Evolutionary variation in salinity tolerance among species of Killifishes (*Fundulus* spp.)

By
Lauren Shea Jonah

A Thesis Submitted to
Saint Mary's University, Halifax, Nova Scotia
in Partial Fulfillment of the Requirements for
the Degree of Master of Science in Applied Science

August 1st, 2019, Halifax, Nova Scotia, Canada

©Lauren Shea Jonah, 2019

Approved: Dr. Anne Dalziel
Supervisor

Approved: Dr. Linda Campbell
Committee Member

Approved: Dr. Genlou Sun
Committee Member

Approved: Dr. Laura Weir
Committee Member

Approved: Dr. William Marshall
External Examiner

Date: August 1st, 2019

Evolutionary variation in salinity tolerance among species of Killifishes (*Fundulus* spp.)

By Lauren Shea Jonah

ABSTRACT

Little is known about how salinity tolerance evolves in fishes. In this study I measured salinity tolerance, and candidate mechanisms underlying tolerance, in Common Killifish (*Fundulus heteroclitus)*, Banded Killifish (*Fundulus diaphanus)* and clonal *F. diaphanus* x *F. heteroclitus* F1 hybrids. To quantify tolerance, killifish acclimated to near isosmotic water (10 ppt) were transferred to 0 ppt and 32 ppt and acclimated in a stepwise manner to 60 ppt. All fish could maintain homeostasis at 0 – 32 ppt, but *F. heteroclitus* have an increased tolerance to 60 ppt compared to *F. diaphanus* and F1 hybrids, which displayed an intermediate salinity tolerance compared to that of the parental species. I also found that *F. heteroclitus* upregulated candidate hypersaline saltwater tolerance genes (*cftr*, *claudin-10c* and *claudin-10f*) to a greater extent than the two less tolerant groups, suggesting that increased tolerance in *F. heteroclitus* is associated with their unique ability to upregulate these genes.

August 1st, 2019

ACKNOWLEDGEMENTS

This project would not have been possible without all the immense support I received from numerous people. First and foremost, I would like to thank my supervisor, Dr. Anne Dalziel. I knew from the first Skype interview that your lab would be a great fit and I would like to thank you for creating such an accepting and enriching lab environment. It's also safe to say that this project would not have happened without your guidance. From the conception of this project, to the many hours spent in your office, the long days and nights of sampling fish, to its completion you have helped me every step of the way. Most importantly, you have taught me how to be a well-rounded scientist, and I am forever grateful for that. I could not have asked for anything more from a supervisor.

Next, I would like to thank my statistical resource guru and committee member, Dr. Laura Weir, for her immensely helpful feedback and for going beyond what is expected from a committee member to assist with this project. I must thank Caila Henderson, our animal care technician, who also went above and beyond what was necessary to make sure my fish were healthy and cared for. Next, I would like to thank my committee members; Dr. Genlou Sun and Dr. Linda Campbell as well as Dr. Bill Marshall, my external examiner, for their time, suggestions and valuable feedback. I also must thank NSERC and the Nova Scotia Graduate Scholarship for funding this project.

I would also like to acknowledge all the members of the SMU Fish Lab and my fellow grad students. I could not have gotten through this project without your support over the last two years. I would especially like to thank Anne Haley for not only being a supportive friend for the past 7 years, but for leading me to this lab and guiding me through grad school. I would like to thank Emily Allen, who has been by my side

throughout this degree, for her immense support and friendship. I would also like to thank Tori Cluney for countless laughs, and relentless encouragement and Yayra Gbotsyo for sharing her positive energy and wealth of molecular biology knowledge with me. Additionally, I must thank Maggie Redden, Rachel Corney and Nathalie MacPherson for all their help with fish collections and for making field work fun! All of you in Dalziel/Weir Lab have created an extremely pleasant work environment and you all make the Fish Lab an extremely difficult place to say goodbye to. I will always cherish the time I spent with you.

Additionally, I am very grateful to have grown up in an environment where I was told I could accomplish anything I put my mind to so I must thank my parents, Sandra and Leonard Jonah, as well as my brother, Tristen, for always believing in me. I must also thank Allison Foley for her relentless encouragement and her undeniable kindness throughout this process. A big thank you also has to go out to the fish for making this project possible. Finally, I can't even begin to thank my best friend, roommate and partner, Justin Robichaud, for all of his support and constant reassurance over the past two years. I truly could not have gotten through this degree without you. Thank you for always listening to me and for learning more about killifish than you ever wanted to know.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1. INTRODUCTION

1.1 Coping with environmental stressors

A major goal in comparative animal physiology is to understand how organisms respond to environmental stressors to determine if, and how, the mechanisms used to maintain homeostasis vary across species (reviewed by Somero, 2000; Whitehead, 2012). Organisms may face many environmental stressors, which can be classified as biotic (from interactions with other animals) or abiotic (from interactions with the non-living chemical and physical environmental factors). There are a wide range of abiotic stressors that animals may encounter, such as extremes in temperature, oxygen, pH, salinity and pressure (reviewed by Schulte, 2014). Organisms can respond to these environmental stressors with a wide variety of mechanisms, such as modifying physiological pathways or altering their behaviour (Cossins & Crawford, 2005; reviewed by Schulte, 2014).

Within their lifetime, animals must acutely cope with stressors occurring within a matter of minutes or hours and may also acclimate/acclimatize to longer term changes that persist for days or months. Furthermore, over generations, populations might locally adapt to environmental stressors. Some organisms are better at responding to environmental stressors than others. These 'tolerant' animals often have the ability to sense and beneficially acclimate (via phenotypic plasticity) to changing environmental conditions (Huey et al., 1999). The idea that some animals are more plastic than others has been coined as "plasticity as an adaptation" and implies that these "more plastic" animals have evolved the ability to respond to changing environmental stressors more effectively than less plastic animals (Gotthard & Nylin, 1995; Beaman et al., 2016; Fox et al., 2019). Interestingly, there is not yet strong evidence that there are major metabolic, or

fitness costs associated with such increased plasticity (reviewed by Murren et al., 2015). Thus, the ability to acclimate within a lifetime may often evolve. A major goal in evolutionary physiology is to determine if the mechanisms (e.g. genes, biochemical pathways, physiological traits) underlying physiological acclimation and evolutionary adaptation to environmental changes are often shared within or between species, and if so, under what circumstances (Whitehead, 2012).

1.2 Salinity as an environmental stressor for teleost fishes

Changes in water salinity can impact a number of physical properies, including viscosity, density, temperature, buoyancy and dissolved oxygen. Altering any of these physical properties can interfere with biological processes, and salinity change is a common stressor for fish and other aquatic species, whose internal physiological systems interact very closely with the external environment (Cossins & Crawford, 2005; McCormick et al., 2013, Kültz, 2015). Teleost fishes are a particularily good model system for studying the effects of changes in environmental salinity because of their diversity in salinity tolerance and the extensive genomic resources available, facilitating studies examining the molecular and genetic basis of salinity tolerance (Cossins & Crawford, 2005).

Salinity change is stressful for teleost fishes, because the concentration of solutes present in a fish's extra-and intra-cellular environments (between 250-450 mOsm) differs from both saltwater (~1000 mOsm) and freshwater (~1 mOsm) (reviewed by Edwards & Marshall, 2012). Tissues can only cope with minor disturbances in osmsolality of the extracellular fluid, so this must be regulated in fishes moving between environments of

differing salinities (Edwards & Marshall, 2012). Many fish are able to detect osmotic changes and can in turn adjust their physiology to maintain an internal osmotic balance in environments of variable salinities (reviewed by Gonzalez, 2012; Kültz, 2012 & 2015; Laverty & Skadhauge 2012; Marshall, 2012). Exposure to stressful changes in salinity are predicted to increase in aquatic organisms due to habitat degradation, climate change, and other anthropogenic factors (reviewed by Kültz, 2015). Because salinity fluctuations can affect fish distributions and abundance, understanding how fish respond to salinity changes will aid in our overall understanding of how populations can persist in changing environments (reviewed by Somero, 2010).

1.3 How fishes maintain ion and osmoregulatory homeostasis

Both freshwater and saltwater environments present an ionic and osmoregulatory challenge for fishes, as they must either combat passive ion gain and water loss in saltwater or passive ion loss and water gain in freshwater. In saltwater, fishes actively excrete ions and take up water to maintain homeostasis whereas fish actively take up ions and excrete water in freshwater to maintain homeostasis (reviewed by Edwards & Marshall, 2012).

Fish are categorized based on their abilty to persist in either a wide range of salinities (euryhaline) or only in specific salinities (stenohaline; reviewed by McCormick et al., 2013). Within their lifetime, fish may also vary in their salinity tolerance, as many fish migrate between fresh and saltwater at specific life stages (i.e., diadromy; reviewed by McCormick et al., 2013). Some fish can also live in environments with fluctuating salinities, which is very energetically expensive because they must constantly vary their

physiology and behaviour to maintain homeostasis (Marshall, 2012). Euryhaline teleost fish must often repeatedly switch between salt absorption and secretion, a process that involves both physiological and structural modifications of their cells and tissues (McCormick et al., 2013). Many physiological parameters can be altered immediately, whereas changes in cell and tissue structure can be seen slightly later in the acclimation response (reviewed by Kültz, 2015).

Fish maintain iono- and osmo-regulatory homeostasis by the action of tissues such as the gills, operculum, kidneys and gastrointestinal tract (reviewed by Marshall, 2012; Kültz, 2015). The gills and the operculum are the main sites of ion uptake in freshwater environments and ion secretion in saltwater environments (Edwards & Marshall, 2012). Within these tissues, fish also maintain ionic and osmoregulatory homeostasis by employing transcellular (movement though the cell) and paracellular (movement between cells) transport of ions and water. In ionoregulatory epithelia, fishes use specialized cells called ionocytes, which contain transcellular proteins on both the apical (top, facing the external environment) and basolateral sides (bottom, facing the blood) to transport and regulate ion balance (Evans et al., 2005; Edwards & Marshall, 2012; McCormick et al., 2013). Fish also use paracellular tight junction proteins to adjust the permeability of epithelial tissues. Tight junctions are found in the spaces between ionocytes and/or accessory cells and are composed of numerous transmembrane proteins including occludins, junction adhesion molecules and claudins (Anderson, 2001; Evans et al., 2005; Kolosov et al., 2013; McCormick, 2013). These tight junctions can either act as a barrier to ion movement or can form pores to facilitate the movement of ions between epithelial cells, depending on the characteristics of the constituent proteins (Anderson, 2001).

Over the past few decades, much has been learned about how fish cope with salinity changes acutely and how fish can acclimate over hours to weeks (reviewed by Marshall et al., 2002; Evans et al., 2005; Gonzalez et al., 2005; Marshall, 2012). However, we know much less about how salinity tolerance evolves among populations and species of fishes (but see Whitehead, 2010; Whitehead, 2012; Whitehead et al., 2013; Brennan et al., 2015; Gibbons et al., 2017; Velotta et al., 2017).

1.3.1 Coping with freshwater

1.3.1.1 Acclimation to freshwater

Freshwater has a much lower osmolarity than a fish's internal fluids, so fish must excrete excess water and actively uptake salts against their concentration gradient (Edwards & Marshall, 2012). There is variation in the cell types and specific ion transporters that different fish species employ during freshwater acclimation, but all use the gills as the main site to actively extract ions from the external environment, while excreting excess water through dilute urine (Dymowska et al., 2012; Edwards & Marshall, 2012; Fig. 1.1). During acclimation to freshwater in all fish species, active trans-epithelial Na^+ and Cl^- absorption occurs with the aid of basolateral Na^+/K^+-ATPase (NAK), in conjunction with Na^+/ Cl^- co-transporter (NCC) (reviewed by Kültz, 2015; Fig. 1.1). Additionally, ion secreting cells are withdrawn from the gill epithelium to counter ion loss (Marshall, 2012, Reviewed by Kültz, 2015). The leakiness of tight junctions is also reduced during freshwater acclimation, as fish increase the expression of 'freshwater' occludin and claudin protein paralogs that create barriers between epithelial cells to prevent excess ion loss (Kolosov et al., 2013, Fig. 1.1).

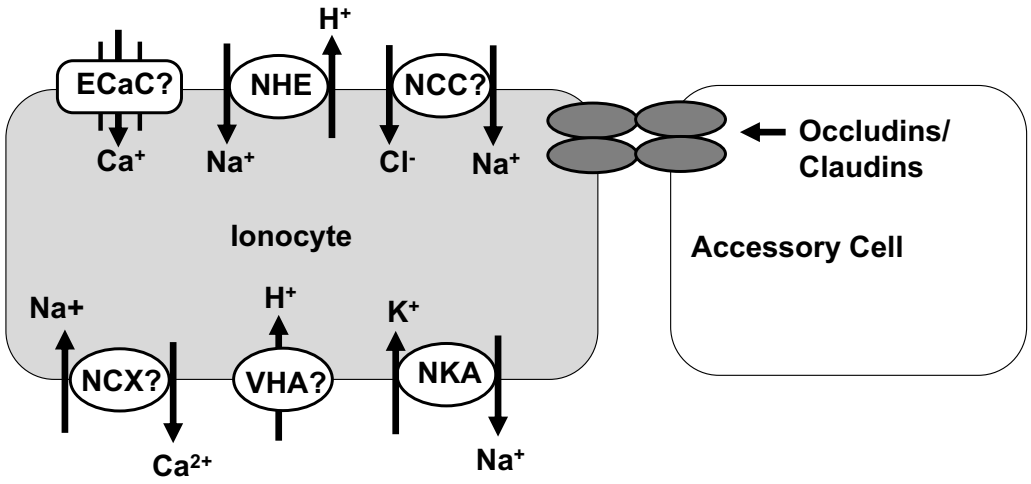

Figure 1.1 Schematic drawing of the proposed ion uptake model for freshwater ionocytes in the gill epithelium of Common Killifish (*Fundulus heteroclitus*). Note that accessory cells that serve as support cells and the apical side of the ionocyte interacts with freshwater and the basolateral side interacts with the blood. Important ion transporters include Na^+-K^+- ATPase (NAK) and a Na+/H+ exchanger (NHE). Transporters with question marks indicate that the role of these proteins is not yet clear and this includes the hypothesized epithelial calcium channel (ECaC; Zimmer et al., 2019), and an apical Na^+-Cl^--co-transporter (NCC). However, killifish do not uptake Cl^- in the gill, so the role of NCC is not clear (reviewed by Dymowska et al., 2012). The Na^+/Ca^{2+}exchanger (NCX) may also function in Ca^{2+} uptake (Zimmer et al., 2019). The role of the vacuolar-type proton pump (V-ATPase) found on the basolateral membrane is also unknown, but it may be involved in freshwater ion uptake (Katoh et al., 2003). Occludins and claudins are tight junction proteins that form junctions between cell types. This diagram is based on the model reviewed by Dymowska et al., (2012) unless otherwise noted. It is important to note that other species of fishes may use different combinations of ion transporters to take up ions in freshwater and the complete molecular mechanisms responsible for ion and acid base regulation are not yet fully understood in killifish (McCormick et al., 2013).

1.3.1.2 Adaptation to freshwater

Most of the current knowledge on salinity adaptation in euryhaline fishes focuses on the evolution of freshwater tolerance from ancestrally marine or anadromous populations (e.g. Whitehead, 2010, DeFaveri et al., 2011; Whitehead et al., 2013; Berg et al., 2015; Divino et al., 2016; Gibbons et al., 2016; Velotta et al., 2017). Multiple species of fish have adapted to freshwater environments, via changes in the capacity to re-model iono-regulatory tissues such as the gills and kidneys (Whitehead, 2010; Hasan, et al., 2017; Velotta et al., 2017). Some adaptive changes include increased upregulation of the genes involved in freshwater acclimation compared to seawater populations when exposed to freshwater [e.g. Alewives (Velotta et al., 2017), e.g. stickleback (Gibbons et al., 2017)], suggesting that genes important for acclimation to freshwater environments could also play a key role in freshwater adaptation (Whitehead et al., 2011; Marshall, 2012; Brennan et al., 2015). As well, the switch to a freshwater lifestyle has often resulted in a reduced ability to tolerate higher salinities (e.g. Brennan et al., 2015; Hasan et al., 2017).

Freshwater adaptation has occurred multiple times within and among species of fish and there is a general understanding of the physiological mechanisms by which this may occur (Ghalambor et al., 2007; Hasan et al., 2017; Velotta et al., 2017). However, we know less about the mechanisms by which saltwater tolerance is lost (or potentially gained) over evolutionary time (e.g. Gibbons et al., 2017; Kusakabe et al., 2017) and far less about how the ability to cope with salinities higher than that of seawater (> 35 ppt) has evolved or been lost (but see Gonzalez et al., 2005; Whitehead, 2010; Gonzalez, 2012; Laverty & Skadhauge, 2012).

1.3.2 Coping with saltwater

1.3.2.1 Acclimation to saltwater

In water with a salinity higher than the fish's internal osmolarity (> 450 mOsM) fish experience osmotic loss of water from their body surfaces and an influx of ions from the more concentrated saltwater (reviewed by Kültz, 2015), and this effect is magnified in hypersaline saltwater (Gonzalez, 2012; Laverty & Skadhauge, 2012). During saltwater acclimation, fish increase drinking rates to take up water, and a number of tissues become modified to prevent excessive water loss and ion gain; these include the gills, kidneys, operculum, and gastrointestinal tract.

Cell water retention increases via intestinal water reabsorption and by decreasing gill epithelium water permeability (reviewed by Edwards & Marshall, 2012; Gonzalez 2012; Laverty & Skadhauge, 2012; Marshall, 2012; Kültz, 2015). Fish must also actively excrete excess ions from epithelial surfaces; this can be done transcellularly via ion-transporters (Fig. 1.2) that become upregulated upon exposure to high salinities (Fig. 1.2; Edwards & Marshall, 2012; reviewed by Gonzalez, 2012; Laverty & Skadhauge, 2012; Marshall, 2012; Kültz, 2015). As well, the leakiness of tight junctions between ionoctyes increases in water hyperosmotic to the fish's tissues to promote the secretion of paracellular Na^+ across the gill epithelium (Kolosov et al., 2013, reviewed by Kültz, 2015). During saltwater acclimation, fish often increase the number of accessory cells and ionocytes to create more junctions between cell types and therefore more pores (tight junctions; Karnaky et al., 1976). In addition to an increase in cellular recruitment, differential regulation of specific tight junction proteins, including claudin isoforms, can

occur during saltwater acclimation and may influence Na^+ excretion (e.g. Tipsmark et al., 2008; Bui & Kelly, 2014; Bossus et al., 2015; Marshall et al., 2018).

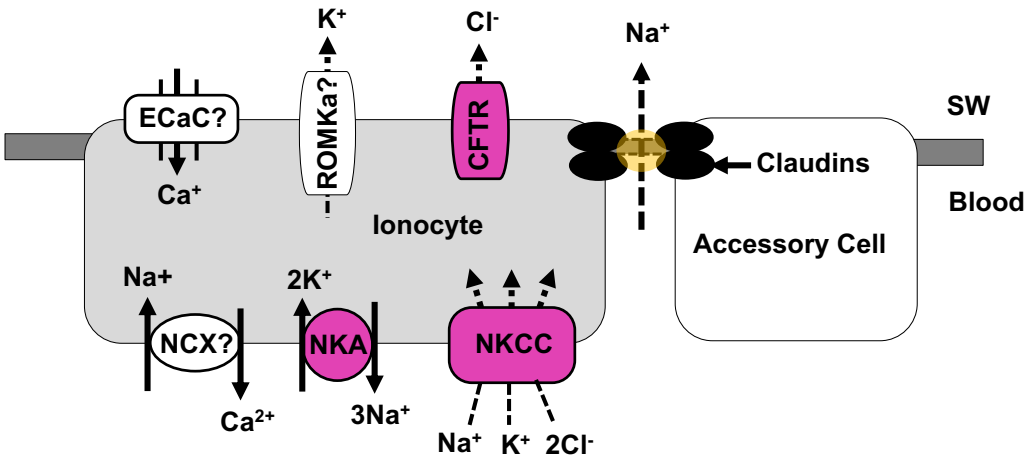

Figure 1.2 Schematic drawing of the ion excretion model for saltwater ionocytes in the gill epithelium in fishes. Accessory cells serve as support cells and increase in number during saltwater acclimation, which increases the number of tight junctions in the gill (e.g. Cozzi et al., 2015). Ion transporters highlighted in pink are predicted to be critical for acclimation to saltwater and include the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), Na^+-K^+- ATPase (NAK) and Na^+-K^+-Cl^--co-transporter (NKCC). Claudins are the key tight junction proteins that regulate paracellular permeability. Basolateral NKA pumps K^+ into, and Na^+ out of, the ionocyte from the blood, while NKCC moves K^+, Na^+, and Cl^- into the ionocyte. The Cl^- concentration then becomes high enough for Cl^- to move out of the ionocyte through CFTR (Marshall & Singer, 2002). Not only does this pathway serve to remove Cl^- from the ionocyte, but it also generates an internal transepithelial membrane potential, coaxing Na^+ out of the ionocyte through tight junctions, allowing fish to overcome the high osmotic gradient (Marshall, 2012). Transporters with question marks indicate that the role of these proteins is not yet clear, and includes the epithelial calcium channel (ECaC), Na^+/Ca^{2+}exchanger (NCX; Zimmer et al., 2019) and the renal outer medullary potassium channel /inward rectifying K^+ channel (ROMKa; Furukawa et al., 2011).This diagram is based on the model reviewed by Evans et al. (2005) and Hwang et al. (2011). Ammonia transport has been excluded from this diagram but see Hwang et al. (2011) for further information.

1.3.2.2 Adaptation to saltwater

We know a great deal about acute responses and short term acclimation to saltwater in fishes such as the Sailfin Mollies (*Poecilia latipinna*), Spotted Green Puffer Fish (*Tetraodon nigroviridis;* Bagherie-Lachidan et al., 2009), Mozambique Tilapia (*Oreochromis mossambicus;* Kültz et al., 1995) and Common Killifish (*Fundulus heteroclitus*; Marshall et al., 2002; Scott et al., 2004 in Laverty & Skadhauge, 2012) (reviewed by Gonzalez et al., 2005; Kültz et al., 2013). However, we know little about how their responses compare to closely related non-tolerant species (but see Kusakabe et al., 2017). At present, we hypothesize that similar mechanisms (as highlighted in section 1.3.2.1) may facilitate adaptation to saltwater. It has recently been suggested that changes in the content (Marshall et al., 2018) and the function (Reid et al., 2017) of tight junction proteins may be especially important for saltwater adaptation.

In fishes there are over 60 claudin isoforms expressed in epithelial tissue, many of which are the result of numerous gene and genome duplications (Loh et al., 2004, Kolosov et al., 2013, Katayama et al., 2017). An increase in the expression of claudin isoforms that form pores in the tight junction complexes of epithelial cells (four claudin proteins make up a pore, two on each side of a tight junction) has been linked to increased salinity tolerance in fishes (Chasiotis et al., 2012, reviewed by Kolosov et al., 2013). For example, claudin-27a proteins were found in the gills of Spotted Green Puffer Fish (*T. nigroviridis*; Bagherie-Lachidan et al., 2009) acclimated to saltwater, and claudin 10s appear to be especially important for salt secretion in numerous species of fish. In particular, *claudin 10-e* has been found to increase in the gills of Atlantic Salmon (*S. salar;* Tipsmark et al., 2008), Japanese Medaka (*O. latipes*; Bossus et al., 2015), Common

Killifish (*F. heteroclitus;* Marshall et al., 2018) and the Spotted Green Puffer (*T. nigroviridis;* Bui et al., 2010, Bui & Kelly, 2011 & 2014) fish during saltwater acclimation. In Japanese Medaka, *claudin-10c*, *10d* and *10f* isoforms were all found to be upregulated in saltwater (Bossus et al., 2015) and *10d* isoforms were also significantly elevated in saltwater acclimated Common Killifish and Spotted Green Puffer Fish (Bui & Kelly, 2014; Marshall et al., 2018).

It remains unclear if selection has acted upon these short-term responses (acclimation) to lead to saltwater adaptation in species with high tolerance to hypersaline environments (but see Kusakabe et al., 2017). One way to better understand how saltwater tolerance evolves is to determine what mechanisms might be critical for survival in highly tolerant species of fish but are lacking in less tolerant congeners.

1.3.3 Coping with very high salinities (> 60 ppt, hypersaline saltwater)

1.3.3.1. Acclimation to very high salinities

Many fish live in hypersaline saltwater environments such as tropical estuaries, mangrove swamps, coastal lagoons and salt marshes and must cope with extremely high salinities of up to 30-50 ppt (reviewed by Laverty & Skadhauge; Sales et al., 2018). The term 'hypersaline saltwater' generally refers to water salinities higher than fish extracellular fluids (250 - 450 mOsm), and I will use the term 'hypersaline saltwater' to refer to any salinity of 45 ppt or higher (following Marshall et al., 2018). Generally, fish cope with hypersaline saltwater in the same manner as saltwater acclimation (section 1.3.2.1); by increasing active excretion of ions in epithelial tissues like the gill and by actively absorbing and retaining water through tissue like the intestine (reviewed by

Gonzalez, 2012; see Weaver et al., 2016 and Schauer et al., 2018). However, these responses are magnified in hypersaline saltwater, leading to an increased demand for energy to maintain homeostasis (Gonzalez, 2012).

During hypersaline saltwater acclimation, the number of ionocytes, accessory cells and tight junctions in epithelial tissue increase to an even greater extent than in saltwater (Lorin-Nebel et al., 2012; Cozzi et al., 2015; Kültz, 2015, Marshall et al., 2018). Accessory cells invade ionocytes in multiple areas along the apical crypt (the cup-shaped apical surface of ionocytes), to a greater extent than during saltwater acclimation (Cozzi et al., 2015). Additionally, the expression of NKA (Weaver et al., 2016), NKCC (Li et al., 2014) and CFTR (Marshall & Singer, 2002) and selected tight junction proteins (ie: claudins) are dramatically increased to combat high internal ion concentrations. The upregulation of pore-forming claudins and CFTR is thought to be especially important to decreasing the strong osmotic gradient that fish must overcome in hypersaline environments (Laverty & Skadhauge 2012; Marshall et al., 2018).

An increase in the expression of particular pore-forming isoforms of claudins that facilitate Na^+ excretion may be critical for ion excretion in hypersaline saltwater (Tipsmark et al., 2008; Bui & Kelly, 2014; Bossus et al., 2015; Marshall et al., 2018). During acclimation to hypersaline saltwater (≥ 45 ppt), but not saltwater (32-35 ppt), the mRNA content of both *claudin-10c* and *10-f* isoforms were found to be significantly elevated in Common Killifish, indicating that these two isoforms might play an important role in hypersaline saltwater acclimation (Marshall et al., 2018). Overall, it is hypothesized that an upregulation of *claudin-10c*, *claudin-10f* and *cftr* may be necessary to overcome the extreme osmotic and ionic gradient in hypersaline saltwater (Marshall et al., 2018). However, the mechanisms underlying the evolution of tolerance to hypersaline

saltwater among populations and species are not yet known. One way to understand how hypersaline saltwater tolerance evolves is to compare hypersaline saltwater tolerance and measure associated candidate mechanisms contributing to tolerance in closely related species of fishes that vary in their capacity to cope with hypersaline saltwater. One such group of fishes that show inter-specific variation in hypersaline saltwater tolerance and can be easily studied in the lab are the killifishes (reviewed by Whitehead, 2007; Whitehead, 2010; Kültz, 2015).

1.4 Killifish as a model organism for studying the evolution of hypersaline saltwater tolerance

The genus *Fundulus* is composed of 38 species of euryhaline teleost fishes that can tolerate salinities ranging from freshwater to extremely hypersaline saltwater environments (0 ppt to 120 ppt) (Fritz & Garside, 1974; Griffith, 1974; Whitehead, 2010). The *Fundulus* genus is an excellent system for studying the evolution of salinity tolerance due to this wide range of salinity tolerances and also the repeated evolution of changes in tolerance in this group (Whitehead, 2010). Having many closely related study species that vary in salinity tolerance is advantageous because there is less overall background genetic variation than in more distantly related species. This allows us to more effectively use the comparative method to link performance to underlying mechanisms and also allows us to interbreed species and use quantitative genetic methods to test genotype-phenotype linkages (Garland et al., 2005; Storz et al., 2015).

The best studied *Fundulus* species, the mummichog or Common Killifish (*Fundulus heteroclitus),* is an estuarine, highly-euryhaline fish which is an important experimental model species for the study of how fish acutely respond to changes in

salinity (reviewed by Marshall, 2012, Kültz, 2015). Additionally, mummichogs can tolerate a wide number of other abiotic stressors including temperature extremes (Garside & Jordan, 1968), hypoxia and toxin exposure (Schulte, 2007). These small fish can also be reared easily in the laboratory setting (Burnett et al., 2007) and there are extensive genomic resources available (Reid et al., 2017). As a result, *F. heteroclitus* has been an excellent model species in which to study the genetic, biochemical and physiological mechanisms underlying acute salinity tolerance and the mechanisms contributing to acclimation (Edwards & Marshall, 2012; Marshall 2012; Kültz, 2015; See Sections 1.2.1.1 – 1.2.1.3).

Recent work by Marshall et al. (2018) has focused on how *F. heteroclitus* can acclimate to hypersaline saltwater (60 ppt). In hypersaline saltwater, *F. heteroclitus* upregulates the mRNA content of *claudin-10c* and *claudin-10f* (pore-forming isoforms) as well as *cftr*, potentially increasing Cl^- excretion and drawing Na^+ out via paracellular pores in tight junctions (Marshall et al., 2018). Additionally, *F. heteroclitus* increase the number of accessory cells and ionocytes present in gill tissue to increase the overall number of tight junctions (Cozzi et al., 2015; See section 1.2.1.3). Overall, these responses are predicted to work synergistically to overcome the high osmotic gradient that exists in hypersaline saltwater (Marshall et al., 2018).

In addition, Reid et al. (2017) conducted genomic analyses of *F. heteroclitus* and found that tight junction proteins (such as claudins) are among the genes that show the highest selective constraints, which is hypothesized to be the result of the key roles that claudin proteins play in salinity acclimation and adaptation in this species. However, it is unclear if these correlated responses are required for an increase in hypersaline saltwater

tolerance and are unique to *F. heteroclitus* compared to less hypersaline saltwater tolerant congeners. One method to further test the hypothesis that increased claudin and *cftr* mRNA content are required to tolerate hypersaline saltwater is to use the comparative method (reviewed by Sanford et al., 2002) and test for associations between the mRNA content of candidate genes and salinity tolerance among species of killifish that differ in salinity tolerance. The Banded Killifish (*Fundulus diaphanus*) is native to freshwater environments and lives in sympatry with the Common Killifish (*F. heteroclitus*) in the Maritimes in salinities ranging from 0-14 ppt (Griffith, 1974; Burnett et al., 2007). Like *F. heteroclitus*, *F. diaphanus* can also tolerate an impressive range of salinities but cannot survive exposure to water higher than 70 ppt (Fritz & Garside, 1975; Ahokas, 1977; Whitehead, 2010). The physiological mechanisms underlying differences in tolerance between *F. heteroclitus*, which can survive at salinities of up to 120 ppt, and *F. diaphanus* are currently unknown.

Within their Maritime range, *F. heteroclitus* and *F. diaphanus* naturally hybridize by forming all female, clonally reproducing, F1 hybrids (Dawley, 1992). Normally the hybrids result from breeding of *F. diaphanus* mothers and *F. heteroclitus* fathers, so have *F. diaphanus* mitochondrial DNA and half of their nuclear genome from each parent (Dawley, 1992; Hernandez Chavez & Turgeon, 2007). While these F1 hybrids normally reproduce clonally, there is evidence suggesting that some hybrids are capable of sexual reproduction (Hernandez Chavez & Turgeon, 2007; Mérette et al., 2009). Additionally, work done on the hybrids has revealed the presence of multiple clonal lineages throughout the Maritimes, suggesting that clonal lineages form repeatedly (Hernandez Chavez & Turgeon, 2007; Mérette et al., 2009; Tirbhowan, 2019). The presence of

natural F1 hybrids also allows us to test for associations between salinity tolerance and candidate physiological mechanisms among *F. diaphanus*, *F. heteroclitus* and hybrids. At present, we lack information on salinity tolerance of F1 hybrids.

F. heteroclitus, *F. diaphanus* and hybrids can all be found in the same regions of Porters Lake, Nova Scotia. This lake has a salinity gradient ranging from 0 ppt at the Northern end the lake to 16 ppt in the Southern end where it connects to the Atlantic Ocean (Mérette et al., 2009). In this thesis, I compared salinity tolerances in these two closely related species of fish that diverged from their most recent common ancestor ~20 MYA (Ghedotti & Davis, 2017) and their F1 hybrids. I then assessed the association between salinity-dependent increases in mRNA content of a set of candidate genes and hypersaline saltwater tolerance. This was done to test the hypothesis that the upregulation of a set of hypersaline responsive genes in the gill (i.e., *cftr*, *claudin 10c* and *10f*) might contribute to evolutionary variation in salinity tolerance among *Fundulus* species.

1.5 Research objectives

This study was designed to compare how Banded Killifish (*F. diaphanus*), Common Killifish (*F. heteroclitus*) and their F1 hybrids cope with salinity changes to better understand the salinity tolerances of this group of fish and then test the mechanisms by which salinity tolerance can evolve in fish. To explore this, I investigated two specific questions:

(1) What is the salinity tolerance of F1 hybrid killifish species and how does this compare to their parental species? To answer this question, I measured and compared salinity tolerance in *F. heteroclitus*, *F. diaphanus* and F1 hybrids by exposing all three species to varying salinities (0 ppt, 10 ppt, 32 ppt, 45 ppt and 60 ppt) and collected samples of white muscle and blood plasma to measure as indicators of how well fish maintain homeostasis. (It is important to note that while hybrid killifish are not technically a separate species, I refer to *F. diaphanus*, *F. heteroclitus* and their F1 hybrids as three different species, following Neaves & Baumann, 2011.) I predicted that *F. heteroclitus* will have increased tolerance at high salinities, by maintaining muscle moisture and plasma ion content values in hypersaline saltwater but may have problems maintaining homeostasis in freshwater [as noted by Fritz & Garside (1974 & 1975), Scott et al. (2004) and Marshall et al. (2018)]. Second, I predicted that *F. diaphanus* will maintain muscle moisture and plasma ion content in freshwater but will struggle to maintain muscle moisture and plasma ion content in salinities above 45 ppt [as noted by Griffith (1974), Ahokas (1975ab) and Dymowska et al. (2012)]. Finally, I predicted that hybrid killifish will display a salinity tolerance intermediate to that of their parents, as I

predicted an additive effect of parental alleles contributing to salinity tolerance in comparison to dominance of either *F. diaphanus* or *F. heteroclitus* alleles.

(2) What candidate mechanisms might limit hypersaline saltwater tolerance among species of killifish? To answer this question, I measured the mRNA content of candidate ion transporter (*cftr*) and tight junction protein (*claudin-10c* & *claudin-10f*) genes predicted to contribute to hypersaline saltwater tolerance in *F. heteroclitus* (Marshall et al., 2018) from gill tissues for all three species at control salinities and hypersaline saltwater (45 - 60ppt). I predicted that the ability to upregulate *cftr* and *claudin10-c* and *claudin-10f* will be associated with the ability to acclimate to and tolerate hypersaline saltwater and will be upregulated to the greatest extent in the most tolerant species, *F. heteroclitus*.

2. METHODOLOGY

2.1 Experimental animal collection

Fish were collected from Porters Lake, Nova Scotia from June 26th, 2017 to October 15th, 2017 (for experiment 1) and from May 31st, 2018 to July 27th, 2018 (for experiment 2) near, or at, sites used by Mérette et al. (2009) (Fig. 2.1, Table A1). Porters Lake has a known salinity gradient ranging from nearly freshwater at the Northern end (0.5 ppt) to brackish water (~16 ppt) at the Southern end near the inlet of the Atlantic Ocean and Lawrencetown Beach, N.S (Mérette et al., 2009). Salinities and temperatures were measured during collections in 2017 and 2018 and can be found in the appendix (Fig. A1-A4). In 2017, I collected 469 *Fundulus* spp. and in 2018, I collected 131 *Fundulus heteroclitus*, 178 *Fundulus diaphanus* and 82 hybrids for a total of 391 fish. During the 2018 field season I tried to limit collection sites to those near Porters Lake provincial park (44.6907, -63.2972), and collection sites #3 and #1 from Mérette et al. (2009) to reduce any potential variation in early-life environmental conditions experienced in collected fish (Fig. 2.1).

All collection methods followed those approved by the SMU animal care committee (Protocol 17-18A2) and Department of Fisheries and Oceans permits (Maritime Region Scientific Collection Permit Licence # 343930) to Dr. Anne Dalziel.

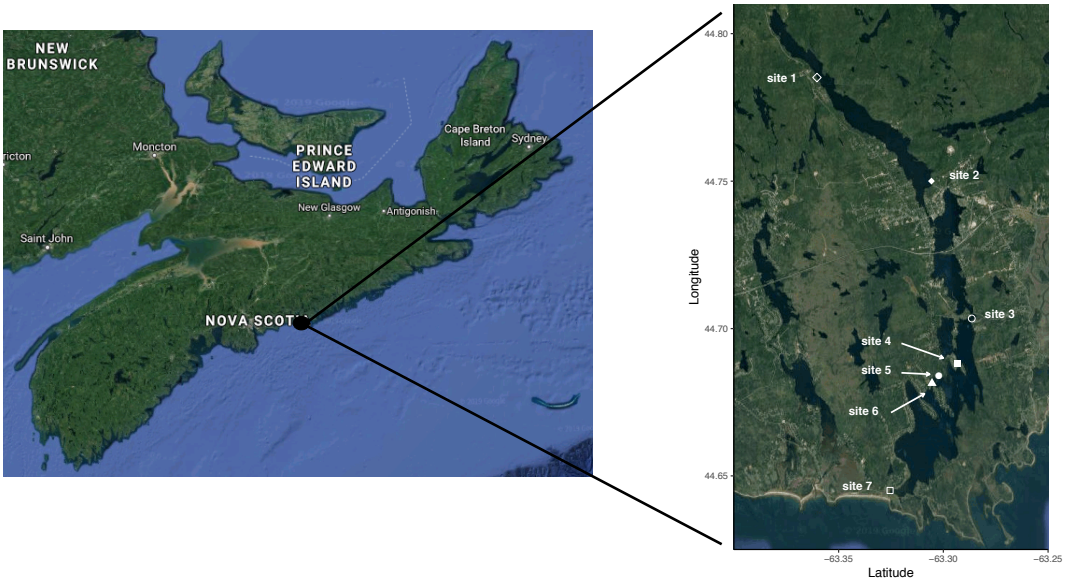

Figure 2.1 Map of Porters Lake, Nova Scotia with sampling locations from 2017 and 2018. Sites 2, 3, & 7 are all sites Mérette et al. (2009) previously sampled. Sites specific to this study are 1, 4, 5 & 6. Image provided by Google Maps©. Co-ordinates can be found in Table A1.

2.2 Experimental animal housing conditions

All fish collected in the field were brought back to the Saint Mary's University aquatic facilities where they were held for a minimum acclimation period of three weeks, in 30 gallon fish tanks with 10 ppt brackish water (artificial salt) made up in dechlorinated Halifax city tap water ([Na^+] 15.9 mg/L; [Cl^-], 9.8 mg/L; hardness as $CaCO_3$ 22.1 mg/L; pH 7.6, Appendix E) at room temperature (17-23 °C) and were maintained at a photoperiod of 9 h:15 h L:D (in 2017) and a photoperiod of 12 h:12 h L:D (in 2018). The 2017 sampling took place in the fall, while the 2018 sampling took place in the summer, so the two photoperiods were chosen to line up with the natural, seasonal, diurnal cycle, and the 12 h:12 h L:D photoperiod is in line with Marshall et al. (2018). Fish were fed a daily diet of flake food (Tetra® TetraMin Tropical Flakes) in the morning, followed by frozen *Mysis* shrimp and bloodworms in the afternoon at a ration of 1-2% body mass, to satiation. I completed water quality testing and water changes on a daily basis while the biofilter was becoming established and then switched to a weekly schedule when the tanks became stabilized. NH_4, NO_3, NO_2 and pH were tested using Nutrafin® and Hagen® test kits. Salinity, temperature and dissolved oxygen were measured with a YSI pro 2030.

It should also be noted that *F. diaphanus* had a higher death rate in comparison to *F. heteroclitus* and hybrids. *F. diaphanus* did not consume flake food so I also fed all fish a diet of bloodworms and mysis, but they were more difficult to maintain in good health, compared to the other two species, as has been noted by other researchers (personal communications to Dr. A.C. Dalziel from Dr. Craig Purchase, Memorial University, and to L. Jonah from Brittney Borowiec, McMaster University).

2.3 Species identification

The three species of killifish can be difficult to differentiate visually, so I used a combination of morphological and genetic methods to identify each species. Mérette (2009) determined that the three species of killifish could be differentated with 90% accuracy using three linear measures (Mérette, 2009). In 2018, fish were measured in the field prior to the salinity tolerance experiments to assign them to one of the three species based upon the morphological identification protocol generated by Mérette (2009). I used Mastercraft calipers and took the three measurements depicted in Fig. 2.2 for each fish while they were in aerated water. Treatment of the animals during the measuring process in addition to tagging (Section 2.3.1) and experimental sampling (Section 2.4 & 2.5) followed Saint Mary's University animal care protocol (protocol # 17-17A3).

This measuring identification method was only completed on fish during fish collections in 2018, but not in 2017. Genetic identifications were used to classify fish in 2017 and to verify the morphological identity for fish collected in 2018. The accuracy of the morphological measuring method was later compared to genotyping results and was found to be 100% accurate for *F. diaphanus* and approximately 90% accurate for *F. heteroclitus* and hybrids, in my study, similar to Mérette (2009) (Tirbhowan, 2019).

I assigned species identity using a genotyping assay designed by Hernandez-Chavez and Turgeon (2007) using DNA extracted from fin clips of fish collected in 2017 and in 2018. Hernández-Chávez & Turgeon (2007) identified three loci containing microsatellites in the nuclear genome (FhCA-1, FhCA-21, Fhe57; Table 2.1) with species-specific alleles in Porters Lake for *F. diaphanus* and *F. heteroclitus* that can be used to differentiate these two species and detect F1 hybrids (fish containing one *F.*

diaphanus and one *F. heteroclitus* allele at all three loci). A third, highly variable locus (FhCA-B103), was used to identify hybrid clonal lineages. Hernández-Chávez & Turgeon (2007) also characterized a restriction fragment length polymorphism of the mitochondrial D-loop to differentiate the *F. diaphanus* and *F. heteroclitus* mitochondrial genomes. I used these primers in addition to newly designed D-loop primers for amplification of the divergent regions of DNA in PCR (Table 2.1) and conducted a restriction digestion of the D-loop PCR product to determine the origin of the mitochondrial genome.

DNA extractions and fish genotyping were performed by Svetlana Tirbhowan as part of her Honours thesis (Tirbhowan, 2019) and by Yayra Gbotsyo. To extract DNA, fin clips were removed from ethanol and blotted on a piece of clean paper towel. Each dry fin clip was transferred to an individually labelled tube and DNA was extracted using either the Gen Elute™ Mammalian Genomic DNA Miniprep kit and (Sigma Aldrich) or the EZDNA® Tissue DNA Kit (Omega Bio-tek), following the manufacturer's instructions. DNA concentration was quantified spectrophotometrically (SpectraMax M3 Spectrophotometer and SpectraDrop Micro-Volume Microplate) and extracted samples were aliquoted and stored at -20 °C. Full details can be found in Tirbhowan (2019).

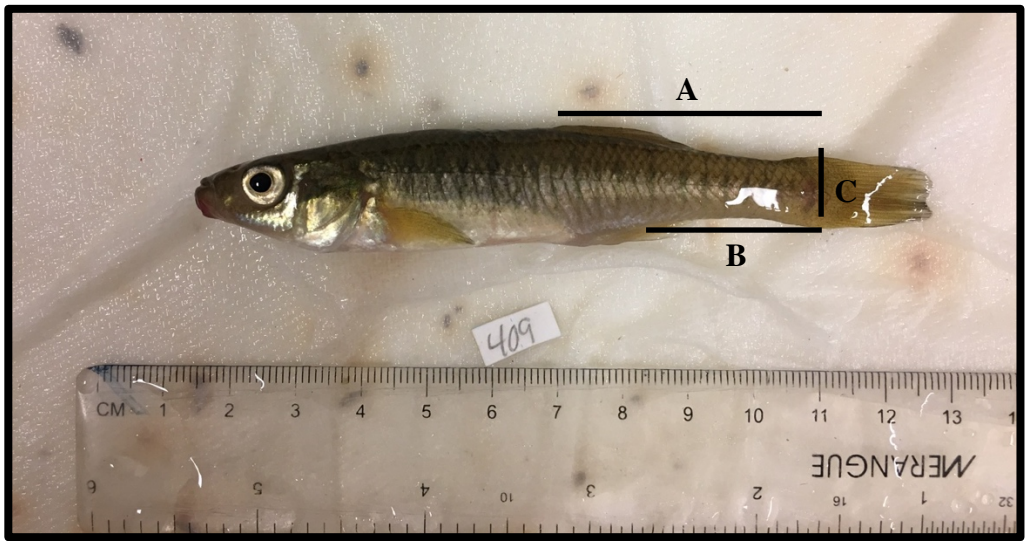

Figure 2.2 Example of the three morphological measurements used to identify and differentiate the three species of killifish (*F. heteroclitus, F. diaphanus* and hybrids) in 2018 (Mérette, 2009). Three measurements were taken on each fish; one from the anterior insertion of the dorsal fin to the dorsal end of the caudal peduncle (A), one from the posterior insertion of the anal fin to the ventral end of the caudal peduncle (B) and one from the dorsal to ventral end of the caudal peduncle (C). Pictured here is an *F. diaphanus.*

Table 2.1 Primers used to genotype *Fundulus* spp.

Locus	F/R	Primer Sequence	Size Range (bp)	Genome	Designed by
FhCA-1	F	5′ -6FAM-GTCCATGCAATGTCGTTCAC-3′	142-183	Nuclear (microsatellite)	Adams et al. (2005)
	R	5′ -GAGGCCAGAAACGCATACAT-3′			
FhCA-21	F	5′ - TAMN-GGTCATTATGGAAAACAGCAACAGATC-3′	144-206	Nuclear (microsatellite)	Adams et al. (2005)
	R	5′-GCTCACTGACACACTGGATTTGGTAGA-3′			
Fhe57	F	5′ - HEX-CTAACTGAACCGCTCACAAGG-3′	131-243	Nuclear (microsatellite)	Hernández Chávez et al. (2007)
	R	5′ -ACTGGTCCACTCTGGCTTC-3′			
FhATG-B103	F	5’- PET-CGGAGCATTGTGATTGTGTTGTTTT-3’	298-436	Nuclear (microsatellite)	Adams et al. (2005)
	R	5’-CCGGGGGACACTTATATGAAATCAGA-3’			
D-loop	F	5′ -TTCCACCTCTAACTCCCAAAGCTAG-3′	441	mitochondrial	Lee et al. (1995)
	R	5′ -CCTGAAGTAGGAACCAGATG-3′			
Fundulus specific D-loop*	F	5’- TTAACCCCCACCCCTAGCTC -3’	660	mitochondrial	Tirbhowan (2019)
	R	5’- GCACTGTGAAATGTCAACTGAA -3’			

PCRs were performed in a total volume of 10-25 μL with 0.8-2 μL of DNA for the nuclear microsatellites and the mitochondrial microsatellites, respectively. All nuclear genome primers were run in multiplex reaction with the following condition: 98 °C for 3 min, 8 cycles at 95 °C for 45 s, 60 °C for 40 s and 72 °C for 40 s; 22 cycles at 55 °C for 40 s, and 72 °C for 40 s; 72 °C for 45 mins; and a final extension at 72 °C for 45 seconds. PCR cycles were as follows for the mitochondrial (D-loop) primers: 95 °C for 3 min; 40 cycles at 95 °C for 45 s, 50 °C for 45 s and 72 °C for 1 min; 72 °C for 5 mins. *Newly designed D-loop primers were used to improve amplification efficiency for PCR assays (Tirbhowan, 2019). As with the original D-loop primer, the PCR product was digested with *Hph*I, which cuts in two sites in *F. diaphanus* and one site in *F. heteroclitus*

2.3.1 Fish tagging

All fish collected in 2017 were designated as *F. diaphanus*, *F. heteroclitus* or hybrids based upon general appearance and then tagged based on this identification, prior to the first salinity tolerance experiment (section 2.4). I tagged all 2017 fish by subcutaneous injection, using a VIE elastomer tagging kit (Northwest Marine Technologies Inc.). Each fish was assigned a unique tag colour, number and body location(s) for later identification. Prior to tagging, fish were anesthetized with 0.5 g of Tricaine methanesulfonate (MS222) and 0.5 g sodium bicarbonate ($NaHCO_3$) in brackish water (10ppt). During this procedure, I also collected a piece of caudal fin from each fish and stored fin clips in 95% ethanol for later DNA extractions to confirm species identity.

For the second salinity tolerance experiment (2018, section 2.5), I assigned a preliminary species identification based upon morphological measurements (as outlined in section 2.4) and tagged each species with a different coloured VIE tag following the methods from 2017. I then conducted the salinity tolerance experiment and collected fin clips for DNA extraction during sampling, following the same methods as in 2017.

2.4. Salinity transfer experiment 1 (freshwater and saltwater) – Summer 2017

The first salinity transfer experiment was completed to test how the three killifish species [Common Killifish (*Fundulus heteroclitus)*, Banded Killifish (*Fundulus diaphanus)* and hybrids] coped with transfer to saltwater (~32 ppt), and freshwater (~ 0 ppt) over a 14-day period. I measured salinity tolerance by collecting and analyzing samples of muscle and blood to use as measures of a fish's ability to maintain osmoregulatory homeostasis (thesis question #1).

I acclimated all three species to brackish water (10 ppt, near isosmotic) for approximately three weeks (Nov 11th – Dec 2nd, 2017). During this time fish were kept on a 9:15LD (light, dark) photoperiod. Feeding, water testing and tank maintenance were the same as described in section 2.1. During the acclimation period all three species had been assigned a unique tag so that they could be mixed within tanks.

After the acclimation period, the first eighteen fish were sampled at 0 hr for baseline measurements (n=6 x 3 species, Fig. 2.3). Then, all remaining fish were transferred into a new tank and placed in one of three salinity treatments; 0 ppt (low salinity), 10 ppt (handling control), or 32 ppt (high salinity). For the tank transfer, fish were removed from their "acclimation" tanks and separated by species into aerated buckets filled with water at 10 ppt. Fish were then placed in a new tank at either 0, 10 or 32 ppt. There were six tanks at each salinity for a total of 18 tanks and fish of all species were mixed within tanks to reduce tank effects. More specifically, four fish of each species (*F. heteroclitus*, *F. diaphanus* & F1 hybrids, for a total of 12 fish), were placed into each of six tanks at 32 ppt and 0 ppt, and five fish of each species were placed in each of six tanks at 10 ppt (Fig. 2.3). This was done so that one fish of each species could be sampled from each tank (six tanks per salinity) at each sampling point to reduce the need to incorporate tank effects into our statistical analysis (Fig. 2.3).

Sampling occurred at 0 hr and at four different time-points after salinity transfer: 8 hrs, 24 hrs, 96 hrs and 14 days. All fish were fasted for 24 hours prior to sampling. These time-points were chosen based on previous studies that investigated short term responses to salinity stress such as changes in water retention, as well as changes in the mRNA content of key ion transporters in *Fundulus heteroclitus* (Scott et al., 2004, Marshall,

2012, Whitehead et al., 2013). Fifty-four fish were sampled at all other time points (n=6 x 3 species x 3 salinities). A total of 234 (6 replicate fish x 3 salinities x 4 time points x 3 species = 216 + 18 fish from control) fish were sampled during the course of the experiment. There were not enough *F. diaphanus* to sample at the 14 day time point, as there was a high mortality of *F. diaphanus* prior to the acclimation period, which reduced my sample size of *F. diaphanus*. It is important to note that these fish were sampled based on preliminary identification, not genetic identification, so although I aimed to have an equal number of species in each treatment, some individuals were mis-identified during sampling.

2.4.1 Physiological sampling procedure

To reduce the effects of sampling order or time of day on any of our measures, I randomly sampled fish to ensure that fish of each species and each salinity treatment were not temporally grouped. At each time point, fish were removed individually from their tank and immediately placed in a lethal dose of anesthetic (2 g Tricaine methanesulfonate, MS222, buffered with 1 g sodium bicarbonate, $NaHCO_3$) in 1 litre of water. I then took standard length and total weight measurements of the fish. Next, I ablated the fish tail with a razor blade and used a hematocrit tube to collect blood from the caudal artery. The hematocrit tube was immediately put on ice and then spun down in a hematocrit centrifuge at 10,000 rpm for three minutes to separate blood plasma from red blood cells. The plasma was stored at -20°C for later analysis of plasma ion content. I also dissected the full gill basket and the right operculum from each fish and placed them in liquid nitrogen to flash freeze the sample before storing it at -80 °C. A 1 cm x 1 cm sample of

white muscle was collected from the caudal region of the fish. The muscle was weighed on an analytic balance to determine the wet mass of the muscle. The muscle was then left to dry at room temperature to a constant mass and then the muscle was re-weighed to calculate muscle water content (Section 2.6). Fin clips were taken from fish after sampling and stored in 95% ethanol for later DNA analyses. The remainder of the fish was flash frozen in liquid nitrogen and then preserved at -80 °C.

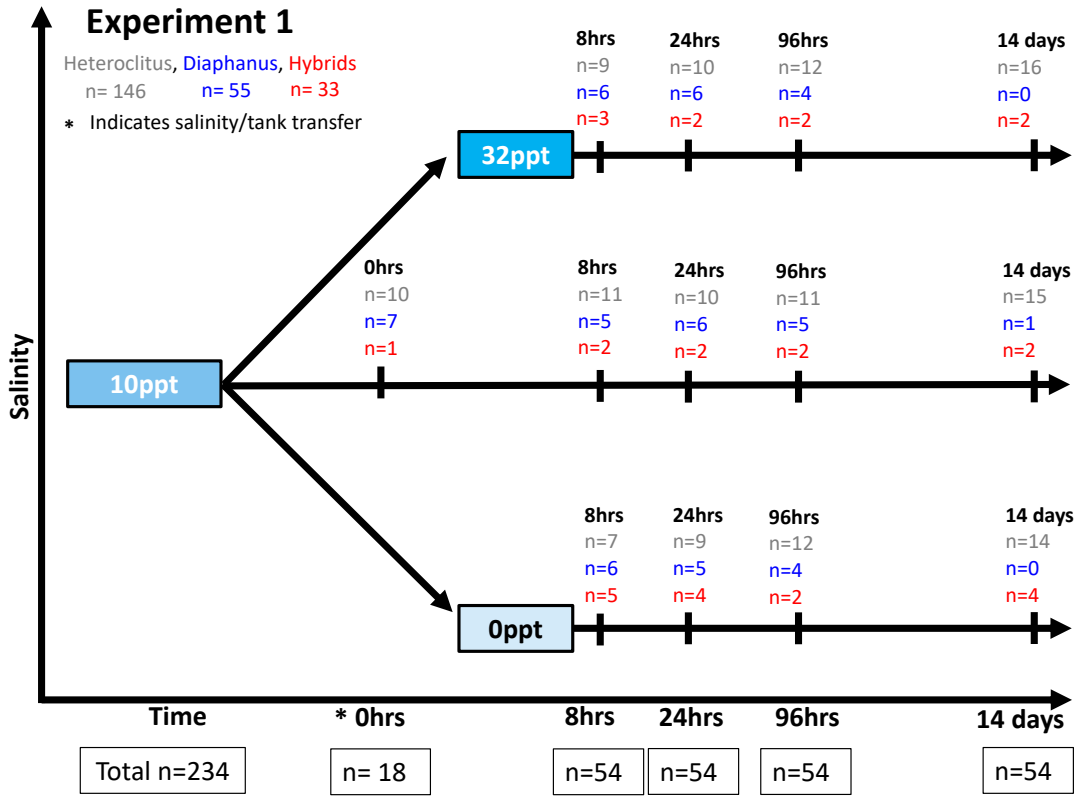

Figure 2.3 Summary of experiment 1 in 2017. There were six tanks at each salinity (0 ppt, 10 ppt & 32 ppt) for a total of 18 tanks. One fish of each species (*F. heteroclitus*, *F. diaphanus* and hybrids) was sampled from each tank during sampling. 18 fish were sampled at time = 0 hrs prior to fish being placed in the experimental tanks (indicated by *). For each experimental sampling point at 32 ppt and 0 ppt there is a time matched control sample at 10 ppt (handling control). Black vertical bars represent a sampling point and the time point and sample sizes are indicated at each point. Though we attempted to have 6 of each species at each salinity and timepoint, molecular genotyping determined that some *F. heteroclitus* were misidentified as both *F. diaphanus* and hybrids during this experiment so the number of each species of fish at each treatment in unbalanced. There is therefore a much smaller sample size of *F. diaphanus* and hybrids than anticipated.

2.5 Salinity transfer experiment 2 (hypersaline saltwater) – Summer 2018

The second salinity transfer experiment was completed to test hypersaline saltwater tolerance among *F. diaphanus*, *F. heteroclitus* and hybrids acclimated to 10 ppt and then transferred to salinities of up to 60 ppt (thesis question #1) and to also assess the mechanisms underlying tolerance in hypersaline saltwater (thesis question #2). I acclimated all three species (*F. heteroclitus*, *F. diaphanus* and F1 hybrids) to brackish water (10ppt, near isosmotic) for three weeks (Aug 1st-27th). During this time fish were also acclimated to a 12:12 LD photoperiod. The photoperiod was changed from 14:10 LD to 13:11 LD on Aug 17, 2018 and then to 12:12 LD on Aug 26th, 2018 to align the diurnal cycle of the fish with that of their natural environment. All fish were fed the same diet as in experiment 1 (2017), and water quality and tank changes follow methods outlined in section 2.1. All three species of fish were previously given species-specific tags based on morphological measures, so were mixed within tanks during acclimation to reduce tank effects.

Additionally, in 2018 all fish were treated for external parasites during the 10 ppt acclimation period. There was a higher presence of external parasites, including leeches (*Myzobdella lugubris*) and sea lice (*Argulus funduli*), which are commonly found in Porters Lake (King, 2009), on fish housed in the lab, in comparison to 2017. I removed the parasites to reduce the potential effect they would have on fish osmoregulatory ability and survivorship, as sea lice can affect gill morphology and leeches could affect overall fish health (Marshall et al., 2008).

I treated my fish for external parasites following the formalin dip procedure of Floyd (1996) as it has demonstrated the ability to remove external parasites from fish without causing unnecessary stress. For each treatment, I added 15 ml of 37% formalin solution to a 15-gallon fish tank filled with 10 ppt water. Formalin displaces oxygen at a rapid rate, so the tank was heavily aerated during treatments. Each formalin bath/treatment lasted a total of 45 minutes. An average number of 12 fish were placed in the formalin solution for each treatment. All formalin baths were completed during a 3-week experimental acclimation period between Aug 9-23rd and only fish that were treated with formalin were used in the experiment 2 (2018).

After the formalin treatment and acclimation period at 10 ppt, my second salinity tolerance experiment began. Each fish was transferred to either 10 ppt (handling control) or 32 ppt (Fig. 2.4). First, 28 fish (n = 9-10 per species) were taken from their 10 ppt acclimation tank and sampled for baseline measurement at 0 hrs (Fig. 2.4). One fish from each tank was then sampled after 24 hrs in 10 ppt (handling control) and 32 ppt (salinity transfer). The fish were then acclimated and transferred to higher salinities, as indicated below and in Figure 2.4, with the exception of the 10 *F. heteroclitus* who remained at 10 ppt (long-term handling control).

Fish were then placed in one of ten tanks at either 10 ppt or 32 ppt (Fig. 2.4) such that there were approximately three to five fish of each species per tank. The fish transferred to 32 ppt tanks were then left at 32 ppt for a second saltwater acclimation period of ten days prior to stepwise transfers to higher salinities up to 60 ppt. I completed this second saltwater acclimation and conducted a step-wise transfer to reduce the stressful nature of a drastic salinity increase and match prior studies (Marshall et al.,

2018) that had acclimated fish to 32 ppt prior to transfer to higher salinities (Fig. 2.4). Additionally, all fish were transferred one tank at a time so that fish that acclimated together were transferred to a new tank together as follows: fish were removed from their tank, placed in an aerated bucket filled with water at the previous salinity and then placed together in a new tank at the desired salinity. Therefore, all three species remained mixed within tanks throughout the experiment.

Fish were then sampled after 24 hrs in 45 ppt and 24 hrs in 60 ppt. *F. heteroclitus* was also sampled after 14 days at 10 ppt (Fig. 2.4). The *F. heteroclitus* in the 10 ppt tanks during the high salinity acclimation were netted and removed from their tanks at the same time that fish were transferred from one salinity to the next to mimic tank transfer on the following transfer days: day 11, 12 and 13 but not at day 10 (Fig. 2.4).

A total of 159 fish were sampled in experiment 2. Six *F. diaphanus* and four hybrids died during the experiment, so I had a lower sample size of those two species at one sampling point (24 hrs at 60 ppt, Fig. 2.4). After the 24hr sampling point at 60 ppt, *F. heteroclitus* were divided into five tanks at 60 ppt instead of 10 to reduce the number of tanks to maintain at 60 ppt.

2.5.1 Physiological sampling procedure

I measured survival, muscle water content and plasma ion content, following the same procedures in experiment #1 (section 2.3.1). In 2018, I also measured hematocrit, which was calculated by measuring the ratio of red blood cells to blood plasma in hematocrit tubes after centrifugation at 10,000 x g for three minutes.

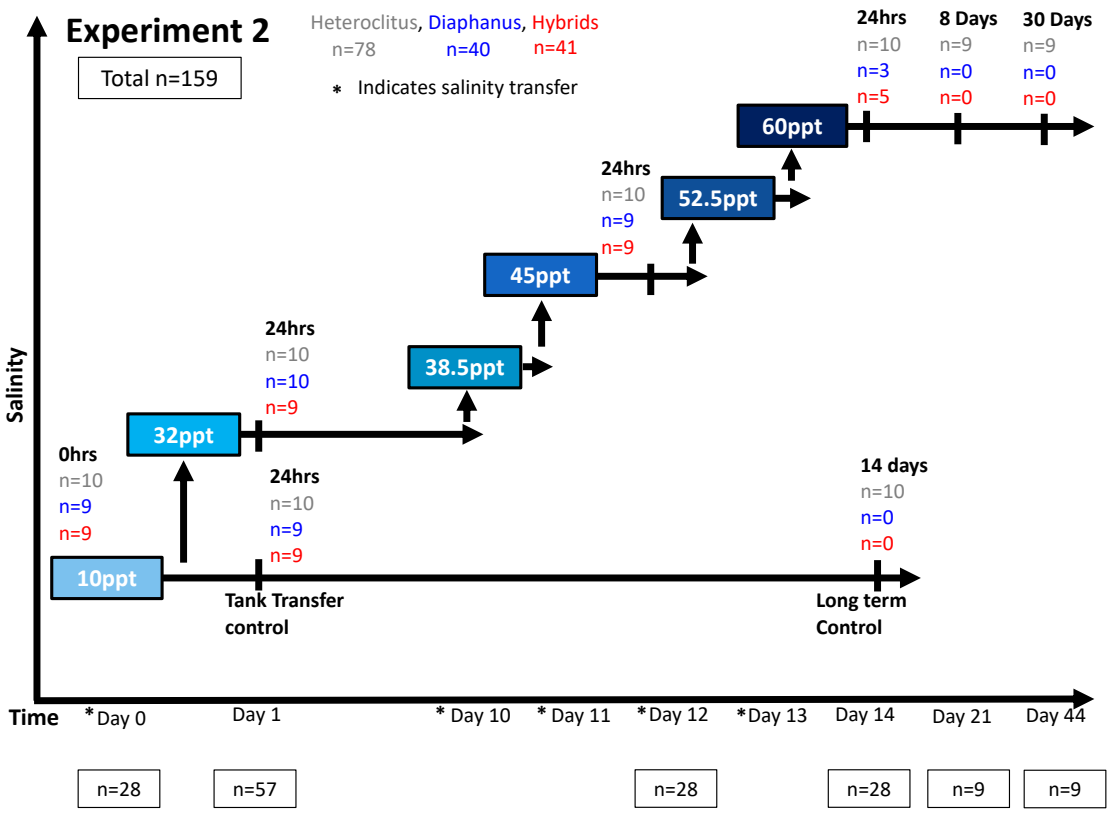

Figure 2.4 Experimental set-up for experiment 2 in 2018. All fish were sampled following the same procedure as in experiment 1 (Fig. 2.4). 28 fish were sampled as baseline controls at time= 0 hrs prior to fish being placed in the experimental tanks. A handling control was completed on day 1 at 10 ppt and a long-term control was completed on day 14 of the experiment. There were 10 separate tanks at each salinity for the fish to be divided between. 28 fish were immediately transferred into 10 ppt and the beginning of the experiment and the rest of the fish were transferred to 32 ppt. The fish then followed a step-wise salinity increase to 60 ppt. After 10 days at 32 ppt fish were transferred to 38.5 ppt tanks. On day 11, they were transferred to 45 ppt tanks, on day 12 to 52.5 ppt tanks and on day 13 to 60 ppt. The * on the x-axis indicates a salinity transfer. Vertical black bars represent a sampling point and the time and sample sizes are indicated at each point.

2.6 Muscle water analysis (Experiment 1 & 2)

A 1 cm x 1 cm sample of wet white muscle was removed from fish and weighed immediately after skin, bone and red muscle was removed. During experiment #1 muscles were air dried for 2 weeks to constant mass and then weighed. During experiment #2 muscles were put in a drying oven for 24 hours at 40 °C and air dried to constant mass, then weighed. I used an analytical balance (Sartorius analytic, model A 120 S) and the weigh-by-difference method to determine the dry weight of the samples for both experiments. The water content was expressed as wet mass minus dry mass divided by wet mass to provide a measure of body hydration (Marshall et al., 2018).

2.7 Plasma ion concentration analysis (Experiment 1 & 2)

Plasma sodium (Na^+), potassium (K^+) and chloride (Cl^-) concentrations were determined using the SMARTLYTE electrolyte analyzer (Diamond Diagnostics). In cases when the volume of the plasma samples were too low to be directly read (< 60 μL), a known volume of Mission Control Level 1 solution (Diamond Diagnostics; Na^+ = 111 mmol/L, K^+ = 1.91 mmol/L and Cl^- = 79 mmol/L) was added to the samples to a final volume of 70 µL. This was used instead of distilled water to keep the values within the range for which this machine has the highest accuracy. I determined the experimental sample concentration by taking the measured concentration, divided by 70 µL, multiplied by the volume of the sample. Any samples under 8 µL were excluded from analysis.

2.8 RNA extraction & mRNA content in hypersaline salt water (Experiment 2, 2018)

2.8.1 Total RNA extraction

RNA extractions were performed on a randomly selected sub-sample of 68 fish from the experimental time points (Fig. 2.4) for which I found significant variation in blood plasma, hematocrit and muscle water content among species, time points or salinities to aid in understanding the molecular mechanisms contributing to hypersaline saltwater tolerance. The four groups I analyzed were: 10 ppt at 0 hr (pre-transfer control), 10 ppt at 24 hr (handling control), 45 ppt at 24 hr, and 60 ppt at 24 hr (Fig. 2.5). For each group, 3-6 samples of each species were selected randomly. Both tissue homogenization and RNA extractions were completed in a randomized order to prevent any batch effects.

To begin extractions, preserved gill tissue from one fish at a time was placed in liquid nitrogen and dissected to isolate the 2nd and 3rd right gill arches, while preserving the rest of the gill for future experiments. The gills were dissected in 500 µL of lysis buffer solution containing a final concentration of 2M DL-Dithiothreitol (Sigma Aldrich) in RNAse free water, made following instructions from the Thermo Scientific Gene JET Purification Kit (Thermo Scientific Gene JET Purification Kit) and isolated gill filaments were then transferred to new tubes with lysis buffer.

The 2nd and 3rd right gill filaments of each specimen were homogenized immediately in 300 µL lysis buffer with a rotor stator homogeniser for three 30 second intervals at 70% power (Variac autotransformer, with 7 mm blade, Kinematic Switzerland model GMpH). Homogenization was then repeated at 30 second intervals at 80% power until little or no filaments were seen in the tube. After homogenizing each sample, I cleaned the rotor for three 30 second intervals using double distilled water. Homogenized gill

tissue was then flash frozen in liquid nitrogen to prevent RNA degradation and ensure all samples were left in the lysis buffer at room temperature for the same amount of time. I was able to collect between 15-25 mg of tissue using this method. The dissection lasted on average four minutes and the combined dissection and homogenization process lasted, on average, eight minutes per sample.

Total RNA was then extracted from the homogenized tissue following the manufacturer's instructions for the GeneJet Purification Kit. However, to reduce genomic DNA contamination, I completed a DNase treatment using the PureLink™ Invitrogen kit during the wash phase of the RNA extraction, following the GeneJet protocol. Specifically, I modified step six of the GeneJet protocol by adding 350 µL of wash buffer #1 to the column and centrifuged each column for two minutes at 12000 x g. The flow through liquid was discarded and then 80 µL of PureLink™ DNase mixture (62 µL H_2O, 10 µL DNase 3 U/µL, and 8 µL DNase buffer) was added to each directly to the spin column membrane and left to incubate at room temperature for 15 minutes. Another 350 µL of wash buffer #1 was added to the column and it was centrifuged at 12 000 x g for one minute and the flow through liquid was discarded. This was the only deviation from the Gene JET protocol. Extracted RNA was divided into three separate 33 µL aliquots, to reduce RNA freeze/thaw cycles and ensure equal sample integrity, and stored at -80 °C.

2.8.2 RNA quanification and quality verification

I assessed RNA quantity by measuring concentration with a spectrophotometer at an absorbance of 260 nm (Spectromax M3, Molecular Devices), using Softmax Pro 7 software, which also measured the absorbance at 230 and 280 nm. To measure RNA concentration, 2.5 µL of each sample was loaded, in triplicate, onto a 24-well MVMP SpectraDrop TM Micro-Volume Microplate (Molecular Devices) with a 0.5 mm cover slip. RNase free water was also included on each plate as a standardized "blank" measurement, in triplicate. If the standard deviation of RNA concentration was observed to be greater than 30% for any sample, the measurements were repeated until the standard deviation was less than 30%. The concentration of RNA ranged from 15.71 ng/µL to 270.32 ng/µL.

I verified RNA purity by measuring $A_{260/280}$ ratios and $A_{260/230}$ ratios. The $A_{260/280}$ ratio is used to assess protein contamination as proteins absorb at 280 nm and nucleic acids absorb at 260 nm (Taylor et al., 2010). The $A_{260/230}$ ratio is calculated to assess phenol or alcohol contamination in samples as phenols absorb at 230 nm (Taylor et al., 2010). The $A_{260/280}$ ratio of 1.8 to 2 indicates that RNA is devoid of protein contamination and an $A_{260/230}$ ratio or 2 to 2.2 indicates that RNA is devoid of alcohol or phenol contamination. All $A_{260/280}$ and $A_{260/230}$ ratios were above 1.5, but were generally similar among samples and groups.

I verified RNA integrity using a Bleach Gel [1% Agarose:Tris-acetate EDTA w/v, 1% Bleach v/v, stained with Ethidium bromide], following methodology of Aranda et al. (2012) (Appendix B). I loaded approximately 1 µg of RNA of each sample per well when I ran the gel in a BioRad Agarose gel electrophoresis system with 1X TAE buffer. I then

used the Bio Molecular Imager® Gel DocTM XR+ imaging system to photograph the gel under UV light. All images were assessed in Image Lab™ (Version 6.0, 2017, Bio-Rad Laboratories, Inc.) to quantify the ratio of 28S rRNA to 18S rRNA (Fig. B). All RNA was of acceptable quality and fell within close range of the 2:1 ratio of 28S: 18S rRNA except for two samples that showed evidence of genomic DNA contamination that were not included in data analysis.

2.8.3 Reverse transcription and cDNA synthesis

Following RNA quantification and quality assessment, complimentary DNA (cDNA) was synthesized from the RNA for all experimental samples using the iScript™ cDNA Synthesis Kit (BioRad) following manufacturer's protocols. The final concentration of RNA added to each cDNA reaction was 12.56 ng/µL. A non-reverse transcribed (NRT) control was run for each individual sample in which all components of the reaction were identical with the exception of reverse transcriptase. Samples were then aliquoted and stored in the freezer at -20°C.

I also completed an extra cDNA reaction to create a pooled sample of cDNA and NRT's for each species following the same specifications as listed in the previous paragraph. The pooled cDNA sample (10 randomly selected individuals of each species) was created for use in the testing of annealing temperature and the generation of standard curves for each primer set following the Bio Rad "MIQE Guidelines", to evaluate the overall qPCR efficiency and compare efficiency between species (Taylor et al., 2010). The pooled cDNA samples were also needed for an inter-plate calibrator (standard curve) for qPCR reactions.

2.8.4 qPCR primers and optimization

Primers for killifish claudin-10 isoforms, cystic fibrosis transmembrane conductance regulator (*cftr*) channel and *18S ribosomal RNA* primers were designed by Marshall et al. (2018) (Table 2.2). Consensus elongation factor 1 alpha (*EF1α*) primers were also designed during this experiment to amplify all three species (Table 2.2).

Primer annealing temperature was first tested following the Bio Rad MIQE Guidelines by running qPCRs for all three species using a (10 °C) temperature gradient during the annealing step of the reaction on a C1000 Touch Bio-Rad Thermal Cycler (Taylor et al., 2010). Standard curves were then generated using a ¼ or ½ serial dilution series for all primer sets (Table 2.2) beginning with a 1/8 or ¼ dilution (0.5 µL or 1 in µL 10 µL qPCR, respectively) using the best annealing temperature. I verified the standard curves by assuring each point on the curve was separated by the appropriate Cq value (1 or 2) for the ½ or ¼ dilution series, respectively (Taylor et al., 2010). I verified I was amplifying a single PCR product by assessing all melt curves and ensuring there was only one amplicon. I also tested all samples with a no cDNA template control as well as a no reverse transcriptase (NRT) to determine the level of background or genomic DNA contamination (See Appendix C for an example melt-curve).

For the *18S* reference gene primer set, I selected the best annealing temperature by analyzing the melt curve for a single amplified product and generated standard curves with acceptable efficiencies (90-110%; Table 2.2; Appendix C). For the claudin and *cftr* primer sets I followed the protocol outlined by Marshall et al. (2018; Table 2.2) and for the *EF1α* reference gene primers I followed the protocol outlined by Scott et al. (2004; Table 2.2), as these conditions amplified a single product and generated standard

curves with acceptable efficiencies (90-110%; Table 2.2). Requirements for good standard curves also include an R^2 value of 0.98-1, as well as having only one product appear as a single peak in the melt curve (Taylor et al., 2010). I obtained reaction efficiencies between 88-108% in my assays and only had one product appear in the melt curve (Table 2.2). For the *cftr*, *claudin-10f* and *18S rRNA* gene, I was only able to generate a standard curve with appropriate reaction efficiencies for two of the three species with the reaction efficiency for one species being slightly lower than acceptable (Table 2.2). The reaction efficiencies were incorporated into calculations of relative mRNA content.

In addition to having a good overall qPCR reaction efficiency, I also verified that all primers bind to *F. heteroclitus*, *F. diaphanus* and hybrids with similar efficiencies, allowing me to compare mRNA content across species (Table 2.2). A difference of 12% or less was considered to be similar reaction efficiency between the three different species. Additionally, I ran samples of each standard curve qPCR product for all three species and primer sets out on an agarose gel to confirm that all primers produce a product of the predicted size and also sent representative samples for sequencing to ensure we amplified the correct product (Table 2.2). We also amplified and sequenced a portion of the *claudin-10c* and *10f* genes from Porters Lake *F. diaphanus* and *F. heteroclitus* to check for mutations in the binding sites of the *claudin-10f* and *claudin-10c* primers that may influence amplification efficiency (von Kursell, 2019; Dalziel, A.C. personal communication). There were no mutations in the primer binding sites between *F. diaphanus* and *F. heteroclitus* for *claudin-10f*. For *claudin-10c*, there was a mutation in the forward primer binding site in *F. diaphanus* compared to the primer, however *F.*

diaphanus showed a higher qPCR efficiency than *F. heteroclitus*, so it does not appear that this mutation impacted overall qPCR efficiency. Unfortunately, our sequenced regions did not include the reverse *claudin-10c* primer, which will be checked in the future by Dr. Dalziel.

Table 2.2 Primers sequences and efficiency for genes of interest and reference genes in qPCR

Gene Product	Accession no. (NCBI)	F/R	Primer Sequence	Product size (BP)	Melt temperature °C (Tm)	Reaction Efficiency (%)
Cldn-10c	XM_012873236.2	F	5'-CGCACGGAGATCACACATAC -3'	87	60	H: 94.6
		R	5'-AGTCTTCCTGGTGGTGTTGG -3'		60	Hy: 91.3
						D: 97.1
Cldn-10f	XM_012873235.2	F	5'-ACTTATATCGGCGGAGCAGA -3'	103	60	H: 93.2
		R	5'- ATAAGCAGTAGGCGGCAAGA-3'		60	Hy: 88
						D:103
cftr	AF000271.1	F	5'- AATCGAGCAGTTCCCAGACAAG-3'	78	52	H: 107.5
		R	5'- AGCTGTTTGTGCCCATTGC -3'		52	Hy: 102.6
						D: 88.9
*18S**	M91180.1	F	5'-TTCCGATAACGAACGAGAC -3'	141	51	H: 92.6
		R	5'-GACATCTAAGGGCATCACAG -3'		51	Hy: 96.5
						D: 88.9
*EF1α**	XM_012859705.2	F	5'- TTA CCT GGT TTA GGG GCA GC- 3'	96	60	H: 100.2
		R	5'- ACC ACG ATG TTG ATG TGG GT- 3'		60	Hy: 97.2
						D: 104.4

* indicates reference genes. H indicates *F. heteroclitus*, Hy indicates hybrid, D indicates *F. diaphanus*. *EF1α* primers were designed during this experiment, all other primers were designed by Marshall et al. (2018). All data was obtained from National Centre for Biotechnology and Information (NCBI). Abbreviations: forward primer sequences (F), reverse primer sequences (R), Claudin (Cldn), Cystic Fibrosis Transmembrane Conductance Regulator (*cftr*), 18S ribosomal RNA (*18S*) and Elongation factor 1 alpha (*EF1α*)

2.8.5 mRNA content of claudin isoforms and *cftr* relative to control genes (*18S rRNA* and *EF1α*)

All cDNA and NRT controls were diluted to a 1/8 concentration, using RNase and DNase free water, for mRNA content analysis and then aliquoted to reduce freeze/thaw cycles and ensure equal sample integrity. qPCR assays used 0.5 µL of undiluted cDNA (as volumes of 1 µL of cDNA or more caused inhibition of the qPCR), 0.3-0.5 µL of each primer (10mM), 5 µL SsoAdvanced™ Universal SYBR® Green Supermix from BioRad and RNase and DNase free water for a total volume of 10 µL. The 18S gene was more concentrated than other samples, so cDNA for 18S and NRT controls were diluted to a concentration of 1/256.

All samples were run in triplicate for each biological replicate sample (individual fish) and each biological replicate was accompanied by a single NRT control. All reactions were assessed following the methodology in section 2.8.4. I calculated genomic DNA contamination using the difference in Cq values between each sample and its NRT control and samples exceeding 2% genomic DNA contamination were excluded from analysis. An inter-plate calibrator consisting of pooled species cDNA samples at dilutions of 1/8, 1/32, 1/128 and 1/512, in triplicate, were included on each plate for each gene. mRNA content for all samples was normalized relative to the inter-plate calibrators, using a calculation provided by Bio-Rad CFX Maestro software (for Mac 1.1, Version 4.1, 2017 Bio-Rad Laboratories, Inc.). These calculations were also completed manually to verify the calibration.

Results were all standardized relative to the combined reference genes of *18S rRNA* and *EF1α*. Scott et al. (2004) used *EF1α* because they found the expression in the

gills does not change following a salinity transfer and Marshall et al., (2018) used *18S rRNA* because its expression was not significantly altered following salinity changes up to 60 ppt. I tested the stability of the reference genes by running two-way ANOVA's with treatment (salinity and sampling time) and species as factors for both *EF1α* and *18S* and found no significant differences in the expression of either reference gene with respect to treatment or species. Additionally, I calculated the M value, or expression stability value, for the two reference genes using Bio-Rad CFX Maestro software and found a value of 0.76, which is below the cut-off value of 1 and fits the assumptions of good reference genes for a heterogeneous sample set (Taylor et al., 2010).

To normalize data to reference genes, I integrated the geometric average of both EF1α and 18S using the Pfaffl equation (Pfaffl, 2001; Bustin et al., 2009; CFX Maestro, 2017). The Pfaffl equation was chosen because the equation allows standardization to multiple reference genes while also taking reaction efficiencies into consideration for each gene. Reaction efficiencies were determined by taking the geometric average of the reaction efficiency for *F. heteroclitus*, *F. diaphanus* and hybrids for each gene from my pooled cDNA sample (Table 2.2). Gene expression calculations were carried out in CFX Maestro software and presented as relative gene expression. This experiment involved multiple controls so normalization to controls occurred during statistical analyses by comparing normalized gene expression of all groups (R Core Team, 2018; See section 2.7 for further details). The Pfaffl equation calculations were also completed manually to verify results.

2.9 Interaction of salinity tolerance and other stressors (copper)

After data collection, I discovered that copper from the chilled de-chlorinated water pipes was leaching into the water used in the aquatic facilities at a much higher level (91-552 μg/L) than the non-chilled, de-chlorinated line (3-41μg/L), resulting in fluctuating copper levels from 3 – 329 ug/L over the course of the experiment (Appendix E). Therefore, a caveat of the experiment was that all fish in both the 2017 and 2018 experiments were exposed to at least some copper (Cu^{2+}). Copper is a known environmental stressor for fish that can decrease survivorship, increase the stress of acclimating to salinity challenges, and reduce the ability to maintain osmoregulatory homoestasis (e.g. Grosell et al., 2007; Ransberry et al., 2015). Fish are normally more sensitive to copper stress in freshwater than isosmotic salinities and fish in saltwater appear slightly more sensitive than fish held at isosmotic salinities (Grosell et al., 2007). This suggests that fish exposed to salinities of 0 ppt, 32 ppt, 45 ppt and 60 ppt in this salinity tolerance experiment were likely more sensitive to potential Cu^{2+} exposure than fish at 10 ppt, with fish at 0 ppt being the most sensitive (Grosell et al., 2007). Fortunately, all three species of killifish were housed in the same tanks, so they were all exposed to equal amounts of copper from the Aquarium Facilities de-chlorinated water. Thus, while copper exposure may be a factor influencing our results and is important to note when comparing to results from other laboratories, we can still compare the effects of salinity among species within our experiment, as all had similar exposures. However, it is possible that in this experiment I measured an interaction between salinity and copper exposure, rather than solely salinity exposure.

While we were not aware that copper levels were as high as they might have been, we were aware that levels fluctuated from 3 - 41µg/L in the non-chilled, de-chlorinated line, so were taking steps to mitigate the effect of copper on experimental fish. First, all water in experimental fish tanks was treated with at least enough Stress Coat (API®), which chelates and binds heavy metals, to bind up to 100 µg/L copper (API® Fish Care Customer Service, personal communication to Dr. A.C. Dalziel). Stress Coat is a nontoxic polymer and also offers mucus layer protection for fish (Brown et al., 2010). Since the maximum amount of copper detected in tanks was 552 µg/L, the stress coat might have reduced the presence of copper in the water.

2.10 Statistical analysis

Generalized Linear Models (GLMs), fitted to a gaussian distribution, were conducted in R with the *nlme* package (Bates et al., 2015) to determine differences between *F. heteroclitus*, *F. diaphanus* and hybrids in all experimental salinities for the 2017 and 2018 data. Species, salinity and sampling time were considered as factors in all GLM's, but for the 2018 data set salinity and sampling time were considered a combined factor as all experimental salinities were sampled after 24 hours, termed "salinity/sampling time". The *emmeans* package (Lenth, 2019). was used for *post-hoc* Tukey multiple comparisons tests on the GLM to compare differences among species at all salinities and sampling times. The *lme4* package (Bates et al., 2015) was used to test for an association between candidate gene expression and plasma osmolarity using a mixed effect model with gene expression and plasma osmolarity at 60 ppt as the fixed effects and species (*F. diaphanus*, *F. heteroclitus* or hybrid) as a random effect.

Plasma osmolarity data and relative gene expression data were transformed using box-cox transformation to improve the normality of the residuals. Muscle water data were non-normally distributed, and arcsin transformation for the percentage data did not improve normality. A Hosmer-Lemeshow Goodness of Fit (GOF) Test was used to confirm that the non-normal data fit the assumptions of the GLM with a gaussian distribution and analysis was completed on non-transformed data. Normality was assessed by plotting residual values. All statistical analyses were conducted with R 3.5.2 (R Core Team, 2018) in RStudio (2017, version 1.1.383) and a significance level of $\alpha=0.05$ was used throughout testing. All figures were created in RStudio using either the *ggplot 2* package (Wickham, 2016) or the *sciplot* package (Morales, 2017).

3. RESULTS

3.1 Experiment 1 – Responses to freshwater (0 ppt) and saltwater (32 ppt)

3.1.1 Muscle moisture content

There were no significant interactions (salinity × species: $F_{4,193} = 1.22$, $p = 0.30$, salinity × timepoint: $F_{6,193} = 1.39$, $p = 0.22$, species × timepoint: $F_{8,193} = 0.22$, $p = 0.99$, species × salinity × timepoint: $F_{10,193} = 0.34$, $p = 0.97$), but a significant difference in muscle water content across species ($F_{2,193} = 3.67$, $p = 0.027$) and across sampling time ($F_{4,193} = 3.12$, $p = 0.016$), but not across salinities ($F_{2,193} = 0.18$, $p = 0.83$). There also were no significant differences in muscle water content between any treatment groups using Tukey *a posteriori* multiple comparisons tests (Fig. 3.1.1). These data indicate that *Fundulus heteroclitus*, *Fundulus diaphanus* and hybrids are all tolerant to acute transfers to 0 - 32 ppt (Fig. 3.1.1).

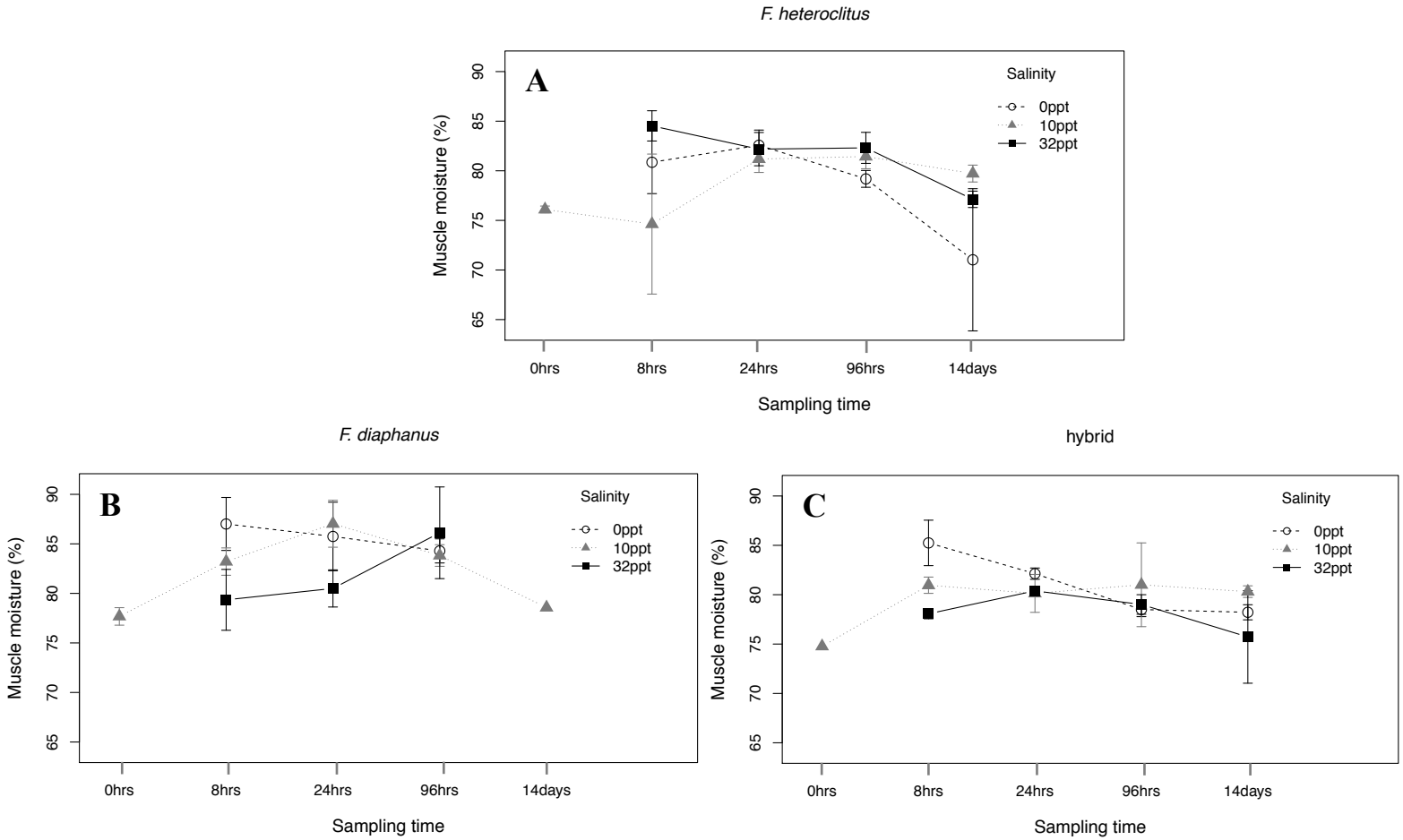

Figure 3.1.1 Effects of transfer from brackish water (10 ppt) to saltwater (32 ppt) and freshwater (0 ppt) on muscle moisture percentage for (A) *F. heteroclitus* (n = 7 - 16 per group), (B) *F. diaphanus* (n = 0 - 7 per group) and (C) hybrids (n = 1 - 5 per group).The points represent the mean and error bars indicate ± one standard error of the mean (S.E.M.). The white circles represent the lowest salinity (0 ppt), the grey triangles represent the control salinity (10 ppt) and the black squares represent the highest salinity (32 ppt).

3.1.2 Plasma ion concentration

There were no significant interactions (salinity × species: $F_{4,96} = 1.25$, $p = 0.30$; $F_{4,96} = 1.59$, $p = 0.19$, salinity × timepoint: $F_{6,96} = 1.27$, $p = 0.28$; $F_{6,96} = 1.08$, $p = 0.39$, species × timepoint: $F_{5,96} = 0.38$, $p = 0.86$; $F_{5,96} = 0.47$, $p = 0.80$, species × salinity × timepoint: $F_{8,96} = 1.38$, $p = 0.22$; $F_{8,96} = 1.43$, $p = 0.20$), but a significant difference in plasma chloride (Na^+) plasma chloride (Cl^-) content across timepoints, respectively ($F_{4,96} = 3.0482$, $p = 0.023$; $F_{4,96} = 3.78$, $p = 0.024$). There were no significant differences between species ($F_{2,96} = 0.52$, $p = 0.60$; $F_{2,96} = 0.71$, $p = 0.50$), nor salinities ($F_{2,96} = 1.26$, $p = 0.29$; $F_{2,96} = 3.14$, $p = 0.05$) for plasma Na^+ and plasma Cl^-, respectively.

There also were no significant differences in plasma Na^+ content between any treatment groups nor plasma Cl^- content between any treatment groups using Tukey *a posteriori* multiple comparisons tests (Fig. 3.1.2 & 3). *F. diaphanus* show a trend of increasing plasma ion content at 0ppt and 32ppt after 96 hours, indicating that they may have some trouble maintaining homeostasis at this timepoint, however; there are no significant differences in plasma ion content between species or salinities. Additionally, hybrids display a trend of elevated plasma Cl^- and Na^+ acutely at 32 ppt, but not after 14 days at 32 ppt. Taken together with the muscle water data, these data indicate that *Fundulus heteroclitus*, *Fundulus diaphanus* and hybrids are tolerant of transfers to 0 - 32 ppt (Fig. 3.1.2 & 3). Plasma potassium (K^+) content was also recorded as a measure of salinity tolerance and can be found in Appendix F.

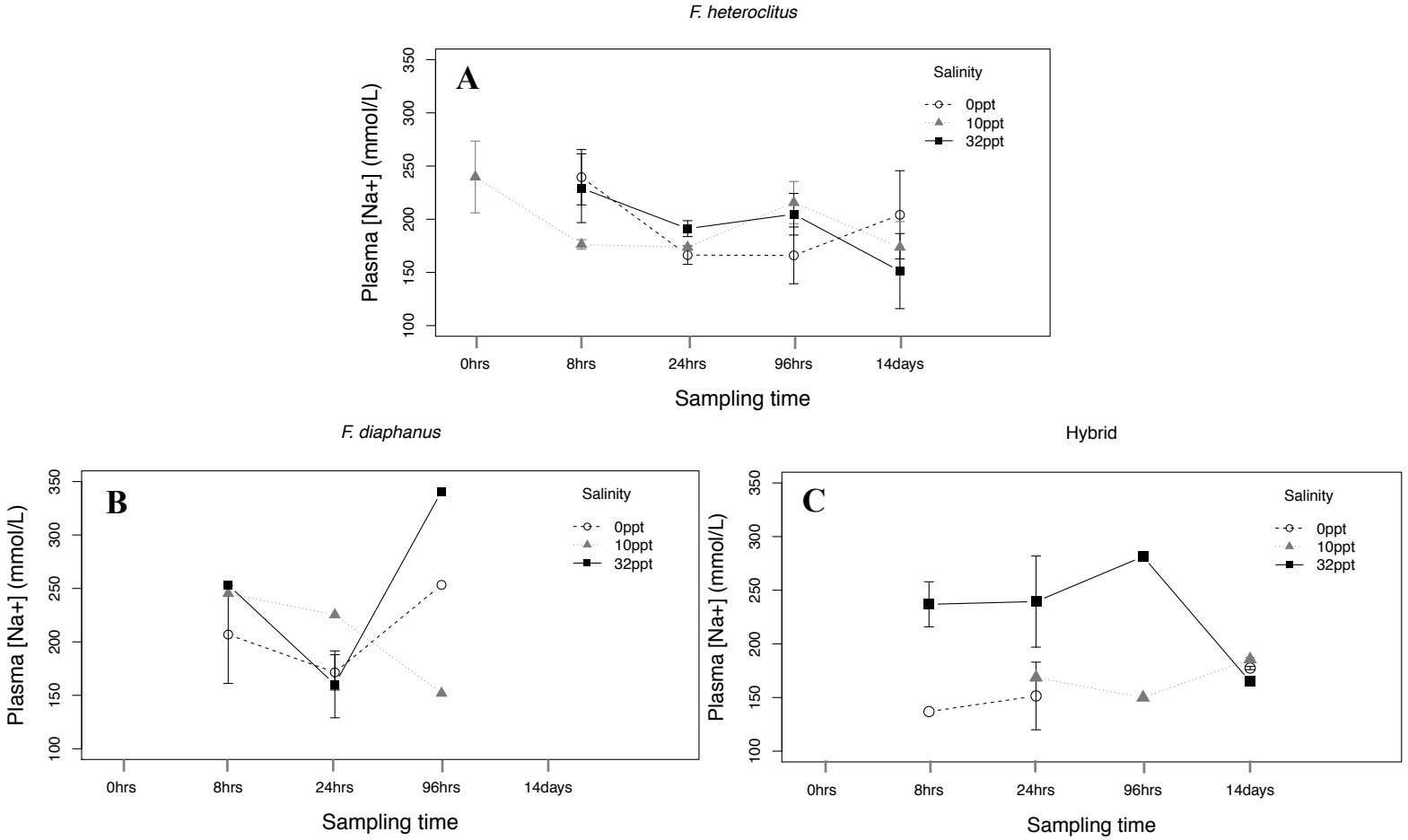

Figure 3.1.2 Effects of transfer from brackish water (10 ppt) to saltwater (32 ppt) and freshwater (0 ppt) on plasma sodium (Na^+) concentration for (A) *F. heteroclitus* (n = 3 - 9 per group), (B) *F. diaphanus* (n = 0 - 2 per group) and (C) hybrids (n = 0 - 2 per group). Data are presented as in Figure 3.1.1.

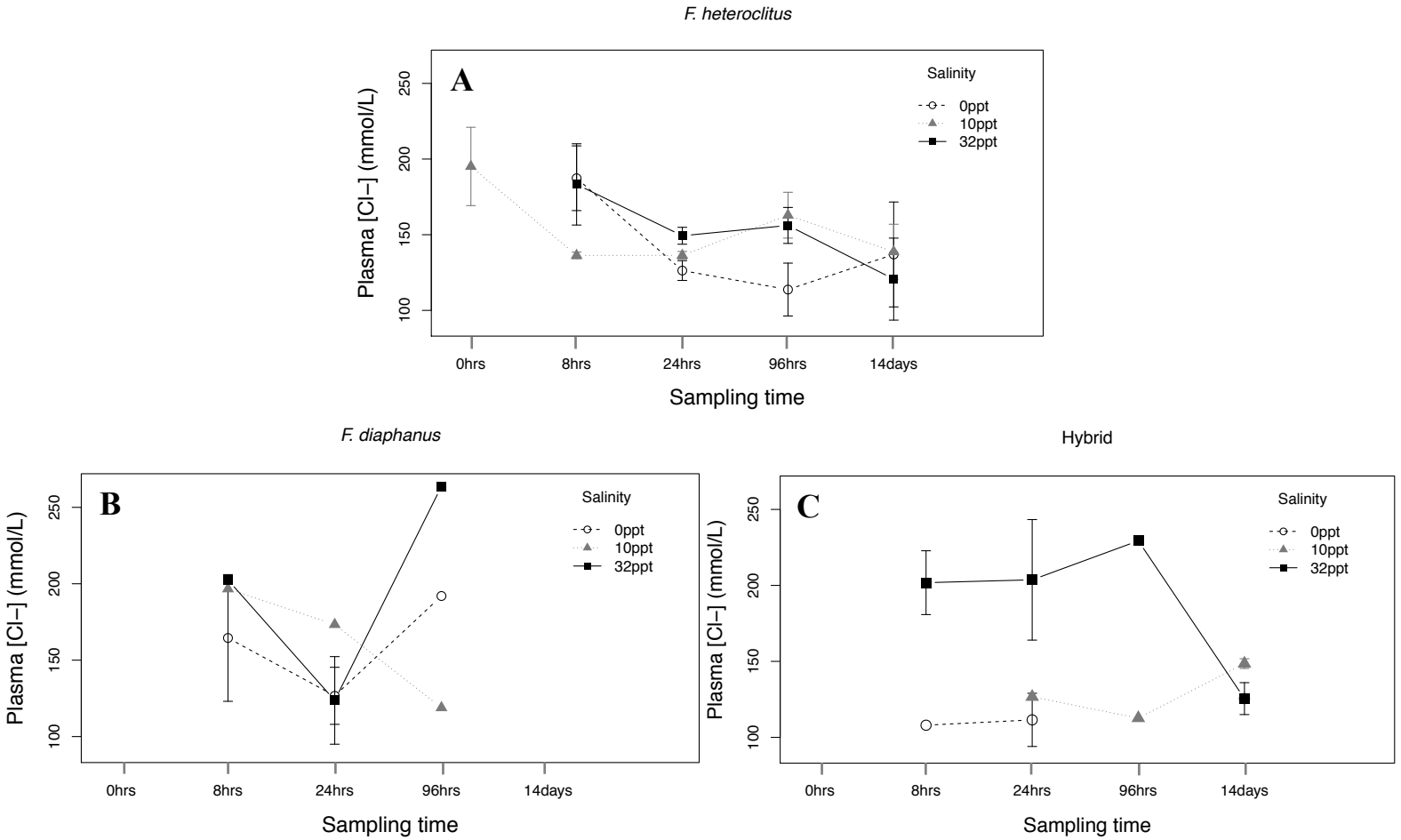

Figure 3.1.3 Effects of transfer from brackish water (10 ppt) to saltwater (32 ppt) and freshwater (0 ppt) on plasma chloride (Cl^-) concentration for (A) *F. heteroclitus* (n = 3 - 9 per group), (B) *F. diaphanus* (n = 0 - 2 per group) and (C) hybrids (n = 0 - 2 per group). Data are presented as in Figure 3.1.1.

3.2 Experiment 2 – Tolerance to hypersalinity (32 ppt – 60 ppt)

3.2.1 Muscle moisture content

There was a significant interaction of salinity/sampling time and species ($F_{8,116}$ = 4.72, $p < 0.001$). There was slight decrease, but no significant change in muscle moisture content for *F. heteroclitus* during salinity increases, indicating that they can maintain osmotic homeostasis (Fig. 3.2.1). There was a significant decrease in muscle moisture content for hybrids at 32 ppt and 60 ppt compared to control (10 ppt, 0 hrs), but not the handling control (10 ppt, 24 hrs). This indicates that hybrids are less effective at maintaining muscle water balance compared to *F. heteroclitus* (Fig. 3.2.1). The muscle moisture content for hybrids at 60 ppt appears to be in between that of both parental species, with half of hybrids more closely resembling the muscle moisture content of *F. diaphanus* at 60 ppt and the other half more closely resembling that of *F. heteroclitus* at 60 ppt. The only species and salinity that showed a significant decrease in muscle water content relative to the handling control (10 ppt, 24 hrs) was *F. diaphanus* at 60 ppt (Fig. 3.2.1). Additionally, *F. diaphanus* at 60 ppt had a significantly lower muscle moisture content compared to all other species, salinities and time points (Fig. 3.2.1). Together, these data indicate that *F. diaphanus* cannot maintain osmotic homeostasis at 60 ppt and have a reduced ability to maintain homeostasis compared to hybrids and *F. heteroclitus*. However, all fish did survive at this salinity.

In addition to the data presented in Figure 3.2.1, I measured muscle moisture content for *F. heteroclitus* at additional sampling points and salinities of 10 ppt for 14 days, 60 ppt for 8 days and 60 ppt for 30 days; these data can be found in Appendix G and were not included in the above statistical analyses in Fig. 3.2.1.

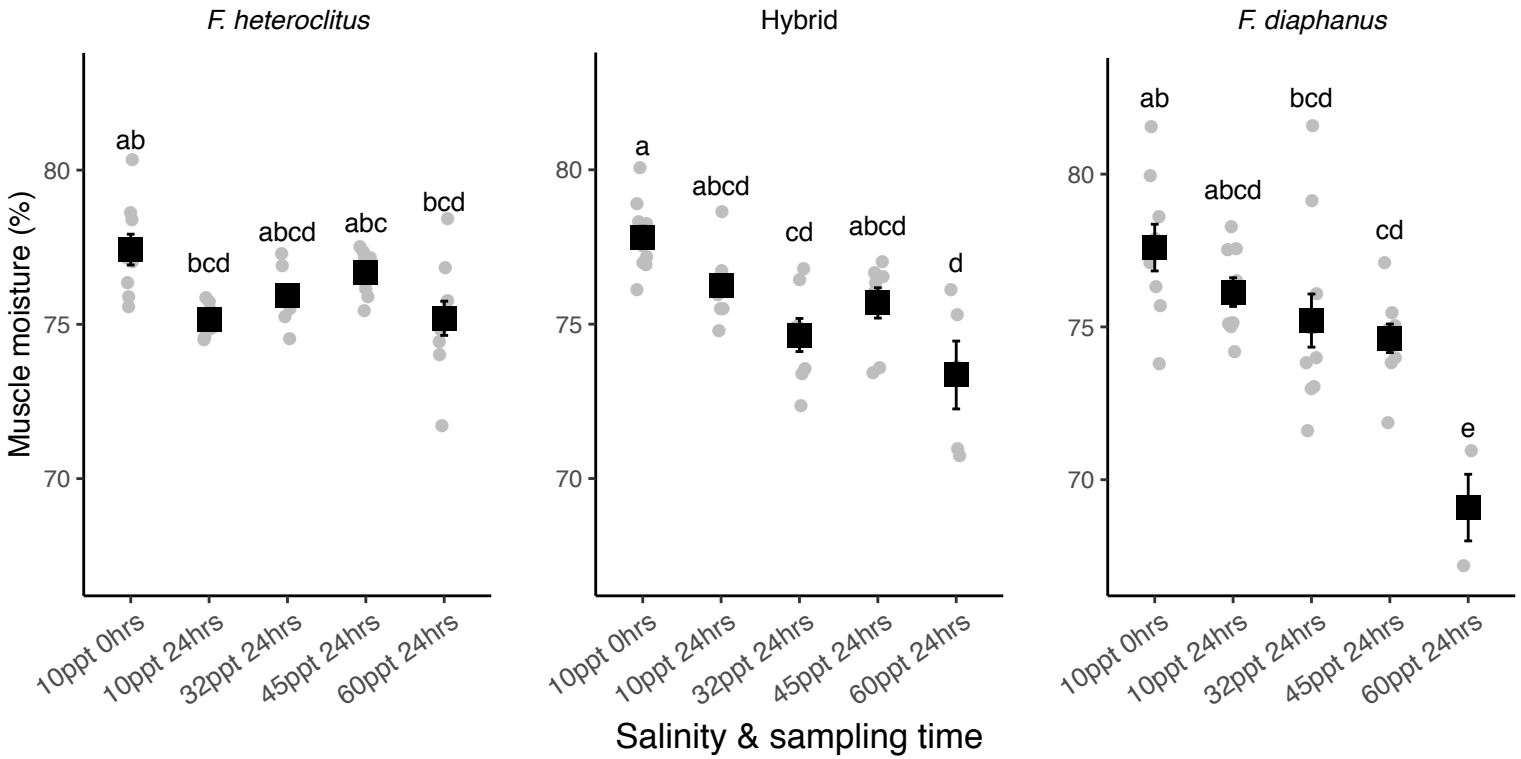

Figure 3.2.1 Effects of transfer from brackish water (10 ppt) to higher salinities (32 ppt, 45 ppt, and 60 ppt) on muscle moisture percentage for *F. heteroclitus* (n = 9 - 11 per group), *F. diaphanus* (n = 3 - 10 per group) and hybrids (n = 5 - 10 per group). Black boxes represent the mean and error bars are ± one S.E.M. Circles represent muscle water percentage for individual fish. Note that 10 ppt at 0 hours is prior to experimental transfers and 10 ppt at 24 hours measures the effect of handling. Significant differences between groups detected by Tukey *a posteriori* multiple comparisons tests are indicated by different letters ($p < 0.05$).

3.2.2 Experiment #2: Plasma ion content

There was a significant interaction of salinity/sampling time and species on plasma Na^+ and plasma Cl^-, respectively ($F_{8, 105} = 7.61$, $p < 0.001$; $F_{8,105} = 4.79$, $p < 0.001$) (Fig. 3.2.2A & B). In particular, *F. heteroclitus* showed no change in plasma Na^+ content after transfer to any experimental salinity (i.e., 32, 45 or 60 ppt) relative to the handling control (Fig. 3.2.2A), but did show a significant increase in plasma Cl^- relative to the handling control at 60 ppt (Fig. 3.2.2D).

There was a significant increase in plasma Na^+ and Cl^- for hybrids and *F. diaphanus* following transfer to 32 ppt or higher, compared to handling controls (10 ppt, 24 hrs), (Fig. 3.2.2B & C, and Fig. 3.2.2 E & F). Together, these data suggest that *F. diaphanus* and F1 hybrids have difficulty excreting ions at high salinities. However, hybrids appear to have a slightly higher tolerance to hypersaline saltwater than *F. diaphanus*, as they were able to maintain plasma Na^+ at a significantly lower level at 60 ppt (Fig. 3.2.2 B & C) and Cl^- to slightly lower level at 60 ppt, although not significantly so (Fig. 3.2.2 E & F).

In addition to the data presented in Figure 3.2.2A - F, plasma Na^+, and plasma Cl^- for *F. heteroclitus* were measured at additional salinities and sampling points; 10 ppt for 14 days, 60 ppt for 8 days and 60 ppt for 30 days, and can be found in the Appendix H. These data were not included in the above statistical analyses. Plasma K^+ and hematocrit were also taken as a measure of salinity tolerance and can be found in Appendix I - L.

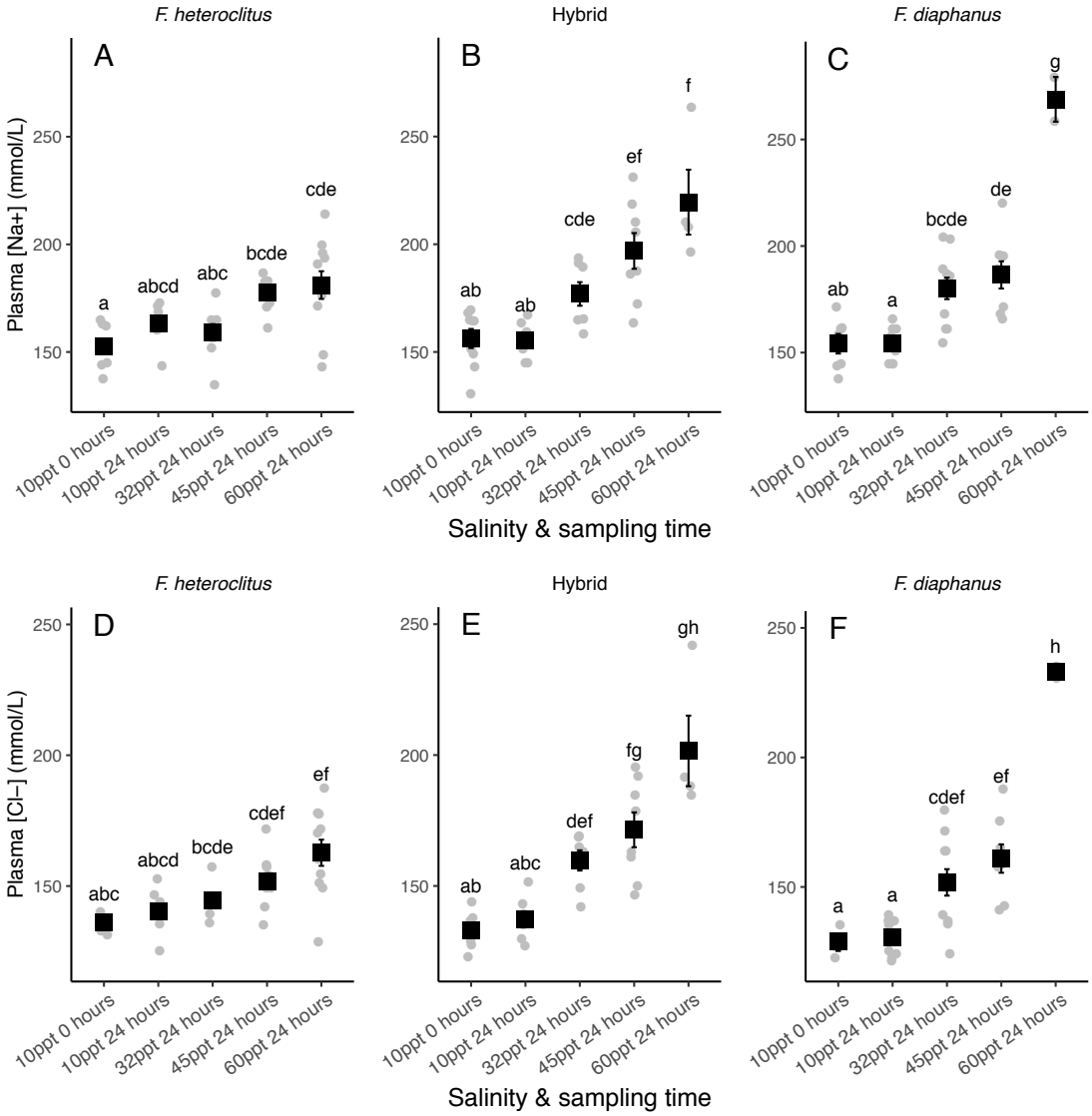

Figure 3.2.2 Effects of transfer from brackish water (10 ppt) to saltwater (32 ppt) and hypersaline saltwater (45 – 60 ppt) on plasma sodium Na^+ (A-C) and chloride Cl^- (D-F) content for *F. heteroclitus* (n = 8 - 11, per group), *F. diaphanus* (n = 2 - 10, per group) and hybrids (n = 4 - 9, per group). Data are presented as in Figure 3.2.1 and post hoc-lettering for A-C are independent of the lettering for D-F.

3.2.3 mRNA content of *cftr* and *Claudin-10c* and f isoforms after transfer to 45 – 60 ppt

There was no significant interaction between species and salinity/sampling time for Cystic Fibrosis Transmembrane Conductance Regulator (*cftr;* $F_{6,51} = 1.63$, $p = 0.16$), *claudin-10c* ($F_{6,53} = 1.17$, $p = 0.34$) or *claudin-10f* ($F_{6,51} = 1.15$, $p = 0.35$). There was a significant effect of salinity/sampling time ($F_{3,51} = 7.35$, $p < 0.001$; $F_{3,53} = 10.1$, $p < 0.001$; $F_{3,51} = 2.81$, $p = 0.048$) and species ($F_{2,51} = 7.9$, $p < 0.001$; $F_{2,53} = 13.3$, $p < 0.001$; $F_{2,51} = 20.6$, $p < 0.001$) on the relative expression of *cftr* and *claudin-10c* and *claudin-10f* respectively (Fig. 3.2.3.1 - 3).

While there was no difference in *cftr* or *claudin-10c* expression in hybrids or *F. diaphanus* with increasing salinity, *cftr* and *claudin-10c* were significantly elevated for *F. heteroclitus* in hypersaline saltwater (60 ppt) relative to 10 ppt, 0 hrs controls (Fig. 3.2.3.1-2). Additionally, *claudin-10f* expression was modestly, but not significantly, elevated for *F. heteroclitus* at 60 ppt, relative to controls (Fig. 3.2.3.3). Together, these data indicate that *F. heteroclitus* upregulate *cftr* and *claudin-10c* in 60 ppt hypersaline saltwater compared to brackish water (10 ppt) controls, but *F. diaphanus* and F1 hybrids between *F. diaphanus* and *F. heteroclitus* do not. However, there were no significant differences in *cftr* or *claudin-10c* expression between *F. heteroclitus*, hybrids and *F. diaphanus* at 60 ppt, or any other common salinity (Fig. 3.2.3.2). *F. diaphanus* do show a significantly higher initial expression of *claudin-10f* in control salinities (10 ppt) compared to hybrids, but do not significantly increase *claudin-10f* expression as salinity increases (Fig. 3.2.3.3). In fact, *F. diaphanus* show the highest expression of each candidate gene at 45 ppt (Fig. 3.2.3A-C, but not significantly so), but a lower expression of these genes at 60 ppt in comparison to *F. heteroclitus*. The expression profile of *cftr*,

claudin-10c and *claudin-10f* for hybrids more closely resembles the expression patterns of *F. diaphanus* compared to *F. heteroclitus*, indicating that hybrids are unable to upregulate the candidate genes in hypersaline salt water (60 ppt).

Overall, the species with the highest salinity tolerance (*F. heteroclitus*) also generally showed the highest expression of *cftr*, *claudin-10c* and *claudin-10f* at 60 ppt indicating that there may be an association between candidate gene expression and hypersaline saltwater tolerance. Though I lacked statistical power, I found there was a weakly negative, but non-significant, association between plasma Cl^- and the relative expression of *cftr* ($F_{1,7} = 0.18$, $p = 0.63$, correlation = -0.55; Fig. 3.2.3.4A) and between plasma Na^+ content and the relative expression of *claudin-10c* and *claudin-10f* at 60 ppt ($F_{1,7} = 0.41$, $p = 0.54$, correlation= -0.52; $F_{1,7} = 0.04$, $p = 0.85$, correlation =-0.52; Fig. 3.2.4B - C).

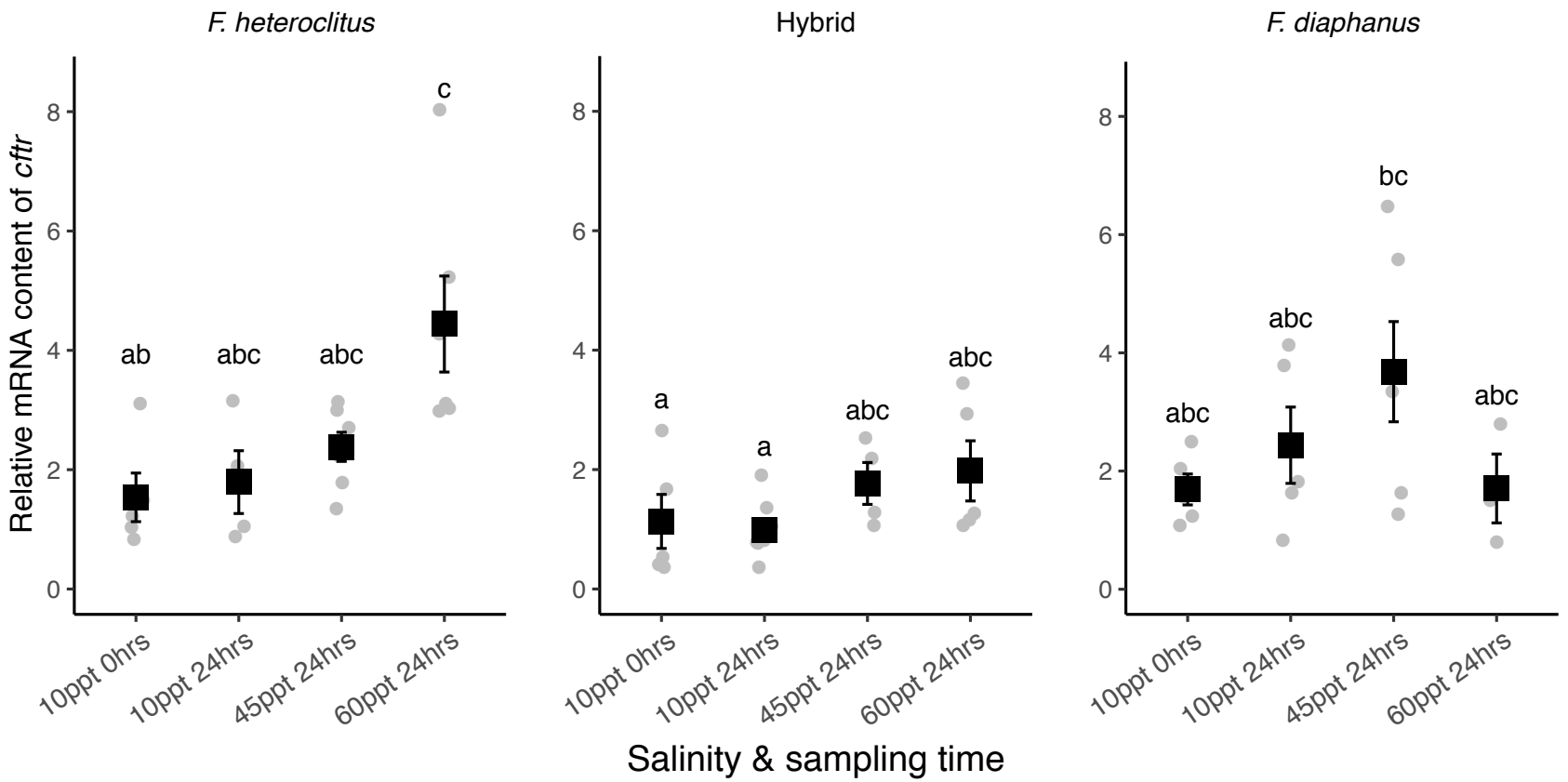

Figure 3.2.3.1 Effects of transfer from brackish water (10 ppt) to saltwater (32 ppt) and hypersaline saltwater (45 – 60 ppt) on the relative mRNA content of Cystic Fibrosis Transmembrane Conductance Regulator (*cftr*) for *F. heteroclitus* (n = 5 - 7, per group), *F. diaphanus* (n = 3 - 6, per group) and hybrids (n = 4 - 7, per group). Expression is relative to *18S rRNA* and *EF1α*. Data are presented as in Figure 3.2.1.

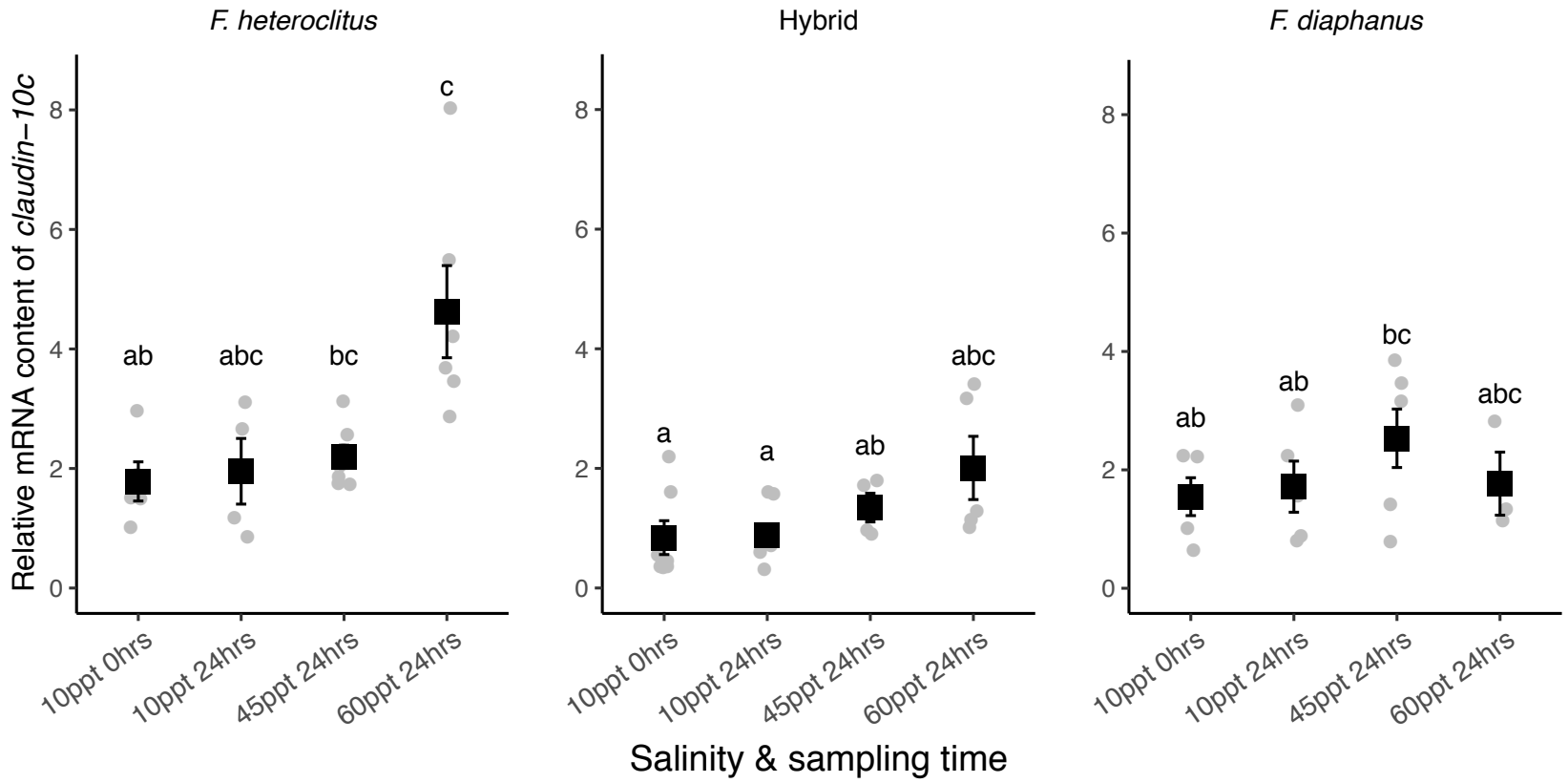

Figure 3.2.3.2 Effects of transfer from brackish water (10 ppt) to a brackish water handling control (10 ppt 24 hrs) and hypersaline saltwater (45 – 60 ppt, 24 hrs) on the reltive mRNA content of *claudin-10c* for *F. heteroclitus* (n = 5 - 7, per group), *F. diaphanus* (n = 3 - 6, per group) and hybrids (n = 4 - 7, per group) relative to *18S rRNA* and *EF1α*. Data are presented as in Figure 3.2.1.

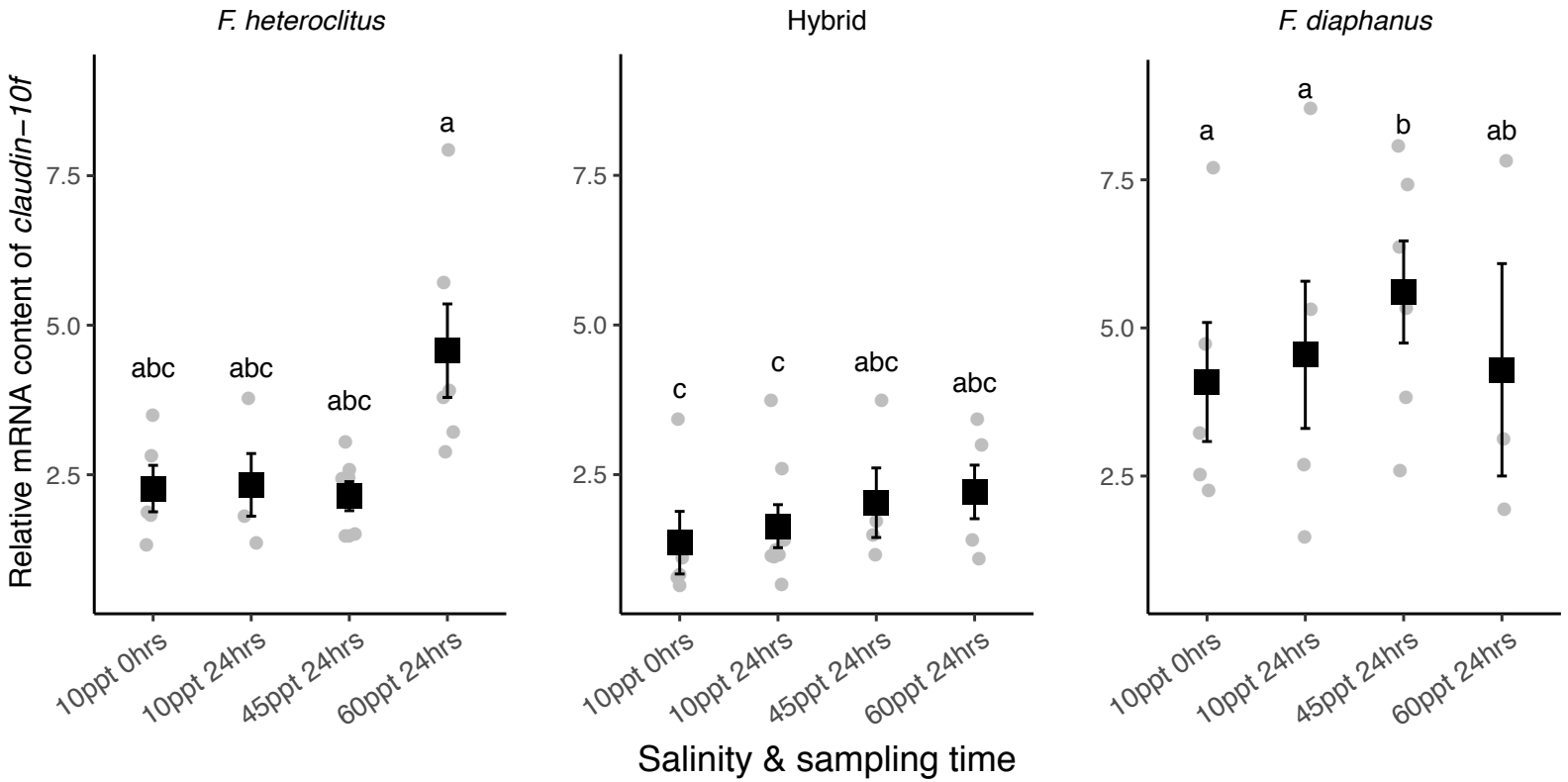

Figure 3.2.3.3 Effects of transfer from brackish water (10 ppt) to saltwater (32 ppt) and hypersaline saltwater (45 – 60 ppt) on the relative mRNA content of *claudin-10f* for *F. heteroclitus* (n = 5 - 7, per group), *F. diaphanus* (n = 3 - 6, per group) and hybrids (n = 4 - 7, per group) relative to 18S rRNA and EF1α. Data are presented as in Figure 3.2.1.

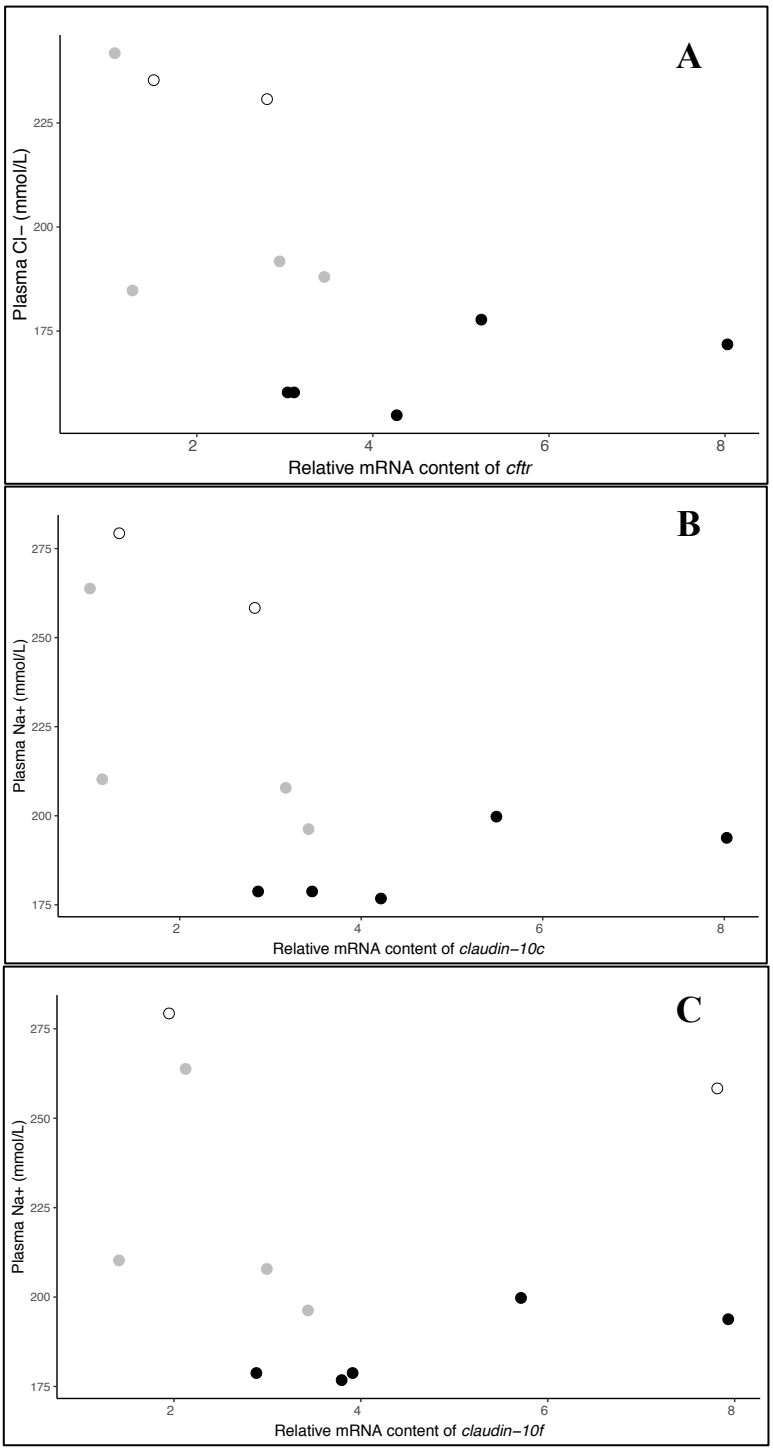

Figure 3.2.3.4 The relationship between (A) plasma Cl^- and the relative expression of *cftr*, (B) plasma Na^+ and the relative expression of *claudin-10c* and (C) plasma Na^+ and the relative expression of *claudin-10f* at 60 ppt for *F. heteroclitus*, indicated by black points (n = 5 per group), *F. diaphanus*, indicated by white points (n = 2, per group) and hybrids, indicated by grey points (n = 4 per group). There was no significant effect of the relative expression of any gene on any plasma ion content.

4. DISCUSSION

We know a great deal about how euryhaline fishes acclimate to freshwater, saltwater, and hypersaline saltwater environments within a lifetime (reviewed by Hwang et al., 2011; Dymowska et al., 2012; Gonzalez, 2012; Marshall et al., 2012; McCormick et al., 2013, Kültz, 2015), and are beginning to understand how populations and species may adapt to freshwater (e.g. Whitehead, 2010, Whitehead et al., 2013; Divino et al., 2016; Gibbons et al., 2016; Velotta et al., 2017) and saltwater (e.g. Brennan et al., 2015; Kusakabe et al., 2017) environments. However, we know little about how hypersaline saltwater tolerance may evolve in fishes. To gain a better understanding of how hypersaline saltwater tolerance evolves, I used a comparative approach to investigate hypersaline saltwater tolerance in *Fundulus heteroclitus*, *Fundulus diaphanus* and their F1 clonal hybrids as a model system. I found that the three species showed different hypersaline saltwater tolerances, and then investigated what mechanisms may contribute to inter-specific variation in hypersaline saltwater tolerance. I found that the ability to upregulate a set of candidate 'hypersaline saltwater tolerance' genes (*cftr*, *claudin-10c* and *claudin-10f*; Marshall et al. 2018) was associated with increased hypersaline saltwater tolerance in *F. heteroclitus* compared to *F. diaphanus* and F1 hybrids.

4.1 Salinity Tolerance of Killifish

Freshwater (0 ppt) and saltwater (32 ppt) tolerance

In my first study (experiment 1), I found that there were no significant differences in salinity tolerance between three species of killifish in water ranging from 0 ppt to 32 ppt, as all were able to maintain homeostasis in muscle water and plasma ion content. *F. diaphanus* showed a high plasma Na^+ and plasma Cl^- content after 96 hours at 32 ppt and

0 ppt; however, there were no significant differences in plasma ion content between species at 32ppt. There was a maximum sample size of two *F. diaphanus* for plasma ion data at all sampling points, which may have skewed the data or reduced our power to detect significant differences between groups. Overall, the plasma ion data and, the more reliable, muscle water data indicate that *F. heteroclitus* and *F. diaphanus* from Porters Lake are tolerant of salinities from 0 to 32 ppt, in accordance with numerous previous studies on these species (Fritz & Garside 1974; Griffith, 1974; Ahokas, 1975ab; Scott et al., 2004; Whitehead, 2010). However, further testing with a larger sample size would be prudent.

Scott et al. (2004) found that northern populations of *F. heteroclitus,* the ecotype used in this study, have an increased freshwater tolerance compared to their southern counterparts. In contrast to my study, Scott et al. (2004) noted that the northern *F. heteroclitus* (collected from New Hampshire) showed decreased plasma Na^+ levels and a reduced ability to acclimate to freshwater acutely, after 24 hours. This discrepancy might have occurred because the current study used de-chlorinated Halifax city water, while Scott et al. (2004) used de-chlorinated Vancouver city tap water. Halifax water has a higher overall ion content of Na^+ (15.9 mg/L vs 0.17 mmol/L), Cl^- (9.8 mg/L vs 0.21 mmol/L), a more neutral pH (7.6 vs 5.8-6.4) and is softer ($CaCO_3$= 22 mg/L vs 30 mg/L) than that of Vancouver water. Having softer water with a greater ion concentration may have reduced the stressful nature of freshwater exposure for our northern Nova Scotia *F. heteroclitus* populations in this experiment and explain a higher freshwater tolerance relative to northern *F. heteroclitus* from New Hampshire when tested in Vancouver (Scott et al., 2004). Additionally, it is possible that Nova Scotia *F. heteroclitus* generally have a

higher overall freshwater tolerance than the Maine populations studied by Scott et al. (2004), which would require a direct comparison of these populations.

I also found that hybrid killifish are tolerant of salinities from 0 ppt to 32 ppt, as hybrids survived and can maintain muscle water balance in saltwater and freshwater; however, I was unable to determine if hybrids displayed an intermediate salinity tolerance phenotype relative to parental species. In general, there are three potential outcomes for hybrid phenotypes in comparison to parental species; hybrids can have phenotypes that are most similar to one of the parental lineages, phenotypes that are outside of the range of parental lineages, or phenotypes that fall somewhere between the two parental types (Birchler et al., 2007). Together these phenotypes might contribute to a hybrid organism's overall fitness. While some hybrid species display a lower fitness than parental organisms (e.g.: copepod hybrids, Burton, 1990), many plants and a number of fishes display heterosis and have a higher fitness compared to parental species (e.g.: cabbage and radish hybrids, Karpechenko, 1927; hybrids of grass carps and bighead carps, Bettoli et al., 1985; hybrids of black crappies and white crappies; Epifanio et al., 1999). Hybrids of *F. diaphanus* and *F. heteroclitus* normally inherit the *F. diaphanus* mitochondrial genome and one allele from each parental species at all loci in the nuclear genome (Dawley, 1992); so, they have one allele from the hypersaline saltwater tolerant *F. heteroclitus*, and one allele from the less hypersaline saltwater and more freshwater tolerant *F. diaphanus* (Griffith, 1974; Scott et al., 2004). Therefore, I predicted that hybrids would display an intermediate tolerance in comparison to parental species due to a hypothesized additive effect of parental alleles in the nuclear genome. However, there were no differences among hybrids or parental species in tolerance to 0 – 32 ppt, so we cannot determine if hybrids are intermediate, or more similar, to either parental species at these salinities.

Additionally, we do not have enough information to rule out the possibility that there is genetic dominance, recessive effects or additivity contributing to the equal freshwater and saltwater tolerance observed in hybrids compared to parental species. Again, increasing our sample sizes by conducting future experiments may help clarify the genetic architecture of salinity tolerance in these fishes. My finding that hybrids can maintain muscle water content in 0 – 32 ppt does suggests that F1 clonal *F. diaphanus* x *F. heteroclitus* hybrids do not experience hybrid dysfunction.

Furthermore, all but one of the hybrids in this study had a mitochondrial genome from *F. diaphanus* (Tirbhowan, 2019), so I could not directly test the influence of the mitochondrial genome and other species-specific maternal factors on salinity tolerance in hybrid killifish. Mitochondria have their own genome that provides control and regulation for metabolic processes associated with adenosine triphosphate (ATP) production and the inheritance of mitochondrial genomes is normally strictly maternal (Blier et al., 2001). Therefore, the mitochondrial genome has the potential to greatly affect offspring phenotypes, especially when the phenotypes are related to energetic processes and require ATP. Myosho et al. (2018), found that maternal factors may have a large effect on hyperosmotic tolerance during early development in *Oryzias Spp*. In particular, Myosho et al. (2018) used genome wide linkage analysis to find two particularly promising candidate maternal genes thought to influence saltwater tolerance in *Oryzias latipes* and the two genes were associated with energy metabolism (phosphofructokinase and acyl-coA dehydrogenase). They hypothesized that "maternal enzymes" associated with these candidate genes are employed to prevent dehydration in developing *Oryzias celebensis* embryos, allowing them to survive in saltwater (>30 ppt) (Myosho et al., 2018).

It is possible that maternal effects, similar to those described by Myosho et al. (2018), may be influencing the observed hybrid salinity tolerance phenotypes in my experiment and that receiving maternal genes from their *F. diaphanus* mothers affects overall saltwater acclimation ability. This especially interesting to consider because key ion transporters that are upregulated during saltwater acclimation in *F. heteroclitus* either directly (NKA) or indirectly (NKCC, CFTR, through secondary active transport) rely on ATP (reviewed by Marshall, 2012). Therefore, the potential for maternal effects on energy metabolism and ion transport during saltwater acclimation should be tested in reciprocal F1 *F. diaphanus* x *F. heteroclitus* and *F. heteroclitus* x *F. diaphanus* hybrids. Future studies could also use genome wide linkage analysis to test for maternal effects in hybrids if we are able to create F2 crosses with hybrids between F. *diaphanus* and *F. heteroclitus*. However, the first step in accomplishing this would be to create *F. heteroclitus* x *F. diaphanus* and male F1 hybrids in a lab setting (individuals not found commonly in the wild), which Fritz & Garside (1974) have found to be possible.

I had a smaller sample size of both hybrids (n = 1- 5) and *F. diaphanus* (n = 5 - 7) compared to *F. heteroclitus* (n = 6 - 12) in my salinity treatments. Furthermore, all of my sample sizes were lower than the n = 10 fish per group used by Marshall et al. (2018) and n = 12 by Gibbons et al. (2017) in similar salinity tolerance studies. Thus, I may also have limited power to detect interspecific differences in salinity tolerance, which should be taken into account when interpreting my results. Following this preliminary study, I compared and investigated salinity tolerance at higher, hypersaline saltwater salinities of 45 ppt and 60 ppt to better characterize how salinity tolerance varies among species.

Hypersaline saltwater tolerance (45 – 60 ppt)

As predicted, *F. heteroclitus* were found to be very hypersaline saltwater tolerant as they showed no change in muscle water percentage or plasma Na^+ from 10 ppt controls after transfer to 45 or 60 ppt. While there was a slight increase in plasma Cl^- relative to the 10 ppt handling control at 60 ppt, *F. heteroclitus* were able to return to control values of plasma Cl^- after 8 days at 60 ppt (Appendix E). This is in agreement with the findings of Marshall et al. (2018), who found that plasma osmolarity returned to homeostasis after 7 days at 60 ppt in *F. heteroclitus*. Unfortunately, I did not have a sufficient sample size to test if *F. diaphanus* and F1 hybrids could return to homeostasis after a longer acclimation period, similar to our finding in *F. heteroclitus* (Appendix E), and this would be interesting to study in the future.

F. diaphanus could not maintain homeostasis after 24 hours at 60 ppt, as they showed significant increases in plasma Na^+ and Cl^- and decreases in muscle moisture content; these data support prior findings that *F. diaphanus* have a narrower salinity tolerance range and reduced hypersaline saltwater tolerance compared to *F. heteroclitus,* but can still survive in salinities up to 70 ppt (Griffith, 1974; Ahokas, 1975ab). I found that F1 clonal hybrid killifish from Porters Lake seem to have an intermediate hypersaline saltwater tolerance in comparison to their parental species. This is in line with other fish hybrids, including the hybrid Mozambique tilapia (Sardella et al., 2004), that have also displayed an intermediate hypersaline saltwater tolerance in comparison to parental species.

Taken together, these data suggest there may be a largely additive effect of salinity tolerance associated alleles, as I would expect hybrids to show a muscle water content phenotype more similar to only one parent if there was a completely dominant

effect of alleles contributing to muscle water retention in hypersaline saltwater. For example, if *F. heteroclitus* alleles were dominant over *F. diaphanus* alleles I would predict that hybrids would show a muscle water content more similar to *F. heteroclitus* than *F. diaphanus*. Though hybrids generally show an intermediate hypersaline saltwater tolerance at 60 ppt, they also appear show a bimodal distribution in muscle water content with half of the hybrids being more similar to *F. heteroclitus* parents and the other half being more similar to *F. diaphanus* parents at 60 ppt. However, plasma ion concentrations for hybrids at 60 ppt do not show the same bimodal distribution as the muscle water data, supporting the hypothesis that *F. diaphanus* and *F. heteroclitus* alleles have an additive effect on hybrid salinity tolerance. In order to truly determine if there is an additive effect of *F. heteroclitus* and *F. diaphanus* salinity tolerance alleles, future work could use QTL mapping in F2 hybrids (e.g. Kusakabe et al., 2017; Rahman et al., 2017). We also do not know if there are differences in *F. diaphanus* and *F. heteroclitus* allele specific mRNA content in hybrid killifish that might contribute to hypersaline saltwater tolerance and the observed intermediate salinity tolerance of hybrids compared to parental species, as we only measured total mRNA content for both alleles (See section 4.2 for a more details).

The F1 hybrid killifish in this experiment are primarily all female clones, that are from a variety of clonal lineages that populate Porters lake, Nova Scotia (Hernandez & Turgeon, 2007; Merette et al., 2009; Tirbhowan, 2019). I was unable to control for clonal lineages in this experiment because hybrids were collected from the wild and not bred in the lab. Fish from five different clonal lineages were used in this study, and 31% of the hybrids belonged to the same clonal lineage (Tirbhowan, 2019). At each salinity we had representative hybrids from a minimum of four different clonal lineages and I found that all hybrids were able to acclimate to and survive at salinities up to 60 ppt. In this study, I

was unable to test if hypersaline saltwater tolerance may differ between clonal lineages as I did not have sufficient sample sizes from each group. Future studies could determine if salinity tolerance differs between clonal lineages of *Fundulus diaphanus* x *F. heteroclitus* F1 hybrids by examining salinity tolerance in larger samples of multiple hybrid clonal lineages.

The potential effect of copper on salinity tolerance

As previously mentioned, the effect of salinity observed in this experiment may not be independent of potential copper toxicity, as the water used in 2017 and 2018 experiments was contaminated by copper ranging from 3 – 552 μg/L. While water was treated with a copper chelator (API® Stress Coat), there is still the possibility copper contamination affected our results. Copper is known to impair fish osmoregulation and growth (Crespo & Karnaky, 1983; Laurén & McDonald, 1987; Grossel et al., 2007), and the effects of copper exposure on killifish osmoregulation and salinity tolerance have been documented (Crespo & Karnaky, 1983; Adeyemi et al., 2013; Ransberry et al., 2015). Other studies have examined the synergistic effect of copper accumulation in the gill (0.9 - 23.4 –μg/L) and mild hypoxia on fish physiology and noted that the effect of both stressors was no more stressful than the effect of either stressor alone (Ransberry et al., 2016). This suggests that the effect of salinity tolerance alone may not have been significantly altered by the addition of copper stress.

It is important to note that most of the copper fluctuations that occurred during this experiment are actually near the range of (freshwater) aquatic environmentally relevant concentrations of copper (0.2 -300 μg/L; USEPA, 2007), suggesting that fish are

likely to experience the maximum potential concentrations of copper noted in this experiment in nature (Ransberry et al., 2016), however this concentration could still impair fish (Blaylock et al., 1984). Though copper concentrations of 150 ug/L in freshwater are lethal for fish such as the Least Killifish (*Heterandria Formosa;* Adeyemi et al., 2013*), F. heteroclitus* can tolerate concentrations of copper up to 200 μg/L without impairment of ionoregualtion in both freshwater and saltwater (Ransberry et al., 2015). In particular, Ransberry et al. (2015) found that exposure to copper concentrations between 50 μg/L – 200 μg/L did not significantly increase ion concentrations for *F. heteroclitus*, indicating that exposure to copper concentrations of 552 μg/L may have only minimally effected salinity tolerance of *Fundulus spp*. in my experiment.

The negative effect of copper on salinity tolerance is also known to be accentuated in freshwater in comparison to saltwater (Blanchard & Grosell 2005; Grosell et al., 2007), but this may not be true for the killifish used in my experiment. Ransberry et al. (2015) found that there were only slight differences in oxidative stress, metabolic responses and copper accumulation in the gills, liver and intestine for *F. heteroclitus* differentially acclimated to freshwater in comparison to saltwater. This suggests that the effects of copper in freshwater (0 ppt) may not be higher than those at higher salinities (10 ppt, 32 ppt, 45 ppt & 60 ppt), but no data is yet available on the physiological effects of copper in the more sensitive *F. diaphanus*.

A few observations from this experiment also suggests that the presence of copper may have only had a minimal effect on the salinity tolerance of experimental fish. Firstly, copper (0.15–0.20 mg/L Cu^{2+}) is often used to treat algae in marine aquaria (Cardeilhac &

Whitaker 1988; Yanong, 2010), but algae were observed in all experimental aquaria throughout the experiment in both 2017 & 2018.

It is known that multiple stressors can reduce a fish's tolerance to a focal stressor, as is the case for *F. heteroclitus* exposed to high temperatures and handling stress (reviewed by Schulte, 2014). If killifish in this experiment had to allocate energy to respond to both salinity and copper stress, I predict they would have a reduced ability to acclimate to hypersaline saltwater when compared to prior studies, due to the high energetic costs of such an acclimation (Gonzalez, 2012). I found that salinity tolerance for both *F. diaphanus* and *F. heteroclitus* fell within ranges documented by other studies (Fritz and Garside, 1974; Griffith, 1974; Ahokas, 1975ab), suggesting that they did not have a reduced ability to acclimate. However, this may not be the case (e.g. Ransberry et al., 2015). *F. diaphanus* were more sensitive to stressors during their housing period in the lab and generally more difficult to maintain, so if copper were to have a negative effect on any fish, we would predict that *F. diaphanus* would be the first and most affected by copper toxicity in combination with salinity stress. The fact that all *F. diaphanus* did not have a clearly reduced salinity tolerance in this experiment compared to prior studies (Fritz and Garside, 1974; Griffith, 1974; Ahokas, 1975ab) suggests that copper likely had a minimal effect on fish in this experiment.

Though there is support of only a minimal effect of copper on salinity tolerance, determining the amount of copper fish were exposed to will reveal the impact of copper on experimental observations of salinity tolerance in this experiment. Future work will expand on this project by measuring copper accumulation in samples of our fish's gills and liver with inductively coupled plasma atomic emission spectroscopy (ICP-AES). We will measure copper accumulation in fish used in the 2017 and 2018 experiments and

compare them to control fish who were not exposed to varying levels of copper but were fed the same diet and kept in reverse osmosis and deionized (RODI) water with artificial salt. Though this may not directly inform of the toxicity of copper for each fish, it could indicate if a significantly higher amount of copper was stored and taken up by the 2017 and 2018 experimental fish relative to control fish (Adeyemi et al., 2013). This will be measured in Fall 2019 at UNB Saint John, as samples of 2019 fish must first be acclimated to the same salinities as previous fish. Additionally, the collected information will be compared to water quality records of the aquatic facilities to get a more comprehensive understanding of copper accumulation in experimental fish (Appendix E). Overall, this will help us understand the degree to which copper may have affected the measurement of salinity tolerance in this experiment.

Though there are indicators and observations to suggest that fish were unaffected by the presence of copper, determining the amount of copper that fish were exposed to will be a critical follow-up experiment to help untangle the relationship between acute copper exposure and salinity tolerance in this experiment (See Section 4.1).

4.2 Mechanisms underlying hypersalinity tolerance in fishes

Changes in mRNA content of candidate genes

Marshall et al. (2018) found that *F. heteroclitus* acclimated to hypersaline saltwater (60 ppt) upregulated specific tight junction isoforms (i.e., *claudin-10c*, *claudin-10f*) and the cystic fibrosis transmembrane conductance regulator (CFTR) and he predicted that their combined upregulation is necessary for hypersaline saltwater acclimation. CFTR is an apical chloride channel that removes Cl^- and generates a gradient to draw Na^+ out of epithelial cells, while claudins *10c* and *10f* appear to form cation

selective pore junctions to allow Na^+ to exit epithelial cells. I therefore predicted that species who cannot maintain osmoregulatory and ionoregulatory homeostasis in hypersaline saltwater (i.e., *F. diaphanus* and hybrids) would show a reduced ability to upregulate pore forming claudins and *cftr*. I measured the mRNA content of these three candidate genes relative to two reference genes [elongation factor 1a (EF1α) and the 18S ribosomal RNA that have been used in isolation in prior studies; Scott et al., 2004; Marshall et al., 2018].

I found that, as in Marshall et al. (2018) hypersaline saltwater tolerance in *F. heteroclitus* was associated with significant increases in the mRNA abundance of *claudin-10c* and *cftr*, and a trend of increased *claudin-10f*. However, an upregulation of these genes did not occur at 60 ppt in less tolerant *F. diaphanus* and hybrids, in agreement with my predictions. This also agrees with other studies that document an association between the upregulation of *cftr* and hypersaline saltwater acclimation (Ouattara et al., 2009; reviewed by Gonzalez, 2012; Cozzi et al., 2015). However, I was unable to detect significant differences in mRNA abundance in *claudin-f* during hypersaline saltwater acclimation to 60 ppt for *F. heteroclitus*, in contrast to Marshall et al. (2018). The difference in findings could be attributed to pre-existing intra-specific differences in salinity tolerance, as we used different populations of *F. heteroclitus* than Marshall et al. (2018). We also had a lower sample size (n = 3 – 6), compared to Marshall et al., 2018 (n = 8), reducing our statistical power. As well, there was a wide variation in the relative mRNA abundance of *claudin-10f* for *F. heteroclitus* at 60 ppt and for *F. diaphanus* at 10 ppt, 45 ppt and 60 ppt (Fig. 3.2.3.3). All three species of fish were collected from the field where they may have been exposed to different salinities during their development, which is a factor that could potentially affect their ability to acclimate to various salinities as

adults (Beaman et al., 2016; Myosho et al., 2018). Though this may not explain why *F. diaphanus* have a higher overall variation in *claudin-10f* mRNA content, previous environmental and developmental conditions could lead to intra-specific differences in salinity tolerance and *claudin-10f* mRNA content.

Though there were no significant differences in *cftr* or *claudin 10c/f* mRNA content between the three species at 60 ppt, nor significant differences in the mRNA content of *claudin-10f* for *F. heteroclitus* relative to controls, the trends in the data do suggest that *F. heteroclitus* can upregulate the three candidate genes to a greater extent than the other two species at 60 ppt (Fig. 3.2.3A - C). Again, I likely did not detect significant differences between species at 60 ppt because of the low statistical power that came from having a smaller sample size (n = 3- 6 individuals per group) than Marshall et al., 2018 (n = 8 per group) and multiple between group comparisons. To increase our ability to detect differences in salinity tolerance between species, future studies could repeat this experiment with a higher sample size at 60 ppt, as I found salinity tolerance varied the most at this salinity.

While both hybrids and *F. diaphanus* do not significantly upregulate *cftr*, *claudin-10c* or *claudin-10f* at 45 - 60 ppt (compared to control salinities), they do show a slight increase with increasing salinity. In particular, *F. diaphanus* mRNA content is somewhat upregulated at 45 ppt, followed by a decline at 60 ppt, while hybrids show continued trend towards slightly increasing claudin and *cftr* mRNA content up to 60 ppt. My current qPCR assays do not differentiate *F. diaphanus* from *F. heteroclitus* alleles in hybrids; however, allele-specific mRNA content can be determined through the design of an additional qPCR assay to selectively amplify species-specific alleles. With this information, I could further test for a correlation between the expression of *F. heteroclitus*

alleles and overall tolerance in F1 hybrids. Additionally, we are uncertain if one of these candidate genes might be more important than others for overall hypersaline saltwater tolerance. Determining which species-specific alleles are more highly expressed in more tolerant hybrids could help us narrow down which specific *F. heteroclitus* genes are critical for hypersaline saltwater acclimation and also the molecular mechanisms regulating differences in mRNA content among species. For example, if *claudin 10-c F. heteroclitus* alleles are more highly expressed in hybrids relative to *F. diaphanus* alleles this will indicate that there is variation in *cis*-regulatory factors, such as transcription factor binding sites, leading to the upregulation of candidate genes in *F. heteroclitus* (e.g. Metzger et al., 2016). Whereas if species-specific allelic expression is the same in F1 hybrids, this is suggestive of *trans* regulatory effect evolution, as both alleles are in the same cellular environment in F1 hybrids. Overall, I hope that future experiments examining allele specific gene expression will help us better understand what limits hypersaline saltwater tolerance in hybrids.

The role of Claudin 10 isoforms in salinity tolerance

Marshall et al. (2018) hypothesized that that there may be salinity-specific roles for claudin 10 isoforms in fish ionoregulatory tissues. This idea corresponds with past studies indicating that claudin isoforms can have tissue specific roles in many vertebrates, including humans (Chasiotis et al., 2012, reviewed by Kolosov et al., 2013, Katayama et al., 2017). In particular, Marshall et al. (2018) found that *claudins 10-e* and *10-d* are the predominant claudin isoforms expressed during saltwater acclimation while *claudins 10-f* and *10-c* are the predominant isoforms expressed in hypersaline saltwater (Marshall et al., 2018). It was hypothesized that different pairings of claudin 10 isoforms (i.e.: *claudin-*

10c/*f* and *claudin-10d/e*) create transcellular pores with distinct properties, facilitation ion excretion across epithelial tissue in different salinities (Marshall et al., 2018).

The results of my study support the hypothesis that *claudin-10f* and *claudin-10c* isoforms generate stable transcellular pores for ion secretion in hypersaline saltwater and are associated with increased tolerance to very high salinities. Interestingly, *F. heteroclitus* showed a similar, approximate, 2-fold increase in the expression of both *claudin-10f* and *claudin-10c* compared to control genes at 60 ppt (Fig. 3.2.3B & C). However, less tolerant species did not show the same trend, as *F. diaphanus* showed a much higher expression in *claudin-10f*, compared to *claudin-10c* at 60 ppt. These data suggest that an upregulation of *claudin-10c* may be especially critical for the evolution of high salinity tolerance in *F. heteroclitus*, as they were the only species who were able to upregulate *claudin-10c* in 60 ppt and were the most tolerant to hypersaline salt water.

Though I have not yet examined the expression of *claudin-10c* and *10-f* in saltwater (32 ppt), nor *claudin-10d* and *10-e*, my findings add support to the hypothesis that there are isoform specific roles for the claudins, as I found that *claudin-10c* and *claudin-10f* were not significantly elevated until 60 ppt in *F. heteroclitus*. *Claudin-10f* and *claudin-10c* upregulation are important for saltwater acclimation in other species of fishes [e.g. Japanese Medaka (*Orizyas latipes)*, Bossus et al., 2015], indicating that further studies should be completed to examine the expression and role of *claudins 10-c*, *10-d*, *10-e* and *10-f* in *F. heteroclitus*, *F. diaphanus* and hybrids at 32 ppt and 60 ppt. By examining changes in the expression of these four claudin 10 isoforms in both saltwater and hypersaline saltwater we further can test if particular isoforms are associated with increased salinity tolerance and determine if the three *Fundulus spp.* show evidence of isoform specific roles for claudin 10 across different salinities.

Another consideration is that differences in salinity tolerance between species may be related to functional differences in *claudins 10-c* and *10-f* in addition to, or instead of, differences in gene expression. To test this, we have sequenced *F. diaphanus* and *F. heteroclitus claudin-10c and 10f* and are examining them for functional differences in *claudin-10c* isoform structure. Von Kursell (2019) found that there were no mutations in *claudin-10c* amino acid sequences at functionally important sites between the two species (von Kursell, 2019). Overall, von Kursell (2019) suggested that differences in hypersaline saltwater tolerance are not related to differences in *claudin-10c* permeability between *F. heteroclitus* and *F. diaphanus*, but other claudin 10 isoforms such as *10-e*, *10-d* and *10-f* should also be tested to determine if the evolution of gene function is associated with differences in salinity tolerance between species.

Other potential mechanisms contributing to salinity tolerance

I found that the ability to upregulate three candidate genes (*cftr*, *claudin-10c* and *claudin-10f*) was associated with increased hypersaline saltwater tolerance in *F. heteroclitus* compared to *F. diaphanus* and hybrids from Porters Lake, Nova Scotia. However, these are only three of many potential candidate 'hypersaline saltwater tolerance' genes. For example, *NKA* and *aquaporin 3* are two other genes that are known to be regulated in an isoform specific manner in fish during hypersaline challenges and may therefore also be important for hypersaline saltwater acclimation and adaptation in *Fundulus spp.* (Laverty & Skadhauge, 2012; Lam et al., 2014). To determine which additional genes are critical for the evolution of hypersaline saltwater tolerance in *Fundulus spp.,* future work should take an 'unbiased' approach to survey transcriptomic variation in both saltwater and hypersaline saltwater among species (e.g. using RNA-seq,

Gibbons et al., 2017). As well, genome scans and quantitative trait locus mapping studies could be used to find new candidate genes underlying salinity tolerance (e.g. Kusakabe et al., 2017). To more directly test if the current candidate genes, and potential new candidates are necessary for hypersaline saltwater tolerance, future work should knock-down or knock-out candidate hypersaline saltwater genes and test the effects on overall tolerance (e.g. Zimmer et al., 2018).

5. CONCLUSION

I found that F1 *F. diaphanus* x *F. heteroclitus* hybrid killifish have an intermediate tolerance to hypersaline saltwater in comparison to the less tolerant *F. diaphanus* and more tolerant *F. heteroclitus*. My measures of tolerance in *F. diaphanus* and *F. heteroclitus* are in accordance with past reports of salinity tolerance for other populations by Griffith (1974), Fritz & Garside (1974), Ahokas (1975ab), Whitehead (2010) and Marshall et al. (2018). These data suggest that alleles from *F. heteroclitus* and *F. diaphanus* that contribute to hypersaline saltwater tolerance have an additive effect in hybrids.

I also found that the ability to upregulate candidate genes (*cftr*, *claudin-10c* and *claudin-10f*) was uniquely associated with increased hypersaline saltwater tolerance in *F. heteroclitus* from Porters Lake, Nova Scotia, and not other, less tolerant *Fundulus* spp.. These data support the hypothesis that upregulating these candidate genes is critical for hypersaline saltwater tolerance (Marshall et al., 2018). By investigating the mechanisms underlying inter-specific differences in hypersaline saltwater acclimation we have increased our overall understanding of how hypersaline saltwater tolerance may evolve.

6. REFERENCES

Adeyemi, J. A., & Klerks, P. L. (2013). Occurrence of copper acclimation in the least killifish *Heterandria Formosa* and associated biochemical and physiological mechanisms. *Aquatic Toxicology*, *130*(1), 51-57.

Ahokas, R. A., & Duerr, F. G. (1975a). Salinity tolerance and extracellular osmoregulation in two species of euryhaline teleosts, *Culaea inconstans* and *Fundulus diaphanus*. *Comparative Biochemistry and Physiology Part A: Physiology*, *52*(3), 445-448.

Ahokas, R. A., & Duerr, F. G. (1975b). Tissue water and intracellular osmoregulation in two species of euryhaline teleosts *Culaea inconstans* and *Fundulus diaphanus*. *Comparative Biochemistry and Physiology Part A: Physiology, 52*(3), 449-454.

Ahokas, R. A., & Sorg, G. (1977). The effect of salinity and temperature on intracellular osmoregulation and muscle free amino acids in *Fundulus diaphanus*. *Comparative Biochemistry and Physiology Part A: Physiology*, *56*(1), 101-105.

Anderson, J. M. (2001). Molecular structure of tight junctions and their role in epithelial transport. *Physiology*, *16*(3), 126-130.

Aranda, P. S., LaJoie, D. M., & Jorcyk, C. L. (2012). Bleach gel: a simple agarose gel for analyzing RNA quality. *Electrophoresis*, *33*(2), 366-369.

Bagherie-Lachidan, M., Wright, S. I., & Scott, K. P. (2009). *Claudin-8* and -27 tight junction proteins in puffer fish *Tetraodon nigroviridis* acclimated to freshwater and seawater. *Journal of comparative physiology B: Biochemical, systemic, and environmental physiology*. *179*(4). 419-31.

Bates, D., Maechler, M., Bolker, B., & Walker, S. (2015). Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software*, 67(1), 1-48.

Beaman, J. E., White, C. R., & Seebacher, F. (2016). Evolution of plasticity: mechanistic link between development and reversible acclimation. *Trends in Ecology & Evolution*, *31*(3), 237-249.

Berg, P.R., Jentoft, S., Star, B., Ring, K.H., Knutsen, H., Lien, S., Jakobsen, K.S. & Andre, C. (2015). Adaptation to low salinity promotes genomic divergence in Atlantic cod (*Gadus morhua L.*). *Genome Biology and Evolution*, *7*(6), 1644-1663.

Bettoli, P. W., Neill, W. H., & Kelsch, S. W. (1985). Temperature preference and heat resistance of grass carp, Ctenopharyngodon idella (Valenciennes), bighead carp, Hypophthalmichthys nobilis (Gray), and their F1 hybrid. *Journal of Fish Biology*, *27*(3), 239-247.

Bio-Rad Laboratories, Inc. (2017) CFX Maestro Software User Guide, Version 1.0. Appendix B, 223-235. Philadelphia Pennsylvania, USA.

Bio-Rad Laboratories, Inc. (2017) iScript cDNA Synthesis Kit protocols. Philadelphia Pennsylvania, USA.

Birchler, J. A., & Veitia, R. A. (2007). The gene balance hypothesis: from classical

genetics to modern genomics. *The Plant Cell*, *19*(2), 395-402.

Blanchard, J., & Grosell, M. (2005). Effects of salinity on copper accumulation in the common killifish (*Fundulus heteroclitus*). *Environmental Toxicology and Chemistry: An International Journal*, *24*(6), 1403-1413.

Blaylock, B. G., Frank, M. L., & McCarthy, J. F. (1985). Comparative toxicity of copper and acridine to fish, Daphnia and algae. *Environmental Toxicology and Chemistry: An International Journal*, *4*(1), 63-71.

Blier, P. U., Dufresne, F., & Burton, R. S. (2001). Natural selection and the evolution of mtDNA-encoded peptides: evidence for intergenomic co-adaptation. *Trends in Genetics*, *17*(7), 400-406.

Bossus, M. C., Madsen, S. S., & Tipsmark, C. K. (2015). Functional dynamics of claudin expression in Japanese medaka (*Oryzias latipes*): Response to environmental salinity. *Comparative Biochemistry and Physiology Part A : Molecular and Integrative Physiology*, *187*(1), 74–85.

Brennan, R. S., Galvez, F., & Whitehead, A. (2015). Reciprocal osmotic challenges reveal mechanisms of divergence in phenotypic plasticity in the Killifish *Fundulus heteroclitus*. *Journal of Experimental Biology*, *218*(8), 1212–1222.

Brown, R. S., Cooke, S. J., Wagner, G. N., & Brad, M. (2010). *Methods for surgical implantation of acoustic transmitters in juvenile salmonids: a review of literature and guidelines for technique*. Pacific Northwest Laboratory, 1-87.

Bui, P.N., Bagherie-Lachidan, M., & Kelly, S.P. (2010). Cortisol differentially alters claudin isoforms in cultured puffer fish gill epithelia. *Molecular and Cellular Endocrinology, 317*(1-2), 120-126.

Bui, P.N. & Kelly, S.P. (2011). Claudins in a Primary Cultured Puffer Fish (*Tetraodon nigroviridis*) Gill Epithelium. *Methods in molecular biology*, *762*, 179-94.

Bui, P.N. & Kelly, S. P. (2014). Claudin-6, -10d and -10e contribute to seawater acclimation in the euryhaline puffer fish *Tetraodon nigroviridis*. *Journal of Experimental Biology*, *217*(10), 1758–1767.

Burnett, K.G., Bain, L.J., Baldwin, W.S., Callard, G.V., Cohen, S., Giulio, R.T., Evans, D.H., Gómez-Chiarri, M., Hahn, M.E., Hoover, C.A., Karchner, S.I., Katoh, F., Maclatchy, D.L., Marshall, W.S., Meyer, J.N., Nacci, D.E., Oleksiak, M.F., Rees, B.B., Singer, T.D., Stegeman, J.J., Towle, D.W., Van Veld, P.A., Vogelbein, W.K.,

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller R., Nolan, T., Pfaffl M. W., Shipley, G. L., Vandesompele, J., & Wittwer, C. T. (2009). The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry*, *55*(4), 611–622.

Burton, R. (1990). Hybrid Breakdown in Developmental Time in the Copepod *Tigriopus californicus*. *Evolution*, *44*(7), 1814-1822.

Cardeilhac, P.T. & B.R. Whitaker. (1988). Copper treatments: uses and precautions. In Tropical fish Medicine. Stoskopf, M.K., ed. The Veterinary Clinics of North America: Small Animal Practice *18*(2): 435–448.

Chasiotis, H., Kolosov, D., Bui, P., & Kelly, S. P. (2012). Tight junctions, tight junction

proteins and paracellular permeability across the gill epithelium of fishes: A review. *Respiratory Physiology and Neurobiology*, *184*(3), 269–281.

Cossins, A. R., & Crawford, D. L. (2005). Fish as models for environmental genomics. *Nature Reviews Genetics*, *6*(4), 324.

Cozzi, R. R. F., Robertson, G. N., Spieker, M., Claus, L. N., Zaparilla, G. M. M., Garrow, K. L., & Marshall, W. S. (2015). Paracellular pathway remodeling enhances sodium secretion by teleost fish in hypersaline environments. *Journal of Experimental Biology*, *218*(8), 1259–1269.

Crespo, S. & Karnaky, K. J., (1983). Copper and zinc inhibit chloride transport across the opercular epithelium of seawater-adapted Killifish *Fundulus heteroclitus*. *Journal of Experimental Biology 102*(1), 337-341.

Dawley, R. M. (1992). Clonal hybrids of the common laboratory fish *Fundulus heteroclitus*. *Proceedings of the National Academy of Sciences of the United States of America*, *89*(6), 2485–8.

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

Divino, J. N., Monette, M. Y., McCormick, S. D., Yancey, P. H., Flannery, K. G., Bell, M. A., Rollins, J.L., von Hippel, F.A. & Schultz, E.T. (2016). Osmoregulatory physiology and rapid evolution of salinity tolerance in threespine stickleback recently introduced to fresh water. *Evolutionary Ecology Research*, *17*(2), 179-201.

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

Edwards, S. L., & Marshall, W. S. (2012). *Principles and Patterns of Osmoregulation and Euryhalinity in Fishes*. *Fish Physiology* (Vol. 32 pp. 2–32). Elsevier Inc.

Epifanio, J. M., Hooe, M., Buck, D. H., & Philipp, D. P. (1999). Reproductive success and assortative mating among *Pomoxis* species and their hybrids. *Transactions of the American Fisheries Society*, *128*(1), 104-120.

Evans, D. H., Piermarini, P. M., & Choe, K. P. (2005). The Multifunctional Fish Gill: Dominant Site of Gas Exchange, Osmoregulation, Acid-Base Regulation, and Excretion of Nitrogenous Waste. *Physiological Reviews*, *85*(1), 97–177.

Fox, R. J., Donelson, J. M., Schunter, C., Ravasi, T., & Gaitán-Espitia, J. D. (2019). Beyond buying time: the role of plasticity in phenotypic adaptation to rapid environmental change. *Philosophical Transactions of the Royal Society B: Biological sciences, 374*(1768), 20180174.

Fritz, E. S., & Garside, E. T. (1974). Salinity preferences of *Fundulus heteroclitus* and *F. Diaphanus* (Pisces Cyprinodontidae): their role in geographic distribution. *Canadian Journal of Zoology*, *52*(8), 997–1003.

Fritz, E. S., & Garside, E. T. (1975). Comparison of age composition, growth, and

fecundity between two populations each of *Fundulus heteroclitus* and *F. diaphanus* (Pisces: Cyprinodontidae). *Canadian Journal of Zoology, 53*(4), 361-369.

Furukawa, F., Watanabe, S., Kimura, S., & Kaneko, T. (2011). Potassium excretion through ROMK potassium channel expressed in gill mitochondrion-rich cells of Mozambique tilapia. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *302*(5), R568-R576.

Garland, T., Bennett, A. F., & Rezende, E. L. (2005). Phylogenetic approaches in comparative physiology. *Journal of experimental Biology*, *208*(16), 3015-3035.

Garside, E. T., & Jordan, C. M. (1968). Upper lethal temperatures at various levels of salinity in the euryhaline cyprinodontids *Fundulus heteroclitus* and *F. diaphanus* after isosmotic acclimation. *Journal of the Fisheries Board of Canada*, *25*(12), 2717–2720.

Ghalambor, C. K., McKay, J. K., Carroll, S. P. & Reznick, D. N. (2007). Adaptive *versus* non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments. *Functional Ecology, 21*(3), 394-407.

Ghedotti, M. J., & Davis, M. P. (2017). The taxonomic placement of three fossil *Fundulus* species and the timing of divergence within the North American topminnows (Teleostei: *Fundulidae*). *Zootaxa*, *4250*(6), 577-586.

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

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

Gonzalez, R. J., Cooper, J. & Head, D. (2005). Physiological responses to hyper-saline waters in sailfin mollies (*Poecilia latipinna*). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 142*(4), 397-403.

Gonzalez, R. J. (2012). The physiology of hyper-salinity tolerance in teleost fish: A review. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology*, *182*(3), 321–329.

Gotthard, K. & Nylin, S. (1995). Adaptive plasticity and plasticity as an adaptation: a selective review of plasticity in animal morphology and life history. *Oikos*, *74*(1), 3–17.

Griffith, R. W. (1974). Environment and Salinity Tolerance in the Genus *Fundulus*. *Copeia*, *1974*(2), 319-331.

Grosell, M., Blanchard, J., Brix, K. V. & Gerdes, R. (2007). Physiology is pivotal for interactions between salinity and acute copper toxicity to fish and invertebrates. *Aquatic Toxicology*, *84*(2), 162-172.

Hasan, M. M., DeFaveri, J., Kuure, S., Dash, S. N., Lehtonen, S., Merilä, J., & McCairns, R. J. S. (2017). Sticklebacks adapted to divergent osmotic environments show differences in plasticity for kidney morphology and candidate gene expression. *The*

Journal of Experimental Biology, *220*(12), 2175–2186.

Hernández Chávez, C., & Turgeon, J. (2007). Asexual and sexual hybrids between *Fundulus diaphanus* and *F. heteroclitus* in the Canadian Atlantic region. *Molecular Ecology*, *16*(7), 1467-1480.

Huey, R. B., Berrigan, D., Gilchrist, G. W. & Herron, J. C. (1999). Testing the adaptive significance of acclimation: a strong inference approach. *American Zoologist, 39*(2), 323-336.

Hwang, P. P., Lee, T. H. & Lin, L.Y. (2011). Ion regulation in fish gills: recent progress in the cellular and molecular mechanisms. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *301*(1), R28-R47.

Karpechenko, G. D. (1927). The production of polyploid gametes in hybrids. *Hereditas*, *9*(1-3), 349-368.

Katayama, A., Handa, T., Komatsu, K., Togo, M., Horiguchi, J., Nishiyama, M., & Oyama, T. (2017). Expression patterns of claudins in patients with triple-negative breast cancer are associated with nodal metastasis and worse outcome. *Pathology International*, *67*(8), 404-413.

Katoh, F., Hyodo, S., & Kaneko, T. (2003). Vacuolar-type proton pump in the basolateral plasma membrane energizes ion uptake in branchial mitochondria-rich cells of killifish *Fundulus heteroclitus*, adapted to a low ion environment. *Journal of Experimental Biology*, *206*(5), 793-803.

King, S. (2009). Ecology and taxonomy of ectoparasites infecting sympatric *Fundulus heteroclitus*, *F. diaphanus* and their asexual hybrid in two Nova Scotian lakes. Masters Thesis in Biology, Saint Mary's University.

Kolosov, D., Bui, P., Chasiotis, H., & Kelly, S. P. (2013). Claudins in teleost fishes. *Tissue Barriers*, *1*(3), e25391.

Kültz, D., Jürss, K. & Jonas, L. (1995). Cellular and epithelial adjustments to altered salinity in the gill and opercular epithelium of a cichlid fish (*Oreochromis mossambicus*). *Cell and Tissue Research, 279*(1), 65-73.

Kültz, D. (2012). Osmosensing. In *Fish Physiology* (Vol. 32, pp. 45–68). Elsevier Inc.

Kültz, D., Li, J., Gardell, A. & Sacchi, R., (2013). Quantitative molecular phenotyping of gill remodeling in a cichlid fish responding to salinity stress. *Molecular & Cellular Proteomics*, *12*(12), 3962-3975.

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

Kusakabe, M., Ishikawa, A., Ravinet, M., Yoshida, K., Makino, T., Toyoda, A., Fujiyama, A. & Kitano, J. (2017). Genetic basis for variation in salinity tolerance between stickleback ecotypes. *Molecular Ecology*, *26*(1), 304-319.

Lam, S. H., Lui, E. Y., Li, Z., Cai, S., Sung, W. K., Mathavan, S., Lam, T. J. & Ip, Y. K. (2014). Differential transcriptomic analyses revealed genes and signaling pathways involved in iono-osmoregulation and cellular remodeling in the gills of euryhaline Mozambique tilapia, O*reochromis mossambicus*. *BMC Genomics*, *15*(1), 921.

Laurén, D. J., & McDonald, D. G. (1987). Acclimation to copper by rainbow trout, *Salmo gairdneri*: biochemistry. *Canadian Journal of Fisheries and Aquatic Sciences*, *44*(1), 105-111.

Laverty, G., & Skadhauge, E. (2012). Adaptation of teleosts to very high salinity. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *163*(1), 1-6.

Lenth, R. (2019). emmeans: Estimated Marginal Means, aka Least-Squares Means. R package version 1.3.3. https://CRAN.R-project.org/package=emmeans

Li, Z., Lui, E. Y., Wilson, J. M., Ip, Y.K., Lin, Q., Lam, T. J. & Lam, S. H. (2014). Expression of key ion transporters in the gill and esophageal-gastrointestinal tract of euryhaline Mozambique tilapia *Oreochromis mossambicus* acclimated to fresh water, seawater and hypersaline water. *PLoS One*, *9*(1), e87591.

Loh, Y. H., Christoffels, A., Brenner, S., Hunziker, W. & Venkatesh, B. (2004). Extensive expansion of the claudin gene family in the teleost fish, *Fugu rubripes*. *Genome Research*, *14*(7), 1248–1257.

Lorin-Nebel, C., Avarre, J. C., Faivre, N., Wallon, S., Charmantier, G. & Durand, J. D. (2012). Osmoregulatory strategies in natural populations of the black-chinned tilapia *Sarotherodon melanotheron* exposed to extreme salinities in West African estuaries. *Journal of Comparative Physiology B*, *182*(6), 771-780.

Marshall, W. S., & Singer, T. D. (2002). Cystic fibrosis transmembrane conductance regulator in teleost fish. *Biochimica et Biophysica Acta: Biomembranes*, *1566*(1-2), 16-27.

Marshall, W. S., Lynch, E. M., & Cozzi, R. R. F. (2002). Redistribution of immunofluorescence of CFTR anion channel and NKCC cotransporter in chloride cells during adaptation of the Killifish *Fundulus heteroclitus* to sea water. *Journal of Experimental Biology*, *205*(9), 1265–1273.

Marshall, W. S., Cozzi, R. R. F., & Strapps, C. (2008). Fish louse *Argulus funduli* (Crustacea: Branchiura) ectoparasites of the euryhaline teleost host, *Fundulus heteroclitus* , damage the ion-transport capacity of the opercular epithelium. *Canadian Journal of Zoology*, *86*(11), 1252–1258.

Marshall, W. S. (2012). *Osmoregulation in Estuarine and Intertidal Fishes*. *Fish Physiology* (Vol. 32 pp. 396–423). Elsevier Inc.

Marshall, W. S., Breves, J. P., Doohan, E. M., Tipsmark, C. K., Kelly, S. P., Robertson, G. N., & Schulte, P. M. (2018). *claudin-10* isoform expression and cation selectivity change with salinity in salt-secreting epithelia of *Fundulus heteroclitus*. *Journal of Experimental Biology*, *221*(1), jeb168906.

McCormick, S. D.,Stephen D., Farrell, A. P., & Brauner, C. J. (2013). *Euryhaline fishes*. Academic Press.

Mérette, D. (2009). Diversité génétique et morphologique des hybrides asexués *Fundulus diaphanus* x *F. heteroclitus*. Master's Thesis in Biology, Univeristé Laval.

Mérette, D., Bourret, A., & Turgeon, J. (2009). Diversity and distribution of gynogenetic

hybrids between *Fundulus diaphanus* and *Fundulus heteroclitus* in Porters Lake (Nova Scotia) in relation to salinity. *Journal of Fish Biology*, *75*(5), 1115–1122.

Morales, M., with code developed by the R Development Core Team, with general advice from the R-help listserv community and especially Duncan Murdoch, (2017). sciplot: Scientific Graphing Functions for Factorial Designs. R package version1.1-1. https://CRAN.R-project.org/package=sciplot

Murren, C. J., Auld, J. R., Callahan, H., Ghalambor, C. K., Handelsman, C. A., Heskel, M. A., Pfennig, D. W., Relyea, R. A., Seiter, S., Snell-Rood, E., Steiner, U. K. & Schlichting, C. D. (2015). Constraints on the evolution of phenotypic plasticity: limits and costs of phenotype and plasticity. *Heredity*, *115*(4), 293-301.

Myosho, T., Takahashi, H., Yoshida, K., Sato, T., Hamaguchi, S., Sakamoto, T. & Sakaizumi, M. (2018). Hyperosmotic tolerance of adult fish and early embryos are determined by discrete, single loci in the genus *Oryzias*. *Scientific reports*, *8*(1), 6897.

Neaves, W. B., & Baumann, P. (2011). Unisexual reproduction among vertebrates. *Trends in Genetics*, *27*(3), 81-88

Ouattara, N., Bodinier, C., Nègre-Sadargues, G., D'Cotta, H., Messad, S., Charmantier, G., Panfill, J. & Baroiller, J. F. (2009). Changes in gill ionocyte morphology and function following transfer from fresh to hypersaline waters in the tilapia *Sarotherodon melanotheron*. *Aquaculture*, *290*(1–2), 155–164.

Pfaffl M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic acids research*, *29*(9), e45.

Ransberry, V. E., Morash, A. J., Blewett, T. A., Wood, C. M., & McClelland, G. B. (2015). Oxidative stress and metabolic responses to Copper in freshwater-and seawater-acclimated Killifish, *Fundulus heteroclitus*. *Aquatic Toxicology*, *161*(1), 242-252.

Ransberry, V. E., Blewett, T. A., & McClelland, G. B. (2016). The oxidative stress response in freshwater-acclimated killifish (*Fundulus heteroclitus*) to acute copper and hypoxia exposure. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *179*(1), 11-18.

Reid, N. M., Jackson, C. E., Gilbert, D., Minx, P., Montague, M. J., Hampton, T. H., Karchner, S. I., Colbourne, J. K., Hahn, M. E., Shaw, J.R., Oleksiak, M.F., Crawford, D.L., Warren, W.C. & Whitehead, A. (2017). The landscape of extreme genomic variation in the highly adaptable Atlantic Killifish. *Genome Biology and Evolution*, *9*(3), 659-676.

RStudio (2017). RStudio: Integrated development environment for R (Version 1.1.383) [Computer software]. Boston, MA. Available from http://www.rstudio.org/

R Core Team (2018). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.

Sales, N. dos S., Baeta, A. S. B. V., de Lima, L. G. & Pessanha, A. L. M. (2018). Do the

shallow-water habitats of a hypersaline tropical estuary act as nursery grounds for fishes? *Marine Ecology*, *39*(1), e12473.

Sardella, B.A., Matey, V., Cooper, J., Gonzalez, R.J. & Brauner, C.J. (2004). Physiological, biochemical and morphological indicators of osmoregulatory stress in California Mozambique tilapia (*Oreochromis mossambicus* × *O. urolepis hornorum*) exposed to hypersaline water. *Journal of Experimental Biology*, *207*(8), 1399-1413.

Schulte, P. M. (2007). Responses to environmental stressors in an estuarine fish: Interacting stressors and the impacts of local adaptation. *Journal of Thermal Biology*, *32*(3), 152–161.

Schulte, P. M. (2014). What is environmental stress? Insights from fish living in a variable environment. *Journal of Experimental Biology*, *217*(1), 23–34.

Scott, G. R., Rogers, J. T., Richards, J. G., Wood, C. M., & Schulte, P. M. (2004). Intraspecific divergence of ionoregulatory physiology in the euryhaline teleost F*undulus heteroclitus*: possible mechanisms of freshwater adaptation. *Journal of Experimental Biology*, *207*(19), 3399-3410.

Schauer, K.L., Reddam, A., Xu, E.G., Wolfe, L.M. & Grosell, M. (2018). Interrogation of the Gulf toadfish intestinal proteome response to hypersalinity exposure provides insights into osmoregulatory mechanisms and regulation of carbonate mineral precipitation. *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics*, *27*(1), 66-76.

Somero, G. N. (2000). Unity in diversity: a perspective on the methods, contributions, and future of comparative physiology. *Annu. Rev. Physiol. 62*(1), 927–937

Somero, G. N. (2010). Comparative physiology: a "crystal ball" for predicting consequences of global change. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *301*(1), R1-R14.

Storz, J. F., Bridgham, J. T., Kelly S. A. & Garland, T. (2015). Genetic approaches in comparative and evolutionary physiology. *American Journal of Physiology – Regulatory, Integrative, and Comparative Physiology 309*(3), R197-R214

Taylor, S., Wakem, M., Dijkman, G., Alsarraj, M., & Nguyen, M. (2010). A practical approach to RT-qPCR—publishing data that conform to the MIQE guidelines. *Methods*, *50*(4), S1-S5.

Tipsmark, C. K., Kiilerich, P., Nilsen, T. O., Ebbesson, L. O. E., Stefansson, S. O. & Madsen, S. S. (2008). Branchial expression patterns of claudin isoforms in Atlantic salmon during seawater acclimation and smoltification. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *294*(5), R1563–R1574.

Tirbhowan, S. (2019). Temporal stability of Killifish hybrid clonal lineages. Honours Thesis in Biology, Saint Mary's University.

USEPA, 2007. Aquatic Life Ambient Freshwater Quality Criteria-Copper 2007 Revision. EPA-822-R-07-001. https://www.epa.gov/wqc/aquatic-life-ambient-freshwater-

quality-criteria-copper-2007-revision

Velotta, J. P., Wegrzyn, J. L., Ginzburg, S., Kang, L., Czesny, S., O'Neill, R. J., McCormick, S. D., Michalak, P. & Schultz, E. T. (2017). Transcriptomic imprints of adaptation to fresh water: parallel evolution of osmoregulatory gene expression in the Alewife. *Molecular Ecology*, *26*(3), 831–848.

von Kursell, A. (2019). Are differences in *claudin-10c* isoform structure and expression associated with variation in salinity tolerance among species of Killifish? Honours Thesis in Biology, Saint Mary's University.

Weaver, P.F., Tello, O., Krieger, J., Marmolejo, A., Weaver, K.F., Garcia, J.V. & Cruz, A. (2016). Hypersalinity drives physiological and morphological changes in *Limia perugiae* (Poeciliidae). *Biology open*, *5*(8), 1093-1101.

Whitehead, A., Winn, R.N., & Crawford, D.L., (2007). *Fundulus* as the premier teleost model in environmental biology: opportunities for new insights using genomics. *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics*, *2*(4), 257–286.

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

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

Whitehead, A. (2012). Comparative genomics in ecological physiology: toward a more nuanced understanding of acclimation and adaptation. *Journal of Experimental Biology*, *215*(6), 884–891.

Whitehead, A., Zhang, S., Roach, J. L., & Galvez, F. (2013). Common functional targets of adaptive micro- and macro-evolutionary divergence in Killifish. *Molecular Ecology*, *22*(14), 3780-3796.

Wickham, H., (2016) ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York.

Yanong, R. P. (2010). Use of copper in marine aquaculture and aquarium systems. *Institute of Food and Agricultural Sciences (IFAS), University of Florida, Gainesville, Florida*.

Zimmer, A. M., Dymowska, A. K., Kumai, Y., Goss, G. G., Perry, S. F., & Kwong, R. W. (2018). Assessing the role of the acid-sensing ion channel ASIC4b in sodium uptake by larval zebrafish. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *226*(1), 1-10.

Zimmer, A. M., Brix, K. V., & Wood, C. M. (2019). Mechanisms of Ca^{2+}uptake in freshwater and seawater-acclimated killifish, *Fundulus heteroclitus*, and their response to acute salinity transfer. *Journal of Comparative Physiology B*, *189*(1), 47-60.

Zuker, M., (2003). Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acid Res*. *31*(13), 3406-15.

APPENDIX

Appendix A

Abiotic factors recorded during the collection of *Fundulus spp.* in 2017 and 2018

Both Salinity and temperature were recorded at multiple collection sites (Table A) in Porters Lake, Nova Scotia over the course of the 2017 and 2018 field seasons. Salinity ranged from 0.3-16 ppt and the temperature of the water ranged from 16.5-30.4 °C during the 2017 field season (Fig. A1, A2). Salinity ranged from 7.1-16.5 ppt and the temperature of the water ranged from 15.3 -28.6 °C during the 2018 field season (Fig. A3, A4).

Table A1 Co-ordinates for sampling locations in Porters Lake, NS in 2017 and 2018.

Location	Year sampled	Latitude and Longitude	Studies including these sites
Site 1	2017 & 2018	44.785135 °N, -63.360340 °W	Current
Site 2	2017 & 2018	44.75001 °N, -63.305684 °W	Mérette et al. (2009), current study
Site 3	2017 & 2018	44.703427 °N, -63.286436 °W	Mérette et al. (2009), current study
Site 4	2017 & 2018	44.68825 °N, -63.29342 °W	Current
Site 5	2017 & 2018	44.684037 °N, -63.302109 °W	Current
Site 6	2017 & 2018	44.681442 °N, -63.305411 °W	Current
Site 7	2017 & 2018	44.645063 °N, -63.325435 °W	Mérette et al. (2009), current study

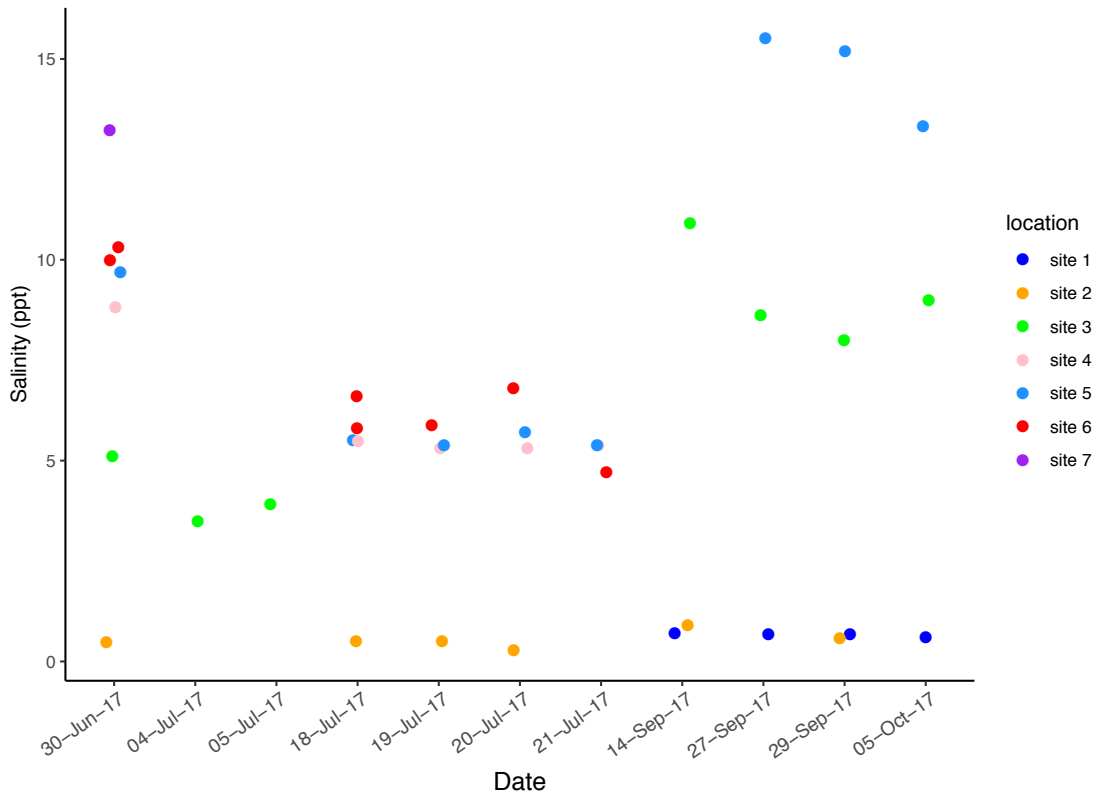

Figure A1. Range of salinities (ppt) at Porters Lake across multiple sampling locations from June 30th, 2017 to October 5th, 2017. Each sampling location is represented by a different colour and experiences a range of salinities across sampling times. Site 1 and 2 are the freshwater sites and all other sites are brackish water sites. Site 3 was the main sampling location and shows a trend of increasing salinity over time and site 5 also shows increasing salinity over time. All other locations show a decrease in salinity over time, except for the freshwater sites in which salinity remained constant. Locations are shown in Figure 2.1 and Table A1 and salinity was measured at depth, approximately 1- 3 meters from the shoreline.

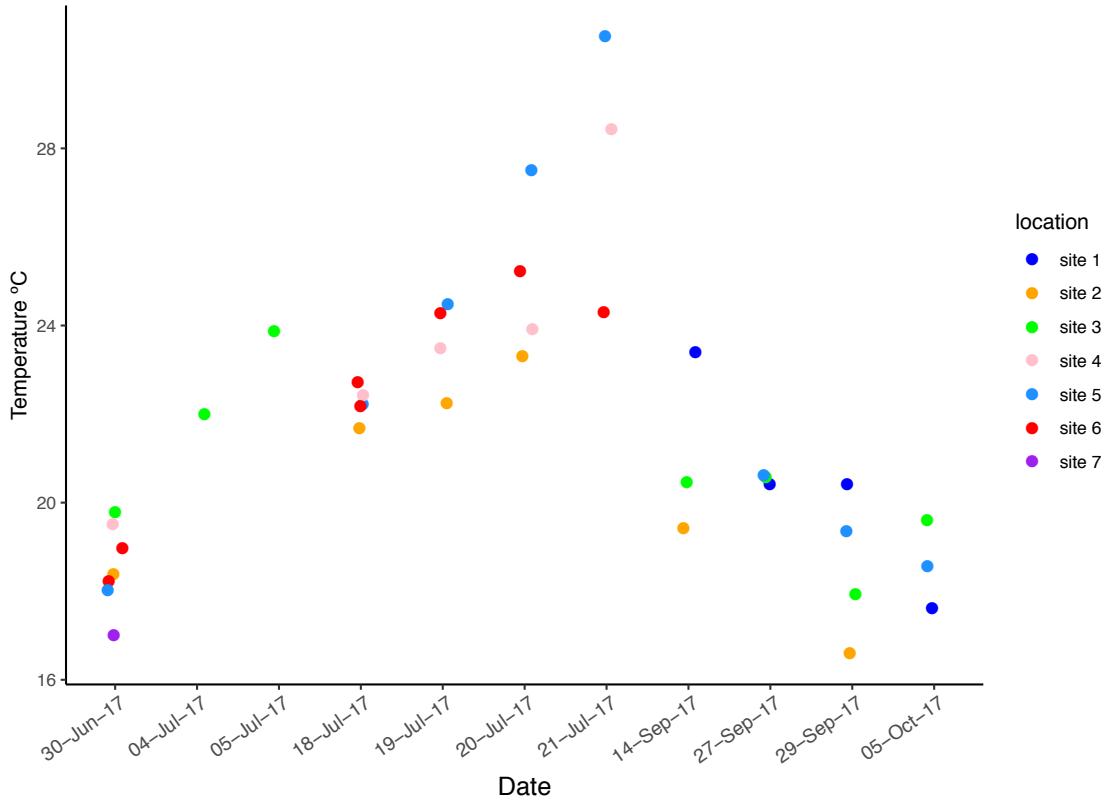

Figure A2. Range of water temperatures (°C) at Porters Lake across multiple sampling locations from June 30th, 2017 to October 5th, 2017. Each sampling location is represented by a different colour and experiences a range of temperatures at across sampling times. There is an overall trend of increasing temperature with time in July and decreasing temperature in the fall months (September-October). Locations are shown in Fig. 2.1 and Table A1 and salinity was measured at depth, approximately 1- 3 meters from the shoreline.

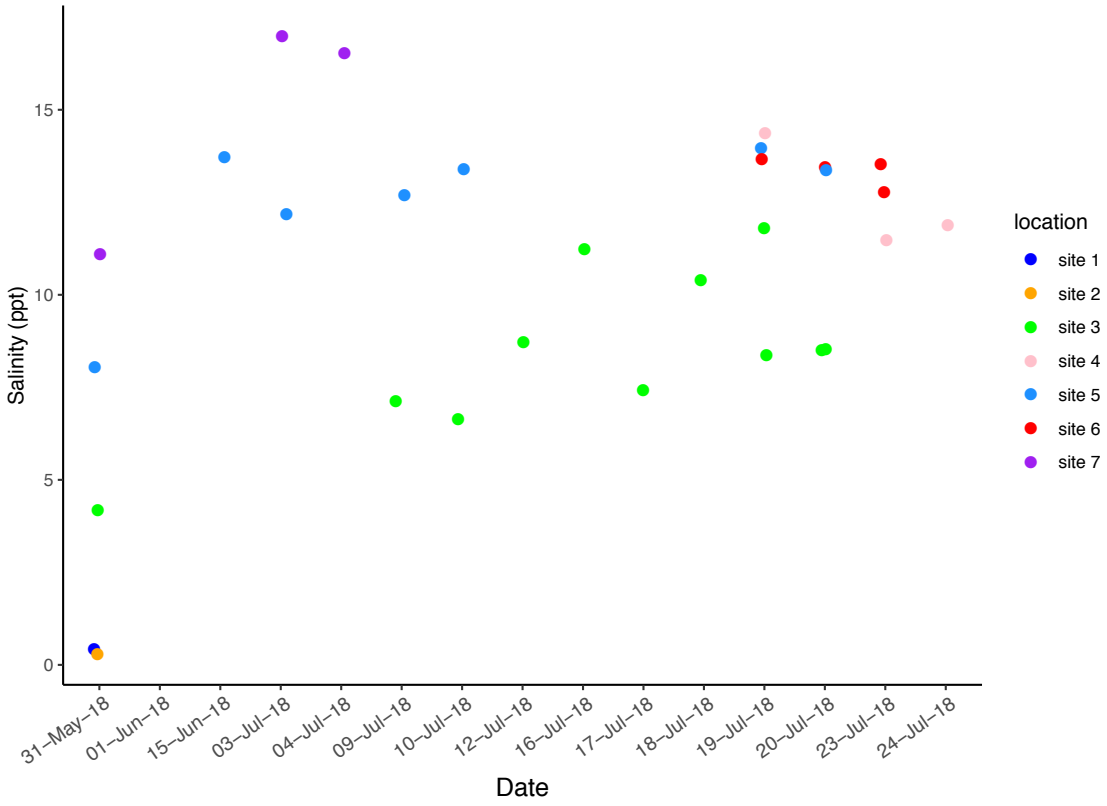

Figure A3. Range of water salinities at Porters Lake across multiple sampling locations from May 31st, 2018 to July 24th, 2018. Each sampling location is represented by a different colour and experiences a range of salinities across sampling times. Site 1 and 2 are the freshwater sites and all other sites are brackish water sites. Site 3 was the main sampling location and shows a trend of increasing salinity over time. Locations are shown in Figure 2.1 and Table A1 and salinity was measured at depth, approximately 1- 3 meters from the shoreline.

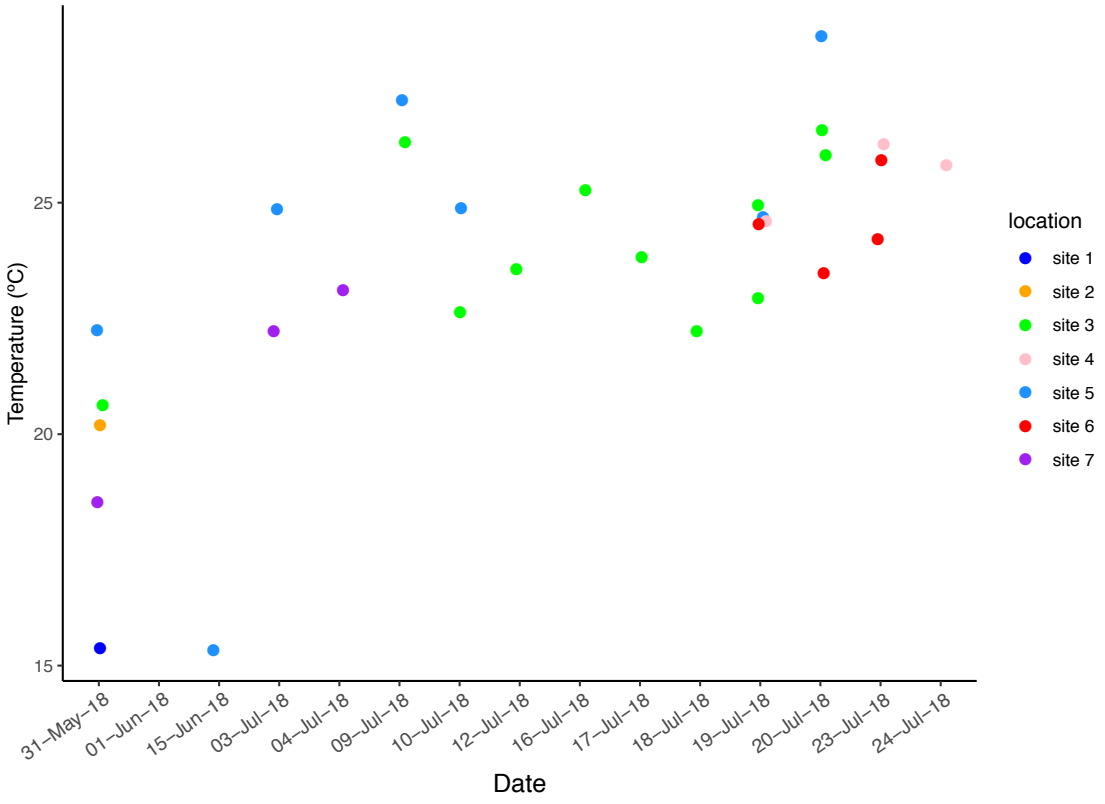

Figure A4. Range of water temperatures (°C) at Porters Lake across multiple sampling locations from May 31st, 2018 to July 24th, 2018. Each sampling location is represented by a different colour and experiences a range of temperatures at across sampling times. There is an overall trend of increasing temperature over time. Locations are shown in Figure 2.1 and Table A1 and water temperature was measured at depth, approximately 1-3 meters from the shoreline.

Appendix B

Assessment of RNA quality using a 1% Agarose Bleach Gel

A bleach gel was used to visualize the quality of the RNA and further test for DNA contamination. DNA contamination is assessed by looking for a large product on the gel which appears as a band near the top of the gel (Taylor et al., 2010) (Fig. B). The quality of RNA can be determined by locating both 28S ribosomal RNA subunit and 18S ribosomal RNA subunit bands on the bleach gel and comparing the ratio of the two bands. The 18S band is smaller than the 28S band and appears near the bottom of the gel (Fig. B). Good quality RNA is indicated by the presence of both bands with intensity and brightness of the 28S band being twice that of the 18S band (ie: ratio of 2:1), (Taylor et al., 2010) (Fig. B). Each gel image was assessed individually in Image Lab™ (BioRad).

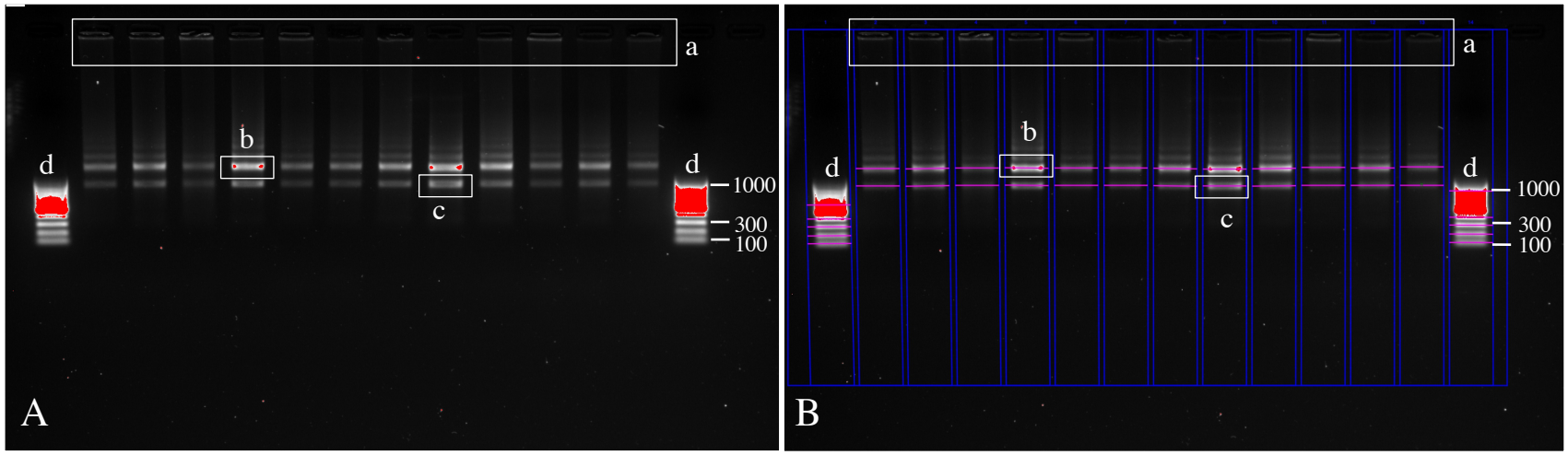

Figure B. Example of a bleach gel used to assess quality of RNA samples before lane and band detection was performed (A) and after (B) (Image Lab™). All wells within the box (a) contain approximately 1 ng of RNA. Genomic DNA contamination would be expected to appear at the top of the gel near the wells (box a). The (28S) ribosomal rRNA sub-unit 28 band (box b) appears higher on the gel than the 18S ribosomal rRNA sub-unit 18 (box c) and with good quality RNA is twice the intensity of the 18S band, as pictured above. A 100bp ladder (well d) (7.5µg) was included on each gel to measure approximate band size.

Appendix C

Example of a melt curve during the generation of a standard curve with qPRC

Melt curves were assessed during each standard curve qPCR and experimental qPCR to assure that levels of background and genomic DNA contamination were not high and to verify the amplification of a single product. An example melt curve generated using CFX Maestro software (BioRad) is pictured in Figure C and demonstrates primers that successfully amplify a single product.

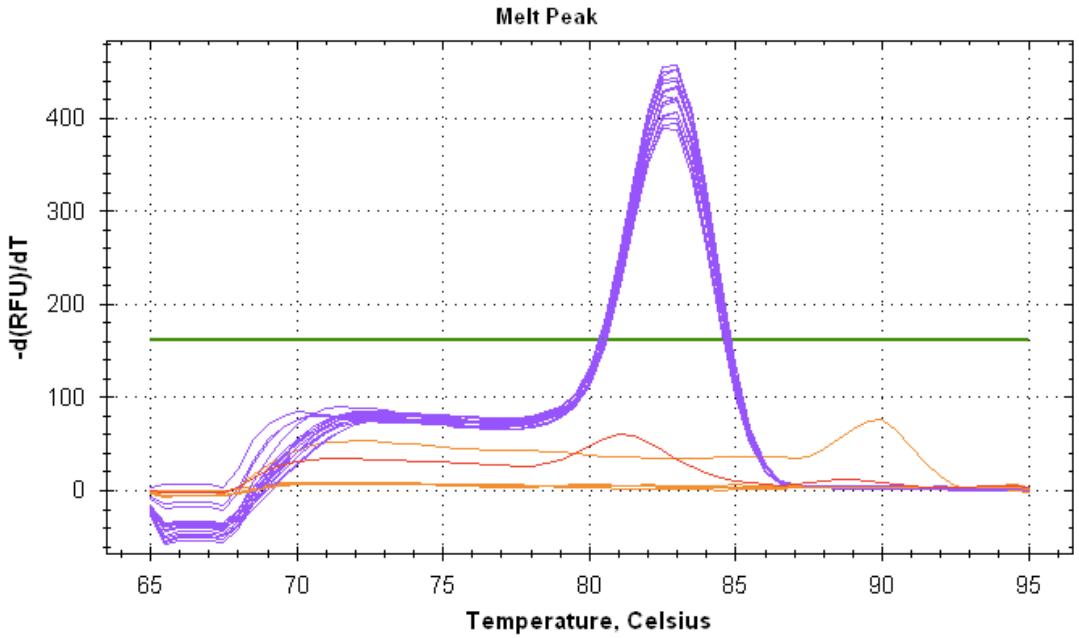

Figure C. Example of a melt curve generated during a standard curve qPCR assay using primers that amplify EF1α. Only one peak appears in the melt curve at ~82.5°C and displays that only one product was amplified and therefore primers are adequate for use in future qPCRs. Additionally, non-reverse-transcribed controls (NRT) and no-template controls (NTC) can be seen as lightly coloured bumpy lines near y = 0 on the graph and confirm that there is no genomic or random contamination in the qPCR assay, respectively. Threshold was manually set and is indicated by a straight line at y = 160. The image was generated in CFX Maestro (BioRad).

Appendix D

Relative Cq values for reference genes

The stability of reference genes was verified by visualizing the relative change in Cq values across experimental treatments. A good reference gene will show no or little change in relative Cq values across experimental treatments. Figure D1 shows the relative Cq values for *18S* across treatments and Figure D2 shows the relative Cq values for *EF1α*.

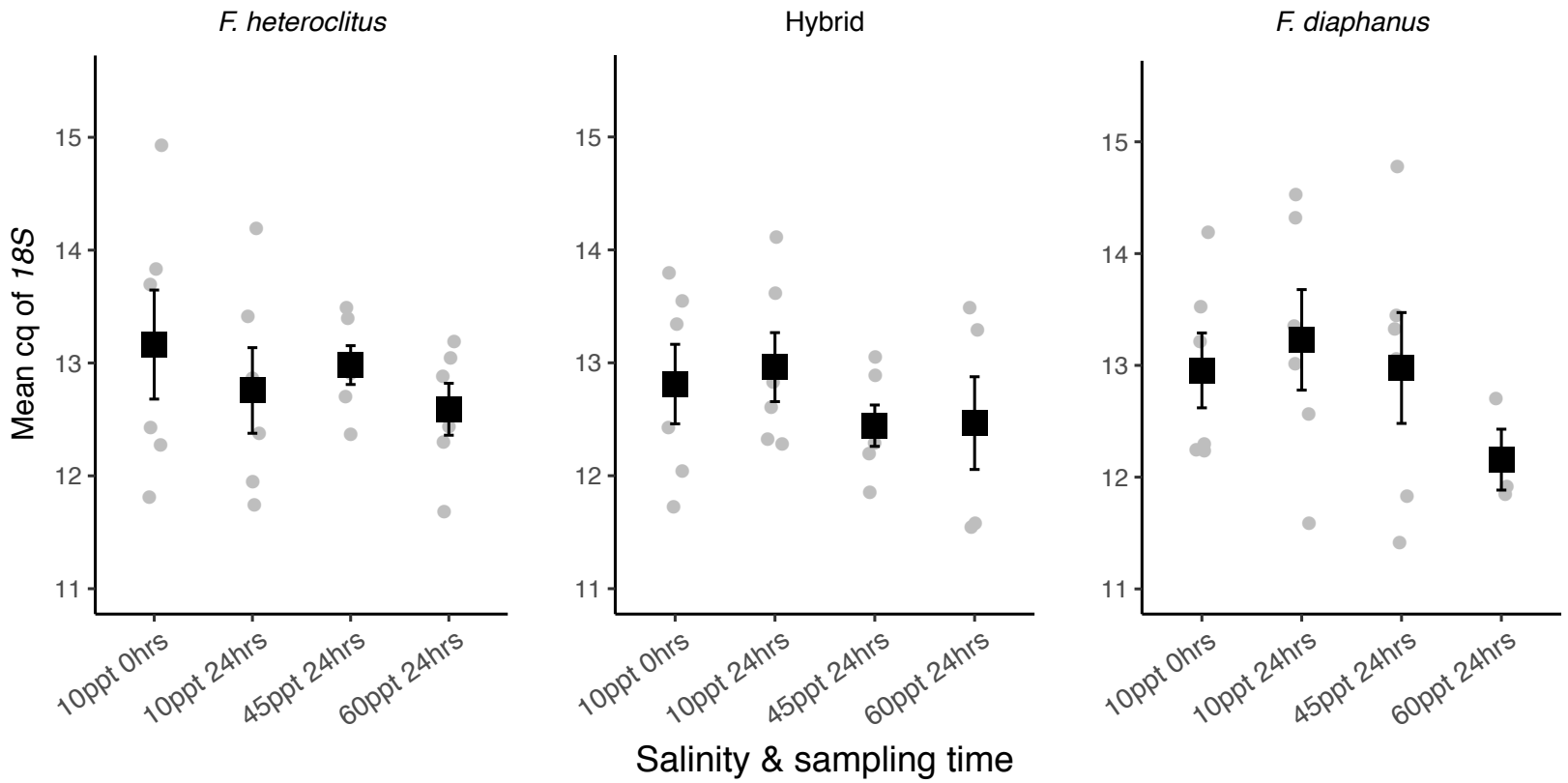

Figure D1. Effects of transfer from brackish water (10 ppt) and hypersaline saltwater (45 – 60 ppt) on the mean Cq of *18S* for *F. heteroclitus* (n = 8 - 11 per group), *F. diaphanus* (n= 3-10 per group) and hybrids (n = 5-10 per group). Data are presented as in Figure D.

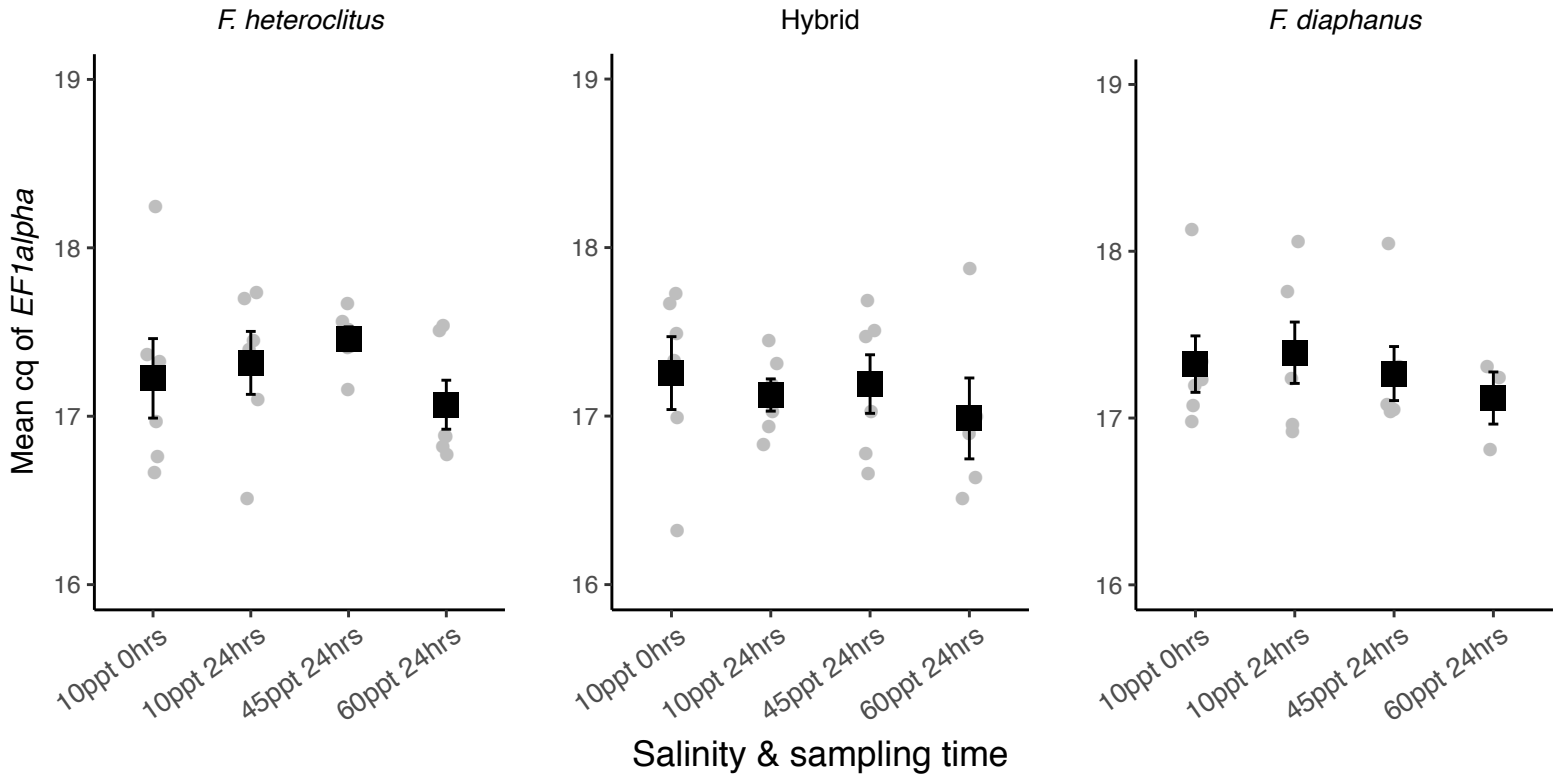

Figure D2. Effects of transfer from brackish water (10 ppt) and hypersaline saltwater (45 – 60 ppt) on the mean Cq of *EF1α* for *F. heteroclitus* (n = 8 - 11 per group), *F. diaphanus* (n= 3-10 per group) and hybrids (n = 5-10 per group). Black boxes represent the mean and error bars ± one S.E.M. and circles represent muscle water content for each fish, with the lightest colour representing the lowest salinity (control salinity of 10 ppt) and the darkest colour representing the highest salinity (hypersaline treatment of 60 ppt).

Appendix E

Water quality report for the Saint Mary's University (SMU) aquatic facilities

Copper was discovered in both water lines in the SMU aquatic facilities with the regular de-chlorinated line showing fluctuations in copper from 3 - 41 μg/L and the chilled de-chlorinated line showing fluctuations in copper from 91 - 552 μg/L (Fig. E). Water from the aquatic facilities de-chlorinated non-chilled line was sent for water testing at the Environmental Services Laboratory vis the Nova Scotia Health Authority on July 7th, 2018, as this was the main line supplying fish tank water. Water quality parameters for the non-chilled dechlorinated line are Listed in Table E and include all necessary information to calculate freshwater copper criterion via the biological ligand model (BLM), or rather compare the effects of copper in water, given water quality parameters (USEPA, 2007). This information could be useful for future comparative studies.

Table E. Water quality parameters measured in SMU aquatic facilities non-chilled dechlorinated line.

Av. Temp	**pH**	**DOC**	**Ca^{2+}**	**Mg^{2+}**	**Na^{+}**	**K^{+}**	**SO_4^{2-}**	**Cl^{-}**	**Alkalinity as $CaCO_3$**
20°C	7.6	1.2mg/L	5.3mg/L	<0.5mg/L	15.9mg/L	<0.5mg/L	9.8mg/L	9.8mg/L	22.1mg/L

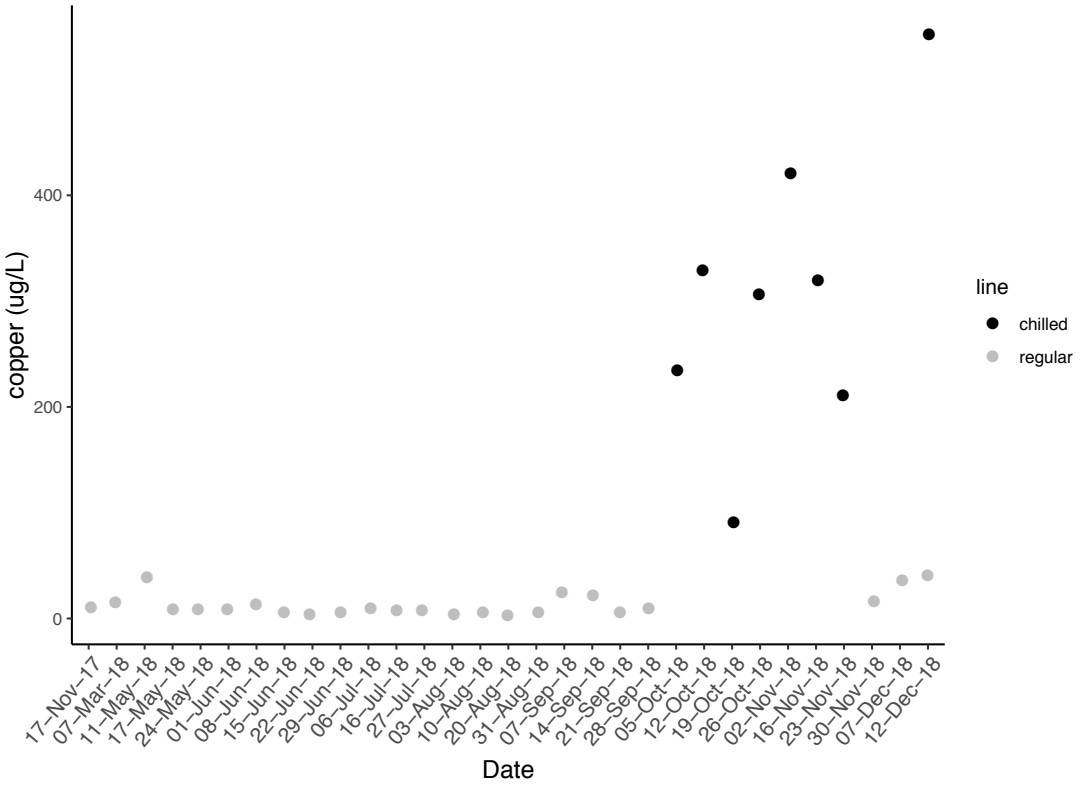

Figure E. Fluctuation of copper in de-chlorinated water lines in the Saint Mary's University (SMU) Aquatic Facilities from Nov 17th, 2017 to December 12th, 2018. This timeline encompasses all experimental timepoints for the 2017 and 2018 salinity tolerance experiments. (Experiment 1: Nov 11th, 2017- Dec 18th, 2017 & Experiment 2: Aug 1st, 2018- Oct 22nd, 2018). The regular water line is indicated with grey circles and the chilled water line is indicated with black circles. Copper fluctuated from 3-41 µg/L in the regular line but was highest in the chilled line (91 - 552 µg/L), which was only first measured on October 5th, 2018. Thus, we do not know the copper concentration during our full experimental acclimation.

Appendix F

Plasma K+ content for *F. heteroclitus*, *F. diaphanus* and hybrids in freshwater (0 ppt) and saltwater (32 ppt)

There was a significant interaction between species × timepoint ($F_{5,96}$ =3.23 , p = 0.009) in plasma K^+ content, but no significant interactions for salinity × species ($F_{4,96}$ = 1.21, p = 0.34), salinity × timepoint: ($F_{6,96}$ = 1.75, p = 0.12), or species × salinity × timepoint ($F_{8,96}$ = 1.06, p =0.40). There was a significant difference in plasma potassium (K^+) content across timepoints ($F_{4,96}$ =4.81, p <0.005). There were no significant differences between species ($F_{2,96}$ = 1.03, p = 0.36), nor salinities ($F_{2,96}$ = 0.52, p = 0.60) for plasma K^+ content.

There were no significant differences in plasma K^+ between any species at any salinity, with the exception of *F. diaphanus* at 32 ppt, 96 hrs which was different from the following treatments (*F. diaphanus* at 32 ppt & 0 ppt, 24 hrs; *F. heteroclitus* at 32 ppt, 14 days & 0 ppt 24 hrs; hybrids at 0 ppt, 24 hrs & 0 ppt & 10 ppt, 14 days; Fig. F). *F. diaphanus* show a trend of increasing plasma K^+ at 0 ppt and 32 ppt after 96 hrs, indicating that they may have some trouble maintaining homeostasis at this timepoint, however; there are no significant differences in plasma ion content between *F. diaphanus* at 10 ppt & 32 ppt nor between species at 32 ppt. Overall, these data indicate that *Fundulus heteroclitus*, *Fundulus diaphanus* and hybrids are tolerant to acute transfers to 0 - 32 ppt (Fig. F).

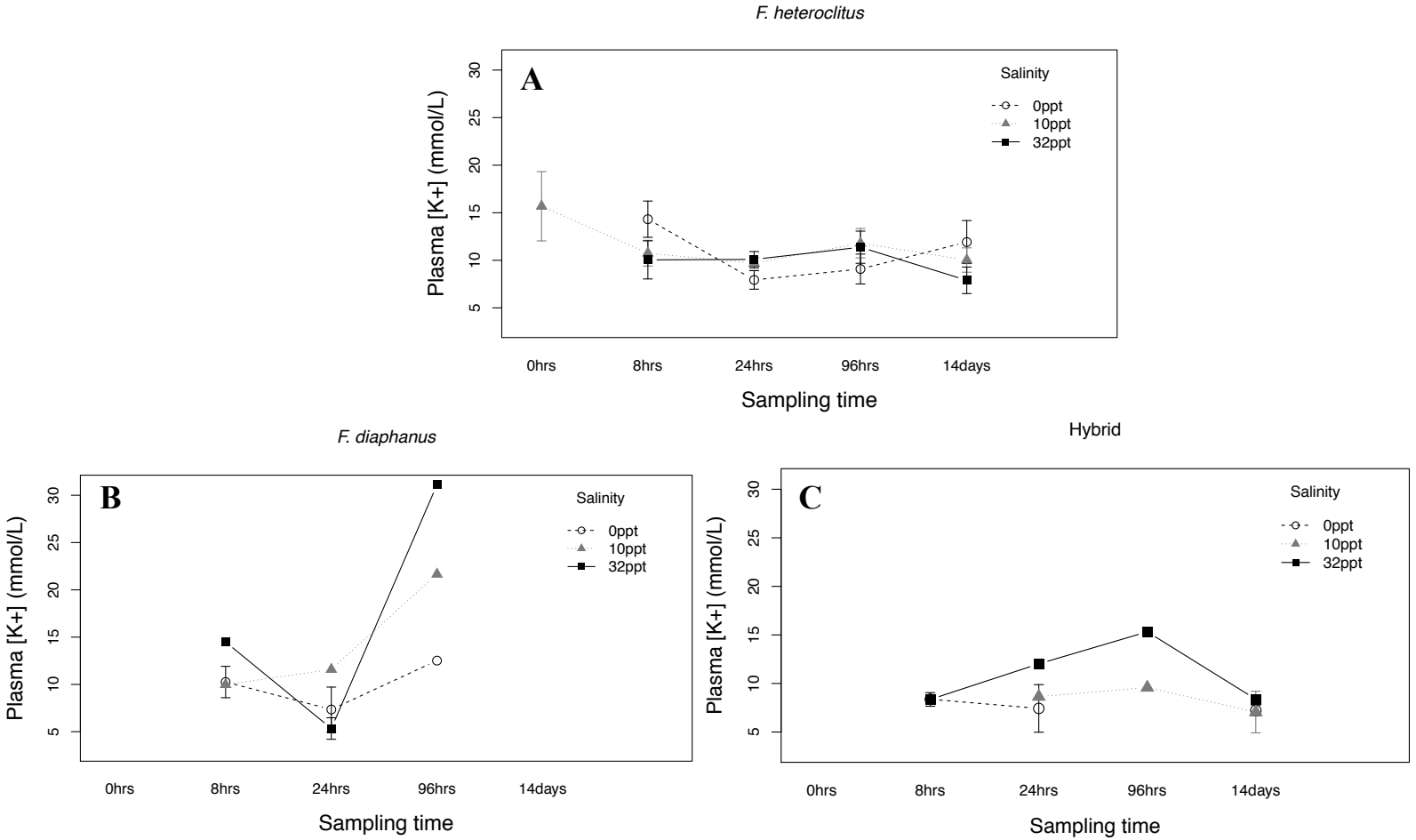

Figure F. Effects of transfer from brackish water (10 ppt) to saltwater (32 ppt) and freshwater (0 ppt) on plasma potassium (K+) concentration for (A) *F. heteroclitus*, (B) *F. diaphanus* and (C) hybrids. The points represent the mean and error bars indicate ± one standard error of the mean (S.E.M.). The white circles represent the lowest salinity (0 ppt), the grey triangles represent the control salinity (10 ppt) and the black squares represent the highest salinity (32 ppt).

Appendix G

Muscle Moisture content for *F. heteroclitus* with prolonged exposure to 60 ppt

Muscle moisture content was measured at additional sampling points in both 10 ppt (14 days, long term-control) and 60 ppt (8 days, 30 days) for *F. heteroclitus* (Fig. G). Muscle moisture data was analyzed using a GLM that contained only *F. heteroclitus* and no other species, followed by Tukey's *a posteriori* multiple comparisons tests. There was a significant effect of salinity & sampling time on muscle water content ($F_{7,68} = 5.68$, $p < 0.001$,) and muscle water returned to control levels after 30 days at 60 ppt, compared to two of three control salinities (10 ppt 24 hrs & 10 ppt 14 days) as well as compared to 32 ppt, 45 ppt and 60 ppt for 24 hrs (Fig. G).

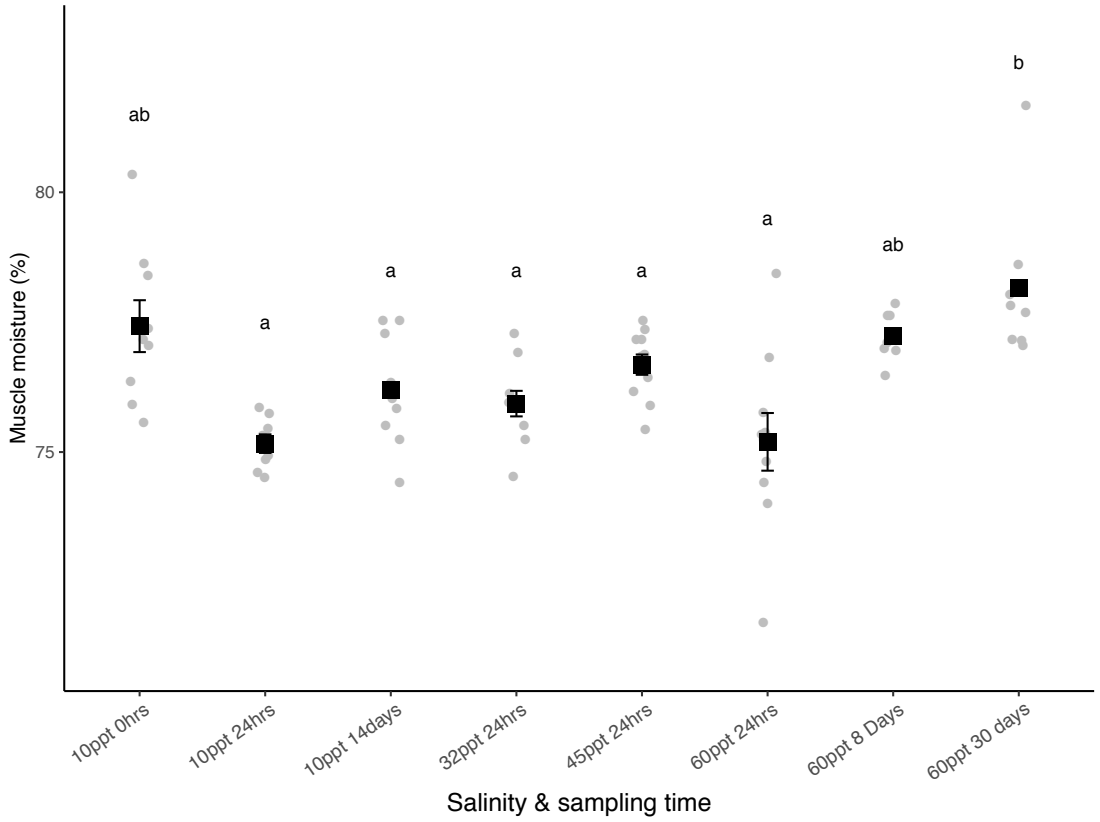

Figure G. Effects of transfer from brackish water (10 ppt) to saltwater (32 ppt) and hypersaline saltwater (45 – 60 ppt) on muscle water content for *F. heteroclitus* (n = 8 - 11 per group). Black boxes represent the mean and error bars ± one S.E.M. and circles represent muscle water content for each fish. Significant differences between groups detected by Tukey a posteriori multiple comparisons tests are indicated by different letters ($p < 0.05$).

Appendix H

Plasma Na+ and Cl- content for *F. heteroclitus* with prolonged exposure to 60 ppt

Both plasma Na^+ and Cl^- were measured at additional sampling points in both 10 ppt (14 days, long term-control) and 60 ppt (8 days, 30 days) for *F. heteroclitus*. As mentioned in Appendix G, data were analyzed with a GLM containing only *F. heteroclitus,* followed by Tukey's *a posteriori* multiple comparisons tests. There was a significant effect of salinity & sampling time on both plasma Na^+ ($p < 0.001$, $F_{7,63} = 5.57$) and plasma Cl^- ($p < 0.001$, $F_{7,63} = 6.70$). There is a significant increase in plasma Cl^- at 60 ppt 24 hrs compared to control salinities and in plasma Na^+ compared to controls of 10 ppt, 0 hrs and 10 ppt, 14 days; however, there is no difference in plasma Na^+ or Cl^- at 60 ppt 30 days compared to control salinities (Fig. H). This indicates that *F. heteroclitus* can acclimate and maintain ionic homeostasis with prolonged exposure to hypersaline saltwater (60 ppt).

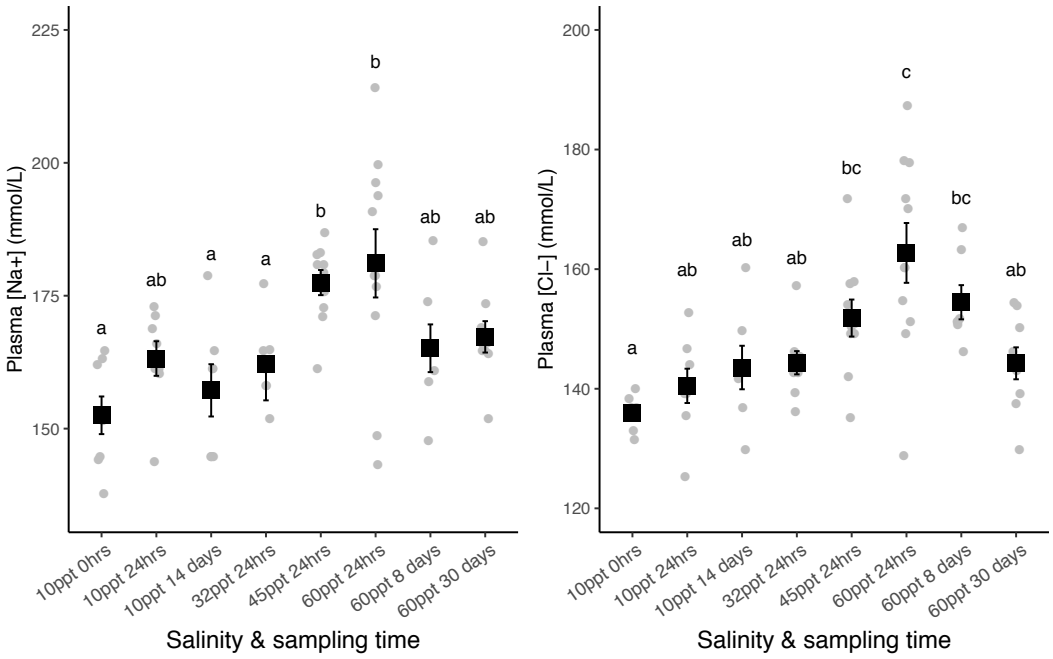

Figure H. Effects of transfer from brackish water (10 ppt) to saltwater (32 ppt) and hypersaline saltwater (45 – 60 ppt) on plasma Na^+ (A) and plasma Cl^- (B) for *F. heteroclitus* (n = 7 - 11 per group). Data are presented as in Figure G.

Appendix I

Plasma K+ content for *F. heteroclitus*, *F. diaphanus* and hybrids in hypersaline saltwater

There is a significant effect of salinity & sampling time ($p < 0.001$, $F_{4,105} = 9.92$) on plasma K^+ content, but no difference between species nor was there a significant interaction. Additionally, there was a high overall variability in plasma K^+ concentration. Data were analyzed with a GLM, followed by Tukey's *a posteriori* multiple comparisons tests.

There were no significant differences in plasma K^+ between any species at any salinity, with the exception of *F. heteroclitus* at 10 ppt, 0 hrs and hybrids at 10 ppt, 0 hrs (Fig. I). Despite having a high plasma K^+ relative to the handling control (10 ppt, 24 hrs, not 10 ppt, 0 hrs), the trends in the data suggest that *F. heteroclitus* can excrete K^+ in hypersaline saltwater and all other experimental salinities.

Additionally, plasma K^+ was significantly elevated for hybrids in 60 ppt compared to one of two control salinities (10 ppt, 0 hrs) and for *F. heteroclitus* in 60 ppt compared to one of two control salinities (10 ppt, 24 hrs). Additionally, both *F. diaphanus* and hybrids showed a trend of increasing plasma K^+ with increasing salinity (Fig. I). This suggests that *F. diaphanus* and hybrids might not be able to excrete K^+ as effectively as *F. heteroclitus* in hypersaline saltwater.

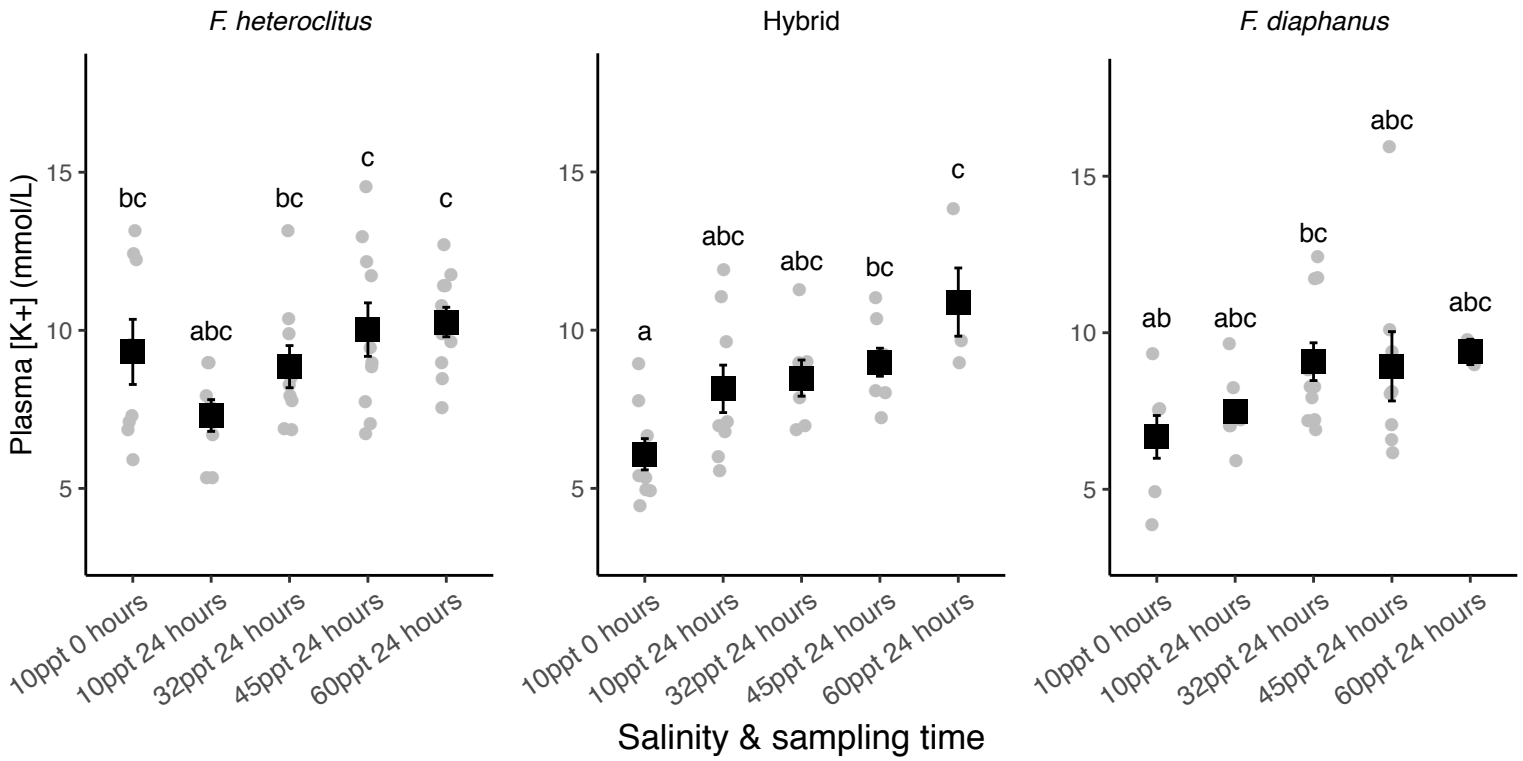

Figure I. Effects of transfer from brackish water (10 ppt) to saltwater (32 ppt) and hypersaline saltwater (45 – 60 ppt) on plasma potassium (K^+) for *F. heteroclitus* (n = 8 - 11 per group), *F. diaphanus* (n = 2 - 10 per group) and hybrids (n = 4 - 9 per group). Data are presented as in Figure G.

Appendix J

Plasma K+ content for *F. heteroclitus* with prolonged exposure to 60 ppt

Plasma K^+ was measured at additional sampling points in both 10 ppt (14 days, long term-control) and 60 ppt (8 days, 30 days) for *F. heteroclitus*. As mentioned in Appendix G & H, data were analyzed with a GLM containing only *F. heteroclitus,* followed by Tukey's *a posteriori* multiple comparisons tests. There was a significant effect of salinity & sampling time on plasma K^+ ($p < 0.0042$, $F_{7,63} = 3.37$), but there were no significant differences in plasma K between experimental salinities (32 ppt, 45 ppt or 60 ppt) at any sampling point compared to control salinity (10 ppt, 0 hrs) (Fig. J.).

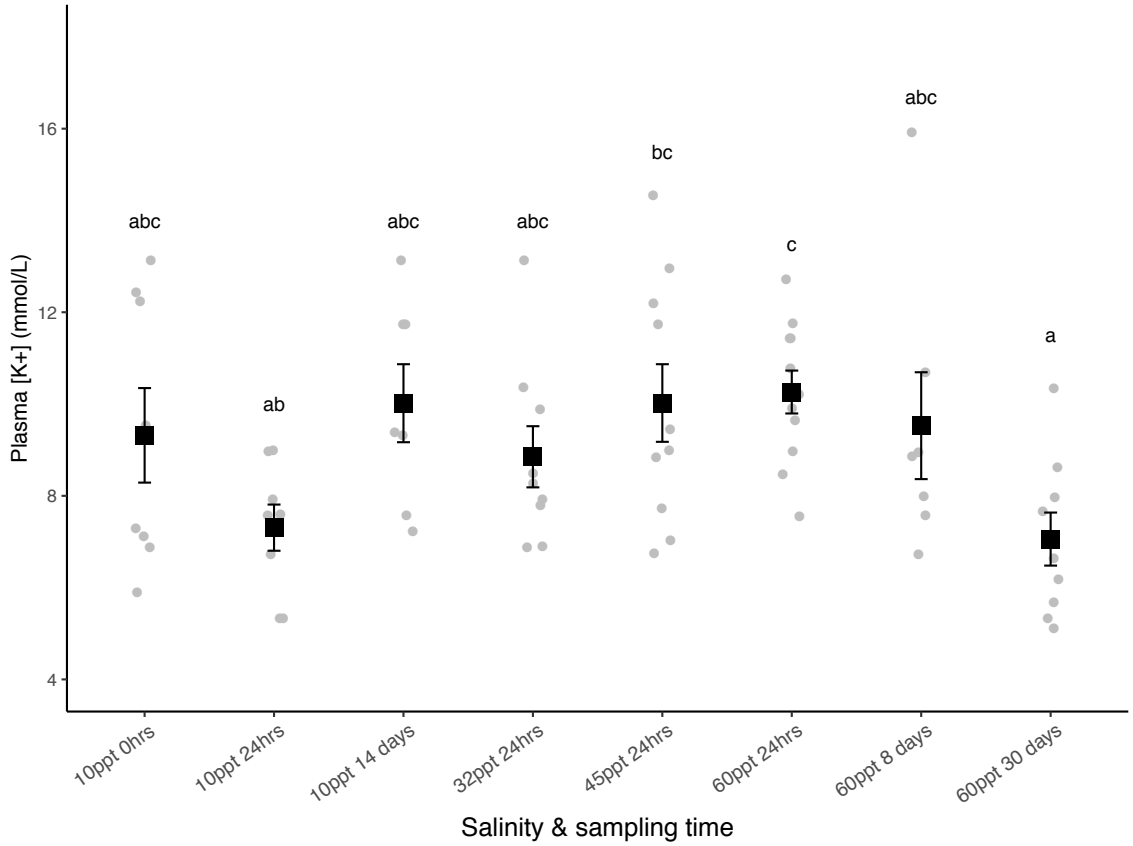

Figure J. Effects of transfer from brackish water (10 ppt) to saltwater (32 ppt) and hypersaline saltwater (45 – 60 ppt) on plasma K+ in *F. heteroclitus* (n = 7- 11, per group). Data are presented as in Figure G.

Appendix K

Hematocrit for *F. heteroclitus*, *F. diaphanus* and hybrids in hypersaline saltwater

Hematocrit was measured for all species after 24 hours at 10 ppt, 32 ppt, 45 ppt and 60 ppt and at 10 ppt for 0 hours. Data were analyzed using a GLM containing all data, followed with Tukey's post hoc test. There was a significant effect of salinity & sampling time on hematocrit ($F_{4,115} = 2.68$, $p = 0.035$) but there were no significant differences in hematocrit at any salinity (32 ppt, 45 ppt or 60 ppt) relative to control salinity (10 ppt, 24 hrs), besides for hybrids after 24 hours at 10 ppt compared to 0 hours at 10 ppt (Fig. K). There were also no significant interactions between species or sampling time. Overall, these data indicate that hematocrit is not significantly altered for any species with increasing salinity (Fig. K).

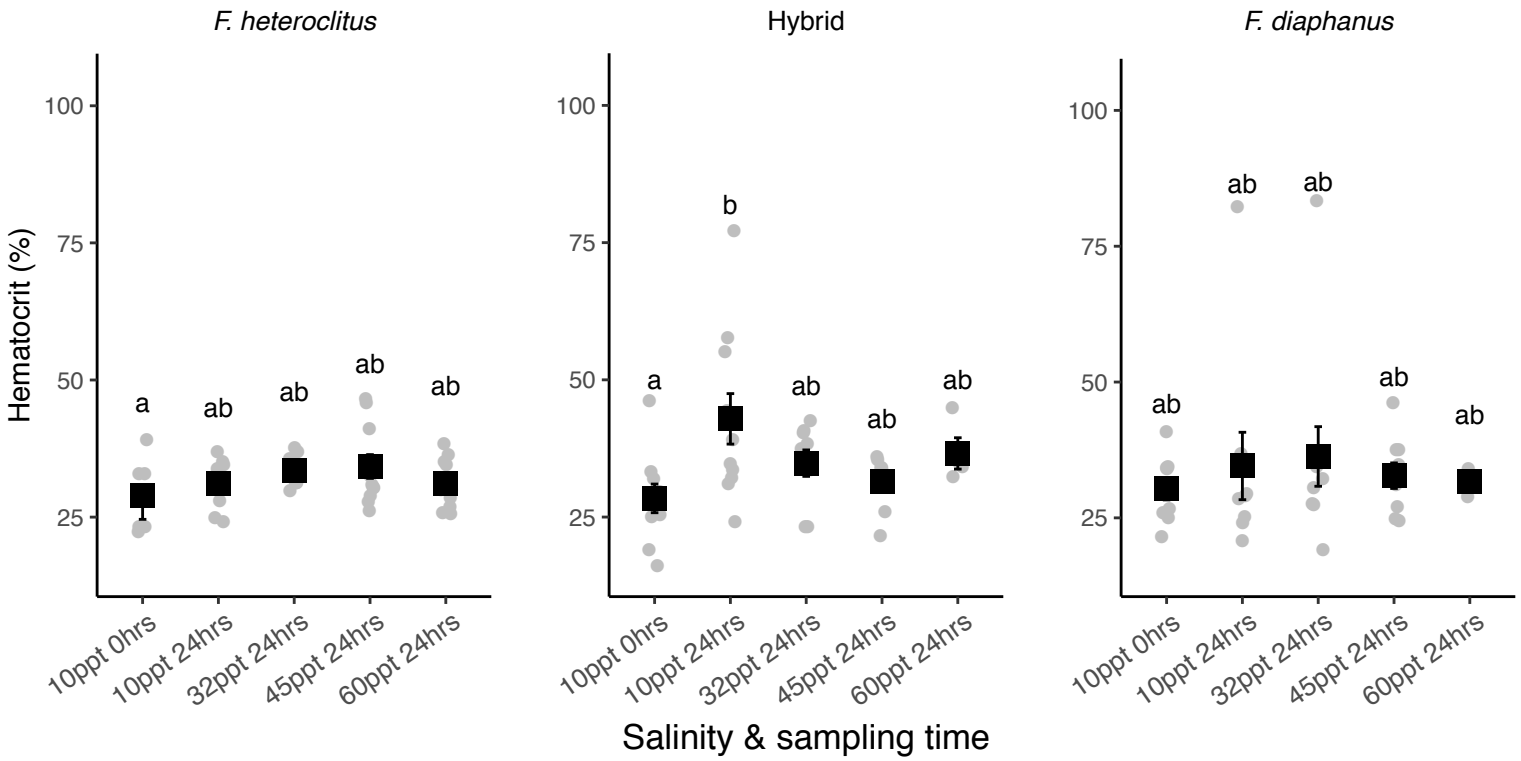

Figure K. Effects of transfer from brackish water (10 ppt) to saltwater (32 ppt) and hypersaline saltwater (45 – 60 ppt) on hematocrit for *F. heteroclitus* (n = 8 - 11 per group), *F. diaphanus* (n= 3-10 per group) and hybrids (n = 5-10 per group). Data are presented as in Figure G.

Appendix L

Hematocrit for *F. heteroclitus* with prolonged exposure to 60 ppt

Hematocrit was measured at additional sampling points in both 10 ppt (14 days, long term-control) and 60 ppt (8 days, 30 days) for *F. heteroclitus*. As mentioned in Appendix G & H, data were analyzed with a GLM containing only *F. heteroclitus*. There were no significant differences in salinity & sampling time on hematocrit ($p = 0.06$, $F_{7,68} = 2.06$) and there were no significant differences in hematocrit between experimental salinities (32 ppt, 45 ppt or 60 ppt) at any sampling point compared to control salinity (10 ppt, 0 hrs) (Fig. L). This indicates that hematocrit is not significantly altered for *F. heteroclitus* with prolonged exposure to 60 ppt.

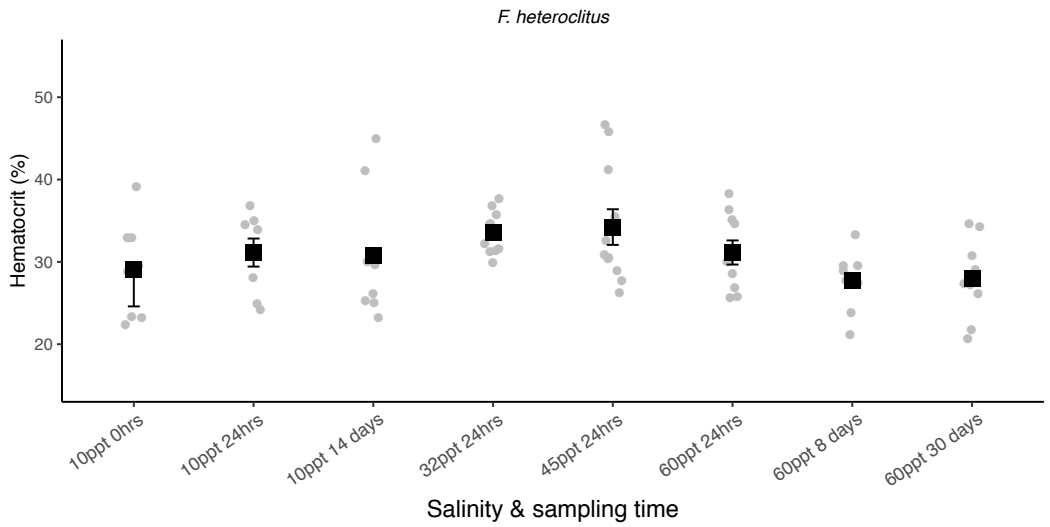

Figure L. Effects of transfer from brackish water (10 ppt) to saltwater (32 ppt) and hypersaline saltwater (45 – 60 ppt) on hematocrit for *F. heteroclitus* (n = 7 - 11, per group). Data are presented as in Figure G.