would be of a smaller magnitude. The present study looked at two different transcripts for the freshwater ionocyte ion transporter NCC2: NCC2-1a and NCC2-1b. NCC2-1b mRNA showed much higher transcription levels than NCC2-1a (*Figure 8*), suggesting that NCC2-1b is the more important transcript in freshwater acclimation in Blackspotted Stickleback and that Blackspotted Stickleback acclimate to freshwater through upregulation of this ion transporter, as Threespine do (Gibbons *et al.*, 2017). When comparing the fold-change in mRNA levels for the NCC2-1b with the RNA-seq data from Gibbons *et al.* (2017), it was noted that the Blackspotted Stickleback had a higher fold-change in NCC2-1b mRNA levels than the Threespine Stickleback. Gibbons *et al.* (2017) found a 5-fold increase in NCC2-1b mRNA in freshwater compared to saltwater (test conditions: 0 ppt and 30 ppt),

compared to the ~500-fold increase observed in the present study. The present study also observed an increase in NCC2-1a mRNA levels in freshwater, with a 6-fold increase from 10 to 0 ppt, compared to the 2-fold increase observed by Gibbons *et al.* (2017) from 30 to 0 ppt. These results contradict the prediction that the Blackspotted Stickleback would show a weaker response to freshwater. Similar upregulation patterns in expression were found in other previous studies where NCC2 was found to be more highly expressed in the gills of freshwater acclimated Mummichog, Mozambique Tilapia and Japanese Seabass (Breves *et al.*, 2020; Furukawa *et al.*, 2014; Inokuchi *et al.*, 2017).

During freshwater acclimation, the genes encoding multi-functional ion transporters expressed in both seawater and freshwater ionocytes have an increased mRNA content in Threespine Stickleback, including ECaC (only freshwater populations), NHE3 and NKA (Gibbons *et al*., 2017). A similar pattern was found in the present study as a significant increase in the expression of NHE3 and NKA after one day of freshwater exposure (*Figure 7B-C*) was found. While the results indicate an elevation in ECaC mRNA levels, no significant increase was detected (*Figure 7A*). On day 4 the expression was somewhat lower for all three ion transporters. This matches the lack of upregulation of ECaC found by Gibbons *et al.* (2017) in marine populations after 3 months of freshwater exposure. The lower gene expression at day 4 could indicate that as time progress other post-transcriptional processes, such as protein localization, increased translation or phosphorylation could play a more important role in maintaining protein function as time progresses (Gamba, 2012). This hypothesis could be tested by designing an experiment examining the prolonged exposure to freshwater, as conducted by Gibbons *et al.* (2017), who studied a 3-month acclimation and also measuring protein content and activity (e.g., Divino et al., 2016). The results on ECaC, NKA and NHE3 mRNA levels on day one of

exposure from the present study are generally on par with Gibbons *et al*.'s (2017) qPCR data in 3 month freshwater acclimated Threespine Stickleback (Gibbons *et al.*, 2017). Gibbons *et al.* (2017) found that NKA and NHE3 remained upregulated ~2.5-fold and 6-fold respectively, in both freshwater and marine Threespine Stickleback ecotypes after 3 months acclimation to freshwater, and I also found upregulation of these transcripts. However, Gibbons et al. (2017) only found ECaC to remain upregulated by about 3-fold in the freshwater population when assessed by qPCR, with no change in the marine Threespine Stickleback population, which was collected from a similar salinity as our Blackspotted fish. The present study found an on average 3-fold increase in ECaC mRNA in marine collected Blackspotted Stickleback in freshwater on day 1 post-transfer, and a 2-fold increase on day 4, but these were not significantly upregulated, similar to Gibbons *et al.* (2017) findings in the Threespine marine population. In addition, the present study found an around 3-fold increase in NKA mRNA levels on day 1 and 4, respectively. As well, there was an average 5-fold increase in NHE3 mRNA levels in freshwater compared to 10 ppt on day 1, and a 6-fold average increase on day 4 in Blackspotted Stickleback. The fold-increase in mRNA content found by Gibbons *et al.* (2017) using qPCR was around 3-fold for NKA and 6-fold for NHE3, however, it is worth noting that these comparisons were made after a 3 month acclimation period. It is interesting to see a similar transcriptional reaction to freshwater transfer in the acute exposure used in the present study compared to the long-term exposure used by Gibbons *et al.* (2017). In addition, the upregulation found in the RT-qPCR assay by Gibbons *et al.* (2017) was supported by RNA-seq data on the same ion transporters (ECaC, NKA and NHE3). The results from the present study indicate that Threespine Stickleback and Blackspotted

Stickleback use similar mechanisms to acclimate to freshwater in the regulation of ion transporters related to both freshwater and seawater.

It was also predicted that the marine Blackspotted Stickleback would not downregulate genes for ion transporters found in seawater ionocytes (e.g. CFTR and NKCC1), in similar fashion as the Threespine Stickleback (Gibbons *et al.*, 2017). However, studies in other teleost fish species have found that CFTR and NKCC1 mRNA content is decreased to reduce the ion export in freshwater (Hebert *et al.*, 2004; Marshall & Singer, 2002). I found that Blackspotted Stickleback could tolerate freshwater, but still found no significant differences in the expression of CFTR and NKCC1 in the freshwater transfer compared to control conditions at 10 ppt. Similarly, prior studies examining ion transporter expression in the gills of Threespine Stickleback transferred from seawater to freshwater also found no significant downregulation in the expression of CFTR (Taugbøl *et al.*, 2014), and NKCC1 (Gibbons *et al.*, 2017). Therefore, these combined data suggest that sticklebacks do not need to transcriptionally downregulate CFTR and NKCC1 to survive in freshwater (Gibbons *et al.*, 2017; Taugbøl *et al.*, 2014; current study), which is quite different from most other teleosts (Breves *et al.*, 2020; Hiroi *et al.*, 2008; Scott *et al.*, 2004). In addition, Divino *et al.* (2016), observed decrease in NKCC1 protein abundance upon freshwater transfer. The lack of downregulation of NKCC1 mRNA levels (Gibbons et al., 2017; Taugbøl et al., 2014; current study), but decrease in NKCC1 protein abundance (Divino *et al.*, 2016) indicate that sticklebacks may use different mechanisms to downregulate NKCC1 than other teleost fish (Breves *et al.*, 2020; Hiroi *et al.*, 2008; Scott *et al.*, 2004).

Therefore, it is concluded that the Blackspotted Stickleback employ similar ion transporters for freshwater acclimation as other species within the sticklebacks (Gibbons

et al., 2017). Further gene expression studies, along with immunohistochemistry assays must be conducted to accurately determine the freshwater ionocyte types in Blackspotted Stickleback and other *Gasterosteidae*. In particular, the reason why there is no transcriptional downregulation of CFTR and NKCC1a expression in sticklebacks (present study; Gibbons et al. 2017; Taugbøl *et al.*, 2014), and the potential post-transcriptional regulatory factors influencing seawater ionocyte ion-transporter function, should be further examined. For example, protein phosphorylation/de-phosphorylation (*e.g.*, Gamba, 2012), protein localization or the role of changes in ionocyte surface area should also be examined.

4.3 Other factors limiting freshwater colonization in Blackspotted Stickleback

The results from the present study indicate that adult Blackspotted Stickleback can acutely tolerate freshwater exposure and possess and can transcriptionally regulate the ion transporters commonly found in gill ionocytes related to freshwater acclimation and could therefore hypothetically inhabit freshwater. As the underlying mechanisms of freshwater acclimation seems to be present in the Blackspotted Stickleback at summer water temperatures and photoperiods, the inability to colonize freshwater is likely to be primarily due to some other factor or combination of factors. The results of Gibbons *et al.* (2016) suggest that Threespine Stickleback growth, and the expression of NKA and NHE3, are reduced at colder (4°C) temperatures in marine populations compared to freshwater populations. A combination of colder temperature and low salinity also lead to higher mortality in European marine Threespine Stickleback ecotypes than freshwater ecotypes (Schaarschmidt *et al.*, 1999), indicating that freshwater ecotypes have evolved increased freshwater tolerance in cold temperatures. It is hypothesized that marine

populations do not have the full capacity to undergo the necessary physiological remodelling required to successfully colonize freshwater when it is cold (Gibbons *et al.,* 2016). In addition, low temperatures in combination with shorter days have shown reduced reproduction ability in Threespine Stickleback (Allen & Wootton, 1982). For the purpose of the present study, the assumption is that Blackspotted Stickleback are more similar to marine Threespine Stickleback ecotypes than freshwater Threespine ecotypes. Thus, I predict that cold winter temperatures in combination with low salinities might restrict freshwater colonization in Blackspotted stickleback, as suggested for Threespine Stickleback (Gibbons *et al*. 2016; 2018).

In addition, Ishikawa *et al.* (2019) suggested that the Blackspotted Stickleback, as well as the Japan Sea Stickleback, cannot cope with the restricted nutrition in freshwater. The study found that freshwater populations of the Threespine Stickleback possess extra copies of the *Fads2* gene compared to some marine populations and the Blackspotted Stickleback that appears to provide them with the ability to better digest fatty acids in less nutritious food that is in freshwater. Ishikawa *et al*. (2019) also found that the *Fads2* in Threespine Stickleback had undergone a transposition, giving it a different chromosomal location than the ancestral location of *Fads2*, suggesting that this relocation of the *Fads2* gene give the Threespine Stickleback the ability to increase the expression of this gene. The advantage of increased fatty acid metabolism in Threespine Stickleback seems to play a key factor in freshwater colonization (Ishikawa *et al.*, 2019). The seemingly lower ability to digest fatty acids in primarily marine Japan Sea Stickleback (and potentially Blackspotted Stickleback) could also be the key to the decreased freshwater acclimation ability observed in juvenile Blackspotted Stickleback. As well, the increased ability to digest fatty acids in the less nutritious food that is in freshwater, could help in the growth

of juvenile fish in freshwater (Campeau *et al.*, 1984; Ishikawa *et al*., 2019). In this short-term study, our fish were fed mysis shrimp, which are known to have a high fatty acid content and may mitigate the negative survival effects of freshwater exposure in Japan Sea stickleback (Ishikawa *et al*., 2019).

5. Conclusion

The present study found that adult Blackspotted Stickleback and white Threespine Stickleback can tolerate an acute freshwater transfer in summer conditions (17-23°C and a photoperiod of 16L:8D). The survivorship was lower in white Threespine Stickleback compared to Blackspotted Stickleback, potentially because of the highly active breeding behaviour of the white Threespine Stickleback (Blouw & Hagen, 1990), but no difference between the salinity treatments (10 and 0 ppt) within the species was observed. Blackspotted Sticklebacks were observed to upregulate the same ion transporters (i.e., NCC2, NKA and NHE3) during freshwater exposure as was found by Gibbons *et al.* (2017) in their study on Threespine Stickleback. Furthermore, the lack of CFTR and NKCC1a transcriptional downregulation is not predicted to be the limiting factor for freshwater colonization in Blackspotted Stickleback, as the euryhaline Threespine Stickleback shows a similar lack of transcriptional downregulation (Gibbons *et al.* 2017; Taugbøl *et al.*, 2014) and instead appears to rely on post-transcriptional mechanisms (Divino *et al.,* 2016). Thus, the specific factors limiting freshwater colonization in Blackspotted Stickleback require further investigation.

Appendix

Appendix A

Table A1. Results from the CFX Maestro™ Software gene stability test using the Reference Gene Selection tool (Bio-rad). Table is including the gene names and the number indicate which primer set was used, ubiquitin (uba-40), L13 ribosomal binding protein (rpl-20), hypoxanthine phosphoribosyltransferase 1 (HPRT-42), eukaryotic elongation factor 1 alpha (eef-37). The evaluation, M value and estimated stability by the software, as well as the number of samples used in the test are included.

Order	Gene Name	Evaluation	Avg M Value	Stability (Ln(1/AvgM))	# Samples
1	uba-40	Acceptable	0.700005597	0.356666948	47
2	rpl-20	Acceptable	0.700005597	0.356666948	47
3	HPRT-42	Acceptable	0.743997502	0.295717602	47
4	eef-37	Unstable	1.08337952	-0.080085341	47

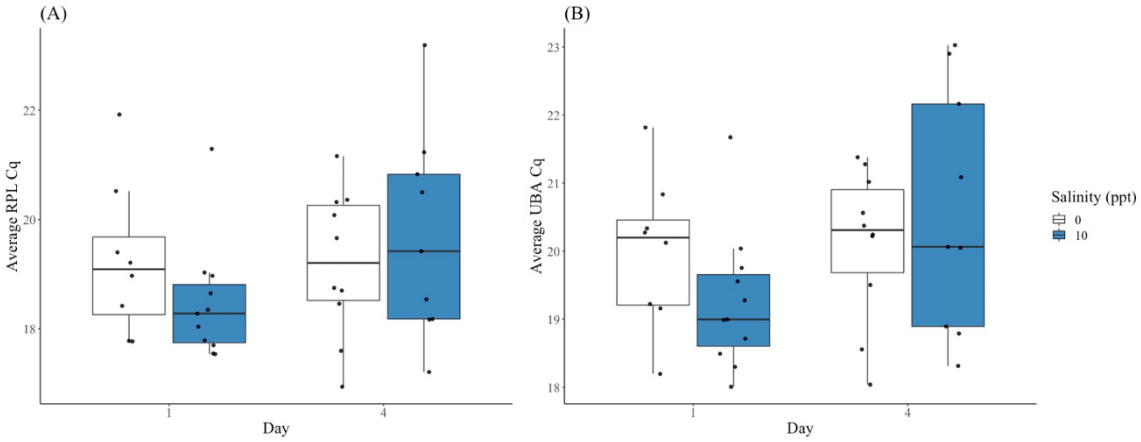

Figure A1. The average Cq (quantitative cycle) for reference genes. (A) Average Cq for L13A ribosomal binding protein (RPL) during the 4-day exposure. n = 11 at 10 ppt on day 1, n = 8 at 0 ppt on day 1, n = 9 at 10 ppt on day 4, n = 10 at 0 ppt on day 4. (B) Average Cq for ubiquitin (UBA) during the 4-day exposure. n = 11 at 10 ppt on day 1, n = 8 at 0 ppt on day 1, n = 9 at 10 ppt on day 4, n = 10 at 0 ppt on day 4.

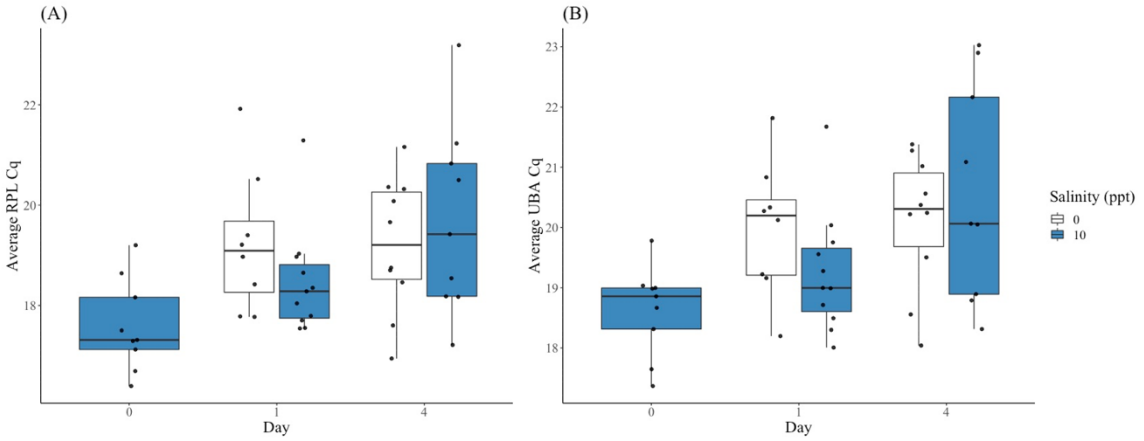

Figure A2. The average Cq (quantitative cycle) for reference genes including the day 0 data points. (A) Average Cq for L13A ribosomal binding protein (RPL). A significant effect by day was found for day 0 and day 4 at 10 ppt ($F = 4.484$, $p = 0.017$, $df = 2$, Tukey *a posteriori*: $p = 0.023$). n = 10 at 10 ppt on day 0, n = 11 at 10 ppt on day 1, n = 8 at 0 ppt on day 1, n = 9 at 10 ppt on day 4, n = 10 at 0 ppt on day 4. (B) Average Cq for ubiquitin (UBA). n = 10 at 10 ppt on day 0, n = 11 at 10 ppt on day 1, n = 8 at 0 ppt on day 1, n = 9 at 10 ppt on day 4, n = 10 at 0 ppt on day 4.

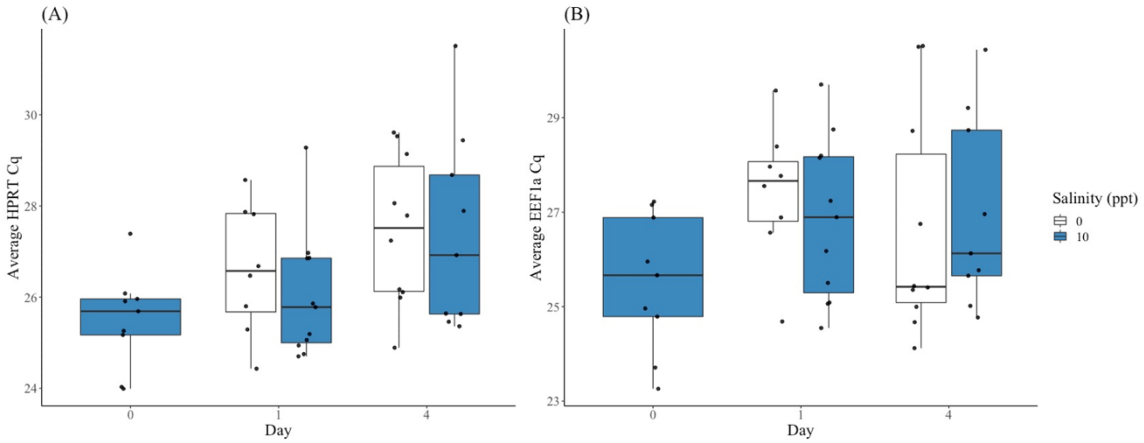

Figure A3. The average Cq (quantitative cycle) for genes included in the gene stability test using the CFX Maestro™ Software Reference Gene Selection tool (Bio-rad). (A) Average Cq for hypoxanthine phosphoribosyltransferase 1 (HPRT). A significant effect by day was found ($F = 4.003$, $p = 0.026$, $df = 2$, Tukey *a posteriori*: $p = 0.058$). (B) Average Cq for eukaryotic elongation factor 1 alpha (EEF1a). As the gene stability test deemed EEF1a "unstable", no statistical analyses were made for this gene.

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